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Comparative prevalence of *Oxalobacter formigenes* in three human populations

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There has been increasing interest in the human anaerobic colonic bacterium *Oxalobacter formigenes* because of its ability to metabolize oxalate, and its potential contribution to protection from calcium oxalate kidney stones. Prior studies examining the prevalence of this organism have focused on subjects in developed countries and on adults. Now using *O. formigenes*-specific PCR, we have compared the prevalence of these organisms among subjects in two remote areas in which modern medical practices have hardly been present with a USA group of mothers and their infants for the first three years of life. Among the Amerindians of the Yanomami-Sanema and Yekwana ethnic groups in Venezuela and the Hadza in Tanzania, *O. formigenes* was detected in 60–80% of the adult subjects, higher than found in adults from USA in this and prior studies. In young children, the prevalence was much lower in USA than in either tribal village. These data extend our understanding of the epidemiology of *O. formigenes* carriage, and are consistent with the hypothesis that the rising incidence of kidney stones is associated with the progressive loss of *O. formigenes* colonization in populations that have been highly impacted by modern medical practices.

Oxalobacter formigenes are gram-negative anaerobes that inhabit the intestinal tract of humans and other mammals^{1,2}. *O. formigenes* is unique in that it utilizes oxalate as its sole carbon and energy source^{3,4}, which it metabolizes into formate and CO₂⁵. In mammals, oxalate is an end-product of metabolism and must be eliminated either in the intestine or via urinary excretion^{6,7}. The renal excretion can lead to nephrotoxicity, including calcium oxalate kidney stones⁸; thus, regulation of oxalate homeostasis has important pathophysiological consequences.

One hypothesis is that intestinal colonization with *O. formigenes* helps protect against calcium oxalate nephrolithiasis^{9,10}. *O. formigenes* utilizes dietary oxalate, reducing the amount absorbed into the circulation, and recent evidence suggests that it induces colonic oxalate transporters (SLC26), acting as a sink for systemic oxalate^{11–14}. Studies comparing adults with recent nephrolithiasis and healthy adults showed a lower rate of *O. formigenes* colonization in the stone formers, consistent with a protective role for the organism^{10,15}. Since ~75% of kidney stones are composed of calcium oxalate¹⁶, and since nephrolithiasis incidence is substantially increasing in both adults and children^{17–19}, a focus on *O. formigenes* may be of interest for therapeutic applications.

Prior studies of the prevalence of *O. formigenes* colonization showed considerable differences, which might reflect true variation or technical issues. Studies in healthy adults suggest prevalence in the United States is between 31–38%^{10,20,21} but as high as 60–77% in Korea, Japan, and India^{22–24}. Such variation could also reflect medical practice differences, as *O. formigenes* is known to be susceptible to many commonly prescribed antibiotics²⁵. In one prospective study, antibiotic treatment resulted in lasting suppression of *O. formigenes*²⁶. Patients

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Sample	N	% Positive
Mothers	40	38
Infants		
Post-Delivery	17	0
Year 1	50	10
Year 2	76	8
Year 3	56	21

Table 1. *O. formigenes* in USA maternal and infant fecal samples by PCR.

Dyad	Mother	Infants						
		Delivery	Month 06	Month 12	Month 18	Month 24	Month 30	Month 36
1	+	-	-	-	+	+	-	+
7	+	-	-	-	-	-	-	-
8	+	-	-	-	-	-	-	-
14	+	-	-	-	-	-	+	-
18	+	-	-	-	-	-	-	-
20	+	-	-	-	-	-	-	-
31	+	-	-	-	-	-	-	-
41	+	-	-	-	-	-	-	+
43	+	-	-	-	-	-	-	-
44	+	-	-	+	-	-	-	-
47	+	-	-	-	-	-	-	-
50	+	-	-	-	-	-	-	-
56	+	-	-	-	-	-	-	-
57	+	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	+
4	-	-	-	-	-	-	-	+
5	-	-	-	-	-	+	-	+
10	-	-	-	-	-	-	+	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	+	-
21	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	+	-
24	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	+	+
30	-	-	-	-	-	-	-	+
32	-	-	-	-	-	-	-	-
33	-	-	-	-	+	-	-	-
35	-	-	-	-	-	-	-	-
36	-	-	-	+	-	-	-	-
37	-	-	-	+	+	-	-	-
38	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	+
45	-	-	-	-	-	-	-	-
46	-	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-
52	-	-	-	+	-	-	-	-
53	-	-	-	-	-	+	-	-
55	-	-	-	+	-	-	-	-
9	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-

