

# Development of an Innovative Method by Optimizing qPCR Technique for Isolating and Determining *Oxalobacter Formigenes* Microbial Load in the Stool of Patients with Urolithiasis

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**Keywords.** *Oxalobacter formigenes*, calcium oxalate urolithiasis, oxalyl CoA decarboxylase, formyl-CoA transferase, quantitative real-time PCR

**Introduction.** *Oxalobacter formigenes*, as a gram-negative anaerobic bacterium, metabolizes oxalate in the intestine by the enzymes oxalyl-CoA decarboxylase (OXC) and formyl-CoA transferase (FRC). Therefore, not only the presence of the bacterium but also microbial load may affect intestinal absorption and urinary exertion. We evaluated the relationship between *Oxalobacter formigenes* load and the formation of calcium oxalate urolithiasis using quantitative molecular methods.

**Methods.** By clinical manifestation and stone analysis, we selected the urine and stool specimens of 73 patients with calcium oxalate urolithiasis. First, the gene regions of the two genes FRC and OXC in *Oxalobacter formigenes* were selected utilizing bioinformatics and specific primers designed for these regions. Following DNA extraction from stool specimens by specific primers of each gene, PCR was carried out and positive samples were sequenced. Then, qPCR was applied to determine the effective load of *Oxalobacter*. Also, biochemical tests were performed to measure the excretion rate of oxalate in urine specimens.

**Results.** In addition to *oxalobacter* identification by PCR, the load of bacteria was quantitatively assessed using qPCR by specific primers for both FRC and OXC gene regions. A significant negative relationship had found between the formation of calcium oxalate urolithiasis and the presence of *Oxalobacter formigenes* in patients with kidney stone disease. The mean excretion of oxalate and citrate in urolithiasis cases were 22.93 and 552.106 mg/24h, respectively. **Conclusion.** The presence of *Oxalobacter formigenes* can highly inhibit the generation of calcium oxalate urolithiasis. Furthermore, molecular techniques are more effective than other methods such as culture for the isolation of this bacterium.

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## INTRODUCTION

Urolithiasis is a common obstructive urinary disorder, and calcium oxalate crystal is the major constituent. Urolithiasis in childhood and adolescence has been increasingly diagnosed, in general population and it is important to evaluate

the etiology.<sup>1-3</sup> The reason for such an increase is not clear, but is associated with some factors. Children with urolithiasis have a wide range of metabolic disorders which should be carefully considered.<sup>4</sup> The urolithiasis is a common, strictly painful, and costly disease with a high relapse

probability.<sup>5-8</sup> The chance of occurrence during the life of a person has been reported to be 1 to 20% around the world.<sup>1-7</sup> Therefore, prevention of relapse is one of the largest challenges for modern urology.<sup>1,9-11</sup> Development of stone in a patient depends on certain physicochemical factors.<sup>7,8</sup> Suspected etiologies are anatomic abnormalities, urinary tract infection (UTI), metabolic disturbances, lifestyle, diet, lack of drinking adequate amounts of fluids and usage of some drugs.<sup>10,12-14</sup> Despite oxalate endogenous generation in the liver as a part of natural metabolism and absorption through oxalate-containing food in the intestine and potential detoxification, mammals do not produce any enzyme for the metabolism of this compound. However, they are dependent on some intestinal bacteria for oxalate absorption that reduce urinary excretion.<sup>15,16</sup> *Oxalobacter formigenes* (*O. formigenes*) is an anaerobic gram-negative bacterium in the intestine of human and other mammals. This bacteria uniquely requires oxalate as the source of energy and carbon.<sup>15,16</sup> It is believed that *O. formigenes* destructs oxalate and prevents its absorption to the circulation by formyl-CoA transferase (FRC) that converts oxalate to active oxalyl-CoA and oxalyl-CoA decarboxylase (OXC) that decarboxylates oxalyl-CoA converting it to formyl-CoA.<sup>15</sup> A mechanism was suggested through which *O. formigenes* stimulates the secretion of oxalate from circulation to intestine.<sup>16</sup> The previous findings showed that both the presence and relative frequency of *O. formigenes* in the intestine may affect the risk of calcium stone formation.<sup>17</sup> Nonetheless, *O. formigenes* presence was highly correlated with decreased urinary oxalate in 55% of the investigations. In a clinical trial recently performed on primary hyperoxaluria patients, *O. formigenes* prescribed as a probiotic for eight weeks did not diminish oxalate urinary excretion.<sup>18</sup> The diagnosis and count of unculturable and fastidious intestinal bacteria are challenging. Although the metagenomics analysis of a new method is under development, its weakness in diagnosis and quantitation of organisms carrying specific genes has led to the consideration of target-based molecular techniques as suitable substitute methods.<sup>19</sup> Applying molecular techniques, such as PCR, real time-PCR, and southern blot with specific DNA segments can improve the sensitivity and specificity of identifying this bacterium

