Bull Environ Contam Toxicol (2012) 88:396–400
DOI 10.1007/s00128-011-0478-6

Contamination Level of T-2 and HT-2 Toxin in Cereal Crops from Aba Area in Sichuan Province, China

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Received: 21 July 2011 / Accepted: 16 November 2011 / Published online: 27 November 2011

Abstract The contamination level of T-2 and HT-2 toxin in cereal crops from Aba area in Sichuan Province of China was investigated by rapid liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The results revealed the high incidence of T-2 and HT-2 toxin and relatively low contamination level in the samples. The incidence of HT-2 toxin was 49.74% and its average level was 3.746 μg/kg. The incidence of toxin was 11.64% and the average level was 0.565 μg/kg. The maximum of T-2 and HT-2 toxin concentration was 3.332 and 34.510 μg/kg, respectively. In addition, contaminated samples not only included homegrown products, but included external purchased rice and flour, which may be attributed to bad storage environment and sanitary conditions.

Keywords Contamination level · Cereal crops · T-2 and HT-2 toxin · HPLC-MS/MS

T-2 and HT-2 toxin belong to non-macrocyclic type A trichothecene produced by *Fusarium fungi* (Turker and Gumus 2009), which are commonly found in various cereal crops predominant grown in tropical and subtropical regions of America, Europe and Asia. HT-2 toxin is the major metabolite of T-2 toxin. The molecular structure of T-2 and HT-2 toxin is shown in Fig. 1. The 12, 13-epoxide ring of T-2 toxin is responsible for its toxic activity and de-epoxidation results in the loss of toxicity. T-2 toxin possesses high thermal and chemical stability and cannot be inactivated in food production and processing. Although insoluble in water, it is highly soluble in most of organic solvents.

It had been proved that the cereal crops contaminated by T-2 toxin may result in potential risks to the health of human and animals. As the most toxic species in trichothecenes, T-2 toxin can inhibit the activity of the immune system as well as the synthesis of proteins, DNA and RNA in vivo and in vitro (Moreno-Reyes et al. 1998). T-2 toxin may be produced in a wide temperature range (0–32°C), the maximum production of which can be reached at temperatures below 15°C. In addition, other factors such as grain defects and moisture content may lead to the production of T-2 toxin. For example, the most suitable moisture for T-2 toxin growth is 13%–22% (Moss 2002).

Although less frequently detected compared to other toxins in grain and other agricultural products, T-2 toxin has received much attention because it has the highest toxicity with relatively high contamination level in all kind of trichothecenes (Mateo et al. 2002). Since HT-2 toxin is a major metabolite of T-2 toxins in vivo and more than 80% of T-2 toxin may be converted to HT-2 toxin and other metabolites (Eriksen and Alexander 1998), HT-2 toxin is more commonly detected than T-2 toxin in infected cereals. Hence a common assessment for T-2 toxin and HT-2 toxin appears reasonable. It had been reported that T-2 toxin may be one of the important suspicious pathogenic factors of Kaschin-Beck Disease (KBD), an osteoarticular disease involving growth and joint cartilage (Cao et al. 2009; Liu et al. 2008; Yan et al. 2010). Hence, it is imperative for a detailed survey about the contaminative status of T-2 toxin in KBD areas. To date no systematical data are available on the distribution and contamination level of T-2 and HT-2 toxin in cereal crops from Aba area in Sichuan Province, one of the most serious KBD endemic

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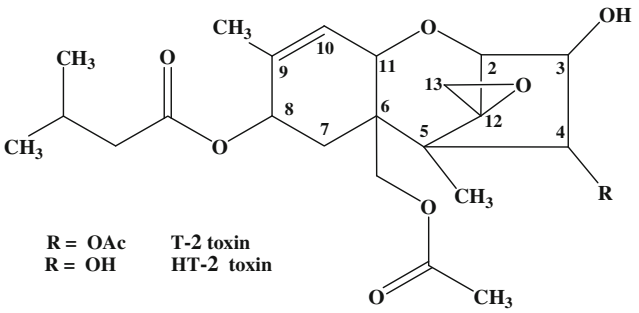

Fig. 1 Chemical structure of T-2 toxin and HT-2 toxin

areas in China. In this paper, we gathered the cereal crops from Aba area and investigated the contamination level and the geographic distribution characteristics of T-2 and HT-2 toxin in the area in detail by a high-performance liquid chromatography-tandem mass spectrometry method (HPLC-MS/MS).

