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Detection of airborne trichothecene-producing *Fusarium* species in chicken houses

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TABLE OF CONTENTS

1. ABSTRACT

One hundred and forty three airborne *Fusarium* isolates in chicken houses belonging to seven *Fusarium* species were analyzed by PCR with *Tri5* gene as a specific marker of mycotoxin product . The result of *Tri5* gene sequence analysis indicates that the PCR amplification products were 89%-96% identical to the previously reported Tri5 genes, which were all amplified from four *F. poae* isolates. T-2 toxin and DON was measured by immunoaffinity column and high performance liquid chromatography in *Tri5*-positive *F. poae* isolates after being cultured at constant and alternating temperatures. The production of T-2 toxin under alternating temperatures was 14 and 53 times higher than those at constant temperature of 8°C and 25°C. No DON was detected under either testing temperature condition. It is concluded that T-2 toxin-producing *F. poae* isolates were present in poultry houses, and the concentration of T-2 toxin produced by *Tri5*-positive *F. poae* isolates was increased under alternating temperatures. The application of *Tri5*-PCR associated with IMC-HPLC is an effective and accurate method for rapid detection of T-2 and DON mycotoxins.

2. INTRODUCTION

The fungal species of the genus *Fusarium* are common molds in animal farm environments (1). The species of *F. avenaceum* (Fr.) Sacc., *F. graminearum* Schwabe and *F. poae* (Peck) Wollenw. have been considered to be the most important trichothecene–producing molds. Trichothecenes are a family of sesquiterpenoid mycotoxins. They are usually divided into four types (A–D) according to their characteristic functional groups (2). The epidemiological surveys have revealed that A and B of trichothecenes are widely distributed in cereals and feed as natural pollutants (2), however the macrocyclic trichothecenes rarely occur in food and feed (2). Deoxynivalenol (DON) and T-2 toxin are examples of the most common type-B and type-A trichothecenes, respectively. They are harmful to both human and animal health by being absorbed through the gastrointestinal tract or skin, resulting in alimentary toxic aleucia, articular cartilage degeneration, skin allergies, diarrhea, cardiac and nerve system damage (3, 4). Moreover, inhalation, ingestion and skin contact with mycotoxins and mycotoxin-producing fungi may cause

Table 1. Details of *Fusarium* isolates collected from the air of the chicken houses

Number	*Fusarium* species isolates	Counts of Fusarium isolates (CFU)	[1]Occurrence (%)	Original codes of collection strains	[2]Depository collection number
1	*F. equiseti*	8	5.59	DF0216 DF0304 DF0309 DF0110	IBE000011 IBE000122 IBE000196 IBE000278
2	*F. graminearum*	44	30.77	DF0109 DF0206 DF0312 DF1002	IBE000005 IBE000124 IBE000129 IBE000135
3	*F. nivale*	3	2.10	DF0912 DF1101 DF1106	IBE000066 IBE000078 IBE000125
4	*F. poae*	4	2.80	DF0416 DF0215 DF1040 DF0591	IBE000029 IBE000075 IBE000132 IBE000138
5	*F. oxysporum*	5	3.50	DF1201 DF0125 DF1104 DF1128	IBE000041 IBE000063 IBE000111 IBE000120
6	*F. solani*	50	34.97	DF0221 DF0415 DF1214 DF1028	IBE000002 IBE000015 IBE000090 IBE000105
7	*F. verticillioides*	29	20.28	DF0931 DF1022 DF1134 DF1108	IBE000009 IBE000082 IBE000106 IBE000127

[1] Occurrence = total count of isolates (143: the sum of *Fusarium* isolates) was divided by the count of each *Fusarium* species and multiplied by 100; [2] The *Fusarium* species collected in this study were deposited in the fungal collection center of Dalian Nationalities University.

infections, allergies and inflammatory reactions for immunocompromised human and animals (5, 6). Inhalation of toxigenic fungi such as aflatoxin-producing *Aspergillus* Micheli ex Link and especially trichothecene-producing *Fusarium* mold may result in more serious damage due to continuous mycotoxin production *in vivo* (7, 8). In a warm and humid environment, the risk of animal or human exposure to mycotoxins is more severe due to favorable environmental conditions (9, 10).