and substitute the previously used methods. Determination of *Oxalobacter* load allows the evaluation of the relationship between bacterial presence and the frequency of calcium oxalate urolithiasis formation. Consequently, we attempted to set up a new method for the first time using the qPCR technique for isolating and determining the load of *Oxalobacter*.

## MATERIALS AND METHODS

### Study Design and Ethical Considerations

The study was performed on the urine and stool specimens of 73 patients with calcium oxalate urolithiasis by clinical manifestation and stone analysis. After obtaining informed consent, an urologist examined the patients, and the samples were taken following the confirmation of diagnosis and completion of the information sheets. This study was approved by the Ethics Committee of Qom University of Medical Sciences, Qom, Iran. The specimens transferred to the Research Center of Qom University of Medical Sciences for molecular and biochemical tests.

### Bioinformatics Analysis and Primer Design

The characteristics of FRC and OXC genes for *O. formigenes* diagnosis were completely detected by diverse soft wares, such as CLC Genomic Workbench 12, Gene Runner, and AlleleID. Then, Blast in the NCBI site was used to assess the specificity of designed primers.

### DNA Extraction and PCR Set Up

*Oxalobacter* DNA was extracted from stool by DNA extraction kit (Favorgen Biotech Corporation, Taiwan) according to the manufacturer instruction. A positive control sample was extracted simultaneously with other specimens and stored at -20°C. The FRC and OXC regions of the extracted DNA from isolates were amplified by two pairs of primers:

FRC.F: 5'CCAGCCTACAACACATTCG3', FRC.R: 5'TCAGAACTTCGTCGGTATGT3' and OXC.F: 5'AATCTAGAGTTGACTGA3', OXC.R: 5'TTGATGCTGTTGATACG3'. PCR was performed using a thermocycler (Applied Biosystems, CA, USA) and 25.12 µL of the reaction mixture (Amplicon, Denmark), 0.8 µL of forward primer (FRC.F or OXC.F), 0.8 µL of reverse primer (FRC.R or OXC.R), 8.15 µL of distilled water, and 3 µL

of DNA. The temperature protocol included a denaturation cycle of 95°C for 5 min, followed by 40 cycles of 94°C for 45 sec, annealing at 56°C for 20 sec, extension at 72°C for 30 sec, and a cycle at 72°C for 5 min. The final product was evaluated by electrophoresis on 1.5% agarose gel using documentation gel (Cambridge, Warwickshire, UK). The purified PCR product of Oxalobacter-positive samples for both FRC and OXC genes were sent to the Bioneer Corporation (Bioneer, Daejeon, South Korea) for sequencing by Sanger method with specific primers.

