CHAPTER 2
Chapter 2: Characterization of Recurrent Kidney Stone Disease

Subjects: Oxalate Quantification and Molecular Imprints of Bacteria in Kidney Stones

2.1 Introduction

Raphids, envelop shaped crystals in plants made of calcium oxalate (chemical formula: CaC$_2$O$_4$ or Ca(COO)$_2$). Similarly found in human kidney in the majority. It is known as beer-stone (a scale that forms on containers used in breweries). These calcium oxalate crystals in the urine confer major part of human kidney stones and impart the toxic effects of ethylene glycol poisoning (Lotan et al., 2005). Kidney stones occur in hyperoxaluria or hypercalcemia condition, but researchers wonder whether it is linked to other factors like age, diet, gender, etc.? (Daudon et al., 2004; Robertson and Peacock, 1980; Zimmermann et al., 2005). Saturation of urine with calcium or oxalate may form the calcium oxalate crystals in hypercalcemia or hypoxemia conditions (Pak et al., 2004). From the population around 10 %, people will experience nephrolithiasis in their lifetime of which 70 % will have recurrences of stones and in that 80 % are composed of calcium oxalate (Finkielstein and Goldfarb, 2006).

High-throughput chromatographic techniques like gas chromatography (Moye et al., 1981; Yanagawa et al., 1983), liquid chromatography (HPLC etc.) (Hönow et al., 1997; Kataoka et al., 1990; Larsson et al., 1982), and ion-pair chromatography (Menon and Mahle, 1983; Robertson et al., 1982) have been developed for analysis of oxalate from biological fluids. Whereas normal estimation methods have some disturbing factors such as pH, sample collection process parameters, the concentration of ascorbic acid, etc. (Mazzachi et al., 1984). The other methods have been developed using enzymatic (Buttery et al., 1983; Costello et al., 1976; Kohlbecker et al., 1979; Obzansky and Richardson, 1983; Potezny et al., 1983; Yriberry and Posen, 1980), colorimetric (Baadenhuijsen and Jansen, 1975; Hodgkinson and Williams, 1972) and isotachophoresis (Baadenhuijsen and Jansen, 1975) assays for analysis of oxalate from biological fluids. All these techniques are
found to be accurate even in presence of oxalate concentration altering bio-
molecules like ascorbic acid. Oxalate oxidase (OO) method was most widely used
among all as the colorimetric method provides accurate, robust and uniform result
at any center over the globe. Many workers have developed HPLC-based protocols
with different parameters like-column, retention time, detector, eluent, etc. For
oxalate estimation from native urine, urine spiked with oxalate and comparative
profile which were evaluated by Zerwekh (Zerwekh et al., 1983), Hesse (Hesse et al.,
1996), and have suggested need for universal method for oxalate analysis. Hence
there is a need for a simple but universal method for estimation of oxalate content
from biological matrices or otherwise previously described methods needs relative
standardization (Maalouf et al., 2011). Here we have proposed new HPLC method for
estimation of oxalate from biological matrices without pre-analysis sample
preparation which would be simple, accurate, precise, sensitive and selective for the
same and useful over the entire globe.

There are two types of kidney stones, classified as into metabolic and infectious.
They are extremely provoking common health complications in people around the
world. These stones formation is utterly linked with bacteria in case of infectious
stones and is considered to be a consequence of a UTI (Ciftçioglu et al., 1999). It has
already been reported the presence of bacteria in nidus of kidney stones
(Tavichakorntrakool et al., 2012), however, the exact role of bacteria in the
nucleation process is poorly understood yet. So, in this regard speculated
hypotheses graphically represented (Figure 2.1) to understand the molecular
methods can trace the role bacteria acting as the mediator for the kidney stone
formation and that.
Figure 2.1: Illustration of speculated hypothesis for the bacterial role in the oxalate kidney stone formation.

