

Rapid identification of *Colchicum autumnale* based on loop-mediated isothermal amplification assay

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23 **Abstract**

24 **Purpose:** *Colchicum autumnale* (Colchicaceae) exhibits toxicity, and accidental
25 ingestion of this plant has been reported in Japan. Identifying the cause of poisoning is
26 important for emergency medical treatment, and a rapid and simple detection technique
27 is required for the identification of the cause of poisoning. In the present study, we
28 developed a rapid and simple method for the detection of *C. autumnale* based on a loop-
29 mediated isothermal amplification (LAMP) assay, a nucleic acid detection method.

30 **Methods** Specific LAMP primers for *C. autumnale* were designed based on the *trnL-trnF*
31 intergenic spacer region. Using the LAMP primers, the LAMP assay was performed at an
32 isothermal reaction temperature of 63 °C.

33 **Results** The LAMP reaction was shown to be specific and highly sensitive to *C.*
34 *autumnale*, given that the assay can be used for 10 pg of purified DNA. Using a simple
35 protocol for on-site detection, the entire procedure from pre-treatment to evaluation
36 required approximately 1 h. Moreover, the LAMP method using a microfluidic device
37 detected multiple genes, including *C. autumnale*-specific DNA regions, at a time.

38 **Conclusions** The currently proposed protocol exhibits good potential as a screening
39 method for *C. autumnale* poisoning in emergency medical care.

40

41 **Keywords:** Colchicum; isothermal amplification; LAMP; accidental ingestion; toxic
42 plants

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Introduction

In many cases, accidental ingestion of toxic plants occurs because of morphological similarity to edible plants. *Colchicum autumnale*, which belongs to the Colchicaceae family, originates in North Africa and is cultivated as a garden plant in Japan. The leaves and bulbs of the plant contain colchicine, which is an alkaloid [1]. In Japan, 22 patients were reported to be poisoned by *C. autumnale* from 2010 to 2019, 10 of whom died [2]. In some cases, the leaves of the plant were accidentally consumed as *Allium victorialis* or *Hosta sieboldiana*, and the bulbs of the plant were ingested as onions (*Allium cepa*) or potatoes (*Solanum tuberosum*). The initial symptoms of *C. autumnale* poisoning include abdominal pain and vomiting [3-5]. In some serious cases, the toxicity of the plant causes multi-organ failure, leading to death. In cases of accidental ingestion of the toxic plant, rapid identification is important for emergency medical treatment. In general, the identification of *C. autumnale* is performed by chemical analyses using high-performance liquid chromatography or liquid chromatography-mass spectrometry [4-7]. Recently, DNA-based analytical methods, such as PCR and real-time PCR, have been proposed to detect toxic plants, including *C. autumnale*. [8-11]. However, these methods consume time and require special equipment. On-site rapid identification of the toxic plant is highly desirable. The loop-mediated isothermal amplification (LAMP) assay is a DNA amplification method performed under isothermal conditions at 60 °C to 65 °C; the amplification efficiency of the assay is 100- to 1000-fold higher than that of PCR [12]. The LAMP method can be performed within a short time using a simple device, such as a block incubator, and has consequently been widely used for nucleic acid detection of viruses, bacteria, and plants [13-17]. In this study, we report an on-site identification method for *C. autumnale* based on the LAMP assay.

Materials and methods

Plant materials and DNA extraction

For the specificity test, a total of 23 plant samples, including *C. autumnale*, *A. victorialis*, *H. sieboldiana*, *S. tuberosum*, and *A. cepa*, were obtained from the botanical garden of Josai University (Table 1).