### qPCR Set Up and Diagnostic Standard Development

In order to set up the qPCR technique, two DNA samples of Oxalobacter FRC and OXC regions sequenced by the Sanger method were used. The amplification of FRC and OXC regions performed by PCR utilizing Step One Plus™ (Applied Biosystem, CA, USA). The reaction solution with the final volume of 12.5 µL consisted of 0.5 µL of each forward and reverse primers (10 pmol/µL), 2.5 µL of reaction mixture HOT FIREPOL® EvaGreen® qPCR Mix Plus at the concentration of 5X (Solis Bio Byne, Estonia), and 2 µL of DNA or PCR product. For PCR, the initial temperature was set at 95°C for 15 minutes for enzyme activation. Denaturation was performed as 40 cycles at 95°C for 30 sec, followed by annealing at 56°C for 30 sec, and extension at 72°C for 40 sec. The purified samples of FRC and OXC genes at high loads were used to determine the limit of detection. Afterwards, the concentration and copy number of DNA molecules were calculated using spectrophotometer and optical density. Next, serial dilutions of DNA were prepared, which was equal

to 1 to 106 copies per mL, and the standard curve was depicted. For the evaluation of the technique, 73 DNA samples extracted from the stool of people with urolithiasis confirmed by the clinical examinations and laboratory tests were prepared and assessed for diagnosis of Oxalobacter.

### Biochemical Analysis

In order to evaluate the excreted oxalate and citrate in urine of patients with calcium oxalate urolithiasis, 24-hour urine samples were collected in 20 mL tubes. The patients did not use vitamin C 24h before the sampling and received their routine diet. The specimens were evaluated by the enzymatic endpoint colorimetric method using OXALATE and CITRATE kits (Biorexfars, Fars, Iran).

### Statistical Analysis

The statistical software MedCalc version 19.0.5 was applied to calculate and compare the sensitivity and the specificity between selected gene regions and applied techniques.

## RESULTS

### General Findings

Age of the participants in this study was 1 to 70 years with a mean of 29.27 years. The mean excretion rate of oxalate and citrate in patients were reported as 22.93 and 552.106 mg/24h, respectively.

### Analysis of FRC and OXC Gene Regions in Primer Design

Characteristics of different genes of *O. formigenes* were completely assessed using recorded sequences in the Gen Bank and bioinformatics tools. The location of designed primers for these two genes of *O. formigenes* is shown in figures 1 and 2. The

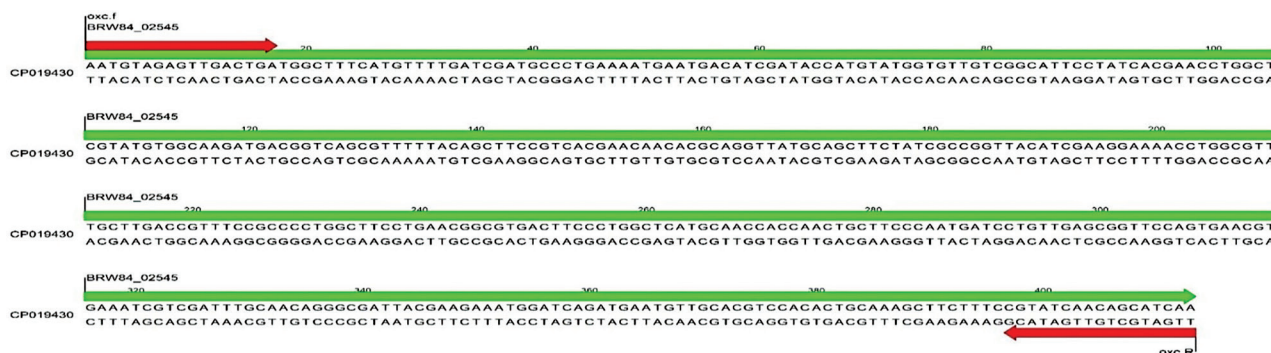
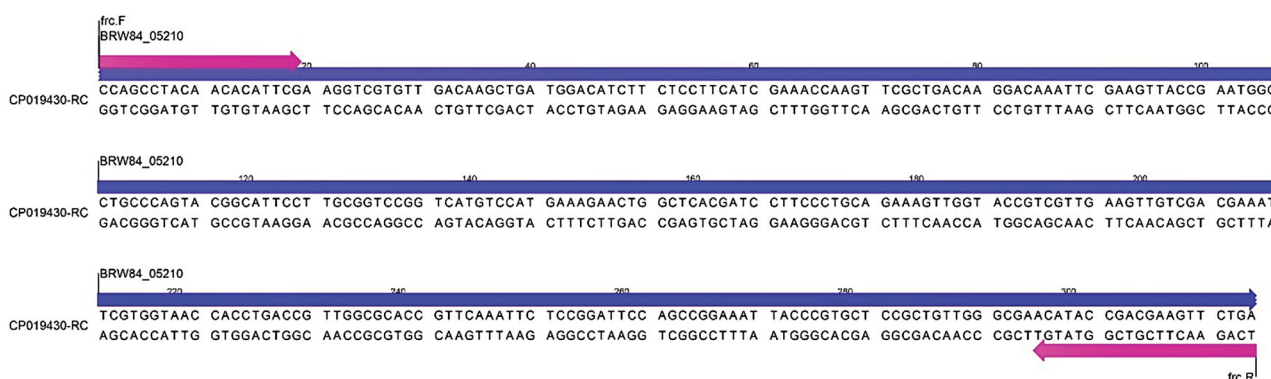