Figure 1. Temporal distribution of *O. formigenes* in mother-infant dyads from USA. Figure represents 199 samples tested from 42 individual children. In total, 19 (45%) children were positive at ≥ 1 time point. Four (10%) children were positive at ≥ 2 time points.

with cystic fibrosis or inflammatory bowel disease, who receive multiple antibiotic courses also have lower *O. formigenes* prevalence than controls^{27,28}. However, little is known about the transmission of *O. formigenes* and its timing, and the factors influencing colonization.

To further address these questions, we examined *O. formigenes* colonization in a small cohort of USA mothers and their infants longitudinally from birth through three years of life. To investigate the hypothesis that modern practices may be impacting *O. formigenes* colonization, in parallel, we examined adults and children in two remote populations: the Hadza, a tribe of hunter-gatherers in Tanzania²⁷, and a group of remote Amerindians in Venezuela²⁹.

Results

***O. formigenes* prevalence in the USA cohort.** Among the 40 women who provided post-partum fecal samples, *O. formigenes* was detected in 15 (38%) (Table 1). Among the 42 infants, a total of 19 (45%) were positive at least once in the multiple determinations, and 4 (10%) were positive at least twice (Fig. 1). *O. formigenes* was only found in children greater than 1-year old and not in infants, and the prevalence appears to increase with age, although study numbers are small.

We then assessed whether there was a relationship between maternal and infant colonization status, using logistic regression with and without adjustments for maternal covariates (age, BMI, and race), calculating Cohen's kappa statistic to examine the agreement between maternal and infant PCR results. We also used a generalized linear mixed model to analyze PCR results with or without adjustment for those covariates as well as maternal antibiotic use prior to pregnancy, during pregnancy, and during delivery. There was no statistically significant correlation between maternal and infant colonization, whether evaluating infant colonization at each time point

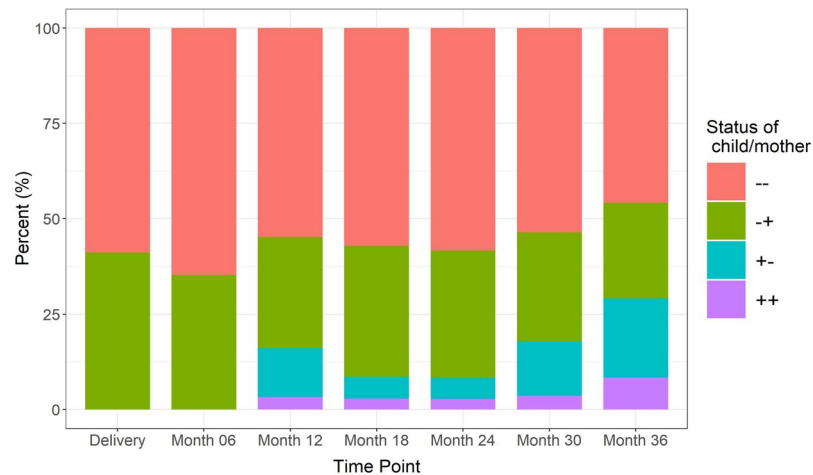


Figure 2. Concordance of maternal and child *O. formigenes* status over the first three years of life. *O. formigenes* detected by *oxc* PCR, as described in the Methods.