Traditionally, mycotoxin-producing fungi were detected by a complex process including fungal isolation and identification, fungal liquid growth and mycotoxin extraction and analysis. In recent years, more and more studies focused on the analysis of trichothecene genes, and demonstrated that *Tri5* genes of trichothecene-producing species of *Fusarium* are conserved (11). The first step of trichothecene synthesis is catalysis by trichodiene synthase, which catalyzes farnesyl pyrophosphate isomerization and cyclition. This gene is clustered with the other 10 genes involved in mycotoxin synthesis, including *Tri5* (trichodiene synthase), *Tri4* and *Tri11* (oxygenases), *Tri3* (acetyltransferase), *Tri6* (a zinc finger transcription factor), and *Tri12* (trichodiene efflux pump). These genes are clustered at the *Tri5* locus. Some of these genes (*Tri*3, *Tri*4, *Tri5*, *Tri*6) have been used in the detection of mycotoxins (eg.T-2 toxin) produced by *F. sporotrichiodes* Sherbakoff (3). Several other putative protein-coding genes (*Tri7, Tri8, Tri9, Tri10*) have also been found at the *Tri5* gene locus (12). Within this gene cluster, the functional changes of the genes involved in trichothecene biosynthesis gene cluster may lead to the production of different toxins (13).

The *Tri5*-PCR method has been applied for the detection and mycotoxin analysis of *Fusarium* species in grains (14). It has also been used to distinguish between toxin and non-toxin producing *Fusarium* species (3). T-2 toxin produced by *Fusarium* species could cause chicken's performance reduction, immune function decline, and blood biochemical changes (15). The task of quick identification of T-2 toxin-producing *Fusarium* species was always considered to be a key to the control of the harmful effects of molds on chickens and workers. The current study was aimed at applying *Tri5*-PCR technique to partially amplify the trichodiene gene from the genomic DNA of airborne *Fusarium* species, to screen for mycotoxin-producing *Fusarium* isolates and to develop a reliable method for the accurate detection of mycotoxin-producing *Fusarium* isolates. The toxin products of *Tri5*-PCR positive isolates and the toxin-producing conditions were further investigated. This study was also performed to determine airborne mycotoxin-producing *Fusarium* levels in order to control the potential exposure risks to mycotoxins in chicken houses.

3. MATERIALS AND METHODS

3.1. Sample Collection

Samples of airborne fungi were collected in December 2006 from the chicken houses of a poultry farm in Dalian, Liaoning province, China. The air samples were collected by using an Andersen-6 six stage air sampler at an air flow rate of 28.3 L/min, 50 cm above ground, and 2 - 4 min per sample.

Table 2. Similarity of the amplified *Tri5* gene product to previously published *Tri5* DNA sequences