Figure 1. Schematic Figure of the Location of Designed Primers for Identifying the FRC Gene of Oxalobacter



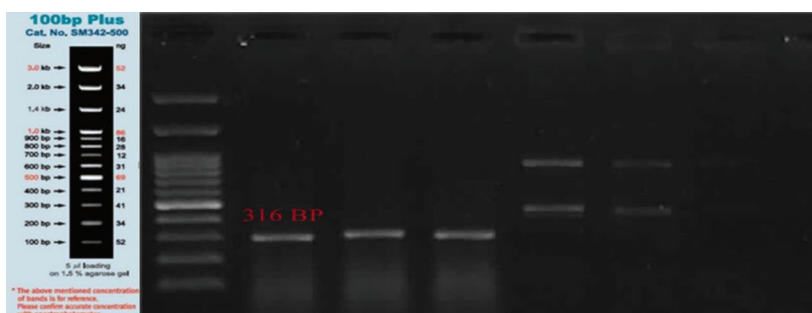
**Figure 2.** Schematic Figure of the Location of Designed Primers for Identifying the OXC Gene of Oxalobacter

sequence of primers designed by Primer-BLAST in the NCBI site was analyzed and found that the primers were specific to the target regions of FRC and OXC genes of Oxalobacter. Therefore, no cross-reaction with any other probable intervention factors was possible.

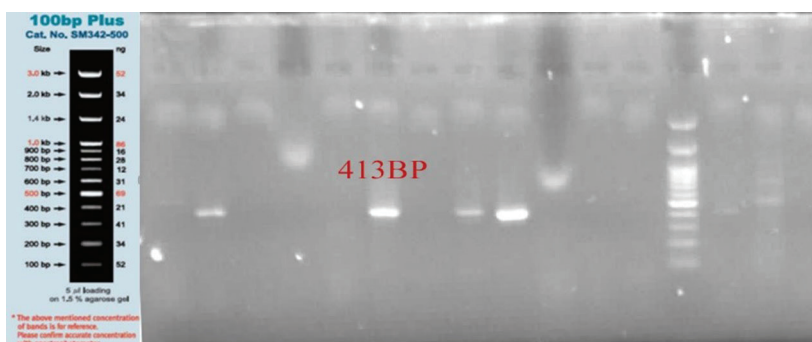
The PCR results for the FRC segment of Oxalobacter in people with oxalate kidney stones demonstrated 16 positive samples based on the 316 bp band (Figure 3). Moreover, PCR for the OXC segment in 73 patients with oxalate urolithiasis revealed one positive sample with a band of 413

bp (Figure 4).