However, some evidences suggest that colonization of bacterial cells inside the nidus would exhibit either renal tubular acid tolerance (Heilberg and Schor, 2006b), degradation of amino acids (Kopple et al., 1978). Some may changing the pH of micro-environment milieu by ammonia production through urease activity (Torzewska et al., 2014). Another possibility explains that bacteria have the ability to inhibit and alter the inhibitors of kidney stone formation (Flannigan et al., 2014). The bacterial imprints in kidney stones have been explored using cultivation and microscopic methods. These studies involved identification of exact bacterial composition of kidney stone and their metabolic activities which are critical points to understand the pathophysiology of formation of kidney stone. The present chapter
intended at characterizing the chemical composition of kidney stones and assesses total bacterial diversity in these stones using 16S rRNA gene clone library approach.

2.2 Materials and Methods

2.2.1 Subjects Enrollment and Sample Collection

Stool samples obtained from 73 Indian subjects were collected under ‘case-control study’ and ‘healthy-cohort study’ collectively. National Centre approved the ‘case-control study’ for Cell Science’s Institutional Ethics Committee (I.E.C). By the Declaration of Helsinki principles; a total of 39 subjects, symptomatic kidney stone diseased (n=24) hereafter referred to as KSD, and healthy control (n=15) hereafter called HLT were involved in ‘case-control study.’ The institutional ethical committee approved the study design, and the protocols followed and informed consent taken from each subject. All study population inclusion of only male candidates and exclusion criteria were the active urinary infection, previous intestinal surgery, and intestinal disease. Moreover, subjects who did not receive any antibiotics or probiotic preparations for the last three months before enrollment were included.

Urine samples after 24-hr, surgically removed kidney stones and fecal samples from each KSD group while 24-hr urine and fecal samples from each HLT group were collected. Fecal samples from the above subjects were collected in a sterile container and stored at -80 °C until they were used for DNA extraction and followed for bacterial diversity and targeted metagenomic analysis. Another study, ‘healthy-cohort study’ (n=34) was undertaken for the isolation of bacteria from healthy subjects, were also added into the further diversity analysis purpose.

All the samples were collected from subjects at Department of Urology, RCSM Govt. Medical College and Endo-urology center, Gune’s Nirmal Nursing Hospital, Kolhapur (Maharashtra) in India. All the voluntarily involved subjects and their metadata from both the studies are mentioned in the Appendix V. For subject characterization, 24-hr urine and surgically removed kidney stone samples were used for oxalate quantification and chemical analysis and microbial imprint analysis respectively from ‘case-control study.’ While the fecal samples were used for Eubacterial diversity,
targeted metagenome, and trans-domain diversity analysis purpose from the both ‘case-control study’ and ‘healthy-cohort study’ for next chapters. Schematic representation of study overview is depicted in Figure 2.2.

![Diagram](image)

**Figure 2.2:** Schematic representation of study overview of the entire thesis. Wherein 24-hr Urine, Kidney stone and Stool samples were collected from the respective subjects for further analysis.

### 2.2.2 Determination of Oxalate in 24-hr (a Day Sample) Urine using HPLC Method

The urine samples of human subjects were collected for method development and analysis over a period of 24-hr (Curhan et al., 2001; Taylor and Curhan, 2008). These urine samples were refrigerated until its analysis. Neither preservative nor any processing was carried out on collected urine sample. It was then processed for analysis to determine oxalate content by High-Performance Liquid Chromatography (HPLC).

Acetonitrile, oxalic acid, methanol, acetic acid and distilled water (LobaChemie Pvt. Ltd. Mumbai, India) of HPLC grade were used. The HPLC system used was a PC based JASCO series comprising of a pump PU-2080 (dual piston with gear driven pump) and a UV-2070 detector. The UV detector used in this HPLC system was a Czerny-Turner Mount Monochromatic with deuterium lamp as the light source. Manual injections were carried out using a Rheodyne injector with a fixed 20 µl external loop. The
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chromatographic and the integrated data were recorded using a Hercule 2000 (interface) computer system. Data processing was carried out using Borwin tool (version 1.5). Spectrophotometric analysis was carried out on Jasco model V-530 UV-Visible double beam high-speed scanning spectrophotometer with a single monochromator with a 1200 grooves/mm concave grating. The detector was Silicon Photodiode (S1337). Light sources used were Deuterium lamp (190 to 350 nm) and a Halogen lamp (330 to 1100 nm). The instrument had wavelength accuracy of ± 0.3 nm and baseline stability of ± 0.001 Abs/hr.

