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ORIGINAL PAPER

Presence of *Oxalobacter formigenes* in the intestinal tract is associated with the absence of calcium oxalate urolith formation in dogs

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Abstract The incidence of calcium oxalate (CaOx) urolithiasis in dogs has increased steadily over the last two decades. A potential mechanism to minimize CaOx urolithiasis is to reduce enteric absorption of dietary oxalate by oxalate-metabolizing enteric bacteria. Enteric colonization of *Oxalobacter formigenes*, an anaerobe which exclusively relies on oxalate metabolism for energy, is correlated with absence of hyperoxaluria or CaOx urolithiasis or both in humans and laboratory animals. We thus hypothesized that decreased enteric colonization of *O. formigenes* is a risk factor for CaOx urolithiasis in dogs. Fecal samples from dogs with CaOx uroliths, clinically healthy, age-, breed- and gender-matched dogs, and healthy non-stone forming breed dogs were screened for the presence of *O. formigenes* by quantitative PCR to detect the oxalyl CoA decarboxylase (*oxc*) gene, and by oxalate degrading biochemical activity in fecal cultures. Prevalence of *O. formigenes* in dogs with CaOx uroliths was 25%, compared to 50% in clinically healthy, age-, breed- and gender-matched dogs, and 75% in healthy non-stone forming breeds. The presence of *oxc* genes of *O. formigenes* was significantly higher in healthy non-stone forming breed dogs than in the dogs with CaOx stones. Further, dogs with calcium oxalate stones and the stone-forming breed-matched controls showed comparable levels of biochemical oxalate degrading activity. We conclude that the absence of enteric colonization of *O. formigenes* is a risk factor for CaOx urolithiasis.

Keywords Calcium oxalate · Urolithiasis · Dogs · *Oxalobacter formigenes*

Abbreviation
PCR Polymerase chain reaction

Introduction

Urolithiasis due to formation of calcium oxalate (CaOx) stones is an increasingly common clinical condition in dogs. The prevalence of CaOx stones in samples submitted to the Minnesota Urolith Center increased from 5.3% in 1981 to >41% in 2007 [23]. CaOx stones present a unique therapeutic challenge since they cannot be dissolved using medical therapy. Despite an array of advanced methods currently available for prevention, CaOx uroliths often recur within 1–3 years, requiring repeated removal to eliminate recurrent clinical signs [19]. Increased oxalate absorption from dietary sources is a risk factor for CaOx urolithiasis [11, 20]. A variety of factors, including dietary calcium, presence of unabsorbed fatty acids and oxalate-degrading microbial flora of the gut, influence the level of free oxalate in the gastrointestinal tract available for absorption [2, 4, 7, 18, 31]. Among the known culturable oxalate-metabolizing bacteria identified from the mammalian gastrointestinal (GI) tract, only *Oxalobacter formigenes* exclusively depends on oxalate for its energy and, therefore, is considered an efficient oxalate degrader in the GI tract [3, 6]. This anaerobe has been cultured from the enteric tract of sheep, pig, rats, and humans [2, 5].

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In human and lab animals, enteric colonization of *O. formigenes* is correlated with the absence of hyperoxaluria and calcium oxalate stones [17, 21, 22, 27, 28, 30]. The risk of recurrent stone formation is reduced by 70% by intestinal colonization of *O. formigenes* in humans and administration of *O. formigenes* or its oxalate-metabolizing enzymes reduced or reversed hyperoxaluria in rats and humans [12–14, 25]. *O. formigenes* colonization also reduced blood oxalate levels in human and rat by generating a trans-epithelial oxalate ion concentration gradient which facilitates enteric efflux of oxalate ions from plasma through the enterocytes, contributing to the enteric clearance of plasma oxalate [9, 10].

Weese et al. [32, 34] reported the presence of *O. formigenes* in healthy dogs and cats through PCR screening but its potential role in the prevention of CaOx urolith formation is unknown. Here we hypothesize that decreased enteric colonization of *O. formigenes* is a risk factor for CaOx urolithiasis in dogs. Thus, we screened for the presence of *O. formigenes* in healthy dogs and those affected by CaOx uroliths. Oxalate degradation was assayed in fecal cultures to predict the functional outcome of increased colonization by oxalate-degrading bacteria.

