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Mycotoxin Detection in Urine Samples from Patients with Chronic Kidney Disease of Uncertain Etiology in Sri Lanka

Biruck Desalegn\(^1\), Shanika Nanayakkara\(^1\), Kouji H. Harada\(^1\), Toshiaki Hitomi\(^1\), Rohana Chandrajith\(^2\), Upul Karunaratne\(^3\), Tilak Abeysekera\(^3\), and Akio Koizumi\(^1\)

for the Chronic Kidney Disease of Uncertain Etiology Consortium\(^4\)

\(^1\)Department of Health and Environmental Sciences, Kyoto University Graduate School of Medicine, Yoshida, Kyoto 606-8501, Japan
\(^2\)Department of Geology, Faculty of Science, University of Peradeniya, Sri Lanka
\(^3\)Nephrology Unit, Teaching Hospital, Kandy, Sri Lanka
\(^4\)A full list of members is provided in the supplementary note.

Correspondence to: Akio Koizumi M.D., Ph.D.
Department of Health and Environmental Sciences, Graduate School of Medicine, Kyoto University, Yoshida Konoe, Sakyo, Kyoto 606-8501, Japan
Tel: +81-75-753-4456; Fax: +81-75-753-4458
E-mail: Akio.Koizumi@z06.mbox.media.kyoto-u.ac.jp
Abstract

This was a screening study that aimed to determine the presence of nephrotoxic mycotoxins in urine samples from patients with chronic kidney disease of uncertain etiology (CKDue) in the North Central Province of Sri Lanka. The percentage detection of aflatoxins (AFLs), ochratoxins (OTs) and fumonisins in 31 patients were 61.29%, 93.5% and 19.4%, respectively. Geometric means of urinary AFLs and OTs were 30.93 ng/g Cr (creatinine) and 34.62 ng/g Cr in CKDue stage 1–2 patients and 84.12 ng/g Cr and 63.52 ng/g Cr in unaffected relatives of patients. In CKDue stage 3–5 patients, geometric means of urinary AFLs and OTs were 10.40 and 17.08 ng/g Cr, respectively. Non-affected relatives of patients (n=6) had comparable levels of these mycotoxins, but healthy Japanese individuals (n=4) had lower levels than in Sri Lanka. The higher detection rate of urinary OTs in Sri Lankans indicates that exposure is common in the region.

Keywords chronic kidney disease of uncertain etiology, Sri Lanka, urine sample, aflatoxin, ochratoxin, fumonisin
High prevalence of chronic kidney disease of uncertain etiology (CKDue) in the North Central Province of Sri Lanka has been reported. The disease predominantly affects male farming communities. Several hypotheses have been made to explain the causal associations between the high prevalence of the disease in the region and existing environmental factors (Chandrajith et al. 2010; Illeperuma et al. 2009).

Mycotoxins, such as aflatoxins (AFLs) (Glahn et al. 1994), ochratoxins (OTs) (Sauvant et al. 2005) and fumonisins (FBs) (Badria et al. 1996) are dietary contaminants that are known to possess nephrotoxicity. Detection of OT associated with the incidence of endemic nephropathy in some regions has been reported (Castegnaro et al. 2005; Domijan et al. 2009). A recent study by Wanigasuriya et al. (2008) has reported that the concentration of OT A in selected food items in the study region was low. Food analysis, in some instances, might not be sufficient to establish a relationship with occurrence of diseases because heterogeneity of toxin distribution over time, and even within a particular food product, casts doubt on the feasibility of sampling plans (Parson et al. 2007). In an attempt to overcome this problem and to validate the actual exposure, we screened urinary excretion levels of AFL, OTs and FBs in patients and their relatives living in a CKD endemic community.

Materials and Methods

Ethical approval for this study was obtained from the Ethical Committee of Kyoto University, Japan and the Ethical Review Committees of the Faculty of Medicine,
University of Peradeniya, Sri Lanka. The urine samples were originally collected at Medawachchiya and Girandrukotte, Sri Lanka in August 2009 (106 patients and 87 unaffected relatives of CKDue patients) and stored at –30°C in the Kyoto University Human Specimen Bank (Koizumi et al. 2009). A total of 41 urine samples, 31 from stage 1–5 CKDue patients, six from unaffected relatives, and four from healthy Japanese individuals as controls, were randomly selected from each stratum. Definition of CKD and further classification of the stages were made according to the Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines. Patients with a history and current treatment of diabetes mellitus, severe hypertension, urological disease of known etiology, glomerulonephritis, or snake bite were excluded. Creatinine concentration in urine sample was measured by enzyme assay using creatinine amidohydrolase (SRL, Tokyo, Japan).