**Preparation of Standard Solutions of Oxalic Acid**

50 mg of HPLC grade pure oxalic acid was weighed accurately. It was then transferred to 50 ml volumetric flask and volume were made up to the mark with pure methanol having concentration 1000 µg/ml. It was then sonicated and used for method development. After method optimization, different solutions of oxalic acid were prepared in mobile phase (Methanol: Acetic acid (0.001N) and in water (50:50, v/v)), both of same strength (1000 µg/ml).

**Selection of Wavelength of Analysis**

The standard stock solution was diluted to 10 µg/ml. The wavelength of analysis of oxalic acid was selected by analyzing 10 µg/ml solution of the same in pure methanol on UV-Visible Double Beam Spectrophotometer over 200 to 400 nm wavelength range. The wavelength of 237 nm was selected having maximum absorbance.

**2.2.3 Chromatographic Method Development for Oxalate Analysis in Urine**

The sample solution of oxalic acid in urine for method development was prepared by mixing 0.1 ml of stock solution of oxalic acid in the mobile phase and 2 ml of urine of healthy human volunteer and diluting it to 10 ml with mobile phase. This solution was injected into the chromatographic system with KYATECH HiQSil C18HS column using Rheodyne injector at a flow rate of 1 ml/min and wavelength of analysis, 237 nm. The oxalic acid was resolved at 2.7 minutes with good retention parameters under above chromatographic conditions. As the concentration of oxalic acid was calculated by single point calibration method, same a sample solution was injected...
under similar conditions for five more times, and area of the oxalic acid peak was recorded using the software. The mean of the area of six readings of oxalic acid in urine was calculated, and concentration of oxalic acid in urine patient was calculated using following formula:

$$\text{Oxalic acid content (µg/ml)} = \frac{\text{Area of oxalate peak}}{\text{Mean of Area of 6 Readings}} \times 10$$

The concentration of oxalic acid in mg / x ml of urine was calculated by multiplying above reading by dilution factor and x.

The quality control samples were prepared from standard stock solution and urine of healthy volunteers and analyzed by the same procedure is given above. Analysis of urine of kidney stone patients and healthy volunteers for oxalate content was carried out by the method explained above without adding 0.1 ml of stock solution of oxalic acid in the mobile phase.

2.2.4 Chemical Compositional Analysis of Kidney Stones by FTIR Method

Total 120 surgically removed kidney stones were collected in sterile containers; surface sterilized with 70 % ethanol, later with sterile distilled water and consequently air dried. Each kidney stone was aseptically pulverized into powdered form, part of which was analyzed by FTIR spectroscopy without KBr method to identify the stone type. Briefly, each part of the stone was placed in the IR beam of the Bruker FTIR-ATR Tensor 37 spectrometer and spectra were obtained in transmittance mode from 4000 to 400 cm⁻¹; 32 scans were averaged with a 4 cm⁻¹ resolution for each spectrum. The resulting spectra obtained for all 120 stones were compared to the spectrum of standard CaOx (Sigma-Aldrich, USA).

2.2.5 Bacterial Imprints Analysis from Kidney Stones by Clone Library Method

Stone powders of all stones derived from a single patient were pooled and used for genomic DNA isolation which was further processed for preparation of 16S rRNA gene clone library. Steps for the traditional clone library preparation were illustrated in Figure 2.3.
**Figure 2.3:** Schematic representation of traditional clone library generation and analysis of clone sequences.

16S rRNA gene was amplified using Eubacteria specific primers: 27F (5′-CCAGAGTTTGATCMTGGCTCAG-3′) and 1490R (5′-GGTTACCTTGTTACGACTT-3′). Following conditions were utilized for PCR amplification: Initial denaturation at 94 °C for 5 min followed by 28 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 0.5 min and extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. The resulting PCR products were cloned and sequenced as explained earlier (Gupta et al., 2012) and good quality clone sequences were obtained. All of these sequences are deposited to NCBI-GeneBank under the accession numbers KM581286-KM581327 and analyzed using EzTaxon database (http://www.ezbiocloud.net/).