Materials and methods

Selection of dogs

Fecal samples were obtained from 20 dogs with idiopathic CaOx urolithiasis at the time of urolith removal. Uroliths were quantitatively analyzed and reported to contain ≥90% CaOx monohydrate or dihydrate or both. Twenty clinically healthy dogs of similar breed, age (±1 year), and gender to the stone-formers were also sampled (Table 1). Medical history, survey radiography, urinalyses, and biochemical profiles were used to exclude dogs with urolithiasis, calcium oxalate crystalluria, or hypercalcemia. Samples were also collected from 20 dogs of non-stone forming breeds (Table 2). All dogs were client-owned and had no history of antibiotic use 2 months prior to sample collection, which was performed in compliance with an approved University of Minnesota IACUC protocol.

Ileal, cecal, colonic and rectal samples were obtained from nine dogs of non-stone forming breeds following humane euthanasia at an animal shelter (Table 2). Samples were obtained within 60 min of euthanasia.

Determination of *O. formigenes* status by PCR

Fecal DNA was extracted from 200 mg of fresh feces using the QIAmp DNA stool kit according to the manufacturer's instructions (Qiagen, Alameda, CA). Primer3 software (National Human Genome Research) was used to design *O. formigenes*-specific primers, forward 5′ GTGTTGTCG GCATTCCTATC 3′ and reverse 5′ GGGAAGCAGTTGG TGGTT 3′, which generated a 214-bp fragment of the oxalyl CoA decarboxylase (*Oxc*) gene. To calculate *Oxc* gene copy number, plasmid pCR4-TOPO (Invitrogen, Carlsbad, CA) containing a 214-bp fragment of *oxc* was generated by

Table 1 List of stone-forming breeds contributing to the study

Breeds	Age	Gender	Total number[a]
Bichon Frise	7	F	2
Chihuahua	11	M	2
Collie	10	M	2
Fox Terrier Wirehair	11	M	2
Jack Russell Terrier	11	M	2
Miniature Schnauzer	10	F	4
Miniature Schnauzer	4–12	M	10
Papillon	7	M	2
Pekingese	6	F	2
Pomeranian	5	M	2
Pomeranian	6	F	2
Shi Tzu	6	F	2
Yorkshire Terrier	6–12	M	6

[a] Number of dogs was evenly divided in each group in every breed between healthy and with stones

Table 2 List of non-stone forming breeds contributing to the study

Sample type	Breeds	Age	Gender	Total number
1	German Shepherd Cross	7–12	F	2
		2	M	1
	Saint Bernard	5–10	M	2
	English setter	5	F	1
	Golden Retriever	5–7	M	2
	Labrador Retriever	5–10	M	3
	German short hair pointer	2	F	2
	Dalmatian	10	M	1
	Standard Poodle	4	M	1
	Clumber spaniel	3	M	1
	Border Collie	11	M	1
	Greyhound	3–5	M	3
2	Pit Bull	1–6	M	4
		4–6	F	3
	German Shepherd Cross	8	M	1
		6	F	1

1 fecal samples, *2* swabs collected from the intestinal tract

standard cloning methods, confirmed by Sanger sequencing (Biomedical Genomics Center, University of Minnesota), purified and quantified (http://www.uri.edu/research/gsc/resources/cndna.html, Rhode Island Genomics and Sequencing Center, University of Rhode Island). Serial dilutions were used to construct a standard curve.

Quantitative PCR (qPCR) was carried out on ABI 7500 Real time PCR system (Applied Biosystems, Carlsbad, CA) using SYBR Green PCR master mix (Applied Biosystem, Carlsbad, CA) with 100 nM primers. Amplification was carried out with enzyme activation at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 23 s, annealing at 60°C for 20 s and extension at 70°C for 40 s. Melting peak of each sample was analyzed to confirm product specificity (83.4–84.5°C).

