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Short Communication

Rapid genetic detection of ingested *Amanita phalloides*

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ABSTRACT

Mushrooms are often poorly digested by humans. Thus, their remains (tissues, spores) may persist in the gastrointestinal tract and can be detected in feces several days after mushroom consumption. In this report, we present protocols for the rapid PCR-based detection of fungal traces in a variety of complex samples. Novel primers were designed to amplify portions of ribosomal DNA from deadly poisonous European members of the genus *Amanita*, namely the death cap (*A. phalloides*), the destroying angel (*A. virosa*) and the fool's mushroom (*A. verna*), respectively. Assay sensitivity was sufficient to discover diluted DNA traces in amounts below the genomic content of a single target mushroom cell. Specificity testing was performed with DNA extracts from a variety of mushroom species. Template amplification was exclusively observed with intended targets and it was not compromised by a vast excess of non-target DNA (i.e. DNA from human and human fecal origin, respectively). A series of experiments was conducted with prepared specimens in order to follow the course of mushroom food processing and digestion. Amplification by direct PCR was successful with raw, fried and digested mixed mushrooms. To improve assay performance with fecal samples, a rapid protocol for sample pre-processing (including water-ether sedimentation and bead beating) and a modified PCR reaction mix were applied. Thereby, it was possible to detect the presence of *A. phalloides* DNA in spiked feces as well as in clinical samples (vomit, stool) from two independent cases of suspected mushroom poisoning.

1. Introduction

"Wild" (uncultivated, field collected) mushrooms are used for human consumption, and because misidentifications (e.g. errors in species determination) do occur, mushroom poisonings continue to be a public health concern in Europe, the USA and several other parts of the world [1–4]. The vast majority of reported fatal intoxications are attributed to a few species of the genus *Amanita*, particularly the death cap (*Amanita phalloides*) [5] which can cause fulminant hepatic failure, renal failure and multi-system organ failure with a high mortality (10–30% in adults) [4,6]. *A. phalloides* is not unique in appearance and has no distinct taste. Thus it can be mistaken for non-poisonous species, and the ingestion of one single mushroom cap may be sufficient to cause death within two to eight days [4–6]. The *A. phalloides* syndrome [7] is characterized by a long (six to 24 h) asymptomatic latency period which is succeeded by severe gastroenteritis (including abdominal pain, nausea, vomiting, diarrhea, dehydration and electrolyte disturbance). Gastrointestinal symptoms typically resolve after 11 to 24 h, while hepatic necrosis progresses.

A variety of toxins (e.g. phallotoxins, virotoxins) have been identified in the deadly poisonous *Amanita* species, but the principal poisonous ingredients are the so-called amatoxins [5,6]. Amatoxins are heat-stable and resistant to cooking, drying and freezing [8]. As little as 0.1 mg/kg body weight can provide a lethal dose [9]. The main mechanism of action is the irreversible inhibition of the DNA-dependent RNA polymerase II, which results in blocked protein synthesis and cell necrosis [10]. In addition, amatoxins may act synergistically with endogenous factors (e.g. tumor necrosis factor) to induce apoptosis [11].

Laboratory methods for the detection of amatoxins exist, including radioimmunoassay [12,13], liquid chromatography–mass spectrometry (LC–MS) [14–16], capillary zone electrophoresis coupled with mass detection [17–19], and enzyme-linked immunosorbent assay (ELISA) [20] – with the latter being currently

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most widely used in clinical settings [21]. Unfortunately, each of these methods has certain practical limitations concerning laboratory requirements (e.g. isotope-work, expensive equipment), time-requirements for sample analysis, specimen types that can be analyzed and diagnostic windows, respectively.

Mushroom species can be identified based on morphological features, but this requires expert knowledge and biological specimens available for morphological diagnosis are often limited, not well preserved and thus frequently unsuitable for a rapid identification [22]. In cases when fungal materials (e.g. tissues, spores) are available, molecular genetic strategies for species identification may provide a promising alternative strategy [23–26]. Kotlowski et al. [23] developed a conventional PCR-based approach for the specific detection of the glyceraldehyde-3-phosphate dehydrogenase (gpd) gene fragment from *A. phalloides*. However, this technique was relatively time-consuming, gpd is a low-copy target [24] which implies low assay sensitivity, and the authors did not present an application to clinical samples. Maeta et al. [25] established a rapid and sensitive real-time PCR methodology for the detection of four poisonous fungal species common in Japan, and demonstrated successful applications with a variety of cooked preparations. Epis et al. [26] published single-plex real-time PCRs for the specific detection of four poisonous mushrooms (including *A. phalloides*) and tested these assays with DNA extracts from a variety of different sources (dried mushrooms, pasta with mushrooms, cooked mushrooms and gastric aspirates mixed with dried mushrooms). Furthermore, the development of a sensitive DNA-based macroarray for the simultaneous (but time-consuming) identification of several selected *Amanita* species was reported [27].

