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Fatal *Amanita muscaria* poisoning in a dog confirmed by PCR identification of mushrooms

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Abstract. Diagnosing mushroom poisoning in dogs can be difficult and often includes identification of suspect mushrooms. Visual identification may be hindered by mastication, oral medications, or poor quality of environmental mushroom samples. Other analytical techniques may thus be necessary to aid in mushroom identification. A 5-y-old neutered male Labrador Retriever dog developed acute onset of vomiting, diarrhea, tremors, seizures, and somnolence. The dog was treated at a veterinary clinic and was briefly stabilized, but died during transport to an emergency clinic. On postmortem examination at the University of Kentucky Veterinary Diagnostic Laboratory, the dog's stomach was full of mushrooms covered with activated charcoal. Mushrooms were damaged, fragmented, and discolored, precluding accurate visual identification. Mushroom pieces were sent to the Department of Plant Pathology at the University of California–Davis for PCR identification; the neurotoxic mushroom *Amanita muscaria* was identified. A qualitative liquid chromatography–mass spectrometry (LC-MS) method was developed to detect ibotenic acid and muscimol, the toxic compounds present in *A. muscaria*. Mushrooms, stomach contents, and urine were analyzed by LC-MS; ibotenic acid and muscimol were detected in all samples. Because identification of ingested mushrooms is sometimes necessary to confirm mushroom poisoning, PCR can identify ingested mushrooms when visual identification is unreliable.

Key words: *Amanita muscaria*; dogs; ibotenic acid; liquid chromatography–mass spectrometry; muscimol; mushroom poisoning; PCR.

Confirming a diagnosis of mushroom poisoning in dogs can be challenging. Ingestions are often not witnessed, and clinical presentations of mushroom poisoning are often nonspecific and possibly attributable to a variety of causes, both toxicologic and non-toxicologic. Additionally, financial limitations may preclude extensive confirmatory testing. Risk of intoxication varies widely between mushroom species. Identification of ingested mushrooms helps determine prognosis and inform treatment decisions.[12] Visual identification of mushrooms relies on observation of growth habitat or substrate, along with macroscopic and microscopic morphologic characteristics of fruiting bodies and spores.[7] Mastication can alter the physical appearance of mushrooms, as can gastric secretions and oral administration of activated charcoal. Visual identification of mushrooms from vomitus or gastric contents is difficult even for experienced mycologists. In cases of human poisoning, PCR methods have been used to identify ingested mushrooms when visual identification was inconclusive or impossible.[5,10]

We describe a fatal case of mushroom poisoning in a dog diagnosed using PCR analysis to identify 5 different species of mushrooms—including *Amanita muscaria*—present in the dog's stomach at postmortem examination. A method was subsequently developed to detect ibotenic acid and muscimol, the toxic compounds present in *A. muscaria*. Both compounds were present in mushroom pieces, stomach contents, and urine, further supporting the diagnosis of *A. muscaria* toxicosis.

A 5-y-old, 38.5 kg, neutered male Labrador Retriever dog developed acute onset of vomiting, diarrhea, and seizures, and was presented to a veterinary clinic for treatment. On arrival at the clinic, the dog had generalized muscle tremors and stuporous mentation. The dog was treated initially with 5 mg of midazolam intravenously. The tremors improved, but facial muscle fasciculations continued. The dog became more somnolent, bordering on comatose.

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Additional treatments included administration of 1.62 mg of atropine subcutaneously, 500 mg of methocarbamol rectally, 300 mL of activated charcoal suspension via orogastric tube, and intravenous lactated Ringer solution (unspecified volume). Tremors and fasciculations subsided. The dog died during transport to an emergency clinic for continued care. Remains were frozen and later submitted to the University of Kentucky Veterinary Diagnostic Laboratory (UKVDL) for postmortem examination.

On gross examination, the stomach contained a large amount of mushroom pieces admixed with black semi-viscous liquid consistent with activated charcoal suspension. The esophagus and trachea contained scant amounts of the same material. The lungs and cerebral meninges were moderately congested. No other gross anatomic lesions were present. Histologically, mild freeze artifact was present in multiple tissues. Airways contained low numbers of macrophages with and without intracellular brown particulate matter. Scattered airways also contained moderate amounts of black, variably sized and shaped particulate matter presumed to be activated charcoal. Subdural congestion and hemorrhage were present in the brain. Hepatic sinusoids and renal interstitial vessels were moderately to severely congested. No other histologic abnormalities were present. Primary differentials were neurotoxic mushrooms, tremorgenic mycotoxins (penitrem A and roquefortine C), and other neurotoxicants.

Stomach contents were analyzed for penitrem A and roquefortine C by liquid chromatography–tandem mass spectrometry at the California Animal Health and Food Safety Laboratory (CAHFS). Neither penitrem A nor roquefortine C was detected (limit of detection [LOD] 25 ppb for each analyte). Mushroom pieces were discolored, severely degraded, and fragile. Even gentle handling caused further damage. Mushrooms varied widely in size, color, shape, and texture, and appeared to be of multiple different species. Visual identification was deemed unreliable. Mushrooms were sent to the Department of Plant Pathology at the University of California–Davis for DNA sequencing.