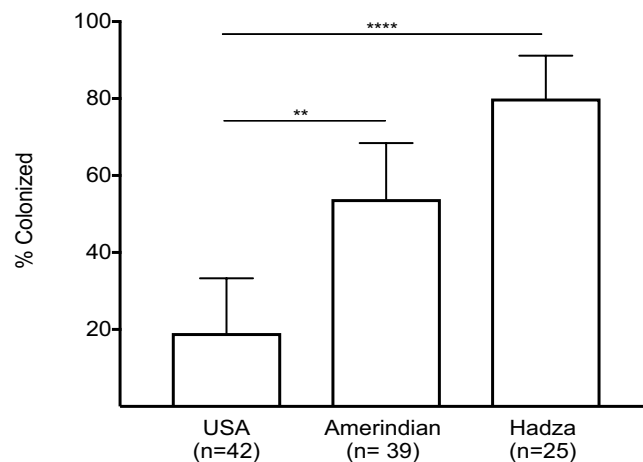


Figure 3. Percent of children less than 5 years old positive for *O. formigenes* in three populations. Only single samples were present from the Amerindian and Hadza children. Multiple samples were tested for the USA children, but only the sample from each child at the oldest age tested was used for the calculation. Percent of children colonized, with 95% CI shown (** $p < 0.01$; **** $p < 0.001$, by Fisher's exact test).

or in infants positive at any point by either model (Fig. 2). Next, we asked whether *O. formigenes* status of the infants was correlated with their delivery method or gender. First, we used Fisher exact test and chi squared tests to assess the correlation between these variables and infants' PCR results. Logistic regression and the generalized linear mixed model were also applied. We found no significant correlation between infant delivery method or gender and their *O. formigenes* colonization status at individual time points or positivity at any time, with or without adjustment for maternal covariates. We also asked whether *O. formigenes* colonization status was correlated with infant antibiotic use. We examined whether antibiotics given perinatally (intravenous antibiotics given to the newborn), systemic antibiotics given during the first, second, or third year of life, or systemic antibiotics given at any point correlated with infants' *O. formigenes* status. Again there was no significant correlation between antibiotic use and colonization at subsequent time points by logistic regression or generalized mixed model with or without covariate adjustment.

***O. formigenes* prevalence in hunter-gatherer populations.** We compared prevalence of *O. formigenes* colonization in the cohort of US children with that in tribal populations from Africa and South America. US children had the lowest prevalence (19%) (Fig. 3); compared with Amerindians (68%; $p = 0.0013$), and with Hadza (82%; $p < 0.0001$, Two-sided Fisher exact test). There were no significant differences in prevalence between the Amerindian and Hadza children. While there was no evidence of colonization in the USA children until 12 months of age (0/17 were positive at 6 months (Fig. 1), *O. formigenes* was detected in some of the youngest subjects sampled in the Hadza and Amerindian populations: the earliest at 9 months and 3 months of age respectively (data not shown). We also examined *O. formigenes* prevalence in adulthood in 68 Amerindian and 116 Hadza subjects. Among the adults, 79% of Amerindians and 55% of Hadza subjects were PCR-positive. Comparing the

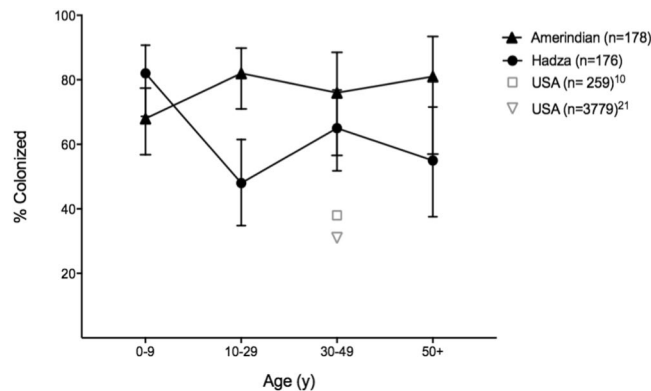


Figure 4. *O. formigenes* prevalence in traditional and USA populations, by age. Positivity for the Hadza and Amerindians determined by end point PCR using *O. formigenes*-specific primers, as described in Methods. Results for two prior US studies for comparison using different methodologies are shown at ~the mean age of the populations tested with the percent colonized indicated; the mean ages were 42 and 46, respectively^{10,21}.

