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Molecular characterization and liquid chromatography-mass spectrometric multiple reaction monitoring-based detection in case of suspected phalloides syndrome poisoning

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ABSTRACT

Background: The most common lethal mushrooms are invariably attributed to amanitas, which contain several types of lethal peptide toxins. During 2015 to 2018, the suspect in phalloides syndrome case reported to the Thai National Institute of Health included 33 patients with 3 deaths. This syndrome is characterized by a long latent period and having two phases of gastrointestinal irritation followed by progressive liver dysfunction.

Objectives: The aims of this study were to identify mushroom samples from four clinically reported cases based on nuclear internal transcribed spacer (ITS) sequence data and diagnose lethal peptide toxins using liquid chromatography (LC)-tandem mass spectrometry (MS/MS) with multiple reaction monitoring (MRM).

Materials and methods: Nucleotide similarity was identified using BLAST search of the NCBI database. Phylogenetic analysis of nuclear internal transcribed spacer (ITS) region was conducted by maximum likelihood method. Mushroom peptide toxins were analyzed using LC-MS/MS with MRM.

Results: Based on BLAST search yielded 98% to 100% of mushroom samples from four clinically reported cases to *Amanita brunneitoxicaria*. Phylogenetic analysis showed all mushroom samples placed closely related to *A. brunneitoxicaria* with a strong bootstrap value (BS=100%). The presence of three lethal peptide toxins in clinical mushroom samples was confirmed by MS/MS spectra acquired from a reference standard, including α-amanitin (*m/z* 919.0, RT=2.157 min), β-amanitin (*m/z* 920.1, RT=2.167 min) and phalloidin (*m/z* 789.3, RT=2.189 min). The product ions of *m/z* 259.3, 259.0 and 330.3 were the most abundant and stable ions for the α-amanitin, β-amanitin and phalloidin analyses, respectively.

Conclusion: This study revealed that the toxic mushrooms ingested by patients were confirmed to be a species of amanitas closely related to *Amanita brunneitoxicaria*. Furthermore, rapid detection using a high-throughput LC-MS/MS with MRM represents an effective method in identifying lethal peptide toxins from poisoning caused by mushrooms.

Introduction

A checklist of Thai Basidiomycetes mushrooms was reported, including 1,978 species.[1] Several mushroom species has proven to be edible and become important in economics. However, the toxicity of some mushrooms species is still inadequate. According to mushroom poisoning case report of Toxicology center (Thai National Institute of Health) were identified approximately 76% for gastrointestinal irritants, 14% for amanitin-containing mushrooms and 10% for alkaloid muscarine-containing mushrooms, respectively.[2] The most common lethal wild mushrooms inhabited are

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amanitas. These mushrooms are sometimes misidentified as their young fruiting bodies; furthermore, there are misconceptions about ethnomycological knowledge such as mushrooms that are ingested by animals are safe for humans and boiling of mushrooms can detoxify toxins.[3] In cases of poisoning, a rapid identification of mushroom samples consumed by the patients is required for appropriate medical treatments. However, the available mushroom samples are not always well-preserved and the standard morphological identification of particular samples needs experienced mycologists. Hence, molecular-based identification as well as LC-MS/MS with MRM method should be employed as tools to identify the poisonous mushroom samples in the absence of morphological characteristics. For molecular marker, the internal transcribed spacer (ITS) region of nuclear ribosomal DNA has successful identification in various groups of mushroom. This locus was proposed as the universal barcode region for fungal identification[4,5] and used in forensic investigations to confirm several cases of mushroom poisoning.[2,3,6,7] Moreover, we developed a rapid and convenient high-throughput method to identify the lethal peptide toxins in fatal case of mushroom poisoning. To analyze the lethal peptide toxins, LC-MS/MS confirmatory method with multiple reaction monitoring (MRM) was performed to measure the product ions of interest.[8] This method could become a practical method for toxicological purpose.

Materials and methods

Clinical mushroom samples and case reports

Mushroom samples obtained from clinically reported cases during 2015 to 2018 with case reports of poisoning were used in this study. In each case hospitalization was required. A total of 36 patients were suspected phalloides syndrome after mushroom ingestion. All patients represented a latent period about 4 to 24 hours that exhibits two phases of gastrointestinal irritations and liver dysfunction. Three deaths occurred in patients due to acute hepatic and renal failure.