### 2.3 Results

#### 2.3.1 Subjects Characterization: Oxalate Content in 24-hr Urine

The different types of studies involving chromatographic, spectroscopic methods stressed on the simple but accurate and universal method for analysis of oxalate in biological fluids particularly urine. Though colorimetric or enzymatic methods were used widely for analysis, the modified HPLC method used in this study was found to be simple with a minimum number of steps involved compared to other methods. After observing the chromatogram, the method followed in this experiment was
found to be more acceptable for oxalate estimation/analysis from urine samples of human volunteers in which most other interfering components in urine were not retained in optimized mobile phase except few.

The sample collection carried out over 24-hr from kidney stone disease and healthy human subjects because sampled urine have detailed information about calcium, oxalate and urinary saturation with oxalate over the time through metabolism (Daudon et al., 2004). Since, every metabolic workup includes measurement of calcium and oxalate concentrations in a 24-hr urine collection, together with other key analytes (Curhan et al., 2001; Taylor and Curhan, 2008). The chromatographic separations on HPLC were performed on a 5 mm KYATECH HiQSil C18HS column (250mm×4.6mm i.d., 5 µm particle size), operating at ambient temperature. The standard set of oxalic acid solution of concentration 100 µg/ml was prepared in methanol (HPLC grade). The development protocol was initiated by analyzing 10 µg/ml solution of oxalic acid in methanol on HPLC system. But the spectrum was not resolved with good retention parameters such as capacity factor, resolution, tailing factor, the number of theoretical plates, etc. Then by using methanol: water (50:50, v/v) mobile phase with good retention parameters resolved the oxalic acid spectrum. Following this 1 ml of oxalic acid solution mixed with the healthy human urine diluted to 10 ml with methanol: water (50:50, v/v) injected into HPLC and chromatogram yield which did not resolve with good retention parameters.

The results of analysis of urine samples were found satisfactory and reliable after several trials when methanol: acetic acid (0.001N) in water (50:50, v/v) mobile phase was used. This combination resolved the oxalic acid in a urine sample at a retention time of 2.705 ± 0.005 minutes (Figure 2.4a). Oxalic acid in urine sample was quantified using 5 mm KYATECH HiQSil C18HS column (250 mm_4.6 mm i. d.) at a flow rate of 1 ml/minute, at a wavelength of 237 nm using methanol: water (50:50, v/v) 0.001 N acetic acid mobile phase.

In most other methods, sample preparation for oxalate content required pre-acidification by acetic acid (Hesse et al., 1996; Zerwekh et al., 1983) but in present protocol, we have used acetic acid as part of mobile phase so no need of sample pre-
processing step. This acetic acid has increased the concentration of oxalate which has been retained in stationary phase.

### 2.3.2 Validation of Chromatographic Method

USFDA guidelines for bioanalytical method validation were followed for the validation of the newly developed method. Following studies were performed for validating the method.

**Selectivity**

Individual specificity, about endogenous urine components, was showed by analysis of series of randomly selected oxalic acid-free urine samples. Figure 2.4a explains the typical chromatograms after analysis of oxalic acid-free urine and urine samples after addition of oxalic acid. The retention time was found to be different for the studied oxalic acid than that of endogenous urine components. It indicates selectivity of a method for elution of oxalic acid in urine.

**Linearity and Range Study**

Table 2.1 shows the calculated response factors were found to be directly proportional to the concentration of analytes over the range tested. The least square method was used to draw the calibration curve which was based on three days study. Table 2.1 has the results of linearity study by regression analysis.
Table 2.1: Results of analysis of quality control samples, linearity and extraction recovery studies of oxalate.

