Field Application of Urinary Zearalenone Concentration Measurements for Monitoring Natural Feed Contamination in Cattle Herds

（尿中濃度を指標とした飼養環境下の牛群における
ゼアラレノン浸潤動態の解明）

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Field Application of Urinary Zearalenone Concentration Measurements for Monitoring Natural Feed Contamination in Cattle Herds

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We hereby recommend that the thesis prepared under supervision by Hiroshi Hasunuma, entitled "Field Application of Urinary Concentration Measurements for Monitoring Natural Feed Contamination in Cattle Herds" should be accepted as fulfilling in part for the degree of Doctor of Philosophy.

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ABSTRACT

Mycoestrogen zearalenone (ZEN) is produced as a second metabolite by many *Fusarium* species, and is a contaminant of animal dietary feeds worldwide. Despite its non-steroidal structure, ZEN activates estrogen receptors, resulting in functional and morphological alterations in the reproductive organs of suffered animals, such as ovarian atrophy, prolonged estrous intervals, persistence of corpora lutea, decreased fertility, and stillbirth. Therefore, ZEN and its metabolites are classified as endocrine disrupter compounds. Moreover, the predominant feature of ZEN distribution in animal feed is its co-occurrence with other *Fusarium* toxins (e.g., trichothecenes and fumonisins) that are detrimental to animal health. Therefore, establishment of a ZEN-monitoring system at the farm level is important. This system must not only detect/monitor ZEN levels in natural feed, but also suppose unknown toxins that may coexist with ZEN. With this in mind, we established a practical method for measuring the concentration of urinary ZEN and its metabolites. This method can be used to monitor natural ZEN contamination of cattle feed. To our knowledge, there are no reports of naturally occurring ZEN contamination in the diet by using the results of urinary ZEN concentrations under farm conditions.

The first series of experiments were conducted to (1) identify the natural source of feed contamination by ZEN, which was suspected to have caused high urinary ZEN concentrations in 1 of our cattle herds, and (2) evaluate the effects of intervention against this source of contamination. As a model, a fattening Japanese Black cattle herd that exhibits high urinary ZEN concentrations was used. Urinary ZEN concentrations of cows fed new rice

straw (n=6) versus those of cows that continued to feed on the old rice straw (n=4) were measured at the start (Day 1) and at 2 weeks (Day 14) after the onset of straw feeding. In addition, ZEN concentrations in the feed and water samples were measured using both the ELISA and HPLC methods. Fungi from rice straw and concentrate feed samples were also isolated and identified. The urinary ZEN concentration (ZEN [pg/mL]/creatinine (Crea) [mg/mL] = pg/mg of Crea) of cows fed new rice straw was significantly ($p < 0.05$) lower (843 pg/mg of Crea) than that of cows fed old rice straw (15951 pg/mg of Crea). On Days 1 and 14, the ZEN concentrations of old rice straw were higher than those of new rice straw. Furthermore, fungal colonies were observed in the culture media obtained from the old rice straw, but not in the culture media from new rice straw or other feed samples. These results indicate that the rice straw fed to the cows was naturally contaminated with ZEN, and that monitoring of urinary ZEN concentrations is a useful tool for detecting cattle exposure to ZEN.

In the second experimental series, we investigated the effects of mycotoxin adsorbent (MA) supplementation in the top dressing of cattle feed and measured the concentrations of urinary ZEN and its metabolites, α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL). Japanese Black cattle herds for breeding (2 herds) and fattening (2 herds) were provided with similar feeding conditions. Two types of MAs were tested and the maximal recommended dose of each MA was supplemented in the feed as a top dressing for 2 weeks. Urine samples were collected from cows ($n = 6–7$) on the first day (Day 0) and 2 weeks after the supplementation period (Day 14). ZEN concentrations were measured by ELISA and liquid chromatography-tandem mass spectrometry methods (LC/MS/MS), and α-ZOL and

β-ZOL concentrations were measured by LC/MS/MS. The concentrations of ZEN and its metabolites were expressed by correcting for creatinine (Crea) as pg/mg Crea. The urinary concentrations of ZEN and its metabolites were variable in all herds, and significant differences were observed between the herds. This result might reflect significant levels of natural ZEN feed contamination at the farm level. The urinary concentrations of ZEN and its metabolites after supplementation with MAs for 2 weeks did not differ significantly. These findings indicate the usefulness of measuring urinary concentrations of ZEN and its metabolites for monitoring the natural ZEN contamination of cattle feed and for objective *in vivo* evaluation of MA function when MAs are employed as feed supplements. Additionally, these results suggest that MA feed supplementation may affect the absorption of mycotoxins in the gastrointestinal tract or limit the binding of MA to mycotoxins.

In conclusion, our field study identified the first case of rice straw ZEN contamination as the original cause of extremely high urinary ZEN concentrations in cattle. The results of our field trials confirm that monitoring urinary ZEN concentrations appears to be a useful tool for predicting animal exposures to ZEN (and possibly other *Fusarium* toxins). By implementation of a urinary ZEN-monitoring system, the benefits of MA supplementation of the diets for farm animal health may be reconfirmed.

GENERAL INTRODUCTION

Mycotoxins are secondary metabolites produced by specific filamentous fungi that are common contaminants of agricultural commodities, and are known to induce a toxic response (mycotoxicosis) when ingested (Binder et al; 2007; Boudra and Morgavi, 2008). Cereal plants have been suggested to be contaminated by mycotoxins in 2 ways: (1) growth of fungi as pathogens on plants or (2) saprophytic growth of fungi on stored plants (Glenn, 2007). Additionally, not all fungal growth results in mycotoxin formation, and detection of fungi does not necessary imply the presence of mycotoxins (Binder et al, 2007). It is well known that naturally contaminated feed on the field could contain many different mycotoxins (more than 300 different mycotoxins have been reported thus far): however, the major class of mycotoxins affecting feedstuffs includes aflatoxins, deoxynivalenol (DON), and zearalenone. These contaminants are present at the highest levels in corn, an ingredient of concentrate feeds (Binder et al, 2007). In the case of farm animals, consumption of a mycotoxin-contaminated feed may induce acute and long-term chronic effects, such as reduced feed intake, feed refusal, poor feed conversion, diminished body weight, increased disease incidence, and reduced reproductive efficacy, resulting in economic losses of each herd (Fink-Gremmels and Malekinejad, 2007; Binder et al, 2007).