DNA extraction and PCR amplification

Fruiting bodies of 2-15 mg were ground in liquid nitrogen. DNA was extracted using the PureLinkTM Plant total DNA purification kit (Invitrogen, USA) according to the manufacturer's instructions. The entire nuclear ITS regions was simultaneously amplified using the universal primers ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3').[9,10] PCR condition was as described previously.[3]

Fungal identification and phylogenetic analyses

The nuclear ITS sequences obtained from the clinical mushroom samples were compared for nucleotide similarity against the GenBank database using BLASTN 2.8.0 server.[11,12] DNA sequences alignment was done using Geneious R8 (http://www.geneious.com/). Ambiguously aligned portions were removed manually. The ITS sequences belonging to core taxa of section of *Phalloidea*e was downloaded from GenBank (Figure 1). These sequences were selected based on current phylogeny of the genus *Amanita*.[13] Phylogenetic analysis was conducted using maximum likelihood (ML) method. ML analysis was performed in RAxML 7.2.6 using the GTRGAMMA model with 25 rate parameter categories.[14] Branch support was estimated by using 1000 bootstrap pseudoreplicates. Only clades that received bootstrap support ≥70% under ML was considered as strongly supported. Phylogenetic tree was depicted using the program FigTree 1.4.3 (http://tree.bio.ed.ac.uk).

Sample preparation and purification

Five grams of clinical mushroom samples were blended and extracted with 20 mL of methanol. The extract was incubated at 65 ^{o}C for 10 min, followed by centrifugation at 14,000 rpm for 5 min. Supernatant (100 µL) was then re-extracted with methanol-chloroform-deionized water (4:2:3, v/v/v). The tube was gently inverted 3 or 4 times followed by 3 min of centrifugation at 14,000 rpm to separate the layers. Polar (lower) phase was transferred to a clean microcentrifuge tube. Supernatant was homogenized with 300 µL of methanol and centrifuged for 3 min at 14,000 rpm. Clear supernatant was decanted to dryness under a stream of nitrogen. Residue was dissolved in acetonitrile and filtered with VertiPure™ PVDF(HL) syringe filters (13 mm, 0.2 µm). The filtrate was then submitted for subsequent analysis.

Peptide toxins detection using LC-MS/MS

Peptide toxins including α-amanitin, β-amanitin, phallacidin and phalloidin obtained from Sigma-Aldrich (St. Louis, USA) were used as standard reference material. Separation of peptide toxins and determination of molecular weight as well as the precursor ion fragmentation were using LC-MS/MS method on Agilent 6495 Triple Quadrupole LC/MS and 1290 infinity LC modules with Agilent MassHunter software (California, USA). In the chromatographic system, a Z0RBAX SB-C18 narrow-bore (21 mm x 150 mm, 3.5 µm) and ZIC-HILIC (20 mm x 2.1 mm, 3.5 µm) columns were used for separation of amatoxins (α-amanitin & β-amanitin) and phallotoxins (phallacidin & phalloidin), respectively. Amatoxins mobile phases were 10 mM ammonium acetate in 0.1% (v/v) formic acid (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B) for the separation of α-amanitin and β-amanitin. Phallotoxins mobile phases were 2 mM ammonium formate in deionized-H_2O (mobile phase A) and acetonitrile (mobile phase B). A program was started with 10% mobile phase A and 90% mobile phase B. Total run time for each sample was 15 min. Flow rate was 0.2 mL/min, and the injection volume was 5 µL. The column temperature was 40°C.

Mass spectrometer was performed in MS/MS mode using a multiple reaction monitor (MRM) to detect a specific precursor ion (MS1) to product ion (MS2) transitions for each analysis. The MS conditions were as follows: gas temperature, 200°C; gas flow, 14 L/min; nebulizer, 40 psi; and capillary voltage, 4,000 V.

Results

Fungal Identification using NCBI-BLAST

The remnant mushroom samples were amplified by PCR based amplification of the nuclear ITS regions. A PCR amplification and purification product was approximately 700 bp long. Result of BLAST search showed the highest pairwise identify for all samples tested with scores ranging from 98 % to 100% identity, which exhibited identical species identification for *Amanita brunneitoxicaria* (KY747436) and the type material of *A. brunneitoxicaria* (NR_151655).