No.	Fungal strains	Origins (base pair size)	Similarity	Genbank accession No.
1	*Fusarium sporotrichioides*	complete trichothecene synthase gene cluster (54488) trichothecene transfer gene cluster (5171), Tri5 gene (1185) Tri5 mRNA (1342)	96% 96% 96% 96%	AF359360 AF364179 AY130293 AY032745
2	*F. poae*	Tri5 gene (1194) Tox5 gene (1194)	94% 94%	AY130294 FPU15658
3	*Gibberella pulicaris*	Tri5 gene (1799)	93%	GBBTOX5
4	*F. pseudograminearum*	complete trichothecene synthase gene cluster (18894)	91%	AY102582
5	*Gibberella zeae*	trichothecene biosynthesis gene cluster (30197) trichothecene gene cluster (18401)	90% 90%	AB060689 AY102584
		complete trichothecene synthase gene cluster (57840) Tri5 gene (3669) Tri5 gene (3747) Tri5 gene (1187) Tri5 mRNA	89% 89% 89% 89% 89%	AF359361 AF508152 AF508153 AY130290 XM_383713
6	*F. asiaticum*	trichothecene gene cluster (18670)	90%	AY102604
7	*F. cerealis*	trichothecene gene cluster (18687)	90%	AY102574
8	*F. culmorum*	trichothecene gene cluster (18936) Tri5 gene (1188)	89% 89%	AY102602 AY130291
9	*F. cortaderiae*	trichothecene gene cluster (18863)	89%	AY102600
10	*F. meridionale*	trichothecene gene cluster (18863)	89%	AY102593
11	*F. austroamericanum*	trichothecene gene cluster (18864)	89%	AY102588
12	*F. mesoamericanum*	trichothecene gene cluster (18552)	89%	AY102598
13	*F. boothii*	trichothecene gene cluster (18540)	89%	AY102597
14	*F. acaciaemearnsii*	trichothecene gene cluster (18717)	89%	AY102578

3.2. Fungal culture and identification

Fungi were cultured on Rose Bengal Chloromycetin (RBC) medium in an illumination incubator for 3-7 days at 25°C. Fungal colonies were counted according to their morphological characteristics under an Olympus stereo microscope. Pure cultures were isolated and confirmed by transferring conidia to standard Petri dishes containing Potato Dextrose Agar (PDA) and cultured for 3-7 d at 25°C. Pure cultures were preserved by vacuum freezing and drying method. *Fusarium* strains were cultured on PDA, Saccharose Nirenberg Agar (SNA), and Potato Sucrose Agar (PSA) for accurate identification. The *Fusarium* species were morphologically identified based on their colonies and reproductive structures according to Leslie and Summerell (16), Gerlach and Nirenberg (17), Nelson (18), Joffe (19) and Booth (20).

3.3. DNA extraction

The *Fusarium* isolates were grown at 25°C for 6-7 days in three 1.5 mL Eppendorf tubes, each containing 0.5 ml liquid glucose yeast medium (GYM, which consisted of: $NH_4H_2PO_4$, 1.0g; KCl, 0.2g; $MgSO_4{\cdot}7H_2O$, 0.2 g; glucose, 10.0 g; yeast extract, 5.0 g; $CuSO_4$ solution which consisted of 0.005 g $CuSO_4{\cdot}5H_2O$ per litre, 1ml; $ZnSO_4$ solution which consisted of 0.01 g $ZnSO_4{\cdot}7H_2O$ per liter, 1 ml; Distilled water, 1000 ml). The genomic DNA was extracted as described by Cenis (21) and the concentration was determined by electrophoresis in 1% agarose gel, staining with ethidium bromide and imaging with a GDS 8000 (UVP, US) transilluminator.

3.4. *Tri5* gene amplification

Genomic DNA was quantified by 2% agarose gel electrophoresis. The total reaction volume of the PCR reaction was 25 μL, containing 1× PCR buffer (1.5 mM $MgCl_2$), 0.4 μM each primer HATri/F (5'-CAGATGGAGAACTGGATGGT) and HATri/R (5'-GCACAAGTGCCACGTGAC), 1 U Taq DNA polymerase (TAKARA Biotechnology Dalian Co. Ltd.), 50 μM of each dNTP, and 50 ng template DNA. The PCR was conducted with an initial denaturation step of 94°C for 75 sec, followed by 35 cycles of 94°C for 15 sec, 62°C for 15 sec, and 72°C for 45 sec, with a final extension step of 72°C for 4 min 15 sec.

3.5. Nucleotide sequencing

DNA sequencing was performed with the primers HATri/F and HATri/R in separate reactions using the Applied Biosystems Prism BigDye terminator v3.1 kit. Sequencing of the *Tri5* gene fragment was conducted by an Applied ABI PRISM 3730 DNA Sequencer (Shanghai Sangon Biotech Inc). Forward and reverse sequences were aligned and the sequences of *Tri5* gene fragment were determined, and then analyzed by BLAST in NCBI (22).