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>% Concentration Estimated (Mean±SD)</th>
<th>% Recovery Estimated (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>85.65±1.4592</td>
<td>90.70±2.4174</td>
</tr>
<tr>
<td>150</td>
<td>87.76±1.4208</td>
<td>89.16±1.8503</td>
</tr>
<tr>
<td>300</td>
<td>87.84±1.0597</td>
<td>92.21±2.1824</td>
</tr>
</tbody>
</table>

Linearity and Range Study

<table>
<thead>
<tr>
<th>Range in ng/mL</th>
<th>Slope</th>
<th>Intercept</th>
<th>SE of Slope</th>
<th>SE of Intercept</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>50-300</td>
<td>3.357×10^{-2}</td>
<td>5.217×10^{-2}</td>
<td>3.024×10^{-5}</td>
<td>4.781×10^{-4}</td>
<td>0.9981</td>
</tr>
</tbody>
</table>

^Average of Five Determinations; ^Standard Deviation; ^Average of Four Determinations; ^Standard Error.

Lower Limit of Quantitation (LLOQ)

LLOQ is the lowest serum concentration of oxalic acid quantified with a coefficient of variation of less than 20%. The LLOQ value of oxalic acid was found to be 49 ng/ml.

Accuracy Study

The recovery study was done by analyzing quality control samples, (includes five determinations per three concentrations) spiked with an analyte.

Extraction Recovery Studies

The recovery represents the efficiency of an analytical method within the variation limit. The recovery in an assay is the detector response obtained from an amount of an analyte added and recovered from the biological matrix (urine). These experiments performed in quadruplicate for an analyte by comparing the analytical results for extracted samples at three concentrations (equivalent to LLOQ, MQC, and HQC) with three unextracted concentrations that represent 100% recovery.

Extraction recovery results for analytes are given in Table 2.1.

Medium quality control concentrations (MQC), 50% of largest concentrations of calibration curve (Linearity Study)
High-quality control concentrations (HQC), 75–90 % of largest concentrations of calibration curve (Linearity Study)

% recovery = (Mean response of extracted samples/Mean response of unextracted samples) ×100.

**Precision Study**

This study was carried out by analyzing the urine samples of three concentrations which were specified in the inaccuracy study for three consecutive days at two different times. This analysis demonstrates the Intra-day and Inter-day precision study. The urine samples were stored in a freezer at -17 °C. The results are shown in Table 2.2.

**Table 2.2**: Result of accuracy and precision studies

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precision</th>
<th>Amount of Pure Oxalic Acid Added</th>
<th>% Concentration Estimated Mean&lt;sup&gt;a&lt;/sup&gt; ±SD&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalate</td>
<td>Intra-Day</td>
<td>T1 5 mg</td>
<td>89.44±2.6215</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2 10 mg</td>
<td>92.46±4.1412</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3 15 mg</td>
<td>91.56±3.0555</td>
</tr>
<tr>
<td></td>
<td>Inter-Day</td>
<td>D1 -</td>
<td>90.57±3.0648</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D2 -</td>
<td>90.25±1.1468</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D3 -</td>
<td>90.28±1.4562</td>
</tr>
</tbody>
</table>

<sup>a</sup>Average of Fifteen Determinations (calculated at three levels 3×5); <sup>b</sup>Standard Deviation; T- time; D-Day.

**Stability Study**

Stability study was carried out by doing an analysis of stock solutions, unextracted urine samples and freshly prepared solutions at different atmospheric conditions and times as shown in the subsequent headings as per guidelines as the mean of 6 readings with its standard deviation (Ferraz et al., 2006).

**Stock Solution Stability**

Five aliquots of standard stock solutions were kept at -17 °C for three days for testing the stock solution stability of oxalic acid. After three days, concentrations were...
analyzed and compared with the fresh stock solution. The percentage recovery of oxalic acid was found to be $99.73 \pm 1.97$ (mean ± standard deviation).

**Freeze and Thaw Stability**

Stability of urine samples along with analytes was recorded at various freeze and thaw cycles by subjecting five aliquots of QC samples at three concentrations (low, middle, and high) unextracted quality control samples to four freeze–thaw cycles. All the samples were investigated after completion of every cycle and compared the experimental concentrations with the nominal values obtained by analyzing fresh samples. The accuracy values of three concentrations in two freeze–thaw cycles were calculated. The percentage recovery of oxalic acid was found to be $99.46 \pm 3.57$ (mean ± standard deviation).