Urine samples were thawed and centrifuged at 15,000 rpm for 10 min to remove any cellular debris, and the supernatant was used for the determination of mycotoxin level. One milliliter of urine was diluted with 3 mL PBS (pH 7.4). The mixed sample was directly passed through analyte-specific immunoaffinity columns (R-Biopharm AG, Darmstadt, Germany) at a flow rate of 1–2 drops/s. The column was washed with 20 mL PBS and air was passed through the column for 1 min. The bound mycotoxin was eluted with 3 mL methanol and the eluate was evaporated to dryness using a nitrogen evaporator. The residue was reconstituted with 100 μL 10% methanol in water, and analyzed for each mycotoxin with the specific competitive ELISA kits (RIDASCREEN FAST Mycotoxins; R-Biopharm AG) using a microplate spectrophotometer (infinite
M200 Pro; Tecan, Tokyo, Japan) at 450 nm. ELISA kits for AFL, OTs and FBs recognized aflatoxins B1, B2, G1, G2 and M1; ochratoxins A, B and C, and fumonisins B1, B2 and B3, respectively. External standards of different concentrations and all urine samples were run in duplicate.

Mean recovery (CV) of fortified samples was 79% (11) for AFLs, 105% (13) for OTs and 92% (15) for FBs. Detection limits were 0.005 ng/mL, 0.005 ng/mL and 0.035 ng/mL for AFLs, OTs and FBs, respectively. For values below the detection limit, half of the limit of detection value was assigned. Mycotoxin concentrations are presented in ng/mL and ng/g Cr (creatinine). Statistical significance of differences was tested by using non-parametric methods ($\chi^2$ test and Wilcoxon two-sample test; $P < 0.05$).

**Results and Discussion**

Study subjects comprised 20 men and 21 women (Table 1). The mean (range) age regardless of disease stage (31, stage 1–5) was 41.32 ± 15.55 (9–65) years, whereas that of unaffected relatives and Japanese controls was 20.67 (6–34) years and 45.25 (42–53) years, respectively.

Results of urinary AFL, OT and FB levels are shown in Table 2. The percentage detection of AFLs, OTs and FBs in patients was 61.29%, 93.5% and 19.4%, respectively. The detection rate of all mycotoxins in stage 1 disease was the highest. Disease stages were classified as early (stage 1 and 2) and late (stage 3–5) for examination of concentration differences during disease progression. Detection rates of AFLs in the early and late stages were 78.57% and 47.06%,
respectively ($\chi^2 = 9.323; P < 0.001$). OTs were detected in all of the urine samples from 14 patients with early stage disease, whereas the rate of detection at the late stage was 88.24% ($n = 17$) ($\chi^2 = 23.516, P < 0.001$). Both AFLs and OTs were detected in all of the relatives of CKDue patients, but only OTs were detected in the Japanese controls.

The highest AFL concentration in urine samples from CKDue patients was 0.8 ng/mL, whereas 90% of the samples had a concentration <0.044 ng/mL (397.1 ng/g Cr). The 90th percentile for OTs was 0.098 ng/mL (60.85 ng/g Cr). The geometric means of urinary AFLs and OTs were 0.033 ng/mL (30.93 ng/g Cr) and 0.037 ng/mL (34.62 ng/g Cr) in the early stage, and 0.008 ng/mL (10.40 ng/g Cr) and 0.012 ng/mL (17.08 ng/g Cr) in the late stage of the disease. Mean concentration difference for urinary OT level was observed between the early and late stages of the disease (Wilcoxon test, $P = 0.008$). In contrast, comparable concentrations of OTs and AFLs were also observed in the unaffected relatives of CKDue patients ($P > 0.05$ compared with all patients). Healthy Japanese individuals, however, had lower levels of OTs (0.007 ng/mL, 8.14 ng/g Cr) than Sri Lankan individuals had.

The small sample size of the control subjects and their characteristic differences with the patients limit the comparability of the results. However, the high detection frequency and urinary levels of OTs and AFLs among CKDue patients and their relatives demonstrated the potential human exposure in the region. Findings were also discussed in relation to similar studies in other countries (Table 3). The average AFL concentration in urine samples from
CKDue patients was markedly higher, by over an order of magnitude, than the level of 0.391 ng/g Cr in the Czech Republic (Malir et al. 2004). An FB exposure study in two Portuguese populations has shown no detectable level in urine samples (Silva et al. 2008) and in Mexico 75% detection frequency was observed (Gong et al. 2008), whereas some level of FBs was detected at the early stage of the disease in the present study.