Table 1 Plant samples and LAMP reactivity

Species	Family
<i>C. autumnale</i>	Liliaceae

<i>A. victorialis</i>	Amaryllidaceae
<i>H. sieboldiana</i>	Asparagaceae
<i>S. tuberosum</i>	Solanaceae
<i>A. cepa</i>	Amaryllidaceae
<i>Lycoris radiata</i>	Amaryllidaceae
<i>Narcissus tazetta</i>	Amaryllidaceae
<i>Angelica acutiloba</i>	Apiaceae
<i>Angelica keiskei</i>	Apiaceae
<i>Cryptotaenia canadensis</i>	Apiaceae
<i>Ophiopogon japonicus</i>	Asparagaceae
<i>Polygonatum odoratum</i>	Asparagaceae
<i>Artemisia indica</i>	Asteraceae
<i>Chimonanthus praecox</i>	Calycanthaceae
<i>Ricinus communis</i>	Euphorbiaceae
<i>Sophora flavescens</i>	Fabaceae
<i>Thymus quinquecostatus</i>	Lamiaceae
<i>Musa × paradisiaca</i>	Musaceae
<i>Ziziphus jujuba</i>	Rhamnaceae
<i>Citrus japonica</i>	Rutaceae
<i>Tetradium ruticarpum</i>	Rutaceae
<i>Curcuma aromatica</i>	Zingiberaceae
<i>Curcuma longa</i>	Zingiberaceae

*n.d. not detected

(N=3, min ± S.D.)

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79 Total genomic DNA was extracted using a NucleoSpin Plant II (Takara, Japan) and
80 quantified using a NanoDrop 1000 instrument (ThermoFisher Scientific, USA). Extracted
81 DNA was diluted to 5.0 ng/μL and used as a DNA template. Optimization of the LAMP
82 assay was performed at 61, 63, 65, and 67 °C using 5.0 ng/μL *C. autumnale* DNA as a
83 positive control and *A. victorialis* as a negative control. LAMP sensitivity was evaluated
84 using 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg of purified *C. autumnale* DNA as a template.
85 Processed samples were prepared according to a previously described method [16].
86 Briefly, *C. autumnale* and *A. victorialis* samples were boiled in water for 5 min or fried
87 with vegetable oil for 3 min. Samples were then treated with artificial gastric solution
88 containing 2.0 g/L sodium chloride, 3.2 g/L pepsin, and 7.0 ml/L 35% HCl for 2 h.
89 Template DNA was extracted using an Easy DNA Extraction Kit version 2 (Kaneka,
90 Japan). Approximately 5 mg of sample was digested with 100 μL of alkaline lysis solution

(Solution A) and heated at 98 °C for 8 min. The heat-treated sample was mixed with 14 μL of neutralization solution (Solution B), and the 10 fold-diluted lysate was used as a DNA template.

LAMP primer design

The LAMP assay targeting the *trnL-trnF* intergenic spacer region was conducted. The *trnL-trnF* intergenic spacer region sequences of *C. autumnale* and *A. victorialis* were obtained from the DDBJ/EMBL/GenBank database (accession no. AJ551352 and KF550177, respectively). The obtained sequences were aligned, and primers specific to *C. autumnale* were designed using PrimerExplore v5 (<https://primerexplorer.jp>). Primer information is listed in Table 2.

Table 2 LAMP primer sequences used in this study

Primer	Sequence (5'-3')	Nucleotide positions*
FIP (F1c-F2)	CTGTCGGTATTGACACGTAGAAC-GAAAAAATGATTAATCGGACGAGA	(423-445) (383-406)
BIP (B1-B2c)	AGGTATTAGGAAAATCCGTCGACT-TAATTGGGGATAGAGGGACT	(459-482) (503-522)
F3	CAAATCATTCATTCCGGAGTC	349-369
B3	TTAGTAAGTCAAAGGGGCTT	523-542

*Nucleotide position is based on DDBJ/EMBL/GenBank database accession no. AJ551352

LAMP assay

The LAMP assay was carried out using an isothermal amplification kit (Nippon Gene, Japan) with a total volume of 25 μL containing 12.5 μL of 2× reaction mix, 1.0 μL of EvaGreen (Biotium, CA), 0.2 μM each of F3 and B3 primers, 1.6 μM each of FIP and BIP primers, and 2.0 μL of genomic DNA as a template. Fluorescence data of the LAMP reaction were obtained using a Thermal Cycler Dice Real Time System Lite (TaKaRa, Japan). The LAMP reaction was carried out for 40 min at a constant temperature of 63 °C. Fluorescence signals were collected at a 1-m intervals. The cycle threshold value was determined and converted to the amplification time. The reactions were carried out in triplicate. Results are expressed as standard deviation (min ± S.D.).

