ABSTRACT: The present study was conducted to 1) identify the natural source of feed contamination by zearalenone (ZEN), which was suspected to have caused persistently increased urinary ZEN concentrations in one of our experimental cattle herds, and 2) evaluate the effects of intervention against this source of contamination. As an experimental model, a fattening Japanese Black cattle herd showing persistently increased urinary ZEN concentrations was identified. Urinary ZEN concentrations of cows fed with new rice straw (experimental group, n = 6) vs. cows that continued to feed on the old rice straw (control group, n = 4) were measured at the start (d 1) and at 2 wk (d 14) after the onset of feeding with straw. In addition, the ZEN concentration in feed and water samples was measured by using both the ELISA and HPLC methods. Furthermore, isolation and identification of fungi from rice straw and concentrate feed samples were performed. The urinary ZEN concentration [ZEN (pg/mL)/creatinine (mg/mL) = pg/mg of creatinine] of cows fed with new rice straw was significantly (P < 0.05) less (843 pg/mg of creatinine) than that of cows fed with old rice straw (15,951 pg/mg of creatinine). On both d 1 and 14, the ZEN concentrations of old rice straw were greater than those of new rice straw. In addition, fungal colonies were observed in the culture media that was obtained from the old rice straw suspected of ZEN contamination, but not in the culture media from new rice straw or other feed samples. In conclusion, our field trials clearly indicate that the rice straw fed to the cows was naturally contaminated with ZEN, and that the monitoring of urinary ZEN concentrations could prove to be a useful tool for detecting the exposure of cattle to ZEN contamination at the farm level.

Keywords: cattle, rice straw, urine, zearalenone

INTRODUCTION

Zearalenone (ZEN) is a nonsteroidal estrogenic mycotoxin produced by Fusarium spp. growing on wheat, barley, maize, rice, and so on. Zearalenone and its metabolites exhibit distinct estrogenic and anabolic properties that affect the reproductive systems of several animals (Kleinova et al., 2002; Fink-Gremmels and Malekinejad, 2007; Minervini and Dell’Aquila, 2008).

Previous studies have suggested that ZEN, along with other Fusarium toxins, may be present in animal feeds (Avantaggiato et al., 2007; Binder et al., 2007). In farm animals, the concentration of ZEN and its metabolites in urine may be used as potential biomarkers of ZEN exposure (Prelusky et al., 1989; Usleber et al., 1992; Kleinova et al., 2002).

Recently, we reported that urinary concentrations of ZEN and its metabolites could be used to monitor the natural ZEN contamination of cattle feed in farms (Takagi et al., 2011). We incidentally detected a fattening cattle herd that repeatedly exhibited significantly greater urinary ZEN concentrations than 3 other fattening herds. We suspected that the feed might have been the source of this difference; however, because we
Animals and Sample Collection

Two herds (herd 1 and 2) of Japanese Black cows, kept for fattening in southern Japan, were included in this study. Herd 1 was composed of 700 cattle and persistently showed significantly greater mean urinary ZEN concentrations than 7 other cattle herds that were studied in our previous report (Takagi et al., 2011). Herd 2 was composed of 120 cattle, which were fed with the same lot of concentrate purchased from the same company as herd 1, but with different straw and water. The content of the feed given to each herd is detailed in Table 1. We first conducted a preliminary examination to reconfirm the urinary ZEN concentrations of the 2 herds by assessing the urine samples of 5 randomly selected cows from each herd, in addition to assessing the ZEN concentrations of the feed and water samples from both herds by ELISA. The results of this preliminary study confirmed that herd 1 contained persistently increased urinary ZEN concentrations, with all urinary samples of herd 1 and only 2 samples from herd 2 showing values above the standard maximum range (4,050 pg of ZEN/mg) of the ELISA kit. In addition, the ZEN concentrations of the rice straw fed to herd 1 were above the standard maximum range values. Therefore, the rice straw fed to the cows of herd 1 was suspected to be the source of the increased urinary ZEN concentrations.