Genomic DNA (gDNA) was extracted from each sample (DNeasy plant mini kit; Qiagen, Hilden, Germany). Briefly, 15 mg from each fungal fruiting body were collected with sterile forceps and placed into a 1.5-mL cryogenic tube, frozen in liquid nitrogen, and macerated. Three sterile glass beads, 400 µL of ethyltrimethylammonium bromide (CTAB) mixture (5% w/v CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 1% w/v polyvinylpyrrolidone [PVP-360]), 2 µL of β-mercaptoethanol, and 4 µL of RNase A were added. Samples were macerated (Vortexer Genie 2; VWR, Radnor, PA) at maximum speed for 5 min and incubated at 65°C for 10 min; the manufacturer's directions for DNA extraction were followed subsequently.

The sequence of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA was determined for 5 different mushrooms isolated from the dog's stomach contents. The ITS region is commonly used for the identification of fungi with ~170,000 sequences deposited in GenBank.[6,14,15] ITS4 and ITS5 had a broad spectrum for fungi, whereas ITS1F and ITS4B had enhanced specificity for basidiomycetes.[6,14] Environmental samples can be contaminated with molds (generally not basidiomycetes); using ITS1F and ITS4B can sometimes enhance mushroom identification. ITS primers used were ITS4 (5'-TCCTCCGCT TATTGATATGC-3'), ITS5 (5'-GGAAGTAAAAGTCGTA ACAAGG-3'), ITS1F (5'-CTTGGTCATTTAGAGGAAG TAA-3'), and ITS4B (5'-CAGGAGACTTGTACACGGTC CAG-3'). PCR reactions consisted of 12.5 µL of 2× Mean Green master mix (Syzygy Biotech, Grand Rapids, MI), 1 µL of each 10 µM primer, and 2 µL of gDNA in a 25-µL reaction. Amplification reactions were performed in a thermocycler (PTC-100; MJ Research, Watertown, MA) under the following conditions: 40 cycles of 94°C for 2 min, 55°C for 2 min and 72°C for 2.5 min, followed by a final extension at 72°C for 10 min. A negative control of water template and a positive control of known fungal DNA were included in the PCR run. Five µL of each sample were visualized by ultraviolet light after electrophoresis on 1.5% agarose gel and staining with ethidium bromide. PCR products were purified (GeneJET PCR purification kit; Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. A 24-µL sample of 40–68 ng/µL of PCR product and 12 µL of each 3 µM primer, ITS4 and ITS5, or ITS1F and ITS4B, were sent for sequencing (Quintara Biosciences, Richmond, CA).

Sequences were manually checked and identified using the NCBI BLAST sequence similarity search program. The 5 mushrooms were identified as *A. muscaria, Suillus americanus, Russula* spp., *Mycena* spp., and *Ganoderma* spp. Identification was based on >98% sequence similarity between 500-bp mushroom sample ITS1F/ITS4B reaction sequences and BLAST database sequences. Additionally, a BLAST search of 1-kb sample sequences from ITS4/ITS5 reactions revealed >99% similarity with sequences of fast-growing mold species *Mucor saturninus* and *M. hiemalis*.

Approximately 70–80% of the mushroom pieces in the dog's stomach were consistent in appearance with the sample identified as *A. muscaria*. Ingestion of *A. muscaria* has been reported to cause anxiety, disorientation, delirium, drooling, vomiting, diarrhea, somnolence, muscle fasciculations, tremors, seizures, and death in dogs.[9,13] The other species identified—*Suillus americanus, Russula* spp., *Mycena* spp., and *Ganoderma* spp.—have not been shown to cause clinical signs in dogs.[12] The presence of *Mucor* species in several samples demonstrates that fast-growing molds can rapidly contaminate environmental samples.

A. muscaria contains the isoxazole derivatives ibotenic acid and muscimol.[1,2] A qualitative liquid chromatography–mass spectrometry method to detect ibotenic acid and muscimol was developed at CAHFS. Both compounds were detected in stomach contents, urine, and mushrooms identified via PCR as *A. muscaria* (LOD 10 ppb muscimol; 500 ppb ibotenic acid). Ibotenic acid is an N-methyl-D-aspartate (NMDA) receptor agonist causing central nervous system (CNS) excitation.[11] Muscimol is a γ-aminobutyric acid

(GABA) agonist causing CNS depression.[4] Muscimol is also a metabolite of ibotenic acid in the dog. As *A. muscaria* poisoning progresses, the relative concentration of muscimol increases. GABAergic anticonvulsants such as midazolam can exacerbate CNS and respiratory depression. Atropine is not recommended in *A. muscaria* intoxications.[12] Midazolam, methocarbamol, and atropine administration likely complicated the clinical presentation in our case. Death appeared to be the result of severe CNS signs as observed by the primary care veterinarian. Aspiration of activated charcoal may have further exacerbated the dog's clinical condition but was not by itself considered the cause of death. Unlike other toxic *Amanita* species, *A. muscaria* does not contain hepatotoxic compounds nor cause liver lesions. *A. muscaria* causes functional neurotoxicity and is not expected to cause specific lesions.[2]