Phylogenetic analysis of mushroom samples consumed by the patients

A matrix of 731 unambiguously aligned nucleotide characters was constructed. ML analysis of the nuclear ITS dataset yielded a tree with the final optimization likelihood of lnL = -3167.926. In this study, we focused on the clinical mushroom samples that suspected phalloides syndrome poisoning and resulted in death of the patients. All clinical mushroom samples are clustered with *Amanita brunneitoxicaria* (BS=100%) and *A. fuligineoides* as a sister taxa (Figure 1). Based on the remnant mushroom samples, its general features include pileus darkest at centre towards greyish at margin, convex to plane; cylindrical stipe, white to pale grey; context stuffed and bulb subglobose with saccate volva (Figure 2).

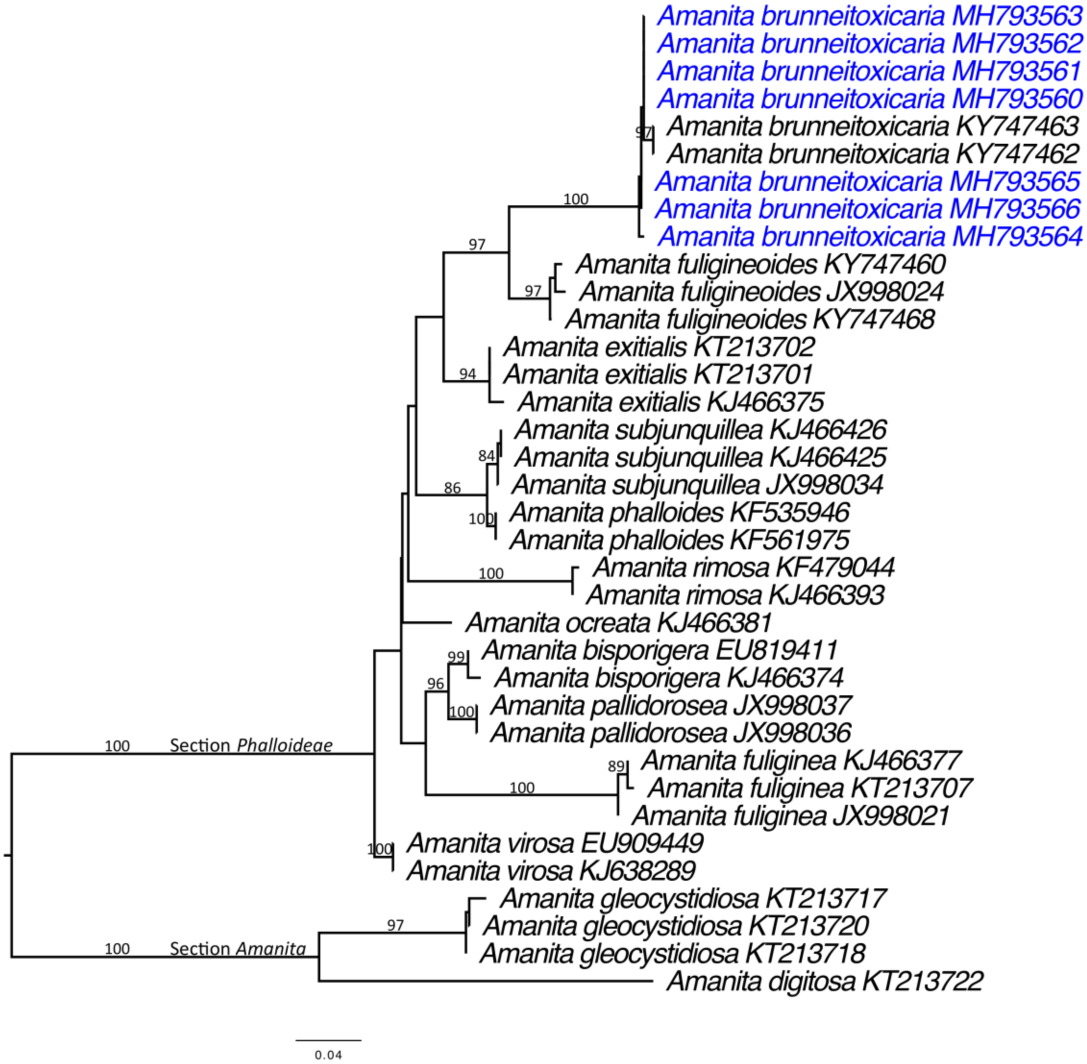

Figure 1 *Best-scoring maximum likelihood tree based on the nuclear ITS rDNA sequences of selected mushroom species of section Phalloideae. Clinical mushroom samples ingested by patients are highlighted. Bootstrap support values performed with RAxML 7.2.6 are given in number above branches. Reference sequences were downloaded from GenBank.*