Materials and Methods

A total of 189 naturally contaminated cereal crop samples were collected from serious KBD endemic families in Zamgtang, Maerkang and Aba County of Sichuan Province in Sept. 2010. To investigate the storage environment influence on cereal quality, the investigated samples not only include homegrown products such as highland barley and broad bean, but also include external purchased rice and flour. In the initial design of sampling methodology, equal quantity of samples should be collected. Actually, there exists some difference in the number of samples in different area, which is dependent on the discrepancy of population density, distribution status of local habitants and geographical complexity of sampling spot. Practically, more samples were collected in the relatively serious KBD region such as Zamgtang County. A total of 98, 67 and 24 samples were gathered in Zamgtang, Aba and Maerkang County, respectively. In Zamgtang County, 44 samples of highland barley, 26 samples of rice and 28 samples of flour were collected; 36 samples of highland barley, 16 samples of rice and 15 samples of flour were collected in Aba County; 5 samples of highland barley, 6 samples of broad bean, 4 samples of rice and 9 samples of flour were collected in Maerkang County. All the samples were air-dried at room temperature, then finely ground and passed through 60-mesh sieve. Finely ground samples should be stored under dry condition at room temperature until pretreatment course.

The stock solution of T-2 toxin (5.0 μg/mL) and HT-2 toxin (1.0 μg/mL) were prepared by dissolving T-2 toxin and HT-2 toxin standard (Sigma-Aldrich Co., USA) in methanol and stored at 4°C. Immunoaffinity column (Bond Elut Mycotoxin) was purchased from Varian Co., USA. Methanol and acetonitrile used for sample pretreatment and HPLC analysis were supplied by Fisher Scientific Co., USA. Ammonium Acetate (HPLC grade, Tianjin, China) was used as received. Deionized water, obtained from Milli-Q apparatus (Millipore Simplicity 185 system, France), was used for all procedures.

A total of 0.5 g finely ground sample was extracted with 4.0 mL of a mixture of acetonitrile/water (80:20, v/v) by blending at high speed for 5 min using a vortex oscillator. After centrifugation at 5,000 rpm for 5 min, the supernatant fluid was slowly pressed through Bond Elut Mycotoxin immunoaffinity column and eluate was collected. 2 mL of acetonitrile/water mixture (80:20, v/v) was used to flush the column and eluate was combined together. The combined eluates were evaporated to dryness by a gentle stream of nitrogen at the temperature of 70°C, and the residue was reconstituted in 0.5 mL of acetonitrile/water (20:80, v/v).

Analysis for T-2 toxin and HT-2 toxin was conducted on 3200 Q-trap triple quadrupole mass spectrometer coupled with an Agilent 1200 series liquid chromatography. The positive ion electrospray mode was adopted for all experiments. Data acquisition was controlled with Analyst 1.4.2 software. The Turbo Ion Spray source was operated at 570°C with the capillary voltage set at 5,500 V. Nitrogen was used as nebulizer gas (55 psi), curtain gas (20 psi) and collision gas (60 psi). The analytes were detected using multiple reactions monitoring (MRM) mode. The chromatographic separation was performed on a Zorbax SB C18 column (150 mm × 2.1 mm × 5 μm, Agilent). The mobile phase consisted of solvent A (methanol) and solvent B (1 mM ammonium acetate aqueous solution). The linear gradient was: 50% A at 0 min, 5% A from 2–4 min, reaching 100% B at 4.30 min, and then 50% B from 4.31 to 7.30 min. The flow rate was 950 $\mu L \cdot min^{-1}$ at 30°C and the injection volume was 15 μL.

Mixed standard solutions of T-2 toxin and HT-2 toxin with concentration change from 0 to 100.0 ng/mL were prepared with acetonitrile/water mixture (80:20, v/v). The peak area of each toxin was plotted against the corresponding concentration and the calibration curves were calculated by linear regression. The limit of determination (LOD) for both T-2 toxin and HT-2 toxin was 0.20 μg/kg determined by S/N equal to 3:1. For testing the recovery, extracts of blank highland barley, broad bean, rice or flour samples were spiked with the T-2 and HT-2 toxin standard solution at levels of 0.2, 1.0, 2.0, 4.0 and 20.0 μg/kg, cleaned up with Bond Elut Mycotoxin immunoaffinity column, and analyzed. Three replicates were performed for each spiked sample extract. The recovery ranges from 57.2% to 91.3% (RSD 9.7%) for T-2 toxin and from 95.2% to 106.8% (RSD 7.2%) for HT-2 toxin. Average recovery for T-2 and HT-2 toxin is 79.5% and 98.3% (RSD 9.9%). The relative standard deviations of the complete method

(analyzing two samples of highland barley, rice, flour and broad bean, naturally contaminated with T-2 and HT-2 toxin; ten replicates each) ranged from 3.47 (HT-2 toxin, highland barley) to 8.02% (T-2 toxin, rice).