two populations by age group (Fig. 4) demonstrated similar rates of colonization except among adolescents and young adults, in which prevalence in the Hadza was significantly lower. Regardless, the prevalence of *O. formigenes* among both healthy Amerindians and Hadza was substantially higher than that of healthy adults in the United States (ranging from 31% to 38%), as reported in three large and independent studies^{10,20,21}, as well as the US mothers in this work.

Discussion

To our knowledge, this is the first study to examine early life *O. formigenes* colonization in more than 20 years and the only study that has examined a cohort of mothers and children longitudinally from birth through the first years of life. The other study of *O. formigenes* colonization in early life was a cross-sectional evaluation of children ages 0–12 in a remote area of the Ukraine where antibiotic use was considered to be limited³⁰. In that study, Sidhu *et al.* found that 72% of children were PCR-positive, with no evidence of colonization before the age of one year, but reaching 90% by age 2; nearly all children appeared to be colonized by age 8, after which rates declined. Similar to the findings in Ukrainian infants, we found no evidence of colonization in children less than one year in our U.S. cohort. This may be explained by *O. formigenes* acquisition from the environment, as infants begin to become more mobile at this age, as proposed³⁰ and demonstrated in rodent models³¹. The lack of association between maternal and infant colonization in this study also supports the hypothesis of horizontal transmission. Alternatively, *O. formigenes* may be acquired at birth from mothers but only at low abundance, blooming later in life with advancement of infants' diet or with maturation of the microbiome and the acquisition of commensal species required for *O. formigenes* colonization success. The current study did not provide any direct support for this hypothesis, but more prolonged cohort studies with a larger number of subjects may be needed to better address this question. All of the children who were found to be *O. formigenes*-positive, also had negative samples at the earliest time points. Our past work^{20,21} showed that the relative abundance of *O. formigenes* in fecal specimens varies over $>3 \log_{10}$. Children with low abundances may be near the threshold of detection, with the variation in positivity that we observed consistent with our prior findings²¹.

Several studies have examined the intestinal microbiome of the Hadza^{29,32–34} and to a lesser extent, the Amerindians of Venezuela³⁵. Since these groups have had only recent exposure to Western medicine, their results provide a window on the pre-industrial intestinal microbiome of humans. Similar to our prior study of a small uncontacted Amerindian population using high throughput sequencing, the prevalence of *O. formigenes* was substantially higher than that of USA subjects³⁵. Now we provide evidence of higher rates in a larger population of both children and adults in remote Amerindian and African populations than in the USA, including those in this study as well as in the literature^{10,21,36}. A possible explanation for this difference is that developments associated with the Westernization, such as changes in diet or antibiotic use may contribute to the loss of this commensal organism. A recent report of the association of kidney stones with prior antibiotic treatments³⁷, across multiple antibiotic classes, is also consistent with that hypothesis.

Limitations of this study include the relatively small number of subjects to address associations with covariates. On average, the subjects in the USA were of higher socioeconomic status, more urban, and better-educated³⁸ than the general population, so they were not fully representative of the USA population. Comparing fecal samples from the immediately post-partum women in the USA to populations of largely non-pregnant women is an intrinsic limitation as well. Individual dietary information was generally not available, so oxalate exposure differences could not be evaluated. Nevertheless, this study used the same *O. formigenes*-specific methodologies to examine specimens across three different populations in large cross-sectional surveys; this is the first report in the literature of such comparisons. In conclusion, *O. formigenes* was present at much lower prevalence in the USA subjects compared to remote indigenous groups, consistent with the hypothesis that *O. formigenes* is a part of the ancestral human gut microbiota, and that it may be disappearing in the context of socioeconomic advances and medical treatments, potentially contributing to the rise in nephrolithiasis.