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3.6. Mycotoxin-producing culture of *Tri5* positive isolates

The *Tri5* positive isolates of *F. poae* (DF0416, DF0215, DF1040, DF0591) (Table 2) were inoculated in GYM (21) and respectively incubated at constant temperatures (8°C or 25°C) for 15 days, and at alternating temperatures of 8°C and 25°C at 12 h intervals. The *Tri5* negative airborne strains were simultaneously inoculated as control.

3.7 DON and T-2 detection

The culture solutions of *Tri5* positive and negative isolates in GYM were filtered through a 0.44 μm nitrocellulose filter. DON or T-2 toxin was isolated and purified by using a DON or a T-2 immunoaffinity column (VICAM Co. Ltd, USA), followed by quantification using an HPLC system (HP1100, Agilent Technology Inc) as described by Sui (23) and Li (24). The T-2 standard was purchased from Sigma (purity more than 99%).

Table 3. Concentration of T-2 toxin in the liquid cultures of *Tri5* positive isolates at different incubation temperatures

[1]Code of isolates	Concentration of T-2 toxin (μg/ml)		
	[2]8°C	[3]25°C	[4]Alternating 8°C and 25°C at 12 h intervals
DF0416	0.103	0.031	1.748
DF0215	0.098	0.031	1.756
DF1040	0.106	0.035	1.754
DF0591	0.102	0.032	1.753
Mean +/- S.D.	0.121+/-0.017	0.033+/-0.002	1.751+/-0.003

[1] Orignal codes of collection strains (Table2) ; [2] Culture at constant temperature of 8°C for 15days; [3] Culture at constant temperature of 25°C for 15days; [4] Culture at alternating 8°C and 25°C at 12h intervals for 15days.

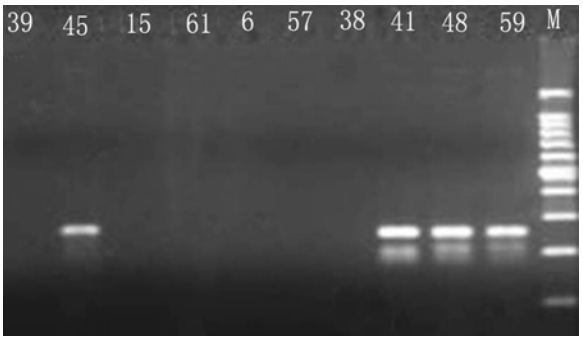

Figure 1. Agarose gel of the amplification products generated by the *Tri5* primers for the 10 *Fusarium* strains of 143 airborne *Fusarium* isolates (Table2) tested. Lanes 45, 41, 48 and 59 are *F. poae* (DF0416, DF0215, DF1040, DF0591); lane 15 is *F. verticillioides* (DF1022); lane 39 is *F. equiseti* (DF0216); lane 61 is *F. graminearum* (DF0109); lane 57 is *F. oxysporum* (DF1128); lane 38 is *F. solani* (DF1214), and lane 6 is *F. nivale* (DF1106). The other lanes without *Tri5* genes are not shown here.

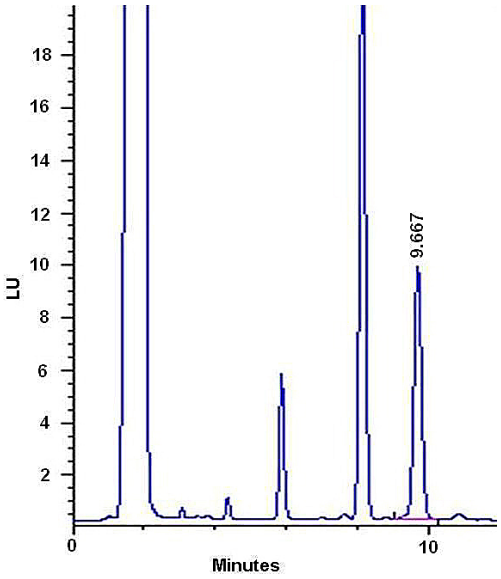

Figure 2. HPLC chromatogram of T-2 toxin standard sample.

