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ORIGINAL ARTICLE

Biomass colonization and bioconversion of the molecular characterized *Oxalobacter formigenes* to mitigate calcium oxlate urolithiasis

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Abstract
Calcium oxalate (CaOx) is one of the common causes of kidney stones and accounts for 40 to 50% of all uroliths in cats. *Oxalobacter formigenes*, an oxalate-degrading intestinal microbiota, has been hypothesized to play a protective role against CaOx urolithiasis due to its capability to degrade oxalate. This study was designed to reveal the association between biomass colonization of *O. formigenes* and clinical occurrence of CaOx urolithiasis in household tomcats. Fifteen tomcats were allocated into three groups (healthy control ($n=5$), static chronic kidney disease (static CKD) ($n=4$), and progressive CKD ($n=6$)) based on diagnosis of CaOx urolithiasis and disease progression. Fecal samples were collected from all tomcats, genomic DNA was extracted, and *oxc*, a gene specific for *O. formigenes*, was quantified using real-time PCR. Additionally, the clinical association between blood serum urea, creatinine, and relative abundance of *oxc* gene among different groups was examined. The *oxc* gene was detected in all cats in various frequency; however, its relative abundance was significantly higher in progressive CKD group compared to static CKD and control groups. In summary, our results suggest a protective role of O. *formigenes* against calcium oxalate urolithiasis only in static CKD. Further studies are required in a larger group of cats to help illustrate the protective role of *O. formigenes* in the pathophysiology of calcium oxalate urolithiasis in cats.

Keywords Biomass · *Oxalobacter formigenes* · Calcium oxalate · Urolithiasis · *Oxc* gene

1 Introduction

Feline lower urinary tract disease is a general term incorporating many different disorders affecting lower urinary tract in cats [1]. Urolithiasis is considered one of the most common diseases of lower urinary tract affections in domestic cats [2]. Feline urolithiasis is a multifactorial condition that includes congenital, familial, and/or acquired pathophysiologic factors. These factors together favor the risk of precipitation of excretory solutes within the urine on an organic matrix to form stones [3]. Additionally, it could be potentially life-threatening and may lead to death from azotemia due to urethral or bilateral ureteral obstruction [4].

Feline urolithiasis is classified based on their mineral composition into calcium oxalate, struvite, urate, cystine, xanthine, silica, and calcium phosphate uroliths. Calcium oxalate (CaOx) urolithiasis accounts 40 to 50% of all uroliths in cats [5]. There are many risk factors for its development such as increased urinary calcium and/or oxalate excretion. Age, sex, and genetic background also have a significant influence on its occurrence. Aged cats of 7–10 years are more susceptible especially Burmese, Himalayan, and Persian cats [6]. Tomcats are more commonly affected than queens and 95% of those were being castrated [7].

The alterations in urinary oxalate levels have a higher effect on the possibility of CaOx formation than the changes in urinary calcium levels [8]. Urinary oxalate originates from two sources; absorbed dietary oxalate and endogenous

Highlights *Oxalobacter formigenes* is a well-characterized oxalate metabolizing bacterium.
Oxalobacter formigenes play a vital role in reducing the exogenous urinary oxalates.
Oxalobacter formigenes has a protective role against CaOx urolithiasis in tomcats.
The oxc gene was detected in higher abundance in progressive CKD in tomcats.

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synthesized in the liver from ascorbate and glyoxylate [9]. The gut lumen is the primary excretory system for degradation of excessive exogenous oxalates. Oxalate metabolizing bacterial species (OMBS) are one of intestinal microbiotas of human, dogs, cats, and rats. Those bacterial species are known to reduce the contribution of exogenous urinary oxalates to the total urinary oxalates [10]. Mammals depends mainly on gut microbiota for oxalate homeostasis, due to their deficiency of specific enzymes needed for oxalates degradation [11, 12].