Based on the preliminary results, we designed a field study to clarify the source of ZEN contamination. For this purpose, cows of herd 1 were allocated either to a control group (n = 4) or to an experimental group (n = 6). All cows of control group continued feeding on the old rice straw that was suspected for ZEN contamination, whereas the cows of the experimental group were fed new rice straw. The urinary samples were collected from these cows by massaging the pudendum at 2 h after the morning feeding. The urinary ZEN concentrations of cows of the experimental group were evaluated before the replacement of the rice straw (d 1) with a new straw lot and repeated at 2 wk (d 14) after replacement by ELISA. Similarly, the urinary ZEN concentration of cows of control group both at d 1 and 14 were evaluated. In addition, approximately 1 kg of rice straw and concentrate feeds, and 50 mL of water samples were obtained to measure the ZEN concentrations. All samples were immediately placed in a cool box, for protection from the light, and transported to the laboratory. After centrifugation at 500 × g for 10 min at room temperature, both urine and water samples were frozen at −30°C, until the analysis of ZEN or creatinine (Crea) concentrations. The feed samples were frozen at −30°C until the analysis of ZEN concentrations and culturing for the detection of fungi.

The general health status of the cattle, such as abnormalities in respiration, appetite, and fecal consistency, was monitored daily throughout the entire experimental period by experienced farm staff. In addition, a veterinarian visited the herd once a day and recorded the general health status and any abnormalities, using previously reported criteria (Matsumoto et al., 2009).

Analysis of ZEN and Crea in Both Urinary and Water Samples

Chemicals and Solvents. Ammonium acetate, HPLC-grade methanol, and acetonitrile were purchased from Wako Pure Chemicals (Osaka, Japan). β-Glucuronidase/arylsulfatase solution was purchased from Merck (Darmstadt, Germany). Sodium acetate was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan), and Tris was purchased from Nakalai Tesque Inc. (Kyoto, Japan). Water for the HPLC assay was purified using a Milli-Q system (Nihon Millipore K.K., Tokyo, Japan).

Table 1. Composition of feed provided to the 2 cattle herds that were kept for fattening purposes (as-fed basis)

<table>
<thead>
<tr>
<th>Herd</th>
<th>Forage feed, kg</th>
<th>Formula feed</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total, kg</td>
<td>Bran, %</td>
<td>Cereal, %</td>
<td>Oil seed meal, %</td>
<td>Other, %</td>
<td>TDN, %</td>
<td>CP, %</td>
</tr>
<tr>
<td>Herd 1</td>
<td>Straw, 2.0</td>
<td>9.0</td>
<td>17</td>
<td>77</td>
<td>5</td>
<td>1</td>
<td>&gt;75</td>
</tr>
<tr>
<td>Herd 2 (control)</td>
<td>Straw, 4.0</td>
<td>9.0</td>
<td>17</td>
<td>77</td>
<td>5</td>
<td>1</td>
<td>&gt;75</td>
</tr>
</tbody>
</table>
Zearalenone concentrations of the urine and water samples were determined by using a commercially available kit (RIDASCREEN Zearalenon, R-Biopharm AG, Darmstadt, Germany), with minor modifications to the manufacturer’s instructions as previously reported (Takagi et al., 2011). In brief, the urine or water samples (0.5 mL) were mixed with 3 mL of 50 mM sodium acetate buffer (pH 4.8). Then, the solution was incubated for 15 h at 37°C in the presence of 8 μL of β-glucuronidase/arylsulfatase solution. Subsequently, the samples were loaded to a C18 solid-phase extraction column (Strata, Phenomenex, Torrance, CA), which had been preconditioned with 3 mL of methanol, followed by 2 mL of 20 mM Tris buffer (pH 8.5)/methanol (80:20). After washing the solid-phase extraction column with 2 mL of 20 mM Tris buffer (pH 8.5)/methanol (80:20) and 3 mL of methanol (40%), the column was centrifuged at room temperature for 10 min at 500 × g to dry the column. Then, the analytes were slowly eluted (flow rate: 15 drops/min) with 1 mL of methanol (80%). The eluate was evaporated at 60°C until it was dry by using a centrifugation evaporator. The dried residue was redissolved with 50 μL of methanol; then, 450 μL of sample dilution buffer was added. The solution was mixed thoroughly, and an aliquot of 50 μL was used for the ELISA assay. To determine ZEN concentrations in the urine or water samples, RIDA SOFT Win (R-Biopharm) was used to calculate the absorbance at 450 nm with a microplate spectrophotometer. Based on the 3 trials, the mean recovery rate of ZEN in the ELISA assay was 84 ± 14%.