Zearalenone (ZEN) is a resorcyclic acid lactone, chemically described as 6-[10-hydroxy-6-oxo-trans-1-undecyl]-β-resorcyclic acid, produced as a secondary metabolite by various *Fusarium* species, including *F. graminearum*, *F. culmorum,* and *F. cerealis,* which are all common soil fungi infecting plants in the early stage of growth and during flowering

(Jimenez and Mateo, 1997; Malekinejad, 2004). Maize is particularly susceptible to infection with *F. graminearum*, thus, the name, zearalenone, refers to the fact that it is found at a high prevalence and concentration in maize (*Zea mays* L.). ZEN has also been found in legumes, oats rice, and sorghum, as well as in various grass species. ZEN concentrations in feed and food samples have been suggested to be the highest in years with heavy rainfall in spring and summer. Conversely, post-harvest contamination is minimal because *Fusarium* species do not grow on stored feed commodities (Malekinejad, 2004), and therefore, the toxin formation predominantly occurs prior to harvest. Previous studies have shown that in case of silage, *Fusarium* species do not survive at a low pH but may remain on the surface, where they may produce ZEN during storage. Based on the worldwide surveillance to evaluate the incidence of mycotoxins in feeds sampled from animal farms or feed production sites, more than half of the samples in Europe were reported to be contaminated (ZEN, DON, and T-toxin as the major contaminants) at levels above the quantification limit of current methods. One-third of the samples from Asia-Pacific regions were positive (as they tended to be contaminated with ZEN, DON, fumonisins, and aflatoxins) (Binder et al, 2007). Therefore, monitoring for the contamination status of mycotoxins in dietary feeds of farm animals, especially at the farm levels, is an essential measure to prevent feed contamination.

Following oral exposure after ingestion with feed, ZEN is rapidly absorbed from the gastro-intestinal tract in monogastric species. In contrast, in ruminants, the fore-stomach flora degrades a significant amount of ZEN and only a fraction reaches the small intestines. Reductase activity has also been found in the intestinal mucosa and its metabolite can be glucuronidated (Huiper-Goodman et al, 1987; Malekinejad, 2004). The fraction of ZEN that

reaches the liver is subjected to the activity of hepatic biotransformation enzymes, which can convert ZEN into α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL). Hydroxylation results in the formation of α-ZOL and β-zearalenol, catalyzed by 3α- and 3β-hydroxysteroid dehydrogenase (HSDs), and glucuronidates the parent compound as well as the 2 metabolites. Conjugation of ZEN and its reduced metabolites with glucuronic acid is then catalyzed by uridine diphosphate glucuronyl transferase (UDPGT). Since the lactone ring of ZEN resembles many structural features of the aromatic ring of estradiol, and apparently fits into binding pocket of mammalian estrogen receptor, ZEN and its metabolites exhibit distinct estrogenic and anabolic properties that affect the reproductive system of several animal species (Kleinova et al., 2002; Fink-Gremmels and Malekinejad, 2007; Minervini and Dell'Aquila, 2008). Additionally, it is thought that the predominant feature of ZEN distribution in animal feed is its co-occurrence with other *Fusarium* toxins such as trichothecenes and fumonisins, and the levels of these mycotoxins can be rather high (Avantaggiato et al., 2007; Binder et al., 2007). Since mycotoxin contamination of animal feeds highly depends on environmental conditions leading to mold growth and toxin production, the inter-annual variability has rendered estimation of mycotoxin contamination a very complex task. It has been strongly emphasized that a mycotoxicosis diagnosis should be established based not only on feed sample analysis but also on parallel investigations of animals that have access to the same feed materials (Binder et al., 2007). Regarding this aspect, the concentration levels of ZEN and its metabolites in urine samples of farm animals, including cattle, are considered potential biomarkers for determining ZEN exposure (Prelusky et al., 1989; Usleber et al., 1992; Kleinova et al., 2002). Therefore, to establish a systematic monitoring system for measuring urinary ZEN concentration, especially at the farm level, a

method must be developed to detect/monitor the natural feed contamination by ZEN and other *Fusarium* toxins that coexist with ZEN.

Based on the above hypothesis, Takagi et al. (2011) recently reported the possible application of measuring the concentrations of urinary ZEN and its metabolites in an attempt to monitor natural ZEN contamination of cattle feed at the farm level. Interestingly, within their series of urinary ZEN monitoring of Japanese Black herds, they detected a fattening cattle herd that showed persistently high urinary ZEN concentrations, which were significantly higher than those of the 3 other fattening herds examined (approximately 18–250 times) for the first time in Japan. However, the source of this extremely high urinary ZEN concentration in the concerned herd remained unclear. To our knowledge, no study has identified the possible presence of naturally occurring ZEN contamination in the diet by measuring urinary ZEN concentrations. Therefore, the experiments described in Chapter 1 aims to identify the original feed source naturally contaminated by ZEN that caused high urinary ZEN concentrations in 1 of the studied cattle herds. In addition, we measured the effects of countermeasures against contamination by feeding a ZEN non-contaminated diet to the herd while monitoring urinary ZEN concentrations.

Several methods have been proposed for the mitigation of mycotoxin contamination of feeds during the preharvest, harvest, and postharvest periods (Kabak et al., 2006; Schatzmayr et al., 2006; Igawa et al., 2007; Zinedine et al., 2007; Boudra and Morgavi, 2008; Murata et al., 2008). Given the various limitations of these methods, it has been suggested that the use of mycotoxin adsorbents (MAs), MA-related mitigating agents, or both, as feed additives is one of the most promising and widely used approaches to reduce the risk of

mycotoxicosis in farm animals (Ramos et al., 1996; Huwig et al., 2001; Sabater-Vilar et al., 2007). To date, classes of substances suggested to be MAs include inorganic mineral clays and zeolites, as well as organic products such as yeast cell wall constituents, activated charcoal, humic acid polymers, and micronized plant fibers (Ramos et al., 1996; Jouany, 2007; Santos et al., 2011). Several *in vitro* and *in vivo* studies of the efficacy of MA have shown beneficial effects of MAs (Avantaggiato et al., 2005, 2007; Korosteleva et al., 2007; Sabater-Vilar et al., 2007). However, to our knowledge, no study in farm animals has confirmed the absorptive capacity *in vivo* by measuring ZEN levels in biological fluids (such as milk, urine, bile, and serum) under field conditions to demonstrate a reduced rate of absorption after MA supplementations. Recently, Takagi et al. (2011) reported a field study that confirmed a significant reduction in the urinary ZEN concentration after a period of MA supplementation (2 weeks) in a dairy herd. They also found a subsequent increase in original ZEN levels after cessation of the MA supplementation. Although the number of examined herds was relatively small, their findings clearly indicate the efficacy of MA in preventing the absorption of ZEN from cattle intestines. Moreover, these results revealed that the objective evaluation of MA supplementation in the feed was sufficient to monitor the urinary ZEN level of the herd. Therefore, the use of bovine urine as a biological sample might be a more useful tool to evaluate the efficacy of MA than *in vitro* MA evaluation methods. However, recent reports suggested the possibility that MA types, as well as application methods, might have an effect on the absorption of mycotoxin in the digestive tract (Kutz et al., 2009). Although MAs have been usually applied as supplements of animal diets as equal mixtures in total mixed rations (TMR) in the dairy industry, another possible method for MA supplementation may be as a top dressing on the feed for individual cattle.

Therefore, for the experiments described in Chapter 2, we performed an objective field study to examine the urinary concentrations of ZEN and its metabolites in beef cattle fed a diet supplemented with an MA product as a top dressing.