4. RESULTS

4.1. Airborne *Fusarium* species

One hundred and forty three airborne *Fusarium* isolates were aquired from 108 airborne samples collected from poultry houses of a chicken farm in Dalian, China in 2006 (Table 1). Seven *Fusarium* species were morphologically identified, namely *F. poae* (Peck) Wollenweber (n = 4), *F. graminearum* Schwabe (n = 44), *F. oxysporum* Schlecht. ex Snyder et Hansen (n = 5), *F. verticillioides* (Sacc.) Nirenberg (n = 29), *F. equiseti* (Corda) Sacc. (n = 8), *F. solani* (Martius) Appel et Wollenw. ex Snyder et Hansen (n = 50) and *F. nivale* (Fr.) Ces. (n = 3). The highest occurrence rate was 34.97% for *F. solani*, and the lowest was 2.10% for *F. nivale* (Table 1). The genomic DNAs of 143 *Fusarium* isolates were successfully extracted.

4.2. *Tri5* - PCR assay

The results of *Tri5*-PCR reaction for the 143 *Fusarium* isolates show that specific fragments of 260 bp (EF547372) were obtained from all four isolates of *F. poae* but not from the other *Fusarium* isolates (Figure 1), suggesting that all *F. poae* isolates were capable of producing trichothecene. Analysis of 50 BLAST hits of the partially amplified *Tri5* gene from *F. poae* using the nucleotide-nucleotide searching option in GenBank indicates that the sequences of the amplified products had 89% to 96% similarity to the other *Fusarium Tri5* genes (Table 2).

4.3. Detection of mycotoxin related to *Tri5* gene

All isolates of *F. poae* were cultured to assay for the production of T-2 toxin and DON by the methods as described in the experimental procedure. The concentrations of T-2 toxin (1.751+/-0.003 μg/ml) produced by the isolates cultured at alternant temperature 8°C and 25°C were 14 and 53 times higher than those treated at constant temperature 8°C (0.121 +/- 0.017 μg/ml) and 25°C (0.033 +/- 0.002 μg/ml) (Table 3, Figure 2, Figure 3, Figure 4, Figure 5). None of the tested *Tri5* negative cultures was found to produce T-2 toxin, and DON was not detected in any of the tested fungal cultures. These results suggest that the airborne *F. poae* isolates are substantial producers of T-2 toxin (1.751 +/- 0.003 μg/ml), and their T-2 toxin producing ability was significantly increased when exposed repeatedly to alternating high and low temperatures.

5. DISCUSSION

The toxicity of trichothecene susquiterpenoids is being increasingly recognized as important in agriculture and the health of immunocompromised human subjects. The traditional method of identifying and detecting environmental toxins produced by *Fusarium* spp. requires laborious procedures including laboratory culture, morphological examination, and fungal liquid culturing to assay for toxin production. These methods are time-consuming and often inaccurate. The screening of fungal isoaltes with trichodiene synthase encoding sequence by means of hybridization to *Tri5* gene cloned from *F. poae*

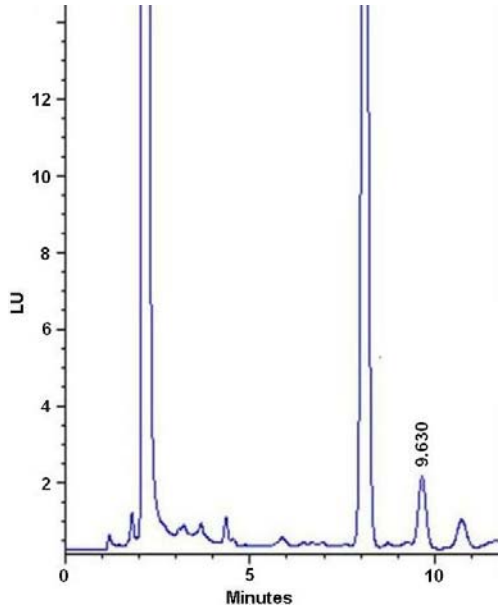

Figure 3. HPLC chromatogram of T-2 toxin from *F. poae* (DF0591) cultured at constant temperature of 8°C for 15 days.