Urinary Crea concentrations were determined by using a commercial kit (Sikarikit-S CRE, Kanto Chemical Co. Inc.) according to the manufacturer’s instructions, and measured with a clinical autoanalyzer (7700 Clinical Analyzer, Hitachi High-Tech, Tokyo, Japan). In the present study, urinary ZEN concentrations were expressed as their ratio to urinary Crea (urinary ZEN/urinary Crea [pg/mg of Crea]) as described previously (Miles et al., 1996; Padilla et al., 2007).

**Analysis of ZEN in Feed Samples.** First, the concentration of ZEN was measured in the feed samples by using a commercially available kit (RIDASCREEN Zearalenon, R-Biopharm AG, Darmstadt, Germany) for screening purposes, with minor modifications to the manufacturer’s instructions. Briefly, representative rice straw and concentrate feed samples were obtained from the farms. Then, the rice straw was chopped into small pieces, after which the rice straw and feed were homogenized. A total of 5 g of aliquot samples were placed in a 50-mL sample tube, to which 25 mL of 70% MeOH was added. The samples were then vortexed and stored overnight in the dark at room temperature. Then, the sample solutions were filtered with filter paper (filter paper 5A, Advantec, Tokyo, Japan), and the filtrate was used to determine the ZEN concentration by using ELISA. Because the ZEN concentration of rice straw derived from herd 1 showed above-range ELISA values in the preliminary assay, the sample solutions were diluted 32-fold with distilled water before the ELISA test.

To confirm the concentrations of ZEN in the feed samples measured by ELISA, the same samples were reanalyzed using HPLC with fluorescence detection, according to the methods of Emoto et al. (2008). Briefly, a sample of 5 g of each feed was extracted in 80 mL of methanol-water (70:30, vol/vol) by vortexing vigorously for 30 min. The extract was filtered with filter paper (Advantec No. 5c), and the supernatant was diluted 5-fold with 0.01 M PBS (pH 7.2). After filtering again with a glass-fiber filter, the solution (10 mL in total) was loaded onto an immunoaffinity column (IAC), which was preconditioned with 10 mL of 0.01 M PBS (pH 7.2), followed by the addition of 10 mL of 0.01 M PBS (pH 7.2) for washing the IAC. Zearalenone was eluted from the IAC with 3 mL of MeOH, and the eluates were evaporated to dryness. The residues were redissolved in 1 mL of acetonitrile-water (40:60, vol/vol). Next, 20 μL of the solution was injected into the HPLC system.

Analyses were performed on the HPLC system, as reported by Emoto et al. (2008). Briefly, chromatographic separation was achieved on a Capcell Pak C8 (4.6 ID × 250 mm, 5 μm; Shiseudo, Tokyo, Japan) at 40°C. The mobile phase consisted of acetonitrile-water (40:60, vol/vol), with an isocratic elution flow rate of 1 mL/min. The fluorescence detector was set to an excitation wavelength of 274 nm and an emission wavelength of 440 nm. Quantification of ZEN in the sample solution was performed by using a calibration curve. The mean recovery rates for ZEN were 94 to 102%.

**Fungal Isolation and Conidiation.** Both the rice straw and concentrate samples from the herds were examined in a preliminary study using Sabouraud agar, and incubated at 25°C for 7 d. Fungal isolation was only conducted from the infected rice straw samples selected from herd 1. The rice straw (5-mm² fragment) was soaked in 1% sodium hypochlorite for 1 min, rinsed 3 times in sterile water, plated on the surface of water agar, and incubated at 25°C for 3 d in the dark. A hyphal tip was then transferred to a potato sucrose agar. Colonies showing typical morphologies for *Fusarium graminearum* species complex on potato sucrose agar, such as purple-red pigmentation and fast growth, were used for conidiation. First, mycelial discs of the colonies were placed onto oatmeal agar plates and incubated at 25°C for 5 d in the dark. Then, the aerial hyphae grown on the plates were removed with a brush, and the plates were further incubated at 25°C for 2 d under black light. The presence or absence in the formation of macroco-
nidia was observed under a light microscope (Nikon Labophot, Tokyo, Japan) at 300× magnification.