Assessment of the sensitivity and specificity of FRC and OXC genes by PCR techniques showed that out of 73 samples positive for oxalate kidney stones, 16 specimens were identified positive for the FRC gene of Oxalobacter, and only one positive for OXC gene. As a result, the FRC gene was found to be 100% sensitive with a lower specificity, compared with the OXC gene (79.17%) (Table 1). Furthermore, the negative predictive value and positive predictive value for the FRC gene were observed as 100% and 6.25%, respectively.



**Figure 3.** Agarose Gel Panel of the 316-bp Product of the Gene Region Amplified by the Primer Pair for *O. Formigenes* FRC



**Figure 4.** Agarose Gel Panel of the 413-bp Product of the Gene Region Amplified by the Primer Pair for *O. Formigenes* OXC

**Table 1.** Statistical Analyses Using MedCalc Software for Determining the Specificity and Sensitivity of the Candidate Genes in PCR (Considering OXC Reference Gene)

Comparison of the Efficiency of the Genes by MedCalc Software	OXC		Total	
	Positive	Negative		
FRC				
Positive	True Positive 1 (100%)	False Positive 15 (20.83%)	16 (21.91%)	PPV 6.25%
Negative	False Negative 0	True Negative 57 (79.16%)	57 (78.08%)	
Total	1 (100%)	72 (100%)	73 (100%)	
Accuracy OXC 79.45% [95% CI: 68.38 to 88.02%]	Sensitivity 100%	Specificity 79.17%	Accuracy FRC	NPV 100%

**Table 2.** Statistical Analyses by MedCalc Software for Determining the Specificity and Sensitivity of the Results of Candidate Genes in qPCR Technique (with OXC as Reference Gene)

Comparison of the Efficiency of the Genes by MedCalc Software	OXC		Total	
	Positive	Negative		
FRC				
Positive	True Positive 2 (66.66%)	False Positive 0	2 (2.73%)	PPV 100%
Negative	False Negative 1 (33.33%)	True Negative 70 (100%)	71	
Total	3 (100%)	70 (100%)	73 (97.26%)	
Accuracy OXC 98.63% [95% CI: 92.6 to 99.97%]	Sensitivity 66.67%	Specificity 100%	Accuracy FRC	NPV 98.59%

**Table 3.** Relationship of *O. Formigenes* Presence and Oxalate Excretion in People with Urolithiasis

U. Oxalate.24h	PCR. FRC		PCR. OXC		qPCR. FRC		qPCR. OXC		Total
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	
< 10	8 (14.03%)	3 (18.75%)	10 (13.88%)	0	11 (15.49%)	0	11 (15.71%)	0	11 (15.1%)
10 to 25	31 (54.38%)	10 (62.5%)	41 (56.94%)	0	40 (56.33%)	1 (50%)	39 (55.71%)	2 (66.66%)	41 (56.2%)
25 to 40	13 (22.8%)	1 (6.25%)	14 (33.33)	1 (100%)	14 (19.71%)	0	14 (20%)	0	14 (19.2%)
> 40	5 (8.77%)	29 (12.5%)	7 (9.72)	0	6 (8.33%)	1 (50%)	6 (8.57%)	1 (33.3%)	7 (9.6%)
Total	57 (100%)	16 (100%)	72 (100%)	1 (100%)	71 (100%)	2 (100%)	70 (100%)	3 (100%)	73 (100%)

### Gene Record

A 316-bp sequence of FRC gene and a 413-bp sequence of OXC of *O. formigenes* were recorded in GenBank with access numbers MN905534 and MN905535, respectively.

### Real-Time PCR

The result of Real-time PCR for 73 samples of calcium oxalate urolithiasis is shown in Figure 5. Out of 73 samples with stone, FRC primer identified the Oxalobacter gene only in two samples, and OXC primer identified only three samples positive for Oxalobacter.