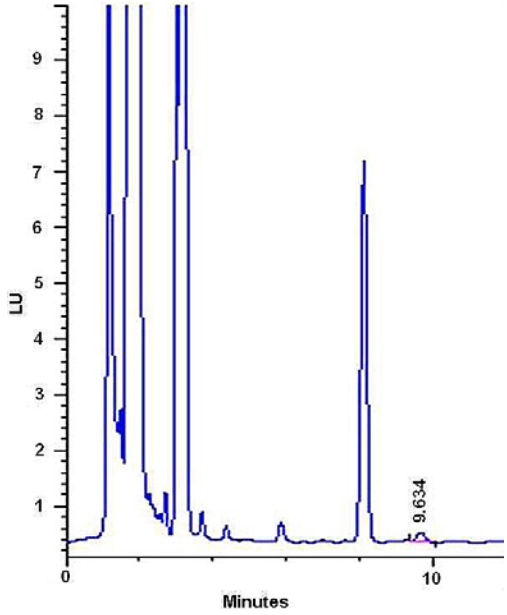

Figure 4. HPLC chromatogram of T-2 toxin from *F. poae* (DF0591) cultured at constant temperature of 25°C for 15 days.

only reveals its genetic capability for trichothecene synthesization and gives no proof for the actual toxin production (11). Therefore, the technique of *Tri5*-PCR associated with immunoaffinity -HPLC analysis was applied in this study to assay for rapid determination of airborne trichothecene-producing *Fusarium* isolates for the detection of toxic metabolites (T-2 toxin and DON). The mass screening of fungal isolates for their genetic capability of trichothecene production could be conducted by the *Tri5*-PCR method.

The test results show that all the *Tri5* positive *F. poae* isolates produced T-2 toxin but not DON. In contrast, the previous studies reported that the *F. poae* isolates from Norway and Poland were not T-2 toxin producers (25). The presence of toxigenic fungi depends on both extrinsic (environmental and geographic conditions) and intrinsic factors (constituents of matrix) (26). For instance, most *Fusarium* isolates from high latitude area (Northern US, Northern Canada, Norway, Russia, and Northern China) produced T-2 and HT-2 toxin, while the *Fusarium* isolates from lower latitude areas, e.g. Egypt and India hardly produced mycotoxin (27). Moreover, *Fusarium* isolates originating from different matrix, such as soil, feed/food and air, might not be equally toxigenic (28, 29, 30). The current investigation reveals the fact that the *F. poae* isolates in the air of poultry houses in Dalian, China (Northeastern China) were T-2 toxin-producers.