Oxalobacter formigenes (*O. formigenes*) is a gram-negative anaerobe and a well-characterized oxalotrophic OMBS [12]. It consumes the oxalate as its sole carbon source and breaks it down into formate and CO2 [13]. *Oxalobacter* has a direct effect with colonic epithelium to prompt enteric oxalate elimination. In a mouse model fed on *O. formigenes* with knockout of genes responsible for endogenous oxalate metabolism resulted in reduction in the degree of hyperoxaluria and renal injury due to high oxalate level [14]. It is hypothesized that intestinal colonization with *O. formigenes* decreases the risk of calcium oxalate nephrolithiasis [12, 15]. Several studies on human founded that a lower abundance of *O. formigenes* in adults with stone compared with healthy adult or patients with recent nephrolithiasis [16]. This hypothesis was confirmed through hyperoxaluria reduction after administration of *O. formigenes* [14]. Moreover, the risk of recurrent stone formation is reduced with intestinal colonization of *O. formigenes* in human and dogs. Similarly, *O. formigenes* was detected in feces of healthy cats [17]. Although the contribution of *O. formigenes* in the pathogenesis of CaOx nephrolithiasis has been hypothesized, there is limited published information about the abundance of *O. formigenes* in the gut, its colonization, and its clinical occurrence of urolithiasis in cat. Therefore, the objective of this study was to reveal the association between biomass colonization of *O. formigenes* and clinical occurrence of CaOx urolithiasis in household tomcats.

2 Materials and methods

2.1 Animals and sample collection

Fifteen household mixed breed (Himalayans and Persian) tomcats were included in this study. Five tomcats with an average age (6.6 ± 1.14) years were assigned as a control group (control, $n = 5$). All tomcats in control group were clinically healthy based on complete physical examination, blood serum biochemical profile, ultrasonographic, and x-ray investigations. The other ten tomcats were diagnosed to have urethral blockage by CaOx urolithiasis. The diagnosis of urethral obstruction in diseased tomcats was based on similar criteria as control group in addition to surgical operation that performed to remove the stone. The diseased tomcats were divided according to the international renal interest society criteria [18] into two groups static CKD, $n = 4$, with an average age (5.37 ± 0.75 year) and progressive CKD, $n = 6$, with an average age (6.25 ± 0.98 year). The classification of CKD was based on measurement of blood serum creatinine concentration and appearance of clinical signs of azotemia. All tomcats in all groups had no history of antimicrobial treatment for 60 days prior to sample collection.

2.2 Clinical, radiographic, and ultrasonographic examination

All cats were clinically examined by measuring body temperature, respiratory, pulse rates, and palpation of superficial lymph node, kidneys, and urinary bladder [19, 20]. Radiographic examination was performed to characterize the uroliths (e.g., location, size, number, shape, and density) using Ficsher imaging system RMX-625R with maximum capacity of 150 kVp and 1250 mAs (Fischer Imaging Company, USA) [2]. To image the urinary bladder, a voltage of 85–109 kVp and 50 mAs was used to scan the posterior portion of the body [21]. Ultrasonographic examinations were performed using Mindray Z5 ultrasound machine (China) with 2-D mode to examine the distended abdomen, kidneys, and urinary bladder for the presence of stone and/or gravels [22]. To optimize ultrasonographic examination quality, the skin was washed with alcohol, and ultrasonographic gel was applied. All ultrasonographic evaluations were conducted in dorsal recumbency position with a longitudinal and transverse plane for easily distinguishing the mineralized material from fecal material [23].

2.3 Sample collection and analysis

2.3.1 Blood sampling and serum biochemical analysis

Blood samples were collected in a plain labelled test from all tomcats via cephalic vein puncture. Blood was left to clot at room temperature for 20 min and clear, non-hemolyzed serum was collected after centrifugation of clotted blood sample at 3000 rpm for 10 min. All serum samples were stored at − 20 °C until further analysis. Blood urea nitrogen (BUN) (mg/dl) and serum creatinine (mg/dl) were assessed calorimetrically using commercial kits (Urea Biosystem kit, Mannheim, Germany) and (Creatinine Biosystem kit, Mannheim, Germany), respectively.