Region	No. subjects	Age: range (y), mean \pm SD	Sex: % female	BMI*: mean \pm SD	Delivery: % vaginal	Race or Ethnicity: (%)	
Tanzania	182	0–80, 28.3 \pm 19.7	48.3	119.5 \pm 3.4	100	Hadza	100
Venezuela	178	0–62, 18.3 \pm 16.2	54.5	18.9 \pm 5.1	100	Sanema	54
						Yekwana	46
New York-Mothers	40	23–40, 35.8 \pm 4.5	100	24.7 \pm 4.3	NA	African-Am	5
						Asian	18
						Hispanic	10
						White	65
						Other	3
New York-Infants	42	0–3, NA [†]	33.3	NA	55	African-Am	5
						Asian	17
						Hispanic	10
						White	67
						Other	2

Table 2. Demographics of the 442 subjects in three locales. *BMI included for subjects over age 5 years. [†]Samples tested at multiple time points over first three years of life.

Methods

Populations tested. All experiments were performed in accordance with relevant guidelines and regulations concerning human subjects (Table 2). United States. Fecal samples were collected from a cohort of mothers and infants in New York City as part of the Early Childhood Antibiotics and the Microbiome (ECAM) study³⁸. The specimens used in this study were obtained under the ECAM protocol, approved by the NYU Langone Medical Center IRB, as reported in detail³⁸. Informed consent was obtained from all subjects, or if subjects were under 18, from a parent and/or legal guardian. Forty of the 52 mothers enrolled in the study during their pregnancy self-collected fecal samples during the first month post-partum (Mean 9 \pm 12 days (SD) post-partum). Fecal samples were collected from their infants within the first postpartum day and usually represented meconium, and then regularly through three years of life. Mothers were provided sterile supplies and instructions for sanitary collection of their own and their infant's stools at home. Fecal samples were immediately chilled to -2 to -8 C, and delivered within 24–48 hours to the laboratory where they were frozen to -80 C. The maternal post-partum (n = 40) and infant delivery (n = 17) samples, as well as those obtained at \sim 6 months (n = 17), 12 m (n = 33), 18 m (n = 38), 24 m (n = 38), 30 m (n = 30) and 36 m (n = 26) were examined for *O. formigenes* colonization.

Tanzania. The Hadza live in north-central Tanzania around Lake Eyasi in the central Rift Valley. There are currently \sim 1,200 Hadza with fewer than 200 following a traditional foraging lifestyle that includes obtaining \sim 80–90% of their caloric needs from hunted and foraged resources. Fecal samples were collected from 182 traditional subjects of the Hadza tribe across wet and dry seasons²⁹. The Hadza are among the last remaining hunter-gatherer populations in Africa, and reside in the eastern Rift Valley of Tanzania near Lake Eyasi³⁹. Traditional Hadza live in camps averaging 20–30 people, with numbers varying according to season and availability of resources. Six camps were represented in this study (Kipamba, Makao, Mwamudu, Onowas, Sengeli, Ukamako)²⁹. Until recently, Hadza had limited access to medical dispensaries and rural hospitals and thus western medications. However, recent encroachment into Hadzaland by pastoralists and other populations has increased access to western medical care – with medical posts within a day's walk from the camps sampled. Permission for the study was obtained from the National Institute of Medical Research (MR/53i 100/83, NIMR/HQ/R.8a/Vol.IX/1542) and the Tanzania Commission for Science and Technology. As per our IRB, Informed Consent was obtained. Prior to sample collection, a brief explanation of the project and its objectives was given in a way that could be easily understood in Swahili via a translator. Each participant then signed a Consent Form either with a signature or thumb print in the presence of a witness to confirm her/his willingness to participate. In the case of minors, consent was provided by a parent or legal guardian²⁹. The number of samples collected was not pre-specified for ensuring adequate statistical power. Subjects ranged from 6 months to 80 years of age; the age of six subjects was unknown and those samples were omitted from related analyses. Of the 182 subjects tested, 8 were pregnant women.

