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

3

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20

21 **Abstract**

22 This was a screening study that aimed to determine the presence of nephrotoxic
23 mycotoxins in urine samples from patients with chronic kidney disease of
24 uncertain etiology (CKDue) in the North Central Province of Sri Lanka. The
25 percentage detection of aflatoxins (AFLs), ochratoxins (OTs) and fumonisins in
26 31 patients were 61.29%, 93.5% and 19.4%, respectively. Geometric means of
27 urinary AFLs and OTs were 30.93 ng/g Cr (creatinine) and 34.62 ng/g Cr in
28 CKDue stage 1–2 patients and 84.12 ng/g Cr and 63.52 ng/g Cr in unaffected
29 relatives of patients. In CKDue stage 3–5 patients, geometric means of urinary
30 AFLs and OTs were 10.40 and 17.08 ng/g Cr, respectively. Non-affected relatives
31 of patients ($n=6$) had comparable levels of these mycotoxins, but healthy Japanese
32 individuals ($n=4$) had lower levels than in Sri Lanka. The higher detection rate of
33 urinary OTs in Sri Lankans indicates that exposure is common in the region.

34 **Keywords** chronic kidney disease of uncertain etiology, Sri Lanka, urine sample,
35 aflatoxin, ochratoxin, fumonisin

36

37 High prevalence of chronic kidney disease of uncertain etiology (CKDue) in the
38 North Central Province of Sri Lanka has been reported. The disease
39 predominantly affects male farming communities. Several hypotheses have been
40 made to explain the causal associations between the high prevalence of the disease
41 in the region and existing environmental factors (Chandrajith et al. 2010;
42 Illeperuma et al. 2009).

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

56

57 **Materials and Methods**

58 Ethical approval for this study was obtained from the Ethical Committee of Kyoto
59 University, Japan and the Ethical Review Committees of the Faculty of Medicine,

60 University of Peradeniya, Sri Lanka. The urine samples were originally collected
61 at Medawachchiya and Girandrukotte, Sri Lanka in August 2009 (106 patients and
62 87 unaffected relatives of CKD patients) and stored at -30°C in the Kyoto
63 University Human Specimen Bank (Koizumi et al. 2009). A total of 41 urine
64 samples, 31 from stage 1–5 CKD patients, six from unaffected relatives, and
65 four from healthy Japanese individuals as controls, were randomly selected from
66 each stratum. Definition of CKD and further classification of the stages were
67 made according to the Kidney Disease Outcomes Quality Initiative (KDOQI)
68 guidelines. Patients with a history and current treatment of diabetes mellitus,
69 severe hypertension, urological disease of known etiology, glomerulonephritis, or
70 snake bite were excluded. Creatinine concentration in urine sample was measured
71 by enzyme assay using creatinine amidohydrolase (SRL, Tokyo, Japan).

72 Urine samples were thawed and centrifuged at 15,000 rpm for 10 min to
73 remove any cellular debris, and the supernatant was used for the determination of
74 mycotoxin level. One milliliter of urine was diluted with 3 mL PBS (pH 7.4). The
75 mixed sample was directly passed through analyte-specific immunoaffinity
76 columns (R-Biopharm AG, Darmstadt, Germany) at a flow rate of 1–2 drops/s.
77 The column was washed with 20 mL PBS and air was passed through the column
78 for 1 min. The bound mycotoxin was eluted with 3 mL methanol and the eluate
79 was evaporated to dryness using a nitrogen evaporator. The residue was
80 reconstituted with 100 μL 10% methanol in water, and analyzed for each
81 mycotoxin with the specific competitive ELISA kits (RIDASCREEN FAST
82 Mycotoxins; R-Biopharm AG) using a microplate spectrophotometer (infinite

83 M200 Pro; Tecan, Tokyo, Japan) at 450 nm. ELISA kits for AFL, OTs and FBs
84 recognized aflatoxins B1, B2, G1, G2 and M1; ochratoxins A, B and C, and
85 fumonisins B1, B2 and B3, respectively. External standards of different
86 concentrations and all urine samples were run in duplicate.

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

94

95 **Results and Discussion**

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

100 Results of urinary AFL, OT and FB levels are shown in Table 2. The
101 percentage detection of AFLs, OTs and FBs in patients was 61.29%, 93.5% and
102 19.4%, respectively. The detection rate of all mycotoxins in stage 1 disease was
103 the highest. Disease stages were classified as early (stage 1 and 2) and late (stage
104 3–5) for examination of concentration differences during disease progression.
105 Detection rates of AFLs in the early and late stages were 78.57% and 47.06%,

106 respectively ($\chi^2 = 9.323$; $P < 0.001$). OTs were detected in all of the urine samples
107 from 14 patients with early stage disease, whereas the rate of detection at the late
108 stage was 88.24% ($n = 17$) ($\chi^2 = 23.516$, $P < 0.001$). Both AFLs and OTs were
109 detected in all of the relatives of CKD patients, but only OTs were detected in
110 the Japanese controls.