On-site detection of the LAMP reaction

Template DNA was prepared from extracted DNA using an Easy DNA Extraction Kit version 2 (Kaneka). Colorimetric detection of double-stranded nucleic acid was performed using leuco crystal violet, which was synthesized from crystal violet, a triphenylmethane dye [18]. An equal volume of 1.5 mM crystal violet dissolved in 20 mM Tris-HCl (pH8.8), 90 mM sodium sulfate, and 15 mM β -cyclodextrin were mixed. The mixture was heated at 50 °C for 30 min, and 5 μ L of the mixture was placed in a 200- μ L tube and evaporated. The colorimetric dye immobilized in the tube was used for the LAMP reactions. The LAMP reaction was carried out in a tube for 40 min at 63 °C in a water bath. After heating, the results were indicated by a color change from colorless to blue.

The LAMP reaction targeting multiple DNA regions was performed using a microfluidic device [19]. A universal plant primer set was used as previously reported [20]. The *C. autumnale*-specific primer set and universal primer set, which consisted of 0.2 μ M each F3 and B3 primers as well as 1.6 μ M each FIP and BIP primers, were immobilized on each reaction chamber. The total volume of the LAMP reaction mixture was 25 μ L, containing 12.5 μ L of 2 $\times$ reaction mix (Eiken Chemical Co., Ltd., Tokyo, Japan), 8 units of *Bst* DNA polymerase, 120 μ M 2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3,6-naphthalenedisulfonic acid, trisodium salt (Dojindo Laboratories, Tokyo), and 2.0 μ L of genomic DNA as a template. The mixed solution was loaded into the inlet. The LAMP reaction was carried out at 63 °C for 60 min using a water bath. After heating, the results were indicated by a color change from dark blue to light blue.

Results and discussion

Optimization of LAMP primers

We aligned the intergenic spacer regions between *trnL-trnF* of *C. autumnale* (accession no. AJ551352), and *A. victorialis* (accession no. KF550177). Based on the alignment data, LAMP primers specific to the sequence of *C. autumnale* were designed (Table 2). The LAMP primers consisted of two outer primers (F3 and B3) and two inner primers (FIP and BIP). The LAMP assay was performed using a set of primers for detecting *C. autumnale*. Fluorescence signals were measured by a real-time PCR device, and the amplification time of the LAMP reaction at several temperatures was calculated. To determine the optimum reaction temperature, the LAMP assay was conducted at 61, 63, 65, and 67 °C (Table 3). Amplification occurred at 19.0 ± 0.2 min, 20.8 ± 0.6 min, and 25.0 ± 1.1 min (reaction time $\pm$ S.D., N=3) at 61, 63, and 65 °C, respectively, but did not

occur at 67 °C. No nonspecific amplification occurred at 40 min at any temperature. Generally, a LAMP reaction becomes specific to a targeted gene when the LAMP reaction temperature is high. Because of a slight difference in reaction time between 61 °C and 63 °C, the optimized LAMP reaction temperature was determined as 63 °C.

Table 3 Optimization of LAMP reaction

Reaction temperature	<i>C. autumnale</i>	<i>A. victorialis</i>
61 °C	19.0 ± 0.2 min	n.d.*
63 °C	20.8 ± 0.6 min	n.d.
65 °C	25.0 ± 1.1 min	n.d.
67 °C	n.d.	n.d.
*n.d. not detected		(N=3, min ± S.D.)

To evaluate the method sensitivity, serial 10-fold dilutions of genomic DNA (10 ng to 10 pg) were used as DNA templates for the LAMP assay. Fluorescence intensity was enhanced by using 10 ng, 1 ng, 100 pg, and 10 pg of DNA template, and with initial rise time of 22.5 ± 1.1 min, 26.0 ± 0.2 min, 29.1 ± 6.0 min, and 30.4 ± 4.0 min, respectively. No amplification was observed with 1 pg of DNA template (Table 4). These results indicate that the LAMP assay is highly sensitive because approximately 3 µg genomic DNA was extracted from 10 mg of *C. autumnale* leaf.