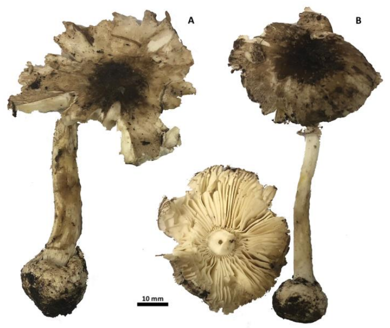

***Figure 2**. The remnant clinical mushroom samples (A) Amanita sp. D344 and (B) Amanita sp. D346.*

Peptide toxins

The reference standard and purified compounds obtained from the clinical mushroom samples were analyzed with MS/MS spectra and the corresponding molecular weights were performed based on the fragmentation of the precursor ion (Figure 3 & 4). The lower limit of quantification was 11 µg/L for α-amanitin, 11 µg/L for β-amanitin, 100 µg/L for phallacidin and 10 µg/L for phalloidin. The ion acquisition timeframe revealed in the range of 100-1,000 (*m/z*). Multiple reaction monitoring (MRM) was performed at unit resolution using a mass transition ion pair (Table 1). The product ions *m/z* 259.3, 259.0, 811.5 and 330.3 were the most abundant and stable ions for the α-amanitin, β-amanitin, phallacidin and phalloidin analyses. Three lethal peptide toxins of α-amanitin, β-amanitin and phalloidin were detected in all mushroom samples (Figure 4).