111 The highest AFL concentration in urine samples from CKD patients was
112 0.8 ng/mL, whereas 90% of the samples had a concentration < 0.044 ng/mL (397.1
113 ng/g Cr). The 90th percentile for OTs was 0.098 ng/mL (60.85 ng/g Cr). The
114 geometric means of urinary AFLs and OTs were 0.033 ng/mL (30.93 ng/g Cr) and
115 0.037 ng/mL (34.62 ng/g Cr) in the early stage, and 0.008 ng/mL (10.40 ng/g Cr)
116 and 0.012 ng/mL (17.08 ng/g Cr) in the late stage of the disease. Mean
117 concentration difference for urinary OT level was observed between the early and
118 late stages of the disease (Wilcoxon test, $P = 0.008$). In contrast, comparable
119 concentrations of OTs and AFLs were also observed in the unaffected relatives of
120 CKD patients ($P > 0.05$ compared with all patients). Healthy Japanese
121 individuals, however, had lower levels of OTs (0.007 ng/mL, 8.14 ng/g Cr) than
122 Sri Lankan individuals had.

123 The small sample size of the control subjects and their characteristic
124 differences with the patients limit the comparability of the results. However, the
125 high detection frequency and urinary levels of OTs and AFLs among CKD
126 patients and their relatives demonstrated the potential human exposure in the
127 region. Findings were also discussed in relation to similar studies in other
128 countries (Table 3). The average AFL concentration in urine samples from

129 CKD patients was markedly higher, by over an order of magnitude, than the
130 level of 0.391 ng/g Cr in the Czech Republic (Malir et al. 2004). An FB exposure
131 study in two Portuguese populations has shown no detectable level in urine
132 samples (Silva et al. 2008) and in Mexico 75% detection frequency was observed
133 (Gong et al. 2008), whereas some level of FBs was detected at the early stage of
134 the disease in the present study.

135 Higher detection of OTs was observed compared with the 61% detection
136 rate among healthy individuals in Hungary and 43% in the endemic nephropathy
137 area in Croatia (Domijan et al. 2009), whereas the detection was comparable with
138 the 88–97.8% in the endemic nephropathy region of Bulgaria (Castegnaro et al.
139 2005). Although the mean OT level in CKD patients in our study was higher
140 than the 0.007 ng/mL in Croatia (Domijan et al. 2009) and 0.013 ng/mL in
141 Hungary (Fazekas et al. 2004), and was comparable to the 0.022 ng/mL in
142 Portugal (Duarte et al. 2010), the urine concentration levels in half of our CKD
143 patients were <0.017 ng/mL ($n = 15$). The potential sources of exposure to OTs in
144 the region need to be clarified.

145 Animal studies have demonstrated the possibility of higher concentrations
146 of OT A in kidney tissues and low levels in the urine (Zepnik et al. 2003).
147 Likewise, an increase in OT A intake in humans in the region of endemic
148 nephropathy did not result in an immediate increase in its elimination (Castegnaro
149 et al. 2005). OT A is characterized by high plasma protein binding potential,
150 therefore, its removal efficiency might be low (Petzinger et al. 2000; Ringot et al.
151 2006), and it is possible that OT A accumulates in renal tissue. It is worth noting

152 that the cumulative effect of long-term consumption of products that contain low
153 levels of mycotoxins could contribute to a gradual deterioration of organ function.

154 This study is believed to be the first to determine the presence of AFLs,
155 OTs and FBs in urine samples from CKD patients and their relatives living in
156 communities with CKD. The higher detection rate of OTs in Sri Lanka has led
157 to a working hypothesis that this mycotoxin could be common in the region,
158 which corroborates the need for further exposure assessment, associated with
159 disease occurrence.

160

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227

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

Disease stages	Sex	Age (yr)
	male/female (Total)	mean (range)
Stage 1 (slight)	3/4	24.14 (9–40)
Stage 2 (mild)	6/1	48 (39–59)
Stage 1–2 (early stage)	9/5 (14)	36.07 ± 15.19 [‡]
Stage 3 (moderate)	3/3	41 (11–60)
Stage 4 (severe)	3/3	47.5 (35–58)
Stage 5 (end stage)	3/2	49.00 (30–65)
Stage 3–5 (late stage)	9/8 (17)	45.65 ± 14.90
Total (CKDue patients)	18/13	41.32 ± 15.55 (9–65)
Relatives of CKDue patients	2/4	20.67 (6–34)
Japanese controls	0/4	45.25 (42–53)

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Table 2. Urine concentration of AFL, OT and FB in CKD patients in Sri Lanka, 2009.