Chapter 1

Natural contamination of rice straw by zearalenone indicated by persistently high urinary zearalenone concentrations in a cattle herd

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 high 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, 1 fattening Japanese Black cattle herd showing persistently high urinary ZEN concentrations was identified. Urinary ZEN concentrations of cows fed with new rice straw (experimental group, n=6) versus cows that continued to feed on the old rice straw (control group, n=4) were measured at the start (Day 1) and at 2 weeks (Day 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 (Crea) [mg/mL] = pg/mg of Crea) of cows fed with new rice straw was significantly ($p < 0.05$) lower (843 pg/mg of Crea) than that of cows fed with old rice straw (15951 pg/mg of Crea). On both Days 1 and 14, the ZEN concentrations of old rice straw were higher 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.

INTRODUCTION

Zearalenone (ZEN) is a nonsteroidal estrogenic mycotoxin produced by *Fusarium* species in plants such as wheat, barley, maize and some other crops including rice. Zearalenone and its metabolites exhibit distinct estrogenic and anabolic properties that affect the reproductive system of several animal species (Kleinova et al., 2002; Fink-Gremmels and Malekinejad, 2007; Minervini and Dell'Aquila, 2008). Zearalenone may occur with other *Fusarium* toxins in animal feeds, resulting in markedly high levels of these mycotoxins (Avantaggiato et al., 2007; Binder et al., 2007).

A diagnosis of mycotoxicosis should be established, not only based on feed sample analysis but also, from parallel investigations of animals that have access to the same feed materials, as well as from postmortem examinations (Binder et al., 2007). The concentration levels of ZEN and its metabolites in urine samples of farm animals, including cattle, are considered as potential objective biomarkers for indicating ZEN exposure (Prelusky et al., 1989; Usleber et al., 1992; Kleinova et al., 2002).

Recently, we reported the possibile practical application of measuring the concentrations of urinary ZEN and its metabolites towards monitoring the natural ZEN contamination of cattle feed at the farm level (Takagi et al., 2011). With this report, we incidentally detected one fattening cattle herd that showed persistently high urinary ZEN concentrations that were significantly higher in comparison to 3 other examined fattening herds (approximately 18 to 250 times). We speculated that feeds were subject to natural contamination from mycotoxins. In the previous report, we only focused on measuring the urinary concentrations of ZEN and its metabolites to investigate the possibility of monitoring

natural feed contamination by mycotoxins, but we did not measure the ZEN concentrations of the feeds of individual herds. Thus, the origin of the extremely high urinary ZEN concentration in the concerned herd remained unresolved. To our knowledge, there are no available reports identifying the possible presence of naturally occurring ZEN contamination to the diet by using the results of urinary ZEN concentrations under farm conditions. Our established urinary ZEN monitoring system might be of practical importance to clarify the relationship between significant levels of infection by *Fusarium* species and natural contamination of ZEN in the identified diet, vis-à-vis significant high urinary ZEN concentrations in herds.

This study aims to identify the original feed source that was naturally contaminated by ZEN, and which possibly caused high urinary ZEN concentrations in one of the studied cattle herds. To accomplish this objective, we used a commercially available ZEN ELISA kit and confirmed the results by performing high-performance liquid chromatography (HPLC) analyses. In addition, the effects of countermeasures against contamination by feeding a ZEN non-contaminated diet to the herd were evaluated by monitoring urinary ZEN concentrations.

MATERIALS AND METHODS

Animals were cared for according to the Guide for the Care and Use of Laboratory Animals (Faculty of Agriculture, Kagoshima University).

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 comprised 700 cattle, and persistently showed significantly higher mean urinary ZEN concentrations than 7 other cattle herds that were studied in our previous report (Takagi et al., 2011). Herd 2 comprised 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 re-confirm 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 high 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 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 high 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

Table 1. Composition of feed provided to the cattle of 2 cattle herds that were kept for fattening purposes.

Herd	Forage feed, kg	Formula feed						
		Total, kg	Bran, %	Cereal, %	Oil seed meal, %	Other, %	TDN, %	CP, %
Herd 1	Straw 2.0	9.0	17	77	5	1	> 75	> 14
Herd 2 (Control)	Straw 4.0	9.0	17	77	5	1	> 75	> 14

(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 while the cows of experimental group were fed with 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 concentration of cows of experimental group were evaluated before the replacement of the rice straw (Day 1) with a new straw lot and repeated at 2 weeks (Day 14) after replacement by ELISA. Similarly, the urinary ZEN concentration of cows of control group both at Day 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 and/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., Japan).

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 (SPE) 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 SPE 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 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) 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 Crea]) as previously described (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, Garmstadt, 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, 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 re-analyzed 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, v/v) 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, v/v). 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 C_8 (4.6 ID × 250 mm, 5 μm; Shuseudo, Tokyo, Japan) at 40 °C. The mobile phase consisted of acetonitrile-water (40:60, v/v), 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 94–102%.

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 days. Fungal isolation was only conducted from the infected rice straw samples selected from Herd 1. The rice straw (5-mm^2 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 days in the dark. A hyphal tip was then transferred to a potato sucrose agar (PSA). Colonies showing typical

morphologies for *F. graminearum* species complex on PSA, 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 days 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 days under black light. The presence or absence in the formation of macroconidia was observed under a light microscope (Nikon Labophot, Japan) at 300 × magnification.

Statistical Analysis

Urinary ZEN concentrations were expressed as mean ± SEM. The urinary ZEN concentrations, conducted on both Day 1 and Day 14, were analyzed with a paired *t*-test, using SPSS (version 16) 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 non-contaminated 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 15951 ± 4178 (pg/mg of Crea) with the old straw (ZEN concentration of the rice straw was estimated to be more than 4536 ng/g from ELISA and 7555 ng/g from HPLC). However, after 14 days 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 Day 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, b). 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 3–5 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 non-significant during the 2-week 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 (Figure 2a and 2b).

Table 2. The results of the preliminary surveillance of Zearalenone concentrations derived from urine, water and straw samples from Herd 1 (experimental) and Herd 2 (control) as measured by ELISA.

	Sampling herd	
Sample	Herd 1	Herd 2
Water (pg/ml)	47.5	80.6
Straw (ng/g)	Over range*	NT
Urine (pg/mg of Crea)		
Cattle 1	456.8**	183.8
Cattle 2	365.2**	106.2**
Cattle 3	277.2**	264.2**
Cattle 4	308.8**	65.9
Cattle 5	284.6**	146.4
Mean	338.5	153.3

*: Zearalenone concentrations measured in the straw by using ELISA were beyond the standard maximum range values (4,050 pg ZEN/mg) of the kit.

**: Urinary zearalenone concentrations measured by using ELISA were beyond the standard maximum range values (4,050 pg ZEN/mg) of the kit, and the urinary ZEN concentrations of the above range sample was calculated to be 4,050 pg/mg, without repeating ELISA measurements by using the diluted samples.

NT: Not determined.

ZEN concentration of distilled water: 61.6 pg/ml measured by using ELISA

Table 3. Results of ZEN concentrations in straw, concentrate, and urinary samples before and after the replacement of rice straw in Herd 1.