Results and Discussion

In Aba area, relatively serious KBD endemic regions include Maerkang, Zamgtang and Aba County, among which Zamgtang County is the most serious KBD endemic area. Hence, 189 cereal crop samples from 15 suburban areas of the three counties were gathered and analyzed.

Analysis results showed that contents of T-2 and HT-2 toxin range from 0.236 to 3.332 μg/kg and from 0.245 to 34.510 μg/kg, respectively. There are 107 samples contaminated by T-2 or HT-2 toxin and total incidence rate arrives at 56.61%, which means that more than half of the samples had been polluted by T-2 or HT-2 toxin in different extent. The incidence rate of T-2 and HT-2 toxin is 11.11% and 49.74%, respectively. Table 1 gives the distribution characteristics of number of different cereal crop samples with T-2 and HT-2 toxin concentration over the limit of detection. As shown in Table 1, highland barley is the main samples polluted by HT-2 toxin. Figures 2 and 3 exhibit the distribution characteristics of contamination levels of T-2 and HT-2 toxin in different cereal crop samples. As seen in Fig. 2, highland barley samples containing HT-2 toxin accounts for very great proportion in all highland barley samples, the number of which far exceeded that of samples containing both T-2 toxin and HT-2 toxin. In addition, the content of HT-2 toxin is obviously higher than that of T-2 toxin in the samples containing both T-2 toxin and HT-2 toxin, whether new harvested samples or stale samples (data not shown). This phenomenon is consistent with the previous finding of Eriksen who attributed the phenomenon to the rapid metabolism of T-2 toxin to HT-2 toxin aroused by the local environmental conditions and the sampling period (Eriksen and Alexander 1998). In addition, the high incidence of T-2 toxin and HT-2 toxin in both new harvested samples and stale samples supports the view that T-2 toxin may grow not only during storage but also may grow on the crop in the field (Park et al. 1996).

The geographical distribution of T-2 and HT-2 toxin in highland barley from Aba area is shown in Fig. 3, which exhibits that the maximum of T-2 and HT-2 toxin contamination appears in Zamgtang and Maerkang County, respectively. The contamination level of T-2 and HT-2 toxin in highland barley shows an ascending trend with the sampling spot moving away from county in Zamgtang and Aba County, with the exceptional spots where relatively better hygienic conditions were found. The sequence for the average contamination level of T-2 toxin in cereal crops is as follows: Zamgtang County > Maerkang County > Aba County (data not shown). The result is in accordance with the distribution characteristics of the serious degree of KBD endemic regions in Aba area. In addition, it should be pointed out that T-2 toxin did not be found in highland barley from Aba County (as shown in Fig. 2).

In Aba area, rice and wheat cannot be cultivated due to the local climate conditions. All of rice and flour samples collected in sampling spots were provided by local

Table 1 Distribution characteristics of sample numbers with T-2 and HT-2 toxin concentration over LOD in different cereal crops from Aba area in Sichuan Province

Region	Number of samples (>*LOD*)							
	Highland barley		Rice		Flour		Broad bean	
	T-2	HT-2	T-2	HT-2	T-2	HT-2	T-2	HT-2
Zamgtang County	3	44	8	0	0	0	0	0
Aba County	0	36	3	0	0	4	0	0
Maerkang County	2	5	2	0	0	0	3	4