Subjects		AFL		OT		FB	
		ng/mL	ng/g Cr	ng/mL	ng/g Cr	ng/mL	μg/g Cr
Stage 1 (<i>n</i> = 7)	Range (n>MDL)	ND–0.800(6)	ND–734.00	0.013–0.360 (7)	17.63–93.90	ND–0.042 (4)	ND–0.14
	Mean	0.359	230.21	0.044	39.67	<MDL	<MDL
	GM	0.092	87.41	0.035	33.33	<MDL	<MDL
Stage 2 (<i>n</i> = 7)	Range (n>MDL)	ND–0.037 (5)	ND–53.05	0.006–0.058 (6)	11.87–74.81	ND–0.036 (1)	ND–0.07
	Mean	0.018	19.58	0.085	65.07	-	-
	GM	0.012	10.95	0.039	35.95	-	-
Stage 1–2	GM	0.033	30.93	0.037	34.62*	-	-
Stage 3 (<i>n</i> = 6)	Range (n>MDL)	ND–0.039 (4)	ND–44.74	ND–0.028 (5)	8.57–41.25	ND–0.130 (1)	ND–0.19
	Mean	0.023	25.57	0.022	21.76	-	-
	GM	0.022	18.75	0.016	19.36	-	-
Stage 4 (<i>n</i> = 6)	Range (n>MDL)	ND–0.800 (4)	ND–991.57	ND–0.019 (4)	ND–34.27	-	-
	Mean	0.140	174.82	0.016	18.75	ND	ND
	GM	0.009	12.71	0.012	17.07	-	-
Stage 5 (<i>n</i> = 5)	Range (n>MDL)	ND	ND	0.010 (4)	ND–27.06	ND	ND
	Mean	-	-	0.044	16.56	-	-
	GM	-	-	0.080	14.72	-	-
Stage 3–5	GM	0.008	10.40	0.012	17.08*	-	-
Stage 1–5	GM	0.012	17.01	0.020	23.50	-	-
Relatives controls (<i>n</i> = 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	-	-
	GM	0.112	84.12	0.085	63.52	-	-
Japanese controls (<i>n</i> = 4)	Range (n>MDL)	ND	ND	0.005–0.012 (4)	4.4–19.40	ND	ND
	Mean	-	-	0.007	9.69	-	-
	GM	-	-	0.007	8.14	-	-

233 ND: not detected; MDL: method detection limit; GM: geometric mean

234 *Wilcoxon test for mean OT concentration difference between early and late stages ($P = 0.008$)

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239 **Table 3.** Urine mycotoxin level in other countries

Mycotoxin Type	Detection rate	Mean (range) ng/gCr	Study subjects	Country	Reference
AFL	61.29%	17.0 (ND–991.6)	CKD patients	Sri Lanka	Present study
	58%	391.0 (19.0–19,219.0) pg/g Cr	General population	Czech Republic	(Malir et al. 2004)
OT A	100%	37.1 (12.4–360.0) pg/mL	CKD patients (early stage)	Sri Lanka	Present study
	88.24%	12.0 (ND–58.2) pg/mL	CKD patients (late stage)	Sri Lanka	Present study
	100%(n=6)	85.0 (32.0–223.0) pg/mL	Relatives of CKD patients	Sri Lanka	Present study
	61%	13.0 (6.0–65.0) pg/mL	Healthy individuals	Hungary	(Fazekas et al. 2004)
	43%	7.0 (5.0–15.0) pg/mL	Endemic nephropathy	Croatia	(Domijan et al. 2009)
	92.20%	22.0 (ND–69.0) pg/mL	General population	Portugal	(Duarte et al. 2010)
	88%	50.8 (1.0–330.0) pg/mL	Endemic nephropathy	Bulgaria	(Castegnaro et al. 2005)
	97.6%	191.7 (1.0–191.0) pg/mL	Endemic nephropathy	Bulgaria	(Castegnaro et al. 2005)
	19.4%	(ND–130.0 pg/mL)	CKD patients	Sri Lanka	Present study
FB	0% (LOD = 5 ng/mL)		General population	Portugal	(Silva et al. 2008)
	75%	70.1 (ND–9312.0) pg/mL	General population	Mexico	(Gong et al. 2008)

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