**Temperature Sensitivity**

Five aliquots of QC samples at low, mid, and high concentration unextracted QC samples were kept at ambient temperature ($15^\circ$C) for 12-hr to determine the temperature sensitivity of the analytes in the urine. Then, the samples were processed and analyzed. The results were analyzed by comparing with the nominal values obtained by analyzing fresh samples. The percentage recovery of oxalic acid was found to be $100.23 \pm 2.25$ (mean ± standard deviation).
Table 2.3: Number of surgical removed stones and oxalate content from respective samples under study.

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Number of Stones</th>
<th>Concentration in µg/ml</th>
<th>Subject ID</th>
<th>Number of Stones</th>
<th>Concentration in µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSD1</td>
<td>7</td>
<td>140.6024</td>
<td>KSD30</td>
<td>4</td>
<td>50.4328</td>
</tr>
<tr>
<td>KSD2</td>
<td>5</td>
<td>70.9542</td>
<td>KSD31</td>
<td>6</td>
<td>120.2586</td>
</tr>
<tr>
<td>KSD3</td>
<td>5</td>
<td>40.0254</td>
<td>KSD32</td>
<td>5</td>
<td>60.9981</td>
</tr>
<tr>
<td>KSD4</td>
<td>4</td>
<td>60.1502</td>
<td>KSD33</td>
<td>4</td>
<td>50.6925</td>
</tr>
<tr>
<td>KSD7</td>
<td>7</td>
<td>80.8047</td>
<td>HLT1</td>
<td>0</td>
<td>2.0875</td>
</tr>
<tr>
<td>KSD9</td>
<td>5</td>
<td>100.6679</td>
<td>HLT2</td>
<td>0</td>
<td>3.8035</td>
</tr>
<tr>
<td>KSD10</td>
<td>5</td>
<td>70.4897</td>
<td>HLT3</td>
<td>0</td>
<td>0.5784</td>
</tr>
<tr>
<td>KSD12</td>
<td>8</td>
<td>50.9875</td>
<td>HLT4</td>
<td>0</td>
<td>2.7721</td>
</tr>
<tr>
<td>KSD13</td>
<td>4</td>
<td>130.8206</td>
<td>HLT5</td>
<td>0</td>
<td>2.1589</td>
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<tr>
<td>KSD14</td>
<td>4</td>
<td>90.5782</td>
<td>HLT6</td>
<td>0</td>
<td>1.9029</td>
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<tr>
<td>KSD20</td>
<td>5</td>
<td>80.2496</td>
<td>HLT7</td>
<td>0</td>
<td>0.8565</td>
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<tr>
<td>KSD21</td>
<td>6</td>
<td>150.2171</td>
<td>HLT8</td>
<td>0</td>
<td>11.4752</td>
</tr>
<tr>
<td>KSD22</td>
<td>4</td>
<td>50.6745</td>
<td>HLT9</td>
<td>0</td>
<td>0.8812</td>
</tr>
<tr>
<td>KSD23</td>
<td>5</td>
<td>50.8762</td>
<td>HLT10</td>
<td>0</td>
<td>0.4785</td>
</tr>
<tr>
<td>KSD24</td>
<td>4</td>
<td>70.9864</td>
<td>HLT11</td>
<td>0</td>
<td>10.1217</td>
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<tr>
<td>KSD25</td>
<td>3</td>
<td>120.9987</td>
<td>HLT12</td>
<td>0</td>
<td>6.7485</td>
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<tr>
<td>KSD26</td>
<td>5</td>
<td>60.3574</td>
<td>HLT13</td>
<td>0</td>
<td>0.8979</td>
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<tr>
<td>KSD27</td>
<td>6</td>
<td>70.6348</td>
<td>HLT14</td>
<td>0</td>
<td>3.2486</td>
</tr>
<tr>
<td>KSD28</td>
<td>5</td>
<td>60.0269</td>
<td>HLT15</td>
<td>0</td>
<td>0.0573</td>
</tr>
<tr>
<td>KSD29</td>
<td>4</td>
<td>50.3985</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Post-Preparation Stability