LOD: limit of detection of the method

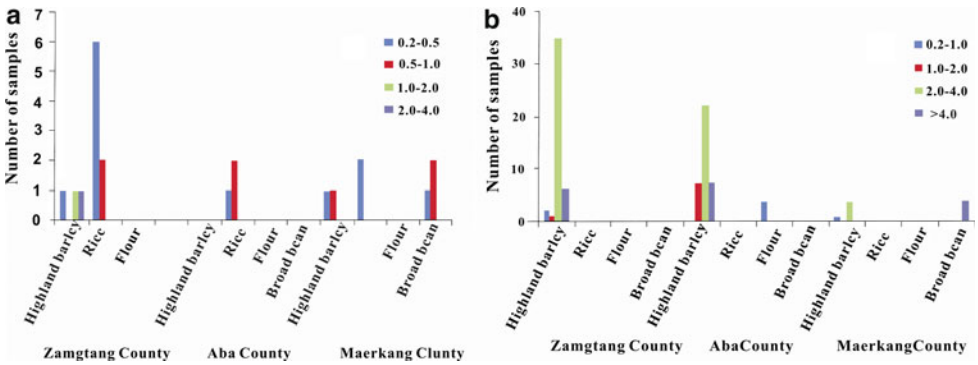

Fig. 2 Distribution characteristics of contamination levels of T-2 (**a**) and HT-2 (**b**) toxin in different cereal crops from Aba area in Sichuan Province

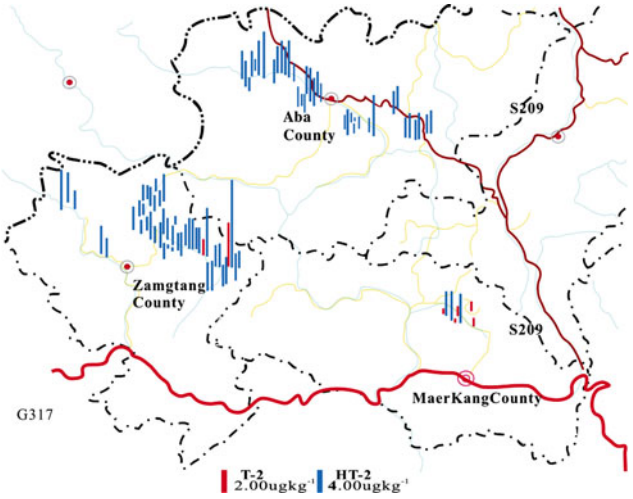

Fig. 3 Distribution characteristics of T-2 toxin and HT-2 toxin in highland barley from Aba area in Sichuan Province

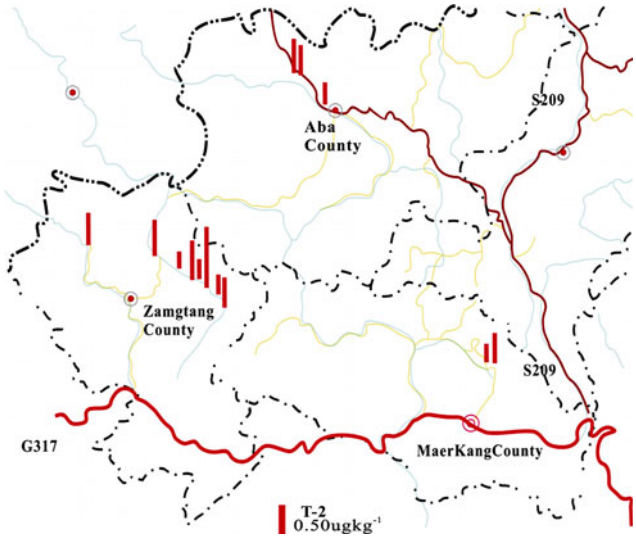

Fig. 4 Distribution characteristics of T-2 toxin in rice from Aba area in Sichuan Province

government which were uniformly purchased from non-KBD region and had been reserved in KBD endemic families for a period of time. The results showed that the contamination rate of T-2 and HT-2 toxin in external purchased cereal crops is 13.97% and 5.75%, respectively. During the investigation, we found that the foodstuffs were placed in the dark, moist, musty and unventilated storage room. It is well known that temperature and moisture conditions during storage are two critical factors affecting fungal infection and toxin synthesis (Edwards 2004; Muller et al. 1998). Storage of commodity under 14% moisture will minimize further fungal growth and production of the T-2 toxin. Moreover, grains kept dried prior to storage may decrease the effects of further contamination (Richard 2007). However, most of samples collected in Aba area were not dry enough for storage. In addition, Hell et al. (2000) once reported that clearing the remains of previous harvested crop residues is one of the basic sanitary measures against storage deterioration. In fact, local habitants in Aba area usually deposited new harvested and stale cereals in the same container, which may increase the chance for the spread of T-2 and HT-2 toxin. The detection results combined with above mentioned local actual situation on sample storage in sampling sites gave us a hint that the cereal storage environment and bad sanitary conditions in Aba area may result in the propagation of T-2 toxin and the contamination of cereal crops by T-2 and HT-2 toxin finally. An intriguing phenomenon is that there is only T-2 toxin contamination in rice samples (as shown in Fig. 4) and HT-2 toxin contamination in flour samples from Aba County (as shown in Fig. 5). The reason for the phenomenon deserves to be better investigated in future studies.