Our previous work showed that the levels of airborne mycotoxins were closedly related to the presence of toxigenic fungi (1). However, the relationship between airborne T-2 toxin or DON and the concentration of airborne *Fusarium* species was not investigated in the current study. The molecular mechanisms that regulate the production of T-2 toxin or DON are still not clear, but it is confirmed that the *Tri5* gene-positive *Fusarium* isolates were potentially producers of tricothecene, especially T-2 toxin. It does not suggest that the presence of the T-2 toxin-producing *Fusarium* species in the air of chicken houses mean the presence of airborne T-2 toxin. On one hand, the occurrence of T-2 toxin-producing *Fusarium* isolates (4/143) in the air of chicken house was much lower than in the maize fields (13/40) (27). On the other hand, the level of T-2 toxin produced by *Tri5* gene-positive *Fusarium* isolates was significantly influenced by environmental conditions, especially temperature. When *Tri5* gene-positive *F. poae* isolates were cultured under an alternating temperature of 8°C and 25°C every 12 h, the T-2 toxin production level was much higher than at constant temperature (8°C or 25°C). All *F. poae* isolates cultured at 8°C or 25°C produced little toxin. The concentration of T-2 toxin produced by *Tri5*-positive *F. poae* could be increased by repeated high and low temperature alternation. These results indicate that the level of T-2 toxin in the chicken house may be very low because the chicken houses were usually kept at a relatively constant temperature. However, mycotoxins are normally considered to be primarily in the mycelia and spores of the toxigenic fungal strains (31, 32, 33, 34, 35). If airborne fungal spores are inhaled into the bronchi and alveoli, they will be lysed and the human body is thereby exposed to their primary and secondary metabolites (5). Inhalation of toxigenic fungi may result in more serious damage due to continuous mycotoxin production *in vivo* (36). “Non-specific” granuloma of the paranasal sinuses and orbit in Northern Sudanese was shown to be the result of infection by toxigenic *Aspergillus flavus* (7). Excretion of aflatoxin by frogs after implantation with toxigenic *Aspergillus flavus* suggests that *A. flavus* retains it aflatoxin-producing ability during

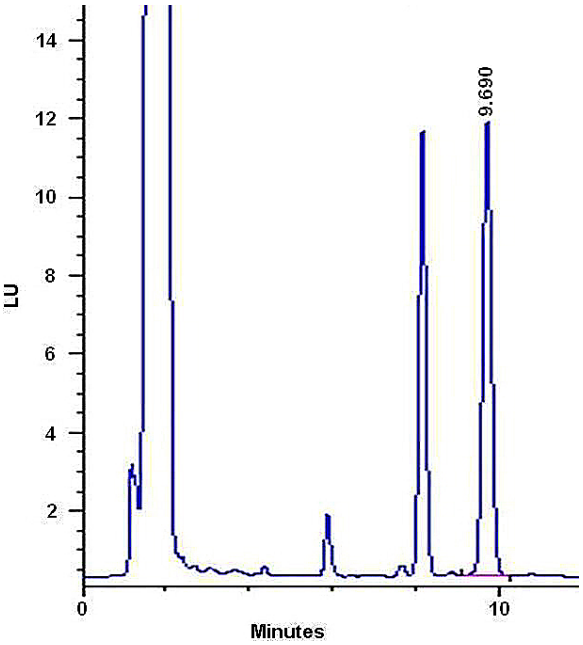

Figure 5. HPLC chromatogram of T-2 toxin from *F. poae* (DF0591) cultured at alternating temperature of 8°C and 25°C at internals of 12h for 15 days

simulated aspergillosis in frogs (8). Therefore, more attention should be provided to the T-2 toxin-producing *Fusarium* species found in the chicken house environment. The PCR technique could be used for fast identification of mycotoxin-producing *Fusarium* species and it would be helpful for developing strategies to avoid or reducing mycotoxin contamination of the chicken raising environment.

6. CONCLUSION

The current study is the first report of screening for toxigenic *Fusarium* isolates in the air of chicken houses by using *Tri5*-PCR coupled with IMC-HPLC. Furthermore, the explored method has never been used for detection of T-2 toxin–producing *Fusarium* isolates in chicken houses. This study takes advantage of PCR technique for the characterization of the toxigenic *Fusarium* species in the animal raising environment. The *Tri5*-PCR technique is faster and more accurate than traditional methods such as laborious subculture and microscopic observation of the airborne spores. The T-2 toxin–producing *Fusarium* isolates can be rapidly detected by *Tri5*-PCR combined with HPLC and T-2 toxin immunoaffinity column. As an effective and fast detection method, it could be widely applied in future investigations of airborne toxins and toxigenic fungi.

7. ACKNOWLEDGEMENTS

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