2.3.2 Fecal samples

Oxalobacter formigenes colonizes the intestine of healthy cats as apart of intestinal microbiota. Thus, fresh fecal

sample was collected for molecular detection of *oxc* gene of *oxalobacter formigenes* [24] from each tomcat in static and progressive CKD groups at the time of urolith removal. Since it is an oxalotrophic bacterium, it has important role in the functioning ecosystem, especially carbon and calcium cycles. It consumes oxalate and produces formic acid and carbon dioxide. Therefore, its colonization hypothesized to decrease the CaOx crystals in urine and the CaOx stone consequently [25]. Additionally, fecal samples were collected from control group at the same time of sampling diseased cats. Samples were placed in a separate sterile labelled container, transferred in an icebox at 4 °C to the laboratory, and then stored in (− 20) until further processing [26].

2.3.3 Genomic DNA extraction and molecular relative quantification (qPCR) of Oxalobacter formigenes

Fecal DNA was extracted from all fecal samples using the QIAamp® Fast DNA Stool Mini kit (QIAGEN Inc., Carlsbad, CA) following the manufacturer's instructions [27]. The concentration of eluted DNA as well as purity was measured using Nanodrop-1000, (Thermo Scientific, USA) and then stored in − 20 °C until examined by PCR. DNA concentration was normalized to 100 ng/µl and used as template for amplification bacterial gene. The oligonucleotide primer of *oxc-gene* was used as a proxy for detection of *O. formigenes* in fecal samples which are shown in Table 1. Real-time PCR for the detection of oxc gene was performed, using Biorad (PTC 1148) with RealMOD™ GH SYBR Green Real-time PCR Master Mix (2x) (iNTRON Biotechnology — Korea) as previously described [28].

2.4 Statistical analysis

Statistical analyses were performed using SPSS version 23.0 (IBM, New York, NY, USA). The data of blood serum urea, creatinine, and relative abundance of *oxc* gene were compared among different groups (control, static, and progressive CKD) using multivariant ANOVA and Tukey–Kramer HSD test for multiple comparison. The values are presented as the mean ± standard deviation (SD). The correlation between blood serum urea, creatinine concentration, and relative abundance OF *oxc* gene among different groups (control, static, and progressive CKD) was examined using Pearson correlation coefficient [29]. The significance level for all analysis was set at 0.05.

3 Results

3.1 Clinical, radiographic, and ultrasonographic examination of diseased cat

Clinical examination of diseased cats revealed similar finding in both static and progressive CKD groups with marked evident of lethargy, continues meowing, frequent visiting of litter box, and urine drippling. Palpation of abdomen revealed tensed urinary bladder and relatively enlarged kidneys. Ultrasonographic examination of distended abdomen in diseased cats revealed mild to severe badly distended urinary bladder and hyperechoic elements dispersed within the anechoic urine (Fig. 1A). The ultrasound waves shadowed a shadow artifact from the bladder wall after being reflected by a hyperechoic structure in some cases. Radiographic examination of blocked tomcats revealed the presence of urolith engaged in the way of the urethra as a small radiopaque structure (Fig. 1B).

3.2 Serum biochemical findings and molecular identification of oxc-gene

Blood serum urea concentration was significantly higher in progressive CKD group and static CKD compared to control groups (Fig. 2A, $P < 0.0001$). No significant differences ($P = 0.124$) were noted in blood serum urea concentration in static CKD group compared to progressive CKD (Fig. 2A, $P = 0.369$). Similarly, blood serum creatinine concentration was significantly higher in progressive CKD group and static CKD compared to control group at (Fig. 2B, $P < 0.0001$). No significant differences were noted in blood serum creatinine in static CKD group compared to progressive CKD group (Fig. 2B, $P = 0.252$).

The *oxc* gene was detected in all tomcats in various frequencies. The relative abundance of oxc-gene in static CKD showed no significant change compared to control group (Fig. 2C, $P = 0.631$). In contrast, the relative abundance of oxc-gene is significantly increased in progressive

Table 1 Oligonucleotide primers for *oxc*-gene of targeted *O. formigenes*

Gene		Primer sequences (5–3)	Annealing temperature	Product (bp)
oxc gene	F	CGACAATGTAGAGTT-GAC TGA (GC content 42.8% and melting temperature 59.5C)	57 °C	164 bp
	R	CGTGTTGTTCGTGACGAA (GC content 52.6% and melting temperature 60.5C)		