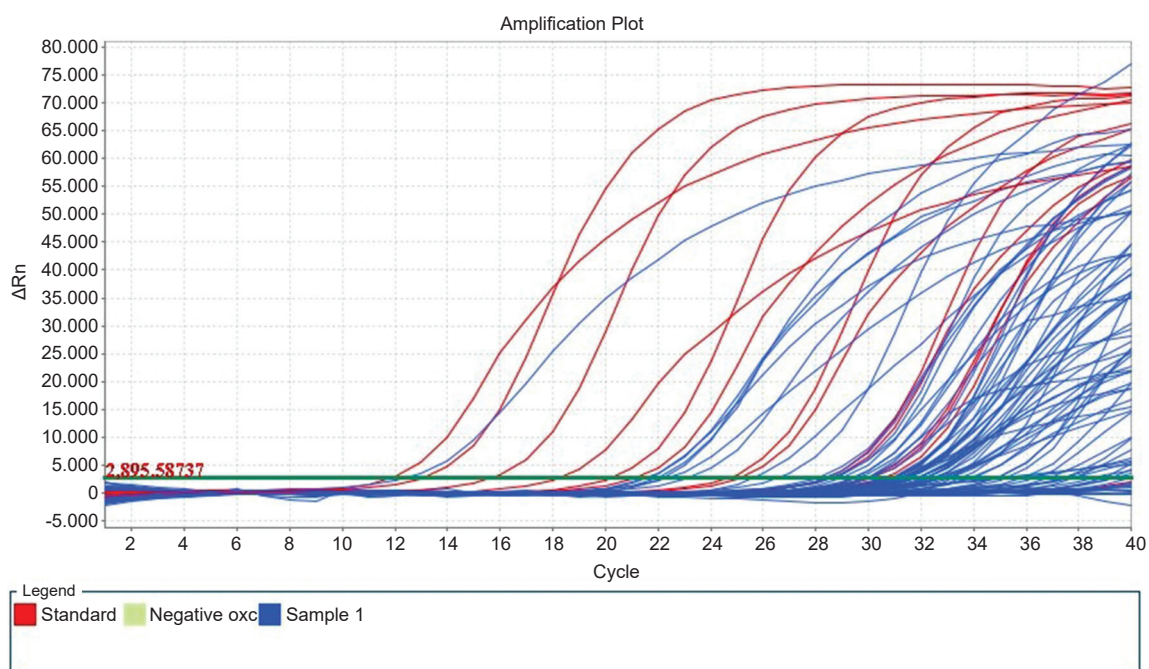
The real-time PCR of seven dilutions showed high efficiency of 97.57 for standards and the slope of -3.382 for standard curve. In addition, the calibration of the curve was approved by  $R^2 = 0.947$ ,

which is very close to 1 (Figure 6).

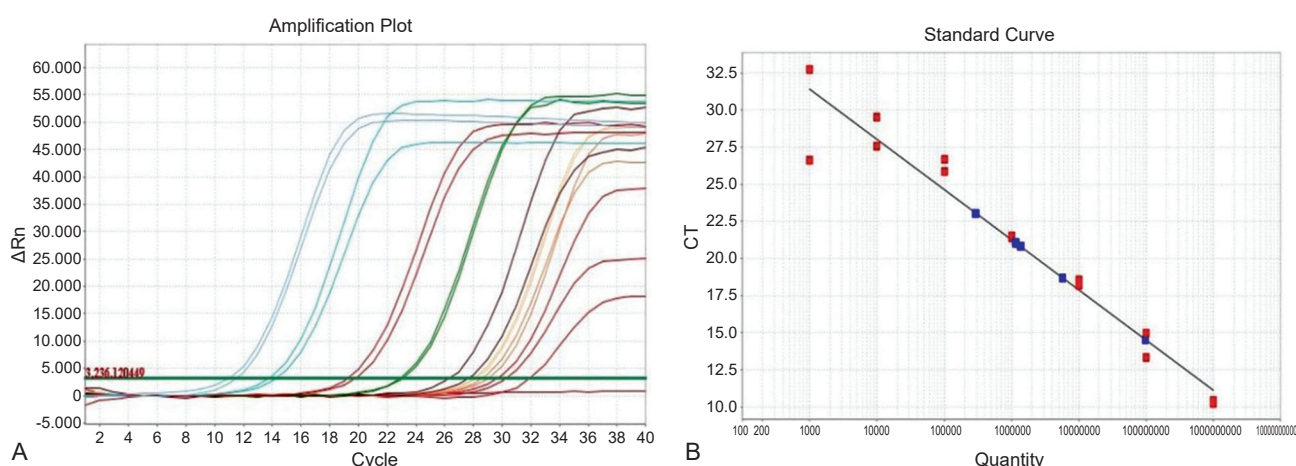
The qPCR revealed the sensitivity and specificity of the FRC gene against the OXC gene as 66.67 and 100%, respectively. These results show lower sensitivity and higher specificity as compared with the PCR method. Furthermore, the positive predictive value for FRC was 100% and the negative predictive value was 98.59.