Determination of *O. formigenes* status by culture

Oxalobacter formigenes culture medium 175-132 supplemented with 10 mM sodium oxalate was prepared as described [3]. Fecal swab extract was anaerobically inoculated and incubated anaerobically at 37°C for 5–14 days. A pure isolate of *O. formigenes* and cecal swabs from pigs were inoculated as positive controls. Broth cultures were centrifuged at 10,000 rpm for 10 min. Oxalate ion concentration of the supernatant was measured by ion chromatography. Fecal cultures that showed complete oxalate depletion were considered as positive for *O. formigenes* and also confirmed by sequencing the 16S rDNA amplicons (Biomedical Genomics Center, University of Minnesota).

Ion chromatography

Samples were diluted tenfold with de-ionized water and analyzed on a Dionex ICS-2000 ion chromatography system consisting of an AS19 analytical column, ASRS 300 suppressor, AS40 autosampler, and integrated dual piston pump and conductivity detector. The eluant was generated by Reagent Free eluant generator system (Dionex, Sunnyvale, CA), which produced a variable concentration KOH eluant, regulated by Chromeleon control software. The control program used a comprehensive anion elution scheme.

Data analysis

Groups were compared by non-parametric Mann–Whitney tests using GraphPad Prism 4.0 (GraphPad Software, Inc. La Jolla, CA) and the crude odds ratio (OR) was calculated by MedCalc software. Differences between groups were considered significant at $p < 0.05$.

Results

Prevalence of *O. formigenes* in dogs

Presence of *O. formigenes* in feces was determined by qPCR amplification of *oxc*. An overall *O. formigenes* prevalence of 50% was observed regardless of CaOx status, and *O. formigenes* abundance was low, in the range of 10^1–10^4 organisms per gram of feces (Fig. 1; Table 3). Ideally, presence of viable *O. formigenes* in oxalate-supplemented fecal culture would result in 100% depletion of oxalate as observed in the pure isolate of *O. formigenes* and in pig cecal culture. However, only two fecal cultures showed 100% depletion of oxalate (Fig. 2).

To determine if *O. formigenes* was more abundant in situ, samples were collected from ileum, cecum, colon and rectum of additional dogs and oxalate degrading activity

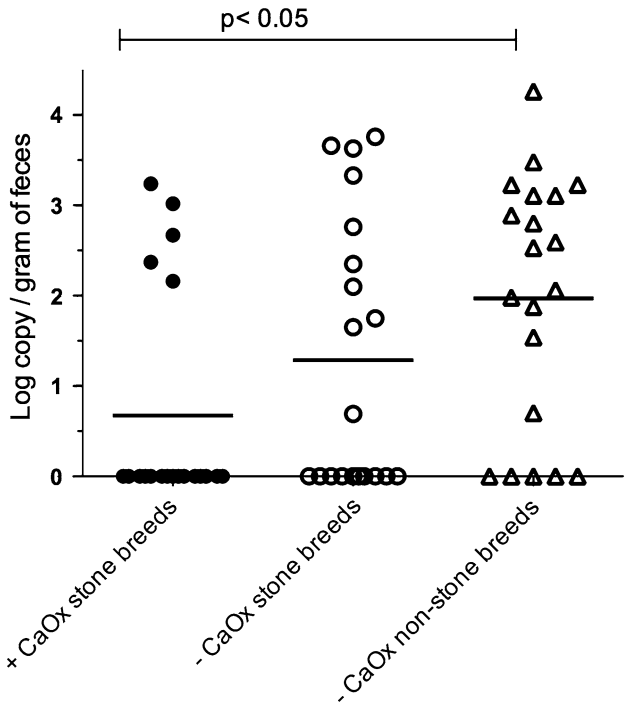

Fig. 1 Fecal qPCR-based screening for *O. formigenes*. The *oxc* gene of *O. formigenes* was quantified in CaOx stone dogs (*filled circle* $n = 20$), breed-matched negative controls (*open circle* $n = 20$) and negative non-stone forming breeds (*open triangle* $n = 20$). *Horizontal line* is the mean