Statistical Analysis

Urinary ZEN concentrations were expressed as mean ± SEM. The urinary ZEN concentrations, conducted on both d 1 and 14, were analyzed with a paired t-test, using SPSS (IBM version 16.0, SPSS Inc., Chicago, IL) statistics software (IBM). The probability values less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

The effects of the intervention on the herd fed with a ZEN noncontaminated diet were evaluated by monitoring the urinary ZEN concentrations of the herd. Because the urinary ZEN concentrations derived from herd 1 showed above-range ELISA values in the preliminary study, the sample solutions were diluted 100-fold with distilled water before the ELISA test. As shown in Table 2, the urinary ZEN concentration of the examined group was 15,951 ± 4,178 (pg/mg of Crea) with the old straw (ZEN concentration of the rice straw was estimated to be more than 4,536 ng/g from ELISA and 7,555 ng/g from HPLC). However, after 14 d of feeding the cows with the new uncontaminated rice straw, the urinary ZEN concentration was significantly reduced to 843 ± 67 (pg/mg of Crea; P < 0.05). In contrast, the urinary ZEN concentrations of the control group from the same herd that continued to feed on the original rice straw lot did not exhibit any reduction in ZEN concentration on d 14, consistently showing extremely high urinary ZEN concentrations.

Fungal colonies were observed in the culture media obtained from the rice straw samples of herd 1, but not in the culture media from the other feed samples of both herds (Figures 1a and 1b). To confirm the presence of fungi in the rice straw, we attempted to isolate the F. graminearum species complex from the rice straw that was used as fodder in herd 1 (Figure 1c). The formation of macroconidia on oatmeal agar showed typical characteristics for the F. graminearum species complex: hyaline, falcate with single foot cells, and 3 to 5 septa (Figure 1d). This observation indicated that the mycotoxins extracted in the preceding analyses were derived from the fungi.

Although the overall general health status between the 2 groups was nonsignificant during the 2-wk period, the examined group showed an improvement in the physical appearance of the buttocks (i.e., fecal discharge remaining on the buttocks) and fecal consistency after feeding with straw that was not contaminated with ZEN (Figures 2a and 2b).

DISCUSSION

We previously reported that the use of commercially available ZEN kits to monitor urinary ZEN concentrations from a small sample volume (0.5 mL) appears to be a useful assessment tool for predicting the exposure of animals to ZEN at the farm level (Takagi et al., 2011). In the previous report, our use of the urinary ZEN monitoring system led to the unexpected detection of a cattle herd with possible mycotoxicosis, showing unusually increased urinary ZEN concentrations. However, our previous study did not evaluate whether the ZEN concentrations of contaminated feeds were above-range because the amount of ZEN in the feed was not known. The results of the present investigation conducted on herd 1 clearly indicated that the cause of the extremely high urinary ZEN concentration was derived from rice straw that was infected with a F. graminearum species complex, resulting from the natural contamination of rice straw by ZEN. Apart from the concerns of mycotoxicosis occurring in concentrate feeds, such as cereals and grains, awareness must be raised that mycotoxicosis may also occur in forage matter, such as grass silage, hay, and straw, because these materials contain increased proportions of mycotoxin ingredients (Binder et al., 2007). To our knowledge, this is the first report indicating a direct relationship of fungal isolation and ZEN detection from the rice straw used as forage for cows, with respect to urinary ZEN concentration in cattle herds at the farm level.

Table 2. Results of zearalenone (ZEN) concentrations in straw, concentrate, and urinary samples before and after the replacement of rice straw in herd 1

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Analytical Method</th>
<th>Day 1</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New straw, ng/g</td>
<td>ELISA</td>
<td>33.7</td>
<td>46.0</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Concentrate, ng/g</td>
<td>ELISA</td>
<td>23.3</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>138.1</td>
<td>86.5</td>
</tr>
<tr>
<td>Urine, pg/mg of creatinine (Crea; n = 6)</td>
<td>ELISA</td>
<td>15,951 ± 4,178&lt;sup&gt;a&lt;/sup&gt;</td>
<td>843 ± 67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old straw, ng/g</td>
<td>ELISA</td>
<td>&gt;4,536&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;4,536&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>7,555</td>
<td>7,223</td>
</tr>
<tr>
<td>Concentrate, ng/g</td>
<td>ELISA</td>
<td>23.3</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>138.1</td>
<td>86.5</td>
</tr>
<tr>
<td>Urine, pg/mg of Crea (n = 4)</td>
<td>ELISA</td>
<td>20,555 ± 2,808</td>
<td>22,300 ± 2,949</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values with different superscripts in the same column differ significantly (P < 0.05).

<sup>1</sup>Experimental group: cattle fed with the new straw lot (noncontaminated rice straw) as roughage from d 1 to 14. Control group: cattle fed with previously old straw as roughage from d 1 to 14.

<sup>2</sup>ND = not detected.