Sample	Analytical Method	ZEN concentrations	
		Day 1	Day 14
Replaced group			
New straw (ng/g)	ELISA	33.7	46.0
	HPLC	ND	ND
Concentrate (ng/g)	ELISA	23.3	37.8
	HPLC	138.1	86.5
Urine (pg/mg of Crea) (n=6)	ELISA	15951 ± 4178 [a]	843 ± 67 [b]
Non-replaced group			
Usual straw (ng/g)	ELISA	4536*	4536*
	HPLC	7555	7223
Concentrate (ng/g)	ELISA	23.3	37.8
	HPLC	138.1	86.5
Urine (pg/mg of Crea) (n=4)	ELISA	20555 ± 2808	22300 ± 2949

Replaced group: Cattle fed with the new straw lot (non-contaminated rice straw) as roughage from Day 1 to Day 14.

Non-replaced group: Cattle fed with normal straw as roughage from Day 1 to Day 14.

*: Zearalenone concentrations in the straw measured by using ELISA were beyond the standard maximum range values (4,050 pg ZEN/mg) of the kit.

a,b: Values with different superscripts in the same column differ significantly ($P < 0.05$).

ND: Not detected.

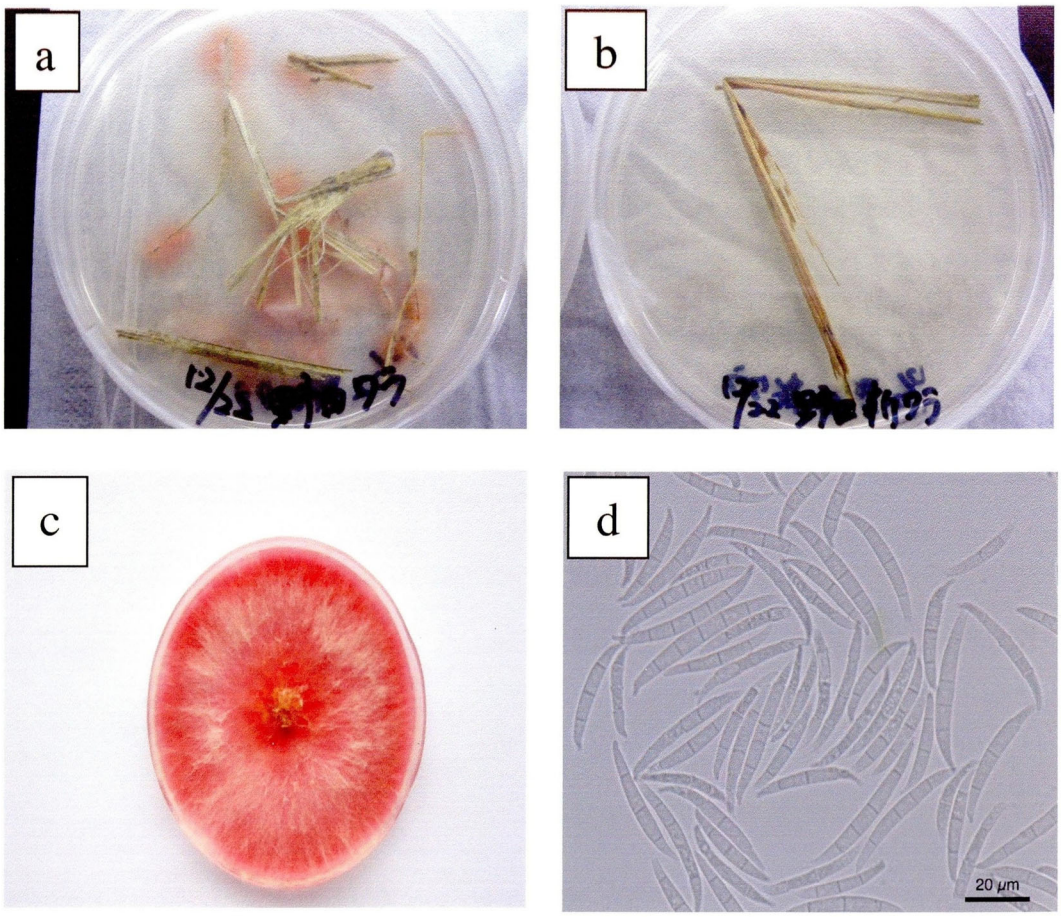

Figure 1. (a) Sabouraud agar culture with colonies showing the typical pink color of zearalenone (ZEN)-contaminated rice straw, derived from Herd 1. (b) Sabouraud agar culture without colonies showing ZEN non-contaminated new rice straw from Herd 1. (c) Potato sucrose agar (PSA) with colonies showing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

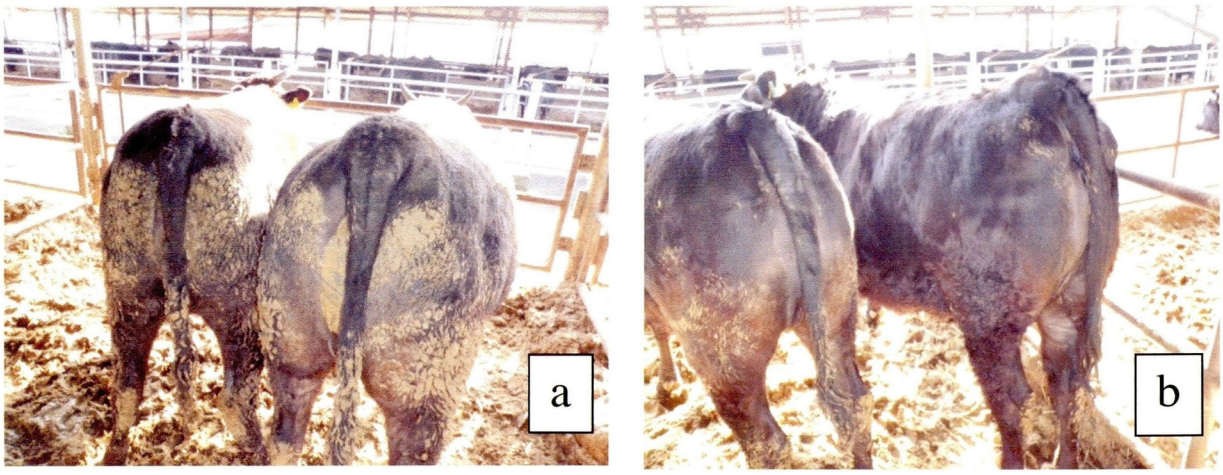

Figure 2. Physical appearance of the cow buttocks in Herd 1 (a) after being fed ZEN-contaminated straw and (b) after being fed ZEN non-contaminated straw during the 2-week study period.

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 high 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, as these materials contain high 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.

In the present study, we evaluated dietary ZEN concentrations by using both modified ELISA and HPLC assays. While 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 straw, because the urinary ZEN/Crea ratio was similar throughout the herd.

Ruminants are known to have relatively lower sensitivity to ZEN exposure than monogastric 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 high urinary ZEN concentrations in Herd 1. Although this previous speculation about the original cause of high 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, lower weight 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 parameters 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 (LC-MS/MS) 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 LC-MS/MS with samples derived from the experimental herd are currently in progress.