Table 4 LAMP sensitivity

Sample	DNA template	Reaction time (min)
<i>Colchicum autumnale</i>	10 ng	22.5 ± 1.1
	1 ng	26.0 ± 0.2
	100 pg	29.1 ± 6.0
	10 pg	30.4 ± 4.0
	1 pg	n.d.*
*n.d. not detected		(N=3, min±S.D.)

The specificity of the LAMP assay for *C. autumnale* was evaluated using edible plants (*A. victorialis*, *H. sieboldiana*, *S. tuberosum*, and *A. cepa*), whose leaves or bulbs are morphologically similar to those of *C. autumnale* (Table 1). Furthermore, we evaluated the LAMP reactivity for randomly collected 18 other plants (Table 1). The samples were fried, boiled and treated with artificial gastric solution (Table 5). Amplification was

observed with processed *C. autumnale* samples. No amplification occurred with processed *A. victorialis* and *S. tuberosum* samples. In actual plant poisoning cases, DNA detection from samples of cooked plant and the patient's stomach contents may be required, where the LAMP assay could be useful.

Table 5 Processed samples

	Non-processed sample	Boiled sample	Fried sample	Artificial gastric solution-treated sample
<i>C. autumnale</i>	+	+	+	+
<i>A. victorialis</i>	-	-	-	-
<i>S. tuberosum</i>	-	-	-	-

On-site detection of the LAMP reaction

To simplify *C. autumnale* detection, the LAMP assay was conducted using raw extracted DNA, as previously reported [16, 17]. In addition, a colorimetric dye was added to the LAMP reaction for visual evaluation [18]. Positive reaction of the LAMP assay was indicated by color change from colorless to blue. Samples of *C. autumnale* leaf, *A. victorialis* leaf, *H. sieboldiana* bulb, *S. tuberosum* bulb, and *A. cepa* bulb were tested using the proposed protocol. Positive reactions were observed in the LAMP assay using samples of *C. autumnale* (Fig. 1). No amplification was observed using samples of *A. victorialis*, *H. sieboldiana*, *S. tuberosum*, and *A. cepa*.

We further evaluated an on-site LAMP detection method based on a microfluidic device targeting multiple genes [19]. *C. autumnale*-specific primer set was placed in reaction chambers 2 and 5, and universal LAMP primer set was placed in reaction chamber 3 as a positive control (Fig. 2). Water was put into reaction chambers 1 and 4 as a negative control. The LAMP reagent and *C. autumnale* DNA were loaded into an inlet port, and the LAMP reaction was conducted at 63 °C for 60 min in a water bath. Positive reactions (light blue) were observed in chambers containing *C. autumnale* (Nos. 2 and 5) and universal primer sets (No. 3), while no amplification (dark blue) was observed in the negative control chamber (Nos. 1 and 4). LAMP reagent showed positive reaction (light blue) for *A. victorialis* sample in chamber 3, while negative reaction (dark blue) in other chambers. It should be noted that there was no evidence of cross-contamination among the reaction chambers. The results indicate that the LAMP detection method using a

microfluidic device can evaluate multiple DNA regions at a time. Fatal plant poisoning is generally caused by *C. autumnale*, *Aconitum*, and *Narcissus tazetta* L. in Japan. Using a microfluidic device with these LAMP primer sets in each chamber, a simple and rapid method for identifying the cause of plant poisoning could be developed.

Conclusions

We have developed a LAMP assay specific to *C. autumnale*. The simplified procedure using unpurified DNA template can be accomplished by approximately 1 h from DNA extraction to detection. The LAMP assay is suitable for on-site detection or less well-equipped laboratories. This rapid, sensitive, highly specific, and convenient method for identifying *C. autumnale* may be used as a reliable screening method for plant poisoning in emergency medical care.