For estimating the stability of oxalic acid in the prepared samples, five aliquots of QC samples at three concentrations (low, mid, and high) were kept at 4 °C for about 4-hr. Then, the analysis was done by comparing the concentrations with the nominal values obtained by analyzing fresh samples. The percentage recovery of oxalic acid was found to be 100.16±3.98 (mean ± standard deviation). Results have been mentioned in Table 2.3 and Figure 2.4.
2.3.3 The Chemical Composition of Surgically Removed Kidney Stones

Total 120 kidney stones were recovered through the surgical methods (Figure 2.5).
Figure 2.5: Photographs of surgically removed actual kidney stones from respective subjects.
All the stones obtained from subjects were analyzed and found to be pure CaOx stones as revealed by FTIR analysis (Figure 2.6). Wave number and transmittance of all the analyzed stones were recorded for the CaOx composition.

Figure 2.6: FTIR spectral analysis of representative kidney stone samples (KSD2 and KSD24) recovered from respective subjects. Modified and reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures, Scientific Reports, Copyright © 2016, Nature Publishing Group.

2.3.4 Molecular Imprints of Bacteria in Kidney Stones

Out of 24 pooled kidney stones, only two samples were positive for 16S rRNA gene amplification (Figure 2.7). As a result, two 16S rRNA gene clone libraries were generated.
Figure 2.7: Gel electropherogram of the PCR for bacterial imprint analysis on 1 % agarose gel. Positive PCR reaction gave approx. 1500 bp of product length as compared to the 1 kb+ DNA ladder.
42 clone sequences were analyzed, and we reported the presence of different bacteria in kidney stones, most of which were belong to the genus *Bacillus* (Table 2.4). Also, bacteria belonging to genera *Acinetobacter, Enterococcus, Leucobacter, Prolinoborus* and *Streptococcus* were also observed in varying distribution in these stones. The genus *Bacillus* includes many spore-forming species, and these endospores can be a reason to initiate the deposition of calcium carbonate (Chutipongtanate et al., 2013). This may be the first report which proves the presence of bacteria other than *Escherichia, Proteus, Staphylococcus, Providencia,* and *Klebsiella* which are commonly associated with the nidus of stone.

**Table 2.4:** Identification of 16S rRNA gene library clones, their putative products as a risk factor for kidney stone formation & oxalic acid metabolism abilities.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Clone Identity</th>
<th>No of Clones</th>
<th>Putative metabolite or activity as potential risk factor†</th>
<th>Oxalic acid metabolism ability‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Acinetobacter lwoffii</em> NCTC 5866¹</td>
<td>2</td>
<td>Acid tolerance</td>
<td>Utilization</td>
</tr>
<tr>
<td>2</td>
<td><em>Bacillus anthracis</em> Ames</td>
<td>5</td>
<td>Surface cell wall protein, Spore coat</td>
<td>Utilization</td>
</tr>
<tr>
<td>3</td>
<td><em>Bacillus anthracis</em> ATCC 14578¹</td>
<td>20</td>
<td>Surface cell wall protein, Spore coat</td>
<td>Utilization</td>
</tr>
<tr>
<td>4</td>
<td><em>Bacillus cereus</em> ATCC 14579¹</td>
<td>1</td>
<td>Surface cell wall protein, Spore coat</td>
<td>Utilization</td>
</tr>
<tr>
<td>5</td>
<td><em>Enterococcus faecium</em> ATCC 19434¹</td>
<td>2</td>
<td>Acid tolerance, Exopolysaccharides</td>
<td>Degradation</td>
</tr>
<tr>
<td>6</td>
<td><em>Leucobacter chromiireducens</em> subsp. <em>chromiireducens</em> L-1¹</td>
<td>4</td>
<td>Acid tolerance, Exopolysaccharides</td>
<td>Tolerance and Utilization</td>
</tr>
<tr>
<td>7</td>
<td><em>Leucobacter kyeonggiensis</em> F3-P9¹</td>
<td>1</td>
<td>Acid tolerance, Exopolysaccharides</td>
<td>Tolerance and Utilization</td>
</tr>
<tr>
<td>8</td>
<td><em>Prolinoborus fasciculus</em> CIP 103579¹</td>
<td>6</td>
<td>Spore coat, Biodegradation of proline</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td><em>Streptococcus anginosus</em> CCUG 39159¹</td>
<td>1</td>
<td>Biofilm formation, Calcium ion aggregation</td>
<td>Degradation</td>
</tr>
</tbody>
</table>