Chapter 2

Monitoring Natural Feed Contamination in Beef Cattle by Measurements of Urinary Zearalenone Concentrations after Oral Administration of Mycotoxin Adsorbents as a top Dressing

ABSTRACT

The aim of this field study was to investigate the effects of supplementation of mycotoxin adsorbents (MAs) in top dressing of cattle feed with respect to concentrations of urinary zearalenone (ZEN) and its metabolites, α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL). Japanese Black cattle herds for breeding (2 herds) and fattening (2 herds) were provided with similar feeding conditions. Two types of MAs were tested and the maximal recommended dose of each MA was supplemented in the feed as a top dressing for 2 weeks. Urine samples were collected from cows (n = 6–7) on the first day (Day 0) and 2 weeks after the supplementation period (Day 14). The concentrations of ZEN were measured by ELISA and liquid chromatography-tandem mass spectrometry methods (LC/MS/MS), and the concentrations of α-ZOL and β-ZOL were measured by LC/MS/MS. The concentrations of ZEN and its metabolites were expressed after correcting for creatinine (Crea). The concentrations of ZEN or metabolites are expressed in terms of pg/mL. This is divided by the Crea concentration in terms of mg/dL and the ensuing parameter is expressed as pg/mg Crea. The urinary concentrations of ZEN and its metabolites were found to be variable in all herds, and significant differences were observed between herds. This might reflect significant natural ZEN contamination of the feed at the farm level. However, the urinary concentrations of ZEN and its metabolites after supplementation with MAs for 2 weeks were not significantly different. These findings indicate the usefulness of measuring concentrations of urinary ZEN and its metabolites, not only for monitoring the natural ZEN contamination of cattle feed at the farm level, but also for objective *in vivo* evaluation of MA function when MAs are employed as feed supplements. Additionally, our results suggest the possibilities that

supplementation of the feed with MA may affect the absorptivity of mycotoxins from the gastrointestinal tract, or limit the binding of MA to mycotoxins. This may depend on the status of mycotoxin contamination of the feed.

INTRODUCTION

Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin produced by *Fusarium* species. This mycotoxin is found in the grains of several monocotyledons, including pasture grasses. Due to their agonistic effects on the estrogenic receptor, ZEN and its metabolites exhibit distinct estrogenic and anabolic properties in several animal species which have several effects on the reproductive system (Kleinova et al., 2002; Fink-Gremmels and Malekinejad, 2007; Minervini and Dell'Aquila, 2008). Additionally, it is generally accepted that the predominant feature of ZEN distribution in animal feed is its co-occurrence with other *Fusarium* toxins such as trichothecenes and fumonisins (Avantaggiato et al., 2007: Binder et al., 2007). On the basis of the recent publication of Binder et al. (2007), it was concluded that the incidence of mycotoxins, which are relevant for animal production, is quite high in animal feed, although an assessment of the relevance of these high levels is difficult to undertake.

Although no technology is currently available that will totally eliminate mycotoxin contamination from the food and the feed chain (Yiannikouris et al., 2006), several approaches for protecting animals from the toxic effects of mycotoxins, including both pre- and post-harvested products, have been conducted (Awad et al., 2010). Among these approaches, supplementation of feed with mycotoxin adsorbents (MAs) has been recognized as an effective and practical addition to the animal production industry (Sabater-Vilar et al., 2007; Kutz et al., 2009). A number of studies have investigated the efficacy of several types of MAs used in the cattle industry, especially in dairy cattle. These reports have included monitoring ZEN and aflatoxin (AF) metabolite concentrations in milk samples (Seeling et al., 2005; Kutz et al., 2009). Recently, we reported the efficacy of monitoring urinary zearalenone

concentrations in cattle herds at the farm level, and indicated that there is a significant reduction of urinary ZEN concentration concomitant with the reduction of somatic cell counts during the period of supplementation of total mixed rations in a dairy cattle herd (Takagi et al., 2011). However, recent reports clearly indicated that not all MA products are equally effective for preventing mycotoxin absorption from the digestive tract (Kutz et al., 2009), which suggested the possibility that types of MAs as well as the methods of applying MAs might have an affect on the absorption of mycotoxin in the digestive tract (Kutz et al., 2009). Although MAs have been usually applied as supplements of animal diets as equal mixtures in total mixed rations (TMR) in the dairy industry, another possible method for supplementation of the MA may be as top dressing on the feed for individual cattle.

Therefore, the objective of this field study was to examine the urinary concentrations of ZEN and its metabolites in beef cattle provided with a diet supplemented with MA product as a top dressing.

MATERIALS AND METHODS

Chemicals and Solvents

Zearalenone was purchased from MP Biomedicals (Heidelberg, Germany). The metabolites α-ZOL and β-ZOL were purchased from Sigma (St. Louis, MO). Ammonium acetate and high-performance liquid chromatography (HPLC)-grade methanol 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).

Herds of Japanese Black Cattle and Sample Collection

Four herds of Japanese Black cows kept for fattening (2 herds) or breeding (2 herds) in Kagoshima Prefecture, Japan, were included in this study. Although the feeding and management systems were different for each herd, all cows were housed indoors, and forage and concentrates (top dressing method) were fed separately. The content of the feeds in each herd are provided in Table 1. In each herd, 5 cows of the same age (fattening category) or similar body weight (breeding category) were selected, and spontaneous urine samples were collected during natural urination following soft massage of the pudendum.

In the present study, 2 types of commercially available MAs were used as supplementation for the diets: MA-A, containing a combination of yeast cell wall, clay, and plant extracts as active ingredients, and MA-B, containing only yeast cell wall as the active ingredient. The MA supplementation was given daily during a 2 week period for the maximal

Table 1. Composition of feed provided to the breeding and fattening purposes herds

Herd	Forage feed, kg	Formula feed						
Purpose		Total, kg	Bran, %	Cereal, %	Oil seed meal, %	Other, %	TDN[1], %	CP[2], %
Breeding								
A	Straw 2.0, Italian 1.0	2.2	57	20	5	18	>64	>14
B	Orchard grass 4.0	2.8	17	39	22	22	>67	>16
Fattening								
C	Straw 1.0	8.0	15	80	3	2	>76	>14
D	Straw 1.5	8.0	19	77	1	3	>74	>11.5

[1] Total digestible nutrients

[2] Crude protein

doses of each MA, according to the manufacturer's instructions. Urine samples were collected from individual cows on the first day of the experiment and at the end of each experimental period, approximately 3 h after morning feeding. The urine samples were immediately stored in a cooling box, protected from light, and transported to the laboratory. After centrifugation at 500 × *g* for 10 min, the samples were frozen at –30°C until analysis. The urinary ZEN concentration was measured by ELISA. Additionally, for confirmation, concentrations of urinary ZEN, α-ZOL, and β-ZOL were measured by LC/MS/MS, and the ZEN/Crea ratio was finally determined.