Higher detection of OTs was observed compared with the 61% detection rate among healthy individuals in Hungary and 43% in the endemic nephropathy area in Croatia (Domijan et al. 2009), whereas the detection was comparable with the 88–97.8% in the endemic nephropathy region of Bulgaria (Castegnaro et al. 2005). Although the mean OT level in CKDue patients in our study was higher than the 0.007 ng/mL in Croatia (Domijan et al. 2009) and 0.013 ng/mL in Hungary (Fazekas et al. 2004), and was comparable to the 0.022 ng/mL in Portugal (Duarte et al. 2010), the urine concentration levels in half of our CKDue patients were <0.017 ng/mL (n = 15). The potential sources of exposure to OTs in the region need to be clarified.

Animal studies have demonstrated the possibility of higher concentrations of OT A in kidney tissues and low levels in the urine (Zepnik et al. 2003). Likewise, an increase in OT A intake in humans in the region of endemic nephropathy did not result in an immediate increase in its elimination (Castegnaro et al. 2005). OT A is characterized by high plasma protein binding potential, therefore, its removal efficiency might be low (Petzinger et al. 2000; Ringot et al. 2006), and it is possible that OT A accumulates in renal tissue. It is worth noting
that the cumulative effect of long-term consumption of products that contain low
levels of mycotoxins could contribute to a gradual deterioration of organ function.
This study is believed to be the first to determine the presence of AFLs, OTs and FBs in urine samples from CKDue patients and their relatives living in communities with CKDue. The higher detection rate of OTs in Sri Lanka has led to a working hypothesis that this mycotoxin could be common in the region, which corroborates the need for further exposure assessment, associated with disease occurrence.

Acknowledgments
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References
environmental implications. Environ Geochem Health. Doi:10.1007/s10653-010-9339-1


Table 1. Baseline characteristics of CKDue patients in Sri Lanka, 2009

<table>
<thead>
<tr>
<th>Disease stages</th>
<th>Sex male/female (Total)</th>
<th>Age (yr) mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1 (slight)</td>
<td>3/4</td>
<td>24.14 (9–40)</td>
</tr>
<tr>
<td>Stage 2 (mild)</td>
<td>6/1</td>
<td>48 (39–59)</td>
</tr>
<tr>
<td>Stage 1–2 (early stage)</td>
<td>9/5 (14)</td>
<td>36.07 ± 15.19†</td>
</tr>
<tr>
<td>Stage 3 (moderate)</td>
<td>3/3</td>
<td>41 (11–60)</td>
</tr>
<tr>
<td>Stage 4 (severe)</td>
<td>3/3</td>
<td>47.5 (35–58)</td>
</tr>
<tr>
<td>Stage 5 (end stage)</td>
<td>3/2</td>
<td>49.00 (30–65)</td>
</tr>
<tr>
<td>Stage 3–5 (late stage)</td>
<td>9/8 (17)</td>
<td>45.65 ± 14.90</td>
</tr>
<tr>
<td>Total (CKDue patients)</td>
<td>18/13</td>
<td>41.32 ± 15.55 (9–65)</td>
</tr>
<tr>
<td>Relatives of CKDue</td>
<td>2/4</td>
<td>20.67 (6–34)</td>
</tr>
<tr>
<td>Japanese controls</td>
<td>0/4</td>
<td>45.25 (42–53)</td>
</tr>
</tbody>
</table>
Table 2. Urine concentration of AFL, OT and FB in CKDue patients in Sri Lanka, 2009.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>AFL</th>
<th>OT</th>
<th>FB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/mL</td>
<td>ng/g Cr</td>
<td>ng/mL</td>
</tr>
<tr>
<td><strong>Stage 1 (n = 7)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (n&gt;MDL)</td>
<td>ND–0.800(6)</td>
<td>ND–734.00</td>
<td>0.013–0.360(7)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.359</td>
<td>230.21</td>
<td>0.044</td>
</tr>
<tr>
<td>GM</td>
<td>0.092</td>
<td>87.41</td>
<td>0.035</td>
</tr>
<tr>
<td><strong>Stage 2 (n = 7)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (n&gt;MDL)</td>
<td>ND–0.037 (5)</td>
<td>ND–53.05</td>
<td>0.006–0.058(6)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.018</td>
<td>19.58</td>
<td>0.085</td>
</tr>
<tr>
<td>GM</td>
<td>0.012</td>
<td>10.95</td>
<td>0.039</td>
</tr>
<tr>
<td><strong>Stage 1–2</strong></td>
<td>GM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (n&gt;MDL)</td>
<td>ND–0.039 (4)</td>
<td>ND–44.74</td>
<td>ND–0.028 (5)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.023</td>
<td>25.57</td>
<td>0.022</td>
</tr>
<tr>
<td>GM</td>
<td>0.022</td>
<td>18.75</td>
<td>0.016</td>
</tr>
<tr>
<td><strong>Stage 3 (n = 6)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (n&gt;MDL)</td>
<td>ND–0.800 (4)</td>
<td>ND–991.57</td>
<td>ND–0.019 (4)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.140</td>
<td>174.82</td>
<td>0.016</td>
</tr>
<tr>
<td>GM</td>
<td>0.009</td>
<td>12.71</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>Stage 4 (n = 6)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (n&gt;MDL)</td>
<td>ND</td>
<td>ND</td>
<td>0.010 (4)</td>
</tr>
<tr>
<td>Mean</td>
<td>-</td>
<td>-</td>
<td>0.044</td>
</tr>
<tr>
<td>GM</td>
<td>-</td>
<td>-</td>
<td>0.080</td>
</tr>
<tr>
<td><strong>Stage 5 (n = 5)</strong></td>
<td>GM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (n&gt;MDL)</td>
<td>ND</td>
<td>ND</td>
<td>0.005–0.012(4)</td>
</tr>
<tr>
<td>Mean</td>
<td>-</td>
<td>-</td>
<td>0.007</td>
</tr>
<tr>
<td>GM</td>
<td>-</td>
<td>-</td>
<td>0.007</td>
</tr>
</tbody>
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Relatives controls (n = 6)