The contamination of T-2 and HT-2 toxin in broad bean samples from Maerkang County was also investigated (As shown in Fig. 6) because broad bean is the main subsidiary foodstuff of local habitants. The results showed that the contamination of HT-2 toxin in broad bean samples is far more serious than that in highland barley samples and the maximum of HT-2 toxin contamination arrived at 34.510 μg/kg.

The average level of T-2 and HT-2 toxin in positive cereal crop samples is 0.565 and 3.746 μg/kg, respectively. Generally speaking, the contamination level of T-2 and HT-2 toxin in the cereal crops from Aba area was lower than that in some countries and regions such as Norway, Germany, Poland and Tibet Autonomous Region of China (Langseth and Rundberget 1999; Schollenberger et al. 2006; Perkowski and Basinski 2002; Haubruge et al. 2003). According to the regulation stipulated by the Scientific Committee on Food of European Union, the combined

Fig. 5 Distribution characteristics of HT-2 toxin in flour samples from Aba County

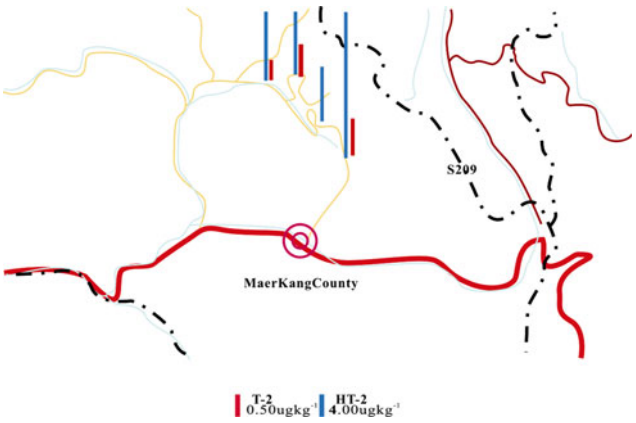

Fig. 6 Distribution characteristics of T-2 and HT-2 toxin in broad bean samples from Maerkang County

tolerable daily intake (TDI) for T-2 and HT-2 toxin is 0.06 μg/kg bodyweight/day. Hence, the tolerable daily intake for T-2 and HT-2 toxin should be in the range of 3.0–4.8 μg based on the fact that the body weight of local habitants is in the range of 50–80 kg. As we all know, highland barley is the staple food in Tibetan family. To evaluate the risk of daily intake of T-2 and HT-2 toxin on Tibetan, the information on the food habits were also investigated which showed that daily intake of highland barley for respective Tibetan changed from 0.50 to 0.80 kg approximately. Hence, the contamination extent of T-2 and HT-2 toxin in Aba area seems to be low and may not endanger the health of local habitants. Although there is no measurable long-term accumulation for T-2 toxin according to the report of JECFA 2001, it cannot be excluded that long-term usage of contaminated cereal crops may result in the accumulation in cartilage cell necrosis of local habitants in Aba area, if T-2 toxin is validated to be the pathogenic factor of KBD in the future research.

Understanding contamination level of hazardous toxins is an important premise for monitoring and evaluating crops quality and personal security in agricultural production region. The contamination level of T-2 and HT-2 toxin in cereal crops from Aba area in Sichuan Province revealed the high incidence rate of T-2 and HT-2 toxin and the relative low contamination level. In addition, polluted samples not only include homegrown products such as barley and broad bean, but also include external purchased rice and flour, which may be attributed to the cereal storage environment and bad sanitary conditions.

Acknowledgments We thank the financial support from China Geological Survey (Grant No. 1212010610918) and the Basic Research Program of National Research Center for Geoanalysis of China (Grant No. 2009CSJ05 and 2009CSJ06). The authors also thank the kind help of Mr. S. G. Luo and Mr. Q. G. Zhao in the course of sample collection.

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