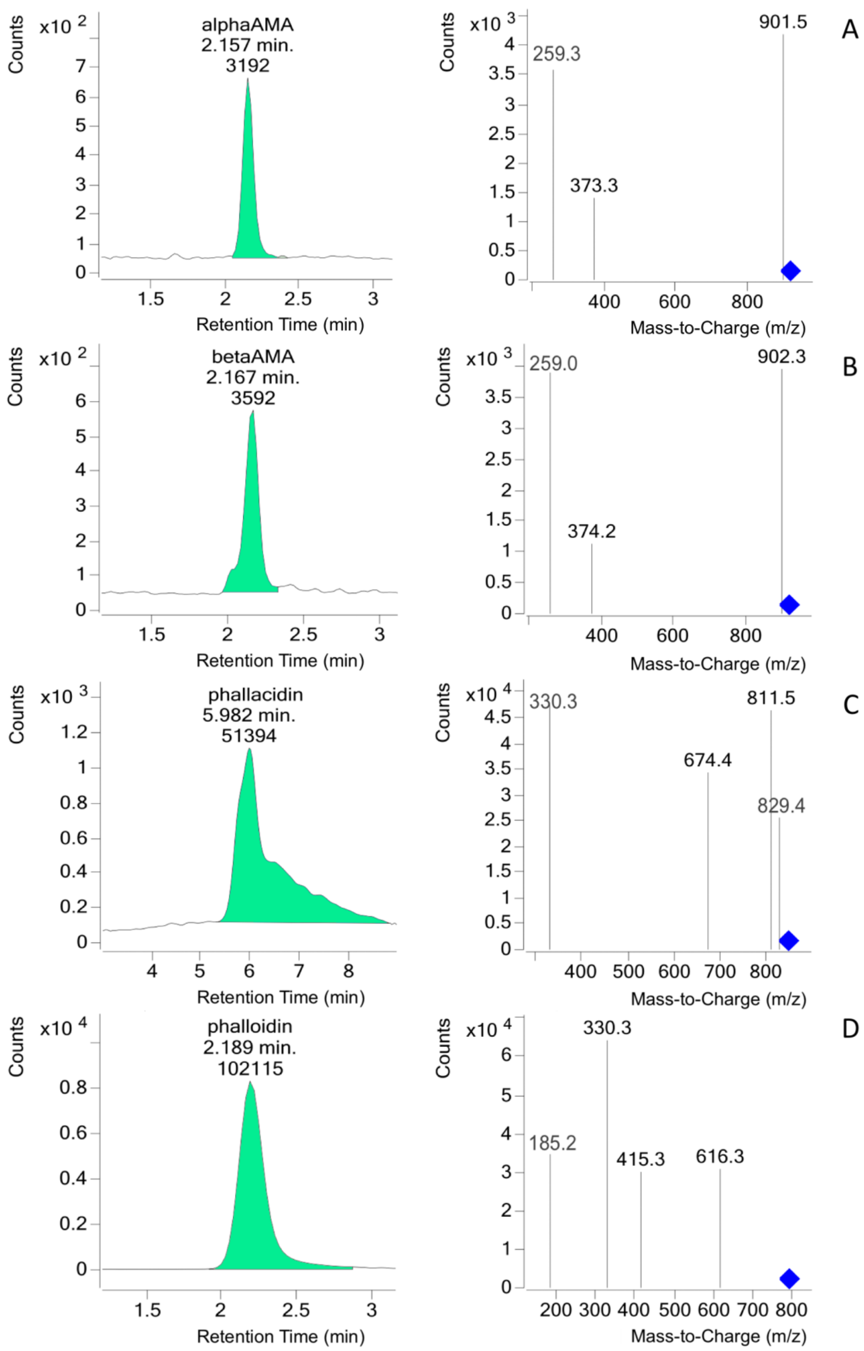

***Figure 3**. Liquid chromatography-tandem mass spectrometric MRM chromatograms of standard lethal peptide toxins (A) alpha amanitin, (B) beta amanitin, (C) phallacidin and (D) phalloidin. Diamond-shape represents the molecular ion.*