Analytical Methods for Determination of ZEN and Crea

As described in detail in our previous report (Takagi et al., 2011), the ZEN concentration in urine was determined using a commercially available kit (RIDASCREEN Zearalenon, R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions with minor modifications. Briefly, a urine sample (0.5 mL) was mixed with 3 mL of 50 mM sodium acetate buffer (pH 4.8). The solution was then incubated for 15 h at 37°C in the presence of 8 μL of β-glucuronidase/arylsulfatase solution. Thereafter, the samples were loaded onto a C18 solid phase extraction (SPE) 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 SPE 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 for 10 min at 500 × *g* to dry the column. The analytes were then eluted at a flow rate of 15 drops/min with 1 mL of methanol (80%). The eluate was evaporated to dryness at 60°C by using a centrifugation

evaporator. The dried residue was re-dissolved in 50 μL of methanol and 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 the ZEN concentration in the urine sample, RIDA SOFT Win (R-Biopharm) was used to calculate the absorbance at 450 nm with a microplate spectrophotometer. The mean recovery rate of the ELISA assay based on the 3 trials was 84 ± 14%. Results of the ZEN analysis were not corrected for the recovery rate.

Urinary Crea concentrations were determined using a commercial kit (Sikarikit-S CRE, Kanto Chemical) 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 are expressed as their ratio to urinary Crea (urinary ZEN/urinary Crea [pg/mg Crea]) as previously described (Miles et al., 1996; Padilla et al., 2007).

To confirm the concentrations of ZEN measured by ELISA, and to measure the ZEN metabolites in bovine urine, the urine samples measured by ELISA were reanalyzed by LC-ESI/MS/MS. The extraction method for the urine samples was rather similar to the above-described ELISA method, with some minor modifications. Briefly, 0.5 mL of a urine sample was mixed with 3.0 mL of 50 mM ammonium acetate buffer (pH 4.8) and 8 μL of glucuronidase/arylsulfatase solution, and incubated for 12 h at 37 °C. After the incubation, 1.5 mL of distilled water was added to each sample solution. The solution (5.0 mL in total) was loaded onto a C18 SPE column, which was preconditioned with 3 mL of 100% MeOH and 2 mL of Tris buffer, followed by the addition of 2 mL Tris

buffer and 3 mL of 40% MeOH. After washing the SPE column with approximately 1 mL of 80% MeOH, the volume of the eluted solution was adjusted to exactly 1 mL. Then, 20 μL of the reconstituted solution was injected into the liquid chromatography-tandem mass spectrometry (LC/MS/MS) system.

LC-ESI/MS/MS analyses were performed according to the procedure described by Takagi et al. (2011) using an API 2000 LC/MS/MS system (Applied Biosystems, Foster City, CA) equipped with an electrospray ionization interface and an HPLC system (1200 Series; Agilent Technologies, Santa Clara, CA). Chromatographic separation with gradient elution using a mobile phase consisting of methanol (A) and water (B) was achieved on an Inertsil ODS-3 (4.6 ID × 150 mm, 5 μm; GL Sciences, Tokyo, Japan). The injected sample volume was 20 μL. For the LC/MS/MS analysis, a multiple reaction monitoring system was used for the transition of ZEN (m/z 317.0-130.5) and α/β-ZOL (m/z 319.0-129.9) in a negative mode.

Statistical Analysis

The results of urinary ZEN concentrations are expressed as means ± SD. The concentrations of urinary ZEN and its metabolites before and after supplementation with MAs in each herd were analyzed by a paired *t*-test using SPSS Statistics software (Version 18, IBM). *P* values less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

Figure 1 shows the results of the ELISA determination of the urinary ZEN concentration of each herd supplemented with 2 different types of MAs (MA-A and MA-B) over the course of 2 weeks for both groups of breeding and fattening cows. In the present study, the urinary ZEN concentration of 5 out of 6 urinary samples collected at Day 14 derived from herd C were beyond the maximal range of the ELISA kit. Therefore, the concentrations of these samples were assigned the concentration value of 4050 pg/mL, which represents the maximal concentration that can be determined by the kit. Significant differences ($P < 0.05$) of ZEN concentrations among the herds were observed. On Day 0, the ZEN concentration was 48.5 pg/mg Crea for herd-A, vs. 213.8 pg/mg Crea for herd-C and on Day 14, the ZEN concentration was 65.6 pg/mg Crea for herd-A, vs. 250.4 pg/mg Crea for herd-C. The urinary ZEN concentration between Day 0 and Day 14 in each of the 4 herds did not differ with respect to the two different MA types used.

Figure 2 shows the results of determining the concentrations of urinary ZEN and its metabolites, α-ZOL and β-ZOL, and total ZEN (ZEN + α-ZOL + β-ZOL) concentrations for each of the 4 herds using the same samples as those of Figure 1, measured by LC/MS/MS. Although the urinary ZEN concentrations of the same samples measured by LC/MS/MS were significantly lower than the ZEN concentrations measured by the ELISA method, the general tendencies of the urinary ZEN concentrations were similar and the urinary ZEN concentration of herd-C was significantly higher than the ZEN concentration of herd-A ($P < 0.05$). Although no significant differences in urinary

concentrations of α-ZOL were observed among the herds, the urinary β-ZOL concentration in herd-C was higher than that of the other herds, and significantly higher than that of herd-A ($P < 0.05$). No significant differences of the concentrations of ZEN and its metabolites were observed between Day 0 and Day 14 within each herd, except for herd-C, which had increased concentrations even with MA supplementation.

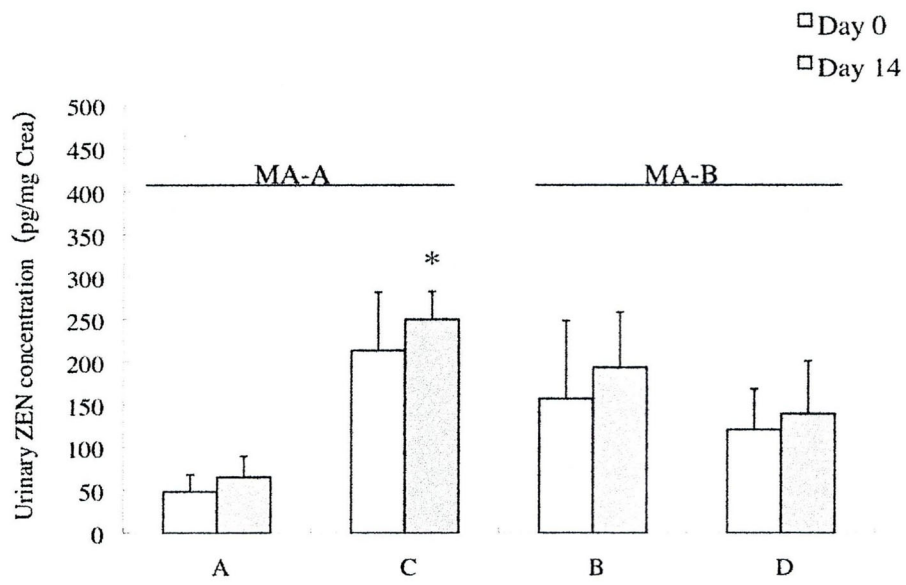

Figure 1. Urinary zearalenone (ZEN) concentrations of each herd in the breeding (A and B) and fattening (C and D) cattle groups measured by the ELISA method. Two types of mycotoxin adsorbents (MA-A and MA-B) were supplemented in the diets as a top dressing for 14 days with urinary sample collection conducted before MA supplementation (D 0) and 14 days during MA supplementation (D 14). Urinary ZEN concentrations of 5 samples from Herd C at Day 14 (asterisk) were given the value of 4,050 ng/L as they were beyond the maximum standard value (4,050 ng/L) of the kit.