Previously, we have demonstrated the feasibility of a rapid direct PCR approach for toxic plant detection with diluted gastric contents from a forensic case [28]. In the present study, a similar strategy was developed for the detection of deadly poisonous European species of the genus *Amanita*, namely *A. phalloides*, *A. virosa* and *A. verna*, respectively. PCR primers were designed that target discriminatory polymorphic sequences located in the nuclear ribosomal DNA. A series of experiments was conducted that follows the course of mushroom food processing and consumption. A variety of test samples, including homogenized mixed mushrooms (raw, fried, digested) and fecal preparations were subjected to analysis by direct PCR. Target amplification by direct PCR was successful with raw, fried and digested mushrooms. With the use of a short protocol for feces pre-processing and a modified PCR reaction mix, we were further able to discover highly diluted *A. phalloides* traces in spiked stool specimens and to provide confirming molecular evidence in clinical cases of suspected mushroom poisoning.

2. Materials and methods

2.1. Fungal materials and preparations

2.1.1. Mushrooms

Dried mushroom voucher specimens (45 isolates from 32 different species) from the herbarium WU [29] of the Institute of Botany, University of Vienna, were provided by I. Krisai-Greilhuber and included: (a) deadly poisonous European members of the genus *Amanita* that contain amatoxins, (b) other poisonous and non-poisonous *Amanita* species, (c) mushrooms that are typical candidates for being mistaken with either of the deadly poisonous Amanitas, and (d) some other popular edible mushrooms [mushroom species names are provided with the electronic supplementary material-1 (ESM-1); voucher specimens are listed in Table 1 of ESM-2]. In addition, fresh *A. phalloides* specimens were provided by G. Frühwirth (Vienna Food Inspection and Market Authority, Municipal Department 59). Cultivated button mushrooms (*Agaricus bisporus*) were obtained from a local supermarket. Fresh basidiomata (fruiting bodies) were stored at −20 °C until further processing.

2.1.2. Mixed raw mushrooms

Raw mushrooms (*A. phalloides* and *A. bisporus*, respectively) were cut and mixed yielding final mass ratios of 1:1, 1:10 and 1:100 (∼1 g total mass, *A. bisporus* in excess). Two milliliters of ATL lysis buffer (Qiagen, Hilden, Germany) were added and homogenization was performed with the IKA ULTRA-TURRAX Tube Drive grinding system (IKA, Staufen, Germany) (for further details see ESM-1).

2.1.3. Mixed fried mushrooms

Chopped mushrooms (*A. phalloides* and *A. bisporus*, respectively) were fried in corn oil for 15 min at 150 °C, mixed yielding final mass ratios of 1:1, 1:10 and 1:100 (∼1 g total mass, *A. bisporus* in excess) and homogenized as described above (further details in ESM-1).

2.1.4. Mixed fried and digested mushrooms

Fried, chopped mushrooms (*A. phalloides* and *A. bisporus*, respectively) were incubated in artificial gastric fluid [30] at 37 °C for 4 h, mixed yielding final mass ratios of 1:1, 1:10 and 1:100 (∼1 g total mass, *A. bisporus* in excess) and homogenized as described above (further details in ESM-1).

2.2. Vomit from a clinical case of suspected mushroom poisoning

The case sample (vomit, a case report is provided in ESM 1) was subjected to centrifugation (2100 × *g*, 5 min). The resulting liquid supernatant (∼35 ml) was decanted. The pellet (incl. remains from ingested mushrooms; ∼0.5 g total mass) was supplied with 0.5 ml water and homogenized using two stainless steel beads and the IKA ULTRA-TURRAX Tube Drive (6000 rpm for 40 s). Small amounts (∼0.1 g) of homogenized pellet were subjected to disruption by bead beating (see below: Section 2.4.2). Aliquots of 5 μl were used as template for direct PCR (modified protocol; see below: Section 2.7.2).

2.3. Fecal specimens

2.3.1. Stool samples from a clinical case of suspected mushroom poisoning

Stool samples were collected from four patients of mushroom poisoning prior to their release from the hospital (a case report is provided in ESM 1). The case samples were stored frozen at −20 °C until further processing.

2.3.2. Stool samples spiked with A. phalloides

A piece from an *A. phalloides* mushroom cap (∼1.6 g) was cut, suspended in 2 ml water, diluted and mixed with aliquots from a clinical stool sample in order to prepare spiked fecal test samples (further details in ESM-1). Spiked fecal specimens were supplied with 2 ml methyl tert-butyl ether (MTBE, Sigma–Aldrich), subjected to vigorous vortexing (30 s), 5 min ultrasonication (Sonorex, Bandelin) and centrifugation (2100 × *g*, 5 min). After decanting the supernatant, a small amount of pellet was subjected to further processing by bead disruption (see below).

2.4. Stool processing prior to direct PCR

2.4.1. Water-ether sedimentation

Feces (up to 1 g) was transferred into IKA-BMT-20-S tubes, supplied with 2 ml of distilled water, and homogenized using five