Fecal samples were self-collected by subjects, frozen in liquid nitrogen within 0–2 hours, and maintained frozen during all transport and storage until processed for analysis in the United States.

Venezuela. Fecal samples were collected from 178 Amerindians in seven villages in the Yanomami-Sanema and Yekwana territory located in the rain forest of the High Caura river basin, from Bolivar state in Venezuela, under Venezuelan Institute of Scientific Research (IVIC) institutional review board (IRB) approval (project Dir0229/10 approval granted to M. Contreras)⁴⁰. Informed consent was obtained according to the approved IRB protocol, first from the community leaders, then from the individual volunteers who were explained the study, and who signed or stamped their thumb print on the consent form. Parents signed to consent their infants and children. Villagers lack a market economy, and live in relative isolation in villages from 15 to 100 people, with access to the closest town by river requiring travel for one week. They maintain the traditional lifestyle of hunter-gatherer-gardeners common in the remote villages in this mountainous region. There are no health posts

in Sanema villages, and one health post in one Yekwana village, while the others receive no medical care, except by infrequent visits from physicians. Amerindian subjects ranged from 3 months to 62 years of age; pregnant women were not sampled. Fecal samples were collected in the field and immediately frozen in liquid nitrogen, and remained frozen until the time of DNA extraction.

PCR methods. DNA extraction from fecal swabs was performed using the PowerSoil DNA Extraction Kit (MoBio, Carlsbad CA), per the manufacturer's protocol. All extracted DNA was stored at -80 C . Primers were designed based on the sequence alignment of the first 500 bp of the *oxc* gene of *O. formigenes*. The specificity of these primers [5'-ATGTAGAGTTGACTGATGGC-3' (forward) and 5'-TTGATG CTGTTGATACG-3' (reverse)] was confirmed by examining their homology with all other bacterial sequences deposited at the National Center for Biotechnology Information Basic Local Alignment Search Tool website (<http://www.ncbi.nlm.nih.gov/BLAST>). PCR was performed using Taq DNA Polymerase and 10x CoralLoad PCR buffer (Qiagen, Valencia CA). Each 50 μl reaction contained 1 μl of extracted DNA, 0.2 μM of each primer and 6.3 μM DNTPs. PCR cycling conditions included an initial denaturation at 95 C for 3 min and 39 cycles of denaturation at 95 C for 30 s, annealing at 54 C for 30 s, and extension at 72 C for 1 min. PCR products were analyzed by electrophoresis using 2% agarose gels.

Statistical Analysis. For the USA studies in which more extensive metadata were available, logistic regression was applied to explore the relationship between the *O. formigenes* status of each infant and of their mother. Analyses were performed with and without adjustment for the mother's covariates (including age, BMI, race, antibiotic use prior to pregnancy, antibiotic use status during pregnancy, and antibiotics use status during delivery). Due to small sample size, race was defined as a binary variable with white = 1 and nonwhite = 0. Infants' *O. formigenes* status was their status at each time point or being positive at any time. As a sensitivity analysis, the agreement of infant and mother *O. formigenes* status pair also was evaluated by Cohen's kappa test statistic⁴¹. Fisher exact test, chi-squared test and logistic regression with or without adjustment for mother's covariates were applied for testing whether the *O. formigenes* status of infants at each time or being positive at any time was related to their birth method, antibiotic use status, and gender, respectively. The generalized linear mixed model was used to determine whether the infants' *O. formigenes* status during 0–36 months was related to their baseline variates and time dependent covariates such as antibiotic usage, with or without adjustment for their mother's covariates. Time points were converted to 0–36 months, and missing data were omitted.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

A.P., L.N. and M.B. planned the study. M.J., O.N.-A., M.C., O.L., J.L., M.G.D.B. collected and analyzed the specimens. A.P. performed the experiments. A.P., C.W. and H.L. performed the analyses. All authors wrote the manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

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