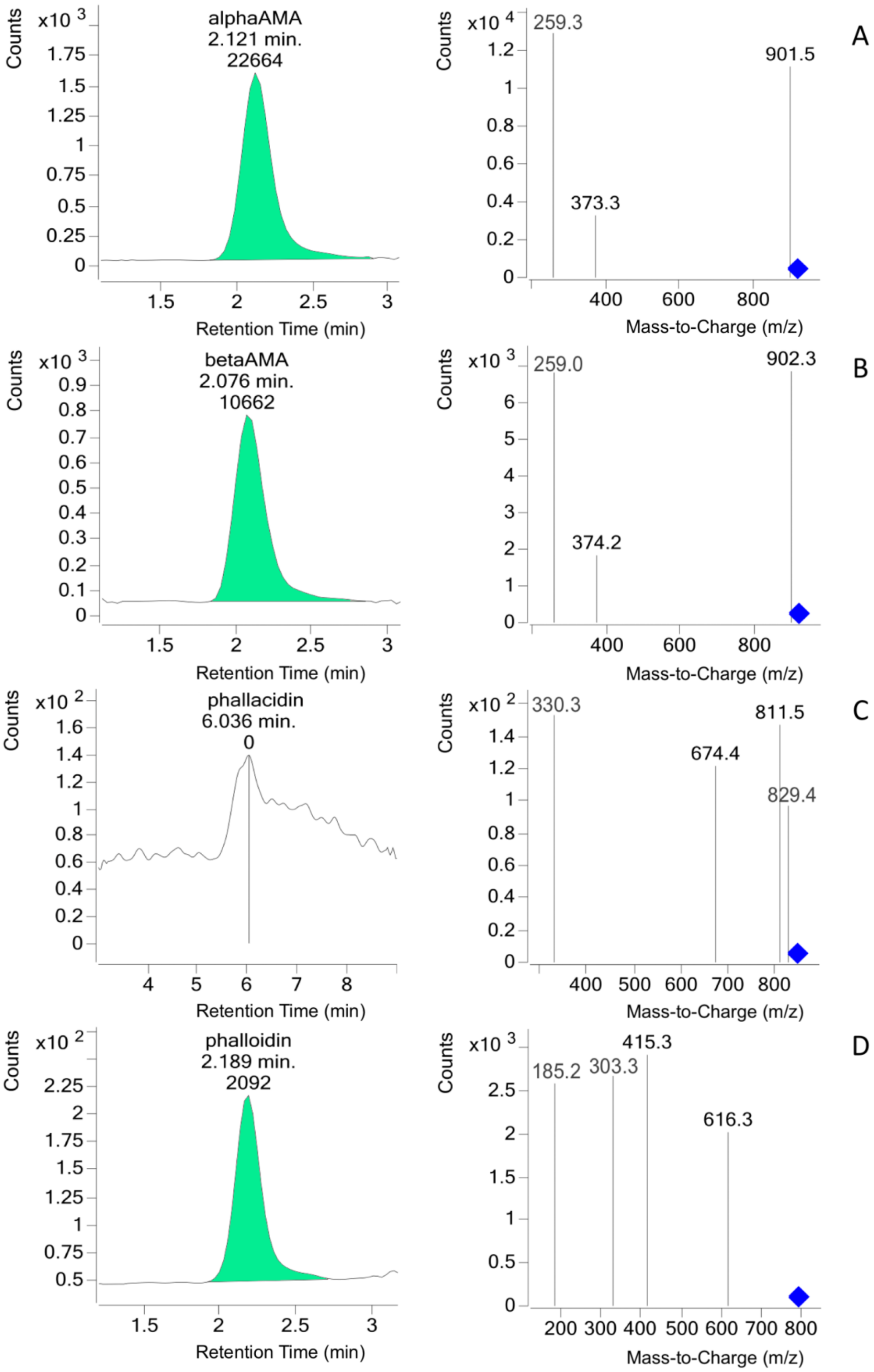

Figure 4 *Liquid chromatography-tandem mass spectrometric MRM chromatograms of lethal peptide toxins obtained from clinical mushroom samples (A) alpha amanitin, (B) beta amanitin, (C) phallacidin and (D) phalloidin. Diamond-shape represents the molecular ion.*

Table 1. Lists of characteristic ions of standard lethal peptide toxins during MRM acquisition.

Compounds	Molecular ion (*m/z*)	Product ions (*m/z*)	Peak area (%)
α-amanitin	919.0	919.0→259.3	3192 (49)
		919.0→373.3	525 (8)
		919.0→901.5	2813 (43)
β-amanitin	920.1	920.1→259.0	3592 (52)
		920.1→374.2	369 (5)
		920.1→902.3	2968 (43)
phallacidin	847.3	847.3→330.3	46803 (31)
		847.3→674.4	31610 (21)
		847.3→811.5	51394 (34)
		847.3→829.4	21241 (14)
phalloidin	789.3	789.3→185.2	50013 (21)
		789.3→330.3	102115 (42)
		789.3→415.3	43275 (18)
		789.3→616.3	45387 (19)

Discussion

Food poisoning by amatoxins-containing mushroom is found abundantly in the genus *Amanita* (Basidiomycota). This genus was traditionally divided into seven sections including *Amanita, Amidella*, *Caesareae*, *Lepidella, Phalloideae*, *Vaginatae* and *Validae*.[15] The most toxic species belongs to the sections *Amanita* and *Phalloideae* such as *Amanita bisporigera*, *A. brunneitoxicaria*, *A. exitialis, A. gleocystidiosa*, *A. phalloides* and *A. virosa*.[3,13,16–19] Both sections synthesize controlled cyclic peptide toxins which divided into amatoxins, phallotoxins and virotoxins.[20,21] Amatoxins are bicyclic octapeptides with an indole-(R)-sulphoxide bridge, while phallotoxins and virotoxins contains bicyclic heptapeptides with an indole/thio-ether bridge and monocyclic peptides with 2-methylsulphonyltryptophan, respectively.[20,21] These toxins can function in different ways. Amatoxins are specific inhibitors of RNA polymerase and prevent subsequent protein synthesis, whereas phallotoxins and virotoxins form complexes with actin and blocks microfilaments depolymerization of the cytoskeleton from liver and muscle cells.[13–15]