Ethics declarations

Conflict of interest

There are no financial or other relations that could lead to conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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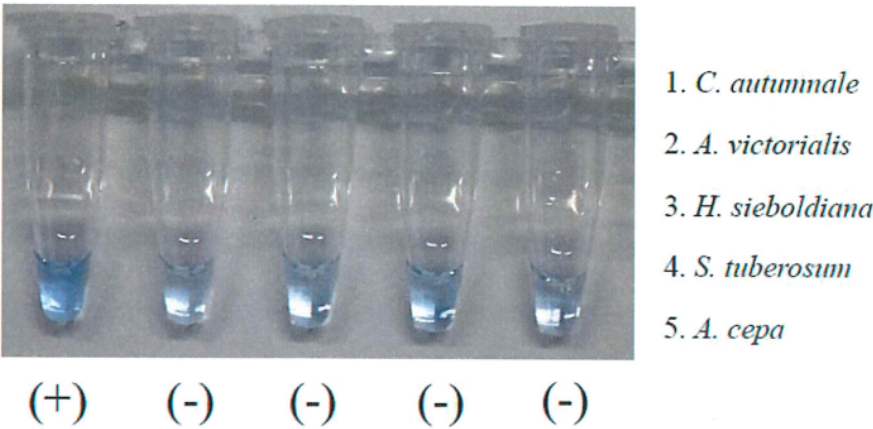
287 Figure Legend

288 Figure 1 On-site detection of LAMP reaction. On-site detection of LAMP reaction was
289 applied to 1: *C. autumnale*, 2: *A. victorialis*, 3: *H. sieboldiana*, 4: *S. tuberosum*, and 5: *A.*
290 *cepa*. LAMP reagent turned blue in the *C. autumnale* sample, but remained colorless in
291 the other species samples.

292 Figure 2 (a) The LAMP detection method based on a microfluidic device. *C. autumnale*-
293 or *A. victorialis*-specific primer set was placed in reaction chambers 2 and 5, and universal
294 LAMP primer set was placed in reaction chamber 3 as a positive control. Water was
295 placed in reaction chambers 1 and 4 as a negative control. (b) For *C. autumnale*, LAMP
296 reagent exhibited positive reaction (light blue) in chambers 2, 3, and 5, whereas it
297 exhibited negative reaction (dark blue) in chambers 1 and 4. (c) For *A. victorialis*, LAMP
298 reagent exhibited positive reaction (light blue) only in chamber 3 and negative reaction
299 (dark blue) in other chambers.

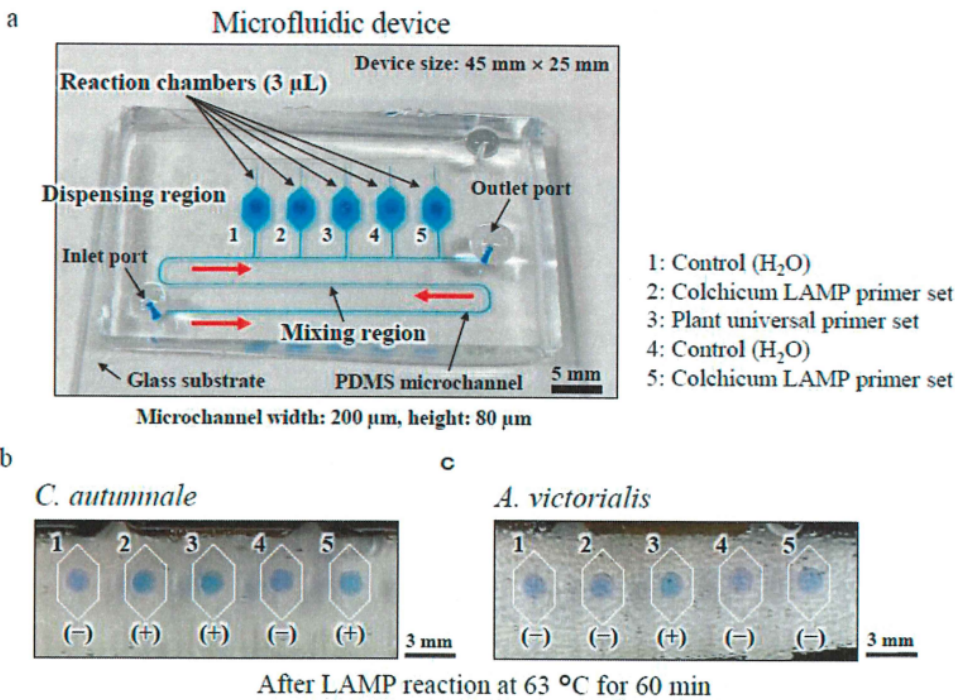
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Fig1



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Fig. 2



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