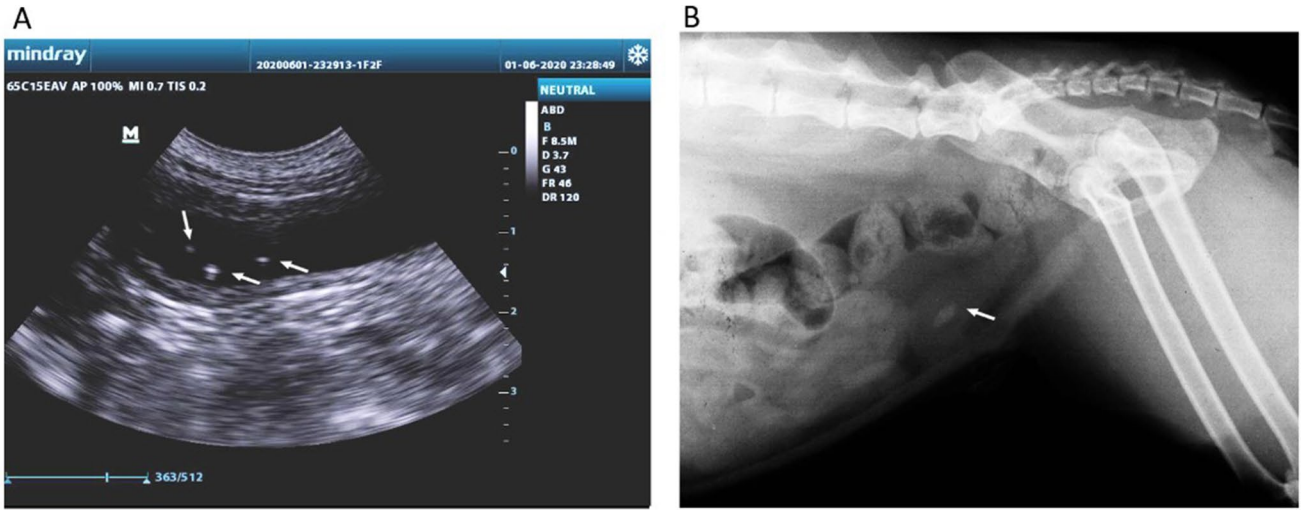

Fig. 1 **A** Ultrasonographic examination of the urinary bladder of blocked tomcat showing distended urinary bladder with multiple gravels which confirmed to be calcium oxalate by urine analysis. **B** Radiographic image of the urinary bladder of blocked tomcat showing distended urinary bladder with multiple small stone (arrow) in urethra which confirmed to be calcium oxalate by urine analysis