### Evaluation of the Oxalate and *O. Formigenes* Presence in Individuals with Urolithiasis

Our PCR and qPCR of FRC and OXC genes for *O. formigenes*, show that the presence of bacterium does not have a significant relationship with oxalate excretion. In other words, most people with kidney stones had normal oxalate excretion in both the presence and absence of this bacterium.



**Figure 5.** Linear Amplification Plot of the Positive Samples for *O. Formigenes* Using the Selected Primers



**Figure 6.** A) Linear Amplification Plot of 1:10 Serial Dilutions of the Diluted PCR Product of *O. Formigenes* Gene Region; B) Standard Curve of 1:10 Serial Dilutions of the Diluted PCR Product of *O. Formigenes* Gene Region

## DISCUSSION

In this report, we aimed to resolve the important scientific issues concerning the role of *O. formigenes* and its microbial load in prevention of calcium oxalate urolithiasis by setting up a qPCR method. The OXC and FRC genes in *O. formigenes* have unique and highly preserved regions that allow amplification by specific primers.<sup>20</sup> The designed primers could well amplify two gene regions of 316 and 413 bp in the FRC and OXC genes, respectively.

The set up PCR method for the OXC gene showed 72 out of 73 samples with calcium oxalate

stone as negative and only one case was revealed positive. Also, we showed that the FRC gene on the DNA extracted from the stool of these 73 samples resulted in 16 positive and 57 negative cases for *O. formigenes*. Therefore, the inverse relationship between the presence of Oxalobacter and the formation of calcium oxalate urolithiasis with both FRC and OXC genes is well clear.

Discrepancy between the results obtained on the same samples and different genes, in addition to the lack of the same efficiency and optimization of the PCR reaction on FRC and OXC gene, can be due to

the high copy number of the FRC compared with the OXC region. It also shows a higher specificity of OXC primers to the target area. Siener *et al.*<sup>21</sup> studied on the OXC gene of *O. formigenes* in 37 patients with calcium oxalate stone, and found 30% positive (11 cases) by PCR. Kwak *et al.* detected *O. formigenes* in the fresh and frozen stool samples of healthy people and patients with calcium oxalate kidney stones by PCR. They concluded that *O. formigenes* is easily and efficiently detected by a PCR-based diagnosis approach. In addition, they found that *O. formigenes* load was significantly lower in urolithiasis patients as compared with the healthy individuals.<sup>22</sup> Recently, diets containing probiotics were recommended for urolithiasis patients and one of the microorganisms utilized in probiotics is *O. formigenes*. Measuring *O. formigenes* load by qPCR might provide more valid data for diagnosing the role of diet oxalate on hyperoxaluria. Also, the nutritional supplements can be modified for each patient considering the number of *O. formigenes*.<sup>23</sup> According to the results of the polymorphisms of the candidate gene regions, OXC and FRC genes have a low polymorphism and are highly conserved making them suitable for *Oxalobacter* identification. In addition to *O. formigenes* isolation by both FRC and OXC genes, the qPCR method in the present study managed to evaluate the infective dose of bacteria using a relative and absolute quantification without cloning the target region. The qPCR results for FRC gene revealed fewer positive cases than PCR method, although the values reported by these two methods did not differ significantly from the specific primers of the OXC gene. The results provided by the two genes FRC and OXC indicate that the selection of the gene region in the diagnosis and determination of the infectious load of the virus is controversial. In a study on 27 patients with urolithiasis, the qPCR results for the OXC gene showed that *O. formigenes* infectious load was significantly lower in individuals with the history of kidney stone as compared with people affected for the first time.<sup>24</sup> Although qPCR method for measurement of *O. formigenes* seems expensive, this technique can save patients with urolithiasis relapse or metabolic disorders, such as hypercalciuria and hyperoxaluria. Accurate *O. formigenes* assessment can assist planning for more accurate treatment. A prospective study on patients under upper endoscopy and biopsy showed that