Table 3 Prevalence of *O. formigenes* determined by *oxc* gene PCR

O. formigenes status	CaOx stone-formed dog % (*n*)	Breed-matched control % (*n*)	Non-stone forming breed % (*n*)
Positive	25 (5)	50 (10)	75 (15)
Negative	75 (15)	50 (10)	25 (5)

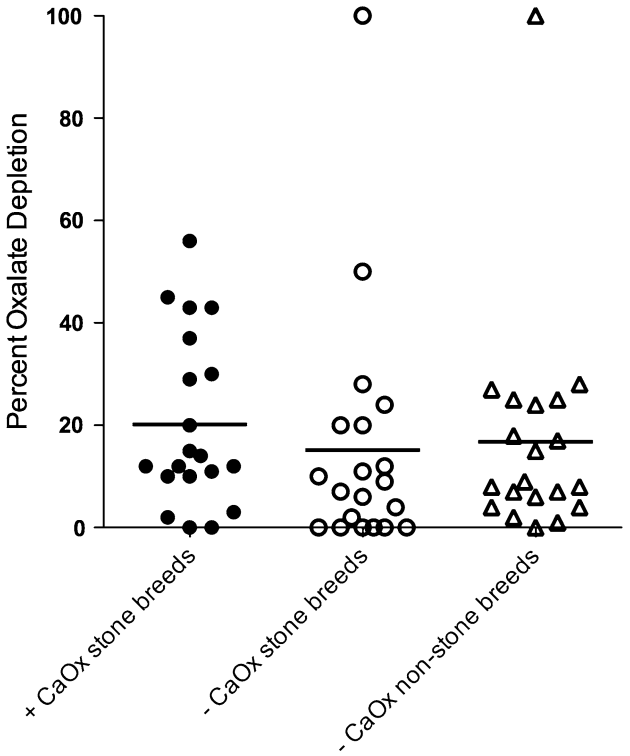

Fig. 2 Oxalate degrading activity in fecal cultures. Fecal samples collected from CaOx stone dogs (*filled circle* $n = 20$), matched-control dogs (*open circle* $n = 20$) and dogs belonging to non-stone forming breeds (*open triangle* $n = 20$) were cultured in oxalate-supplemented anaerobic medium for 14 days and oxalate concentrations were analyzed by ion chromatography. *Horizontal line* is the mean

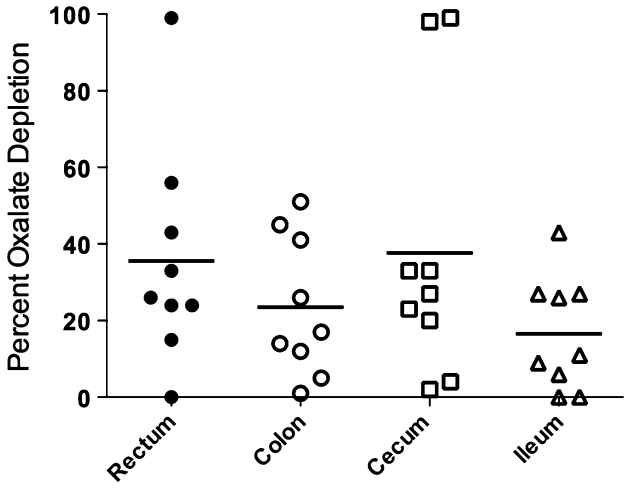

Fig. 3 Oxalate degrading activity in the intestinal tract. Gastrointestinal tract contents were collected from euthanized, healthy dogs ($n = 9$), from the following regions: rectum (*filled circle*), colon (*open circle*), cecum (*open square*) and ileum (*open triangle*). The contents were inoculated into oxalate-supplemented anaerobic medium to determine oxalate depletion. Cultures from all four regions showed comparable levels of oxalate depletion, with highest oxalate degrading cultures from cecal and rectal regions. *Horizontal line* is the mean