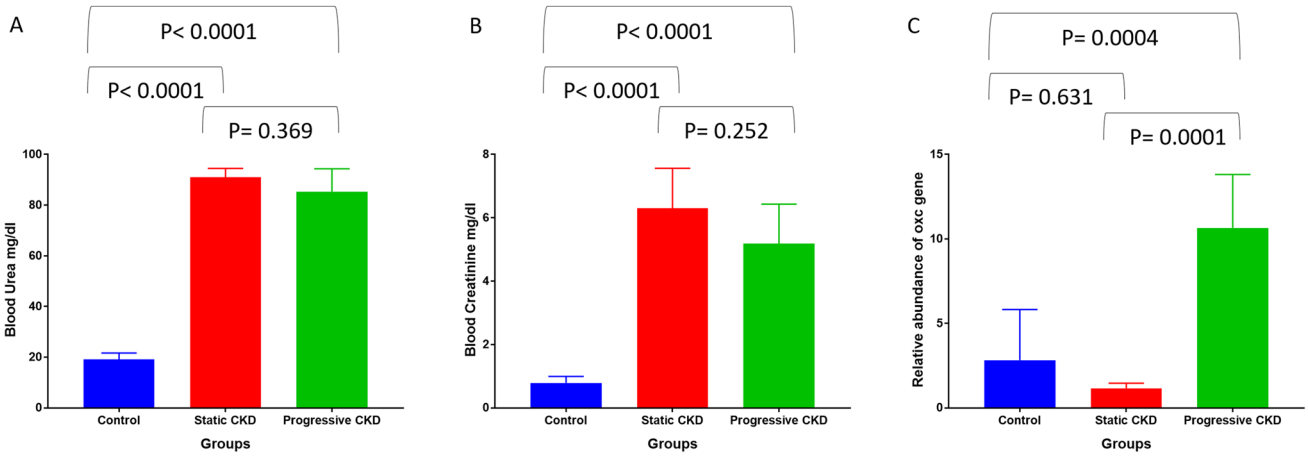

Fig. 2 The result of multivariant ANOVA for serum creatinine (**A**), urea concentration (**B**), and relative abundance of oxc gene measured by q-PCR (**C**) among control, cases with urolithiasis (static and the progressive CKD group)

CKD compared to the control and static CKD groups (Fig. 2C, $P = 0.0004$ and 0.0001), respectively.

To further evaluate the clinical association between colonization of *O. formigenes* and clinical representation of CaOx urolithiasis in household tomcats, the correlation between blood serum urea, creatinine concentration, and relative abundance OF *oxc* gene among different groups (control, static, and progressive CKD) was analyzed using Pearson correlation coefficient (Fig. 3). The correlation analysis between the investigated molecular and biochemical biomarkers revealed a positive correlation between blood serum urea, creatinine concentration, and relative abundance of oxc gene (Fig. 3A and B).

4 Discussion

Urolithiasis in domestic cats is considered one of the most common diseases of lower urinary tract disease in cats [30]. Despite the widespread dietary management and using urolith-preventive diets, CaOx uroliths become a primary etiological stone in domestic cats in the last decades [31, 32]. In this study, we focused on one of the oxalotrophic bacterial that might help protection against CaOx stones. All included tomcats were considered a high risk for development of stone [33]. Additionally all included cats were tomcats which are more commonly affected with

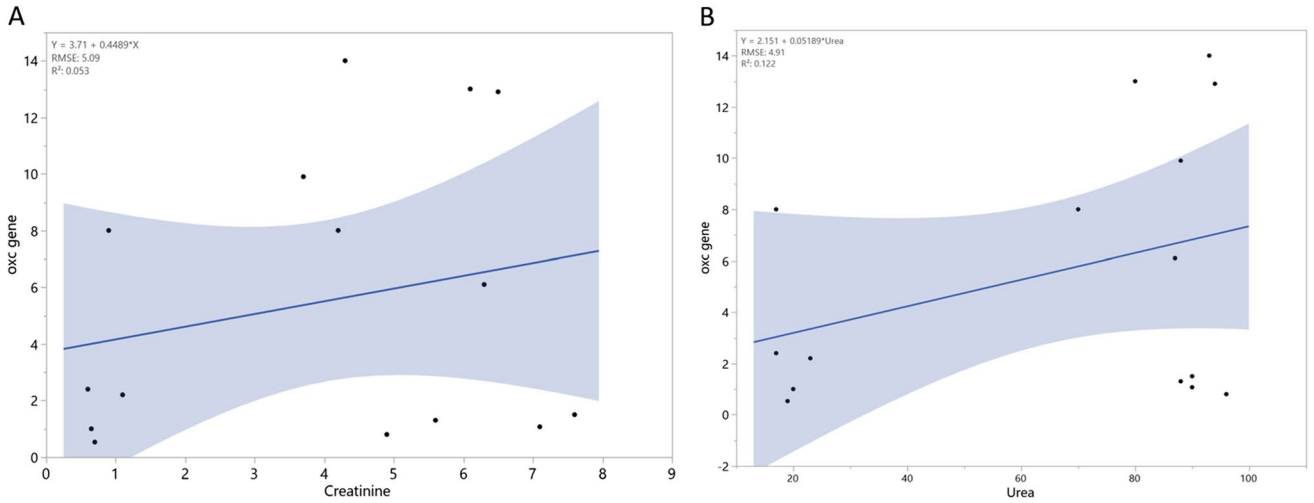

Fig. 3 **A** Correlation analysis between serum urea concentration and relative abundance of oxc gene of *O. formigenes* in feces. **B** Correlation analysis between serum creatinine concentration in and relative abundance of oxc gene of *O. formigenes* in feces

urethral obstruction compared to queens due to their long and narrow urethra [7].