Ingestion of toxic mushrooms can cause significant morbidity and mortality. Dogs are particularly susceptible to mushroom toxicosis given their indiscriminate eating habits and a tendency to roam. In cases of suspected mushroom poisoning, establishing a definitive diagnosis can be difficult. In the few published veterinary case reports of *A. muscaria* poisoning, 1 dog was observed eating mushrooms, 1 dog vomited mushrooms, and, in 2 separate cases, partially eaten mushrooms were found in the dogs' environments.[9,13] In all 4 cases, mushrooms were visually identified as *A. muscaria*. In one case, ibotenic acid and muscimol were detected with high-performance liquid chromatography (HPLC) in both serum and urine (LOD 6 ppb for ibotenic acid; 24 ppb for muscimol). In another case, HPLC detected ibotenic acid and muscimol in serum, but only detected muscimol in urine.[13]

A. muscaria is an ectomycorrhizal fungus native to conifer and deciduous forests throughout the Northern Hemisphere. In North America, these mushrooms are commonly found on the ground beneath pine, spruce, birch, and eastern hemlock. The typical growing season is late June through November, although the season may extend through February in California.[8] *A. muscaria* is hallucinogenic and has long been used by humans for spiritual and recreational purposes.[3]

According to the Toxicology Reports and Poison Case Registry of the North American Mycological Association (NAMA), *A. muscaria* and its relative *A. pantherina* are the mushrooms most commonly ingested by animals, particularly dogs (https://www.namyco.org/toxicology_reports.php). This might be because dogs are attracted to the reportedly fishy odor of these mushrooms. Also, human recreational use of *A. muscaria* can lead to these mushrooms being stored indoors and unsecured. In our case, the dog stayed inside a large fenced yard with a sizeable wooded area behind the owners' house. The owners were unaware of any mushrooms growing in the yard, but they had not been in the wooded portion for some time. The owners denied use of *A. muscaria* by family members, guests, or themselves. The source of mushrooms in this case was not definitively determined.

In most cases of mushroom poisoning reported to NAMA, mushrooms are identified visually. This is not always possible, especially when mastication or orally administered treatments such as activated charcoal have distorted the appearance of the mushrooms and spores. PCR analysis can be completed in 12 h and may be a useful tool in these cases. Very little sample (15 mg of each mushroom for the method described herein) is required for PCR, and the analysis is unaffected by most treatments administered to the patient. PCR can be used to analyze various matrices including fresh, cooked, and partially digested mushrooms, and is worth considering as a laboratory tool in cases of mushroom ingestion in which visual identification is unreliable or inconclusive.

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References

1. Bowden K, Drysdale AC. A novel constituent of *Amanita muscaria*. Tetrahedron Lett 1965;12:727–728.
2. Bowden K, et al. Constituents of *Amanita muscaria*. Nature 1965;206:1359–1360.
3. Crundwell E. The unnatural history of the fly agaric. Mycologist 1987;1:178–181.
4. Curtis DR, et al. Bicuculline, an antagonist of GABA and synaptic inhibition in the spinal cord of the cat. Brain Res 1971;32:69–96.
5. Epis S, et al. Molecular detection of poisonous mushrooms in different matrices. Mycologia 2010;102:747–754.
6. Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol Ecol 1993;2:113–118.
7. Hall AH, et al. Mushroom poisoning: identification, diagnosis and treatment. Pediatr Rev 1987;8:291–298.
8. Lincoff GH. The Audubon Society Field Guide to North American Mushrooms. New York, NY: Knopf, 2005:539–540.
9. Lindberg H, Holmgren A. Two lethal cases of *Amanita muscaria* ingestion in dogs. Clin Toxicol 2012;50:289.
10. Maeta K, et al. Rapid species identification of cooked poisonous mushrooms by using real-time PCR. Appl Environ Microbiol 2008;74:3306–3309.
11. McLennan H, Lodge D. The antagonism of amino acid-induced excitation of spinal neurones in the cat. Brain Res 1979;169:83–90.
12. Puschner B. Mushrooms. In: Peterson M, Talcott PA, eds. Small Animal Toxicology. 3rd ed. St. Louis, MO: Elsevier Saunders, 2013:680–698.
13. Rossmeisl JH, et al. *Amanita muscaria* toxicosis in two dogs. J Vet Emerg Crit Care 2006;16:208–214.
14. Schoch CL, et al. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. Proc Natl Acad Sci U S A 2012;109:6241–6246.
15. White TJ, et al. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, et al., eds. PCR Protocols: A Guide to Methods and Applications. San Diego, CA: Academic Press, 1990:315–322.