was measured. Two out of nine dogs showed 100% oxalate depletion in cecal swabs. One of the two animals also showed 100% oxalate depletion in the rectal swab sample (Fig. 3). Quantitative PCR showed an elevated copy number of *O. formigenes Oxc* gene in the 5 cultures that showed 100% oxalate depletion. Presence of *O. formigenes* in the oxalate-depleted cultures was confirmed by 16S rDNA sequencing. However, pure colonies of *O. formigenes* were not isolated from the positive cultures by roll tube culture despite numerous attempts. Our data show that *O. formigenes* is present in the intestinal tract of the dogs. It appears that its culture requirements are more stringent than for porcine *O. formigenes*, which was readily culturable using identical conditions.

Prevalence of *O. formigenes* and incidence of CaOx uroliths

Prevalence of *O. formigenes* in dogs with CaOx uroliths was 25% ($n = 20$), whereas the prevalence in breed-, age- and gender-matched clinically healthy dogs was 50% ($n = 20$) (Fig. 1; Table 3). In clinically healthy, non-stone forming large breeds, prevalence of *O. formigenes* was 75% ($n = 20$). The prevalence is significantly higher in non-stone forming healthy large breeds compared to dogs with CaOx uroliths ($p < 0.05$). Although the prevalence of *O. formigenes* was numerically higher in healthy matched-control animals compared to the CaOx dogs, the difference was not statistically significant (Fig. 1).

The crude OR between CaOx and healthy matched-control dogs was 3.0 (95% confidence interval: 0.8–11.05). The crude OR between the CaOx dogs and the non-stone forming large breeds was 9.0 (95% confidence interval: 2.21–36.59). Based on the crude OR, absence of *O. formigenes* enteric colonization increases substantially the risk of CaOx stone formation.

Quantitative PCR results also showed that, among the *O. formigenes*-positive samples, the mean copy numbers of *oxc* genes were equivalent in all groups, ranging from $10^{2.9} \pm 10^{2.5}$ to $10^{3.5} \pm 10^{3.1}$ copies per gram of feces. There was no significant difference in the oxalate degrading activity between the healthy, matched controls and urolith dogs (Fig. 2). Complete oxalate depletion was only achieved in two fecal samples; one from a matched control and one from a non-stone forming large breed. Extensive variation was observed in the oxalate depletion among the fecal cultures, which may indicate the presence of other oxalate-metabolizing bacteria.

Discussion

We report here the first association between gut *O. formigenes* colonization and CaOx urolith formation in dogs. Specifically, *O. formigenes* is positively associated with healthy dogs, especially in non-stone forming breeds. The differences in the prevalence of *O. formigenes* were significant between CaOx dogs and non-stone forming breed dogs. These findings indicate that absence of gut colonization with *O. formigenes* is a risk factor for CaOx urolithiasis. The overall *oxc* copy numbers were similar and relatively low among all three groups of animals, suggesting a lack of quantitative relationship between the bacterial load and CaOx urolith development.

In humans and other mammals, presence of *O. formigenes* is assessed by both fecal culture and non-culture-based methods. Prevalence of fecal *O. formigenes* in healthy humans is 38% but only 17% of subjects afflicted with CaOx uroliths have *O. formigenes* [14]. Although *O. formigenes* mainly colonizes the cecum and colon, fecal samples are considered the best non-invasive clinical sample in live animals. However, a selective media for *O. formigenes* has not been described. Therefore, presence of viable *O. formigenes* is generally detected by the depletion of oxalate ions in an oxalate-supplemented culture medium. Based on culture methods, *O. formigenes* in humans is present in a range between 3×10^5 and 3×10^8 CFU/g of feces, suggesting that 10^5 bacteria per gram is the lower detection limit [2]. Detection of *oxc* gene is an indirect way of detecting *O. formigenes* in fecal samples that avoids a stringent anaerobic growth requirement [24, 26, 29, 32]. *Oxc* is also present in other oxalate-degrading bacteria, but sequence variations among bacterial genera allowed amplification specifically from *O. formigenes*. The majority of humans who harbor *O. formigenes* had fecal colonization of less than 4×10^4 CFU/g [24].