<sup>3</sup>Zearalenone concentrations in the straw measured by using ELISA exceeded the standard maximum range values of the kit.
In the present study, we evaluated dietary ZEN concentrations by using both modified ELISA and HPLC assays. Whereas cross-validation analysis of ZEN concentrations from the same feed samples using both ELISA and HPLC assays was not conducted in the present study, the concentrations derived from both analyzes were similar. Thus, the results of both the ELISA and HPLC methods show a direct relationship between the 2 methods. This outcome could further validate the effectiveness of the commercially available ELISA kit for the concomitant monitoring of ZEN concentrations in urine and feed. Moreover, the concentration of urinary ZEN in individual cattle from herd 1 indicates that ZEN is likely to be uniformly distributed within the rice

Figure 1. a) Sabouraud agar culture with colonies showing the typical pink color of zearalenone (ZEN)-contaminated rice straw, derived from herd 1. b) Sabouraud agar culture without colonies showing ZEN noncontaminated new rice straw from herd 1. c) Potato sucrose agar with colonies showing typical purple-red pigmentation morphologies for *Fusarium graminearum* species complex. d) Formation of macroconidia on oatmeal agar showing the typical characteristics of *F. graminearum* species complex.

Figure 2. Physical appearance of the cow buttocks in herd 1 (a) after being fed zearalenone-(ZEN) contaminated straw and (b) after being fed ZEN noncontaminated straw during the 2-wk study period. Color version available in the online PDF.
straw, because the urinary ZEN/Crea ratio was similar throughout the herd.

Ruminants are known to have relatively less sensitivity to ZEN exposure than nonruminant animals, because the presystemic elimination of ZEN by their microbial rumen flora seems to reduce the internal dose of ZEN (Seeling et al., 2005; Fink-Gremmels and Malekinejad, 2007). Rumen acidosis occurs in beef and dairy cattle fed for high production, resulting in rumen stasis, and the destruction of a large percentage of the normal rumen microflora (Kersting and Thompson, 1999). Thus, in our previous report (Takagi et al., 2011), we suggested microflora failure in the rumen as one possible cause of increased urinary ZEN concentrations in herd 1. Although this previous speculation about the original cause of increased urinary ZEN concentrations may be rejected based on the results of the present study, we observed a dramatic improvement in the physical appearance and fecal consistency of the cows after replacing the rice straw. These results lend support to the possible occurrence of rumen stasis, and the destruction of a large percentage of the normal rumen microflora, as a result of extremely high ZEN concentrations. Moreover, various reports describe the co-occurrence of ZEN with other Fusarium toxins, particularly deoxynivalenol (DON) in animal feeds (Avantaggiato et al., 2007; Richard, 2007). It is well known that DON leads to symptoms such as vomiting, diarrhea, reduced BW gain and feed intake, and immunosuppression (Rotter and Prelusky, 1996). Although ZEN is only known as a nonsteroidal estrogenic mycotoxin, it has been recently reported that feeds naturally contaminated with Fusarium mycotoxins could affect metabolic variables and immunity in dairy cows, especially through the effect of DON contamination (Korosteleva et al., 2007, 2009). Therefore, one possible reason for the significant changes in the general physical appearance of cattle after the replacement of rice straw is the effects of DON on ZEN-contaminated rice straw, which was not measured in our present study. Further investigations are required to clarify this hypothesis in conventional farms. Hence, we plan to measure both ZEN and DON in urinary samples by using liquid chromatography coupled with tandem mass spectrometry on the same herd, using the herd as a practical example of a contaminated model.

In conclusion, our field study identified rice straw as the original cause of extremely high urinary ZEN concentrations in the cows of herd 1. Binder et al. (2007) noted that, to date, studies on the occurrence of mycotoxin in animal feed are primarily focused on commodities such as grains and cereals, with limited research being conducted on the contents of other forage materials, such as grass silage, hay, and straw. Our results clearly support this notion, indicating that the clinical case of natural ZEN contamination may be common in conventional cattle herds. In addition, our study demonstrates that the commercially available ZEN ELISA kit may be useful for measuring dietary ZEN, in addition to its intended use for measuring urinary ZEN. The combination of these 2 types of measurements may generate a useful tool for monitoring the ZEN exposure of animals at the farm level. Further studies for simultaneously measuring both urinary ZEN and DON concentrations by liquid chromatography coupled with tandem mass spectrometry with samples derived from the experimental herd are currently in progress.

LITERATURE CITED