| Range (n>MDL)          | 0.020–0.800 (6) | 5.9–1000.00 | 0.032–0.223 (6) | 28.63–278.00 | ND–0.093 (1) | ND–0.14 |
| Mean                   | 0.298 | 249.09 | 0.104 | 88.95 | ND–ND | ND–ND |
| GM                     | 0.112 | 84.12 | 0.085 | 63.52 | ND–ND | ND–ND |

Japanese controls (n = 4)

| Range (n>MDL)          | ND   | ND   | 0.005–0.012(4) | 4.4–19.40 | ND–ND | ND–ND |
| Mean                   | -    | -    | 0.007 | 9.69 | ND–ND | ND–ND |
| GM                     | -    | -    | 0.007 | 8.14 | ND–ND | ND–ND |

ND: not detected; MDL: method detection limit; GM: geometric mean
*Wilcoxon test for mean OT concentration difference between early and late stages (P = 0.008)
<table>
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<tr>
<th>Mycotoxin Type</th>
<th>Detection rate</th>
<th>Mean (range)</th>
<th>Study subjects</th>
<th>Country</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>AFL</td>
<td>61.29%</td>
<td>17.0 (ND–991.6) ng/gCr</td>
<td>CKDue patients</td>
<td>Sri Lanka</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>58%</td>
<td>391.0 (19.0–19,219.0) pg/g Cr</td>
<td>General population</td>
<td>Czech Republic</td>
<td>(Malir et al. 2004)</td>
</tr>
<tr>
<td>OT A</td>
<td>100%</td>
<td>37.1 (12.4–360.0) pg/mL</td>
<td>CKDue patients (early stage)</td>
<td>Sri Lanka</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>88.24%</td>
<td>12.0 (ND–58.2) pg/mL</td>
<td>CKDue patients (late stage)</td>
<td>Sri Lanka</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>100% (n=6)</td>
<td>85.0 (32.0–223.0) pg/mL</td>
<td>Relatives of CKDue patients</td>
<td>Sri Lanka</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>61%</td>
<td>13.0 (6.0–65.0) pg/mL</td>
<td>Healthy individuals</td>
<td>Hungary</td>
<td>(Fazekas et al. 2004)</td>
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<td></td>
<td>43%</td>
<td>7.0 (5.0–15.0) pg/mL</td>
<td>Endemic nephropathy</td>
<td>Croatia</td>
<td>(Domijan et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>92.20%</td>
<td>22.0 (ND–69.0) pg/mL</td>
<td>General population</td>
<td>Portugal</td>
<td>(Duarte et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>88%</td>
<td>50.8 (1.0–330.0) pg/mL</td>
<td>Endemic nephropathy</td>
<td>Bulgaria</td>
<td>(Castegnaro et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>97.6%</td>
<td>191.7 (1.0–191.0) pg/mL</td>
<td>Endemic nephropathy</td>
<td>Bulgaria</td>
<td>(Castegnaro et al. 2005)</td>
</tr>
<tr>
<td>FB</td>
<td>19.4%</td>
<td>(ND–130.0 pg/mL)</td>
<td>CKDue patients</td>
<td>Sri Lanka</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>0% (LOD = 5 ng/mL)</td>
<td>General population</td>
<td>Portugal</td>
<td>(Silva et al. 2008)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>75%</td>
<td>70.1 (ND–9312.0) pg/mL</td>
<td>General population</td>
<td>Mexico</td>
<td>(Gong et al. 2008)</td>
</tr>
</tbody>
</table>