antibiotics for *Helicobacter pylori* infection result in a continuous reduction in intestinal colonization of *O. formigenes*.<sup>25</sup> Furthermore, the lack of *O. formigenes* in women can be due to the repeated antibiotic usage for urinary tract infections, which have been proved to be accompanied by elevated oxalate excretion.<sup>26-28</sup> The role of *O. formigenes* in the growth of calcium oxalate stone has been shown, however the relationship between this bacterium and hyperoxaluria is less known.<sup>27</sup> Nonetheless, it is expected that oxalate destruction by *O. formigenes* may reduce oxalate urinary excretion, and several studies related to the lack of *O. formigenes* to the higher excretion of oxalate in urine.<sup>28,29</sup> Some other studies did not report a significant difference in the oxalate excretion of patients with positive and negative tests for *O. formigenes*.<sup>26-31</sup> In the present investigation, among 73 people with oxalate stone, most individuals with positive (62.5%) and negative (54.38%) PCR results for the FRC gene had oxalate excretion in the normal range of 10 to 25 mg / 24h. Moreover, 56.94% of the cases with a kidney stone that had negative PCR results for the OXC gene had oxalate excretion in the normal range of 10 to 25 mg / 24h. These results are also valid for both genes examined by qPCR. Our results are similar to the Federici *et al.* study that the relative frequency and total count of *O. formigenes* were not different among patients with urolithiasis, hyperoxaluria and people that had normal urinary oxalate excretion.<sup>19</sup> Knight *et al.* studied 38 cases of calcium oxalate urolithiasis and concluded that absorption was not significantly different between individuals with or without bacterium.<sup>31</sup> Siener *et al.* evaluated *O. formigenes* intestinal colonization on plasma oxalate concentration in idiopathic kidney stone patients and revealed that the mean plasma oxalate in patients without *O. formigenes* (5.79  $\mu\text{mol/L}$ ) was more than three times of patients with bacterial colonization (1.7  $\mu\text{mol/L}$ ).<sup>21</sup>

Overall, these findings show that intestinal oxalate absorption is not balanced by *O. formigenes*. There is not yet sufficient knowledge concerning with the mechanism of *O. formigenes* colonization in patients with idiopathic oxalate calcium urolithiasis. Nonetheless, a major limitation is that none of the studies were performed under controlled conditions, including diet, antibiotic consumption, and genetic factors. The current investigation had limitations; the most important one was lack of a

nutritional diet. Moreover, the small size of the study population prevents generalization despite valuable and valid results.

## CONCLUSION

Colonization of *O. formigenes* does not occur or is very low in patients that have repeated calcium oxalate urolithiasis. We believe that the accurate measurement of *O. formigenes* load by qPCR is one of the main reasons for elevated urolithiasis rate and adding probiotics containing *O. formigenes* to the diet of these cases can be considered as a treatment and control approach for oxalate kidney stones.

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