Similar to previous study, the clinical signs of the diseased tomcats were typical urethral blockage presentation [34]. The ultrasonographic examination confirmed that the distended abdomen was due to distended urinary bladder. The diagnosed lethargy may be attributed to electrolyte disturbance caused by impaired kidney function as confirmed by the biochemical analysis of serum urea and creatinine [35]. The ultrasonographic examination revealed many hyperechoic elements dispersed within the anechoic urine and shadow artifact due to accumulation of stone in the distended bladder. Additionally, our diagnosis was confirmed by radiographic imaging of the caudal part of the abdomen that revealed a small radiopaque stone engaged within the penile urethra in the diseased tomcats.

In the current study, the affected cats with urolithiasis were divided into static CKD and progressive CKD groups according to the IRIS staging for CKD (2013) that depend on evaluation of serum biochemical biomarkers such as urea and creatinine [36]. In this study, blood urea and creatinine concentration were significantly higher among progressive CKD and static CKD compared to control group ($P < 0.0001$) and this attributed to the impaired kidney function and confirms the kidney damage in diseased groups [34, 37].

The intestinal tract is a diverse and complex ecosystem [27]. Gut microbiota and its ecological and functional characteristics are thought to be useful to the host health [38]. Therefore, estimating the different factors that modulate the gut microbial composition is very important in determining which strategies could be used to maintain the host health. The main objective of this study was to reveal the association between biomass colonization of *O. formigenes* and clinical occurrence of CaOx urolithiasis in household tomcats. Real-time PCR assay was used to determine the relative abundance of oxc gene which is the proxy for molecular identification of *O. formigenes* and CaOx urolithiasis clinical cases [11, 17].

The relative abundance of oxc gene was not significantly higher in control group compared to static CKD. This result supports the suggested hypothesis that *O. formigenes* presence in the intestinal tract may be act as a protecting factor against calcium oxalate urolithiasis [17]. The high rate of *O. formigenes* has the capability to degrade oxalate and decrease the oxalate absorption, and therefore, decreasing the risk of calcium oxalate urolithiasis. In contrast, the relative abundance of oxc-gene is significantly increased in progressive CKD compared to the control and static CKD groups. The higher relative abundance of *O. formigenes* within the progressive CKD group may be explained by the role of gut–kidney axis which play an important role in modulation of gut microbiota and signaling the increase of *O. formigenes* abundance to metabolize the increased oxalate in progressive CKD [16].

While the findings of this study were important, the study limitations should be considered. This study was conducted on a small number of cats in each group, though it is considered the first studies in diseased tomcats. Therefore, further studies are required in a larger group of cats to help determine the protective role of *O. formigenes* in the pathophysiology of feline calcium oxalate urolithiasis before definitive conclusions can be made. Moreover, evaluation the relative abundance of *O. formigenes* using microbiome analysis was beyond the scope of this study. Despite these study limitations, the results of this study provide important

information that could be of great importance to feline urinary tract health.

5 Conclusion

This is the first study to describe the clinical association between colonization of *O. formigenes* and clinical representation of CaOx urolithiasis in household tomcats. In this study, we observed a significantly higher relative abundance of *O. formigenes* within the progressive CKD group compared to control and static CKD groups, suggesting a protective role of *O. formigenes* against calcium oxalate urolithiasis only in static CKD. Therefore, we recommend future studies to be carried out on larger number of clinical cases in longer time to strengthen the reliability of results.

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Author contribution MD, MZ, NG, MN, YH, RS, and MA conceived and designed the experiment; MD, MA, and MZ conducted the experiment and data analysis; MD, MZ, NG, MN, YH, RS, JCM, RS, MA, and RR prepared the manuscript. All authors approved of the manuscript.

Availability of data and material Not applicable.

Code availability Not applicable.

Declarations

Ethics approval Animal studies have been approved by ethical committee. The research was performed in accordance with the ethical standard laid down in the 1996 declaration of Helsinki and its later amendments.

Consent to participate All authors agree to participate in the current work.

Consent to for publication All authors agree to publish the findings of current research.

Conflict of interest The authors declare no competing interests.

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