In Thailand, several poisoning cases of amanitas have been recorded including *Amanita digitosa*, *A. exitialis, A. fuliginea*, *A. gleocystidiosa*, *A. pyriformis* and *A. virosa*.[3,19,23] Our previous studies showed that the species containing amatoxins and phallotoxins were *Amanita exitialis*, *A. fuliginea* and *A. gleocystidiosa*.[3,23] In addition, we also found a highest alpha-amanitin concentration in *A. gleocystidiosa* of the section *Amanita*. Outbreaks of above toxic *Amanita* species were found in the northern and northeastern part of Thailand.[3] In this study, we focused on four major mushroom poisoning cases during 2015 to 2018. These cases were suspected phalloides syndrome poisoning. The first poisoning case (August 2015) reported from Yasothon Province included 7 patients and 1 death. The second confirmed case (July 2017) from Mahasarakham Province included 12 patients and 1 death. The last two cases (May to June 2018) reported from Ubon Ratchathani and Chanthaburi Provinces included 14 patients and 1 death. The remnant mushroom samples harvested by the patients were delivered to the Toxicology Center (National Institute of Health, Department of Medical Sciences). Based on their morphology, the remnant mushroom samples were primarily identified as the genus *Amanita*. However, the mushroom samples are not well preserved for species level diagnosis. Thus, we used the molecular techniques to help clarify their species. The nuclear ITS marker is one of the universal DNA barcode marker has been applied in various group of mushrooms for toxicological and clinical purposes.[2,3,7,24–27] Comparison with the ITS sequences deposited in GenBank showed that all mushroom samples were closely related to *Amanita brunneitoxicaria*. These sequences matched BLAST reference sequence with scores ranging from 98 % to 100% identity. Our phylogenetic analysis confirmed that the toxic mushrooms ingested are genetically similar to *A. brunneitoxicaria*. This species was a newly discovered species in Thailand.[13] Its general features include small to medium pileus, dark greyish brown, darkest at centre, non-striate margin; gill free, white; stipe cylindrical, white to greyish white; bulb subglobose with saccate volva and the presence of alpha-amanitin.[13] In northeastern region, amanitas are favourite food source and very common in local market during the wet season including *Amanita princeps*, *A. javanica, A. hemibapha* and *A. vaginata.* According to the poisoning case report revealed that most cases of poisoning occur in people who are not familiar with the local mushroom. Most of the cases revealed that the patients ate wild mushrooms that were thought to be the edible *A. vaginata*. Toxic *Amanita brunneitoxicaria* and *A. vaginata* belong to different sections. Morphologically, *A. brunneitoxicaria* is similar to *A. vaginata* in having small to medium cap with dark greyish brown, stipe cylindrical with white to greyish white; however, *A. brunneitoxicaria* can be distinguished from *A. vaginata* by its non-striate of pileus margin.

So far, several liquid chromatography methods have been used for the detection of mushroom peptide toxins.[2,3,8,28,29] Here, we analysed lethal peptide toxins via multiple reactions monitoring (MRM) using tandem mass spectrometer with electrospray (ESI) source operating in positive mode. The MRM method was performed on a triple quadrupole mass spectrometer. The precursor ions of interest was preselected with the mass filter and induced to fragment of product ions by collision-induced dissociation (CID). Our result confirmed that three lethal peptide toxins of α-amanitin, β-amanitin, phallacidin were found in all mushroom samples ingesting by patients. The most abundant and stable product ions obtained from MRM-MS analysis were *m/z* 259.3, 259.0 and 330.3, respectively. According to Duffy, amanitins are potently toxic to humans with an approximate lethal dose of α-form 0.1 mg/kg of body weight which can be found in a single mushroom sample.[30] Based on our findings *Amanita brunneitoxicaria* contained α-amanitin with an average level of 0.04 to 0.5 µg/g wet weight. Having ingested the toxic *A. brunneitoxicaria*, all patients showed the first latent period within 4 to 14 hours of gastrointestinal phase, which is characterized by nausea, vomiting, abdominal pain and cramps as well as severe secretory diarrhea. The second latent period (14-24 hrs.) starts with the first signs of liver and renal damage. The clinical symptoms described here were similar to those of phalloides syndrome.[3,20,30]

In conclusion, we suggest that molecular based diagnostics as well as LC-MS/MS method are suitable for the rapid detection of toxic *Amanita* samples, independent of their complete morphology. These methods can separate the lethal peptide toxin-producing mushroom species; thus, they are urgently needed for the appropriate medical treatments of mushroom poisoning cases. Furthermore, the toxic *Amanita brunneitoxicaria* provided a new informative data for clinical studies.

Conflict of interest

The authors declare that there is no conflict of interest.

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