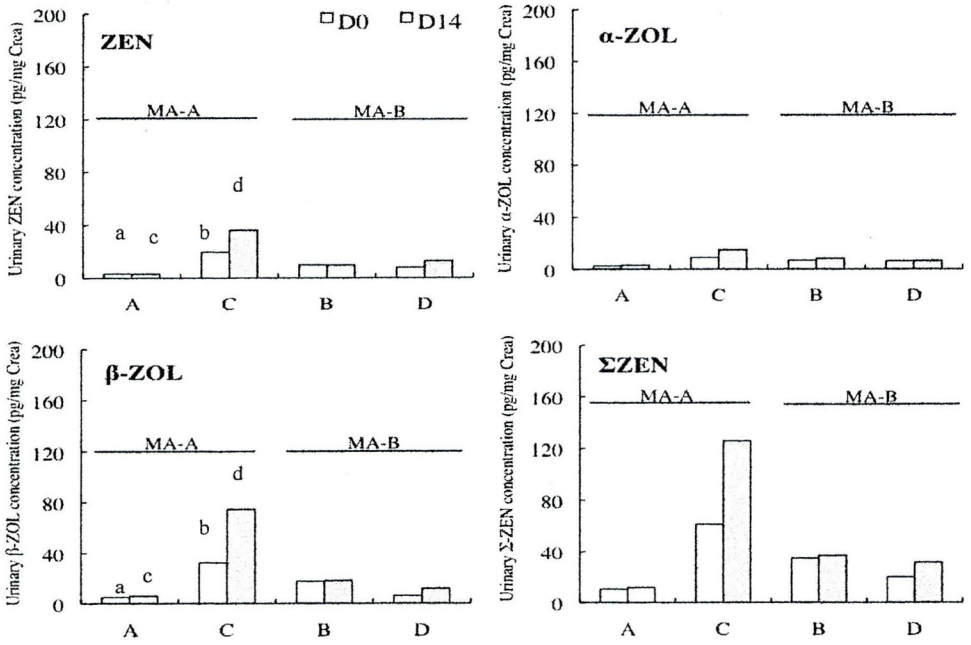

Figure 2. Urinary zearalenone (ZEN), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), and total ZEN (ΣZEN; ZEN + α-ZOL + β-ZOL) concentrations of each herd in the breeding (A and B) and fattening (C and D) cattle groups measured by LC/MS/MS method. Two types of mycotoxin adsorbents (MA-A and MA-B) were supplemented in the diets as a top dressing method for 14 days periods with urinary sample collection conducted before MA supplementation (D 0) and 14 days after MA supplementation (D 14).

a–b, c–d: Concentrations were significantly different ($P < 0.05$).

DISCUSSION

Detection of ZEN and its metabolites from urinary samples of cattle has been reported to be possible via several methods such as ELISA, GC/MS, and LC/MS/MS; these methods have also been suggested to be useful tools for residue control programs (Plasencia et al., 1990; Usleber et al., 1992; Dusi et al., 2009). Recently, we reported for the first time that exposure to ZEN via naturally contaminated feed could be monitored at the farm level by measuring urinary ZEN concentrations (Takagi et al., 2011). Additionally, in the same report, we confirmed a significant decrease of urinary concentrations of ZEN and its metabolites after supplementation of the feed with an MA product at the farm level. This indicates the efficacy of the urinary ZEN monitoring system for the objective evaluation of MA supplementation. In the present study, the effect of supplementation of MA in feed as top dressing was investigated based on measurements of urinary concentrations of ZEN and its metabolites at the farm level. Our results indicated that there are no reduction in the concentrations of urinary ZEN and α- and β-ZOL after supplementation with MAs in all 4 herds. This indicates the possibility that the method of supplementation, i.e., uniform mixing with feed as TMR versus a top dressing on the feed, may affect the prevention of the absorption of ZEN and its metabolites from the gastrointestinal tracts of cattle.

Several methods have been proposed for mitigation of mycotoxin-contaminated feeds during pre-harvest. These involve selection of resistance varieties, improved field management, and use of biological and chemical agents. Furthermore, harvest, and postharvest methods involve improving the drying and storage conditions, and the use of

natural and chemical agents as well as irradiation processes (Kabak et al., 2006; Schatzmayr et al., 2006; Igawa et al., 2007; Zinedine et al., 2007; Murata et al., 2008). Given the various limitations of these methods, it was suggested that the use of MAs or related mitigating agents, or both, as a feed additives is one of the most promising and widely used approaches to reduce the risk of mycotoxicoses in farm animals (Ramos et al., 1996; Huwig et al., 2001; Sabater-Vilar et al., 2007). In turn, for evaluation of the efficacy of MA, there have been severalreports of both *in vitro* and *in vivo* studies on the beneficial effects of MA (Avantaggiato et al., 2005, 2007; Korosteleva et al., 2007; Sabater-Vilar et al., 2007; Kutz et al., 2009). Sabater-Vilar et al. (2007) assessed the binding efficiency of 20 different binders through *in vitro* bioassays and confirmed the usefulness of the method as a rapid screening protocol for potential mycotoxin binders. With respect to *in vivo* evaluation of dairy cows, Korosteleva et al. (2007) confirmed the effectiveness of supplementation of glucomannan MA to feed which was naturally-contaminated with *Fusarium* mycotoxin in order to prevent metabolic and immune deficiencies of cows. However, as previously proposed by other studies (Prelusky et al., 1989; Sabater-Vilar et al., 2007), to confirm the absorptive capacity *in vivo*, it would be necessary to establish an *in vivo* monitoring system by measuring various biological fluids (such as milk, urine, bile, and serum) in farm animals under field conditions to demonstrate a reduced rate of absorption. In our previous study (Takagi et al., 2011), it was clearly indicated that MA-A supplemented as TMR (theoretically equally mixed with the feed diets) was effective in preventing the adsorption of ZEN from the intestines of dairy cattle. Thus, as a preliminary study, we examined whether the method of supplementing MA within the feed diets may have an effect on the urinary concentrations of ZEN and its metabolites. In the present study, the MAs were supplemented as top dressing metho, and no effects of