† Based on literature survey, NA- Not Any report found

### 2.4 Discussion

#### 2.4.1 Elevated Level of Oxalate and CaOx Containing Kidney Stones are the Characteristics of Hyperoxaluria
The method developed was found to be with less error, appropriate and highly sensitive for quantification of oxalate from the urine of kidney stone diseased patient and healthy volunteers. The method did not entail pre-analysis sample processing as acetic acid is part of the mobile phase, used to concentrate oxalate in biological matrices. Our method will be very useful for estimation of oxalate from other biological matrices with a few optimizations. Oxalate content of hyperoxaluria conditions in different geographical studies tended to greater than 40 mg/24-hr (23.1 µg/ml) (Glew et al., 2014). This study was also in accordance and comparable to the previous hyperoxaluria reports wherein greater than 40 µg/ml for KSD, and lesser than 11.4 40 µg/ml for HLT in the tested subjects. Table 2.3 depicts the generated oxalate content data which was used for characterization of the gut microbial diversity of recurrent stone patients and healthy male subjects in comparison using oxalotrophic bacterial colonization pattern and oxalate content in urine.

2.4.2 Bacterial Imprints inside the Nidus may Clue for its Lithiasis Role

Growing evidence support the fact behind the development metabolic as well as infectious kidney stones causing due to microbial cells or their metabolic products. This is the first study to report the presence of bacteria in surgically removed metabolic stones and attempts to relate them with the recurrent episodes of CaOx stones. Molecular analysis (16S rRNA gene clone library) and taxonomic characterization of bacteria is a gold-standard approach to study the total bacterial diversity of a variety of samples (Rajendhran and Gunasekaran, 2011). In recent time, the technique has also been implemented to characterize the bacterial diversity associated with gallstone (Peng et al., 2015; Swidsinski et al., 1995). Hence we have adopted this approach in this study to characterize CaOx stone associated bacterial diversity.

Table 2.4 shows the details about the bacteria present in nidus of kidney stone possess one or more capacity which may make them potential contributor in the progression of recurrent oxalate kidney stone. Our results are strengthened by another in-vitro study, in which role of Gram-positive and Gram-negative bacteria in
lithiasis of CaOx stones has been dealt in mechanical and illustrative ways (Chutipongtanate et al., 2013). Surprisingly, we did not detect any common Enterobacteriaceae members such as *E. coli* in all stone studied. So there could be many more bacteria associated (listed in Table 2.4) with the development of metabolic stone especially in the case of recurrent episodes of stones. Conversely, further studies on a large number of samples are required to show whether members of Enterobacteriaceae are absent in all the cases or they are present along with bacteria that we were able to detect in the stone.

Bacterial presence in stones indicates that in hyperoxaluric condition, bacteria are not only found in the kidney but they may participate in the formation of kidney stones and other urological diseases (Whiteside et al., 2015). Hence this kind of studies is supportive of finding out the exact bacterial composition of stone and are also essential to predict the stone type as hypothesized earlier. Furthermore, the study suggests the need for combinatorial approach may be in the form of antibacterial therapy along with surgical procedures to provide an exact remedy for recurrence of stones.

In summary, hyperoxaluria condition was characterized by recurrent kidney stones episodes, found to have multiple oxalate-containing kidney stones. Whereas in Indian scenario urine volume were found to be less, and the crystalluria is may be the main culprits (Figure 2.8).

![Figure 2.8: Summarization of hyperoxaluria condition in the ‘case-control study’ subjects.](image)