Our results show similar levels in positive dogs by PCR and demonstrated the reduced sensitivity of culture, as only 2 of 60 were positive. Intra-fecal variation is observed in shedding of *O. formigenes* in humans and dogs [24, 33]. However, our finding of a 50% prevalence is intermediate between the 37 and 89% rates previously reported in dogs [32, 33]. Therefore, we conclude PCR is substantially more sensitive than culture, possibly due to either lack of viable fecal *O. formigenes* or very low levels of the bacterium.

Feces may not be an ideal sample for culture of *O. formigenes*, a strict anaerobe. Therefore, *O. formigenes* culture also was attempted from various areas of the GI tract. In cecal cultures, 22% (2 of 9) of dogs were culture positive for *O. formigenes*. One of the dogs also yielded a positive fecal culture. The findings indicate that cecum, where anaerobic conditions predominate, is a better site from which to obtain viable *O. formigenes*.

The potential use of oxalate-degrading bacteria, particularly *O. formigenes*, as novel therapeutics against CaOx urolithiasis has been explored in humans and laboratory rodents with encouraging outcomes [8–10, 12, 13, 25]. In dogs, such novel therapeutics for CaOx urolithiasis have not been explored, largely due to the lack of preliminary screening studies on gut microflora or oxalate-catabolizing bacteria. The potential variability among numerous dog breeds also might have contributed to the lack of progress in oxalate-metabolizing bacteria studies. Here, we investigated the risk of calcium oxalate stone urolithiasis in the absence of *O. formigenes* in the intestinal tract by including dogs with CaOx urolithiasis, matched controls and non-stone forming large breeds. Interestingly, the proportion of dogs positive for fecal *O. formigenes* was significantly higher in non-stone forming large breeds compared to the CaOx urolithiasis dogs. It is reasonable that altered oxalate-metabolizing microbes such as *O. formigenes* provide a mechanism to prevent CaOx stones. While many risk factors, including environment, diet, genetic and metabolic disorders, can contribute to the pathogenesis of CaOx urolithiasis, oxalate-metabolizing microbes should be considered as a factor amenable to therapeutic or dietary manipulation. In this study, dogs had no antibiotics 2 months prior to the sample collection. Recent epidemiological studies on humans claim that the *O. formigenes* prevalence is high in individuals who never had antibiotics and therefore future risk factor analysis on studies should include information on usage of antibiotics [15]. Apart from *O. formigenes*, other oxalate-degrading bacteria including *Bifidobacterium* spp. *and Lactobacillus* spp. in the intestinal tract also can prevent oxalate absorption [1]. Future studies need to be focused on global genomic survey approaches to detect oxalate-metabolizing microbial populations in healthy dogs and CaOx stone-formed dogs.

Although the present study sheds light on the prevalence of *O. formigenes* in CaOx stone-formed dogs, there are some limitations to the pathophysiological interpretation of the findings. Urinary oxalate concentrations were not known and the oxalate content of the diets was not available due to the use of client-owned animals. In humans, oxalate hyperabsorbers are colonized with *O. formigenes,* but an oxalate hyperabsorbing phenotype has not been described in dogs [16]. Therefore, the biological implications of oxalate-metabolizing bacteria in hyperoxaluric phenotypes cannot be assessed. The results here will help to elucidate the association between dietary oxalate, urinary oxalate and oxalate-metabolizing bacteria, and the etiopathogenic impact of CaOx urolithiasis in dogs, cats and human.

In conclusion, elevated fecal prevalence of *O. formigenes* in healthy stone forming and non-stone forming dogs supports a potential preventive role for this oxalate-metabolizing bacterium in CaOx urolithiasis. The findings

provide a strong rationale to pioneer novel and non-invasive therapeutic approaches to minimize CaOx urolithiasis in dogs by exploiting oxalate-metabolizing bacteria.

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