urinary concentrations of ZEN and its metabolites were observed in all of the herds examined. Thus, from these results, we propose the following 2 hypotheses to explain the results. Firstly, it was assumed that the method used to supply the MA into the feeds (TMR; theoretically equally mixed MA with diet or top dressing; partially mixed of MA with diet) might have a significant effect on the extent of absorption of mycotoxin from gastrointestinal tracts. In our previous study, significant decreases in the concentrations of urinary ZEN and its metabolites were observed during the MA supplementation period. However, MA supplementation revealed that it was not possible to adsorb ZEN and its metabolites completely from naturally contaminated feeds, even if supplied with a maximum dose of MA to the TMR for a period exceeding 2 weeks as recommended by the manufacturer (Takagi et al., 2011). Although we cannot simply compare the total ZEN concentrations between our previous and present studies because of the differences of breed of cattle and body weights, the minimal concentration of total ZEN in previous report was approximately 400 pg/mg Crea as determined by ELISA and 100 pg/mg Crea as determined by LC/MS/MS. The maximal concentrations of total ZEN in the present study was approximately 250 pg/mg Crea as determined by ELISA and 120 pg/mg Crea as determined by LC/MS/MS. Therefore, another hypothesis is that there is a limited ability of MAs to adsorb the contaminated mycotoxin within the feeds. Additionally, in the present study, we used 2 types of MAs for evaluation as candidate MAs based on our established urinary ZEN monitoring system. Unfortunately, we could not detect any significant differences between the 2 MAs. So far, several types of MAs such as mineral clays, humic substances, and components of yeast cell wall are commercially available (Avantaggiato et al., 2005; Sabater-Vilar et al., 2007). Kutz et al. (2009) recently examined the efficacy of 3 types of MAs in reducing aflatoxin M1 (AF- M1) concentrations

in milk of dairy cows fed an AF-contaminated diet as TMR, and reported the differences in reduction of AF-M1 in milk with respect to the MA products employed. It was concluded that the differences might be due to composition and mechanism of action of the active compounds of the MAs. Therefore, in the future, there may be a requirement to determine the appropriate indications of MAs based on biological samples from animals, such as urine, for proper applications that depend on the mycotoxin contamination status of the diets of each cattle herd. Malekinejad et al. (2005) reported on the differences between species in hepatic biotransformation of ZEN, and demonstrated that β-ZOL is the dominant hepatic metabolite in cattle. In the present study, as shown in Figures 2, the urinary concentration of β-ZOL was found to be generally higher than that of α-ZOL in all cases. Moreover, present results may indicate that, with respect to at least the two MAs used in the present study, there is no tendency of each MA to exhibit a binding affinity for ZEN as well as for α- and β-ZOL.

In conclusion, the results of our field trials confirm that the system for monitoring urinary ZEN concentrations appears to provide a useful tool for predicting the exposure of animals at the farm level to ZEN (and possibly other *Fusarium* toxins). By applying the urinary ZEN monitoring system, it may be reconfirmed that objective evaluation of MA supplementation to the feeds might be possible at the individual farm level, and the method of supplementing the diets with MAs might have an effect on their efficacy. Further studies focusing on evaluation of suitable MAs for each cattle herd based on the urinary ZEN monitoring system are crucial.

OVERALL DISCUSSION AND CONCLUSION

While mycotoxins in animal feeds may cause acute intoxications, their greatest impact is their negative effect on animal performance and productivity. Thus, veterinary practitioners are confronted with difficult differential diagnoses such as subacute mycotoxin intoxications that usually lack specific clinical symptoms. In addition, farm managers, requiring optimal animal production, will question the quality of their feed supplies and expect advice from the veterinary professional on mycotoxin prevention methods, contamination, and treatment of the diseased animals (Malekinejad, 2004). Therefore, an objective assessment method using biological samples collected *in vivo* is necessary for monitoring the mycotoxin contamination/recovery in the cattle and is crucial for assessment of the dietary feeds and feeding management. We previously reported the practical usefulness of measuring urinary concentrations of ZEN and its metabolites not only for monitoring the natural ZEN contamination levels in cattle feed at the farm level, but also for *in vivo* assessment of MA function after supplementing feeds with MA (Takagi et al., 2011). In this thesis, practical application of our established urinary ZEN monitoring system for cattle herd was utilized to gain new insights regarding possible mycotoxin prevention methods at the farm levels.

As described in Chapter 1, we investigated the original feed source that was naturally contaminated by ZEN, which was incidentally detected from monitoring of urinary ZEN concentrations in 1 of the studied cattle herds. The results clearly indicated that the cause of the extremely high urinary ZEN concentration in that cattle herd was derived from rice straw that was infected with a *F. graminearum* species complex, resulting from the natural

contamination of rice straw by ZEN. To our knowledge, this is the first report indicating a direct relationship between fungal isolation and ZEN detection from the rice straw used as forage for cattle.

Recently, studies of mycotoxin occurrence in animal feed have been 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 (Binder et al., 2007). Our results clearly indicated an instance of natural ZEN contamination in a conventional cattle herd. Moreover, these data suggest that the mycotoxin contamination of dietary feeds, especially roughages such as hay and straw, may not be rare at the cattle farm levels. Additionally, our study demonstrates that the commercially available ZEN ELISA kit may be useful for measuring dietary ZEN. While its intended use is for measuring urinary ZEN, this is the first instance of sample screening from multiple cattle herds. Finally, we identified a potential clinical methodology to identify unknown ZEN-contaminated candidates from both the dietary feeds and water used for the cattle herd.

Practical and cost-effective methods of detoxifying mycotoxin-contaminated feed are in great demand. Besides preventive management of dietary feeds during their storing periods in farm conditions, approaches have been employed, including physical, chemical, and biological treatments, to detoxify mycotoxins in contaminated feeds. It has been proposed that a successful detoxification process must be economical and capable of eliminating all traces of toxin without leaving harmful residues and without impairing the nutritional quality of the dietary feeds (Oguz, 2012). As described in Chapter 2, we investigated the effect of MA

supplementation in feed as a top dressing based on measurements of urinary concentrations of ZEN and its metabolites at the farm level. Our results indicated that there was no reduction in the concentrations of urinary ZEN and α- and β-ZOL after supplementation with MAs in all 4 herds. This suggests a possibility that the method of supplementation, whether uniform mixing with feed as TMR or a top dressing on the feed, may affect the prevention of the absorption of ZEN and its metabolites from the gastrointestinal tracts of cattle. Another possibility is that MA may bind to mycotoxins; however, this may depend on the type/degree of mycotoxin contamination of the feeds. Overall, our results clearly indicate a new strategy of *in vivo* examination of the effects of MA supplementation by using a urinary ZEN monitoring system.

In conclusion, the results of these field trials confirm that a system for monitoring urinary ZEN concentrations is a useful tool for predicting the exposure of animals at the farm level to ZEN (and possibly other *Fusarium* toxins). By applying the urinary ZEN-monitoring system, it allows for the objective evaluation of MA supplementation to feeds at the farm level. Moreover, supplementation of diets with MAs may have an effect on ZEN efficacy. Further studies focusing on the collection of a large dataset are needed to enhance the understanding of the relationship between urinary ZEN concentrations and dietary feeds from cattle herds, and for the evaluation of suitable MAs for each cattle herd based on the urinary ZEN-monitoring system.

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