Note

Improved On-Site Protocol for the DNA-Based Species Identification of *Cannabis sativa* by Loop-Mediated Isothermal Amplification

Masashi Kitamura,*[a,b,†] Masako Aragane,[c] Kou Nakamura,[c] Tatsushi Adachi,[d] Kazuhito Watanabe,[e] and Yohei Sasaki[a]

[a] *Laboratory of Molecular Pharmacognosy, Division of Pharmaceutical Sciences, Graduate School of Medical Sciences, Kanazawa University; Kakuma-machi, Kanazawa, Ishikawa 920–1192, Japan:* [b] *Forensic Science Laboratory, Ishikawa Prefectural Police H.Q.; 1–1 Kuratsuki, Kanazawa, Ishikawa 920–8553, Japan:* [c] *Medicinal Plant Garden, Tokyo Metropolitan Institute of Public Health; 21–1 Nakajima-cho, Kodaira, Tokyo 187–0033, Japan:* [d] *Kaneka Corporation; 1–8 Miyamae, Takasago-cho, Takasago, Hyogo 676–8688, Japan: and* [e] *Daiichi University of Pharmacy; 22–1 Tamagawa-cho, Minami-ku, Fukuoka 815–8511, Japan.*

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Cannabis sativa **L. is cultivated worldwide for a variety of purposes, but its cultivation and possession are regulated by law in many countries, necessitating accurate detection methods. We previously reported a DNA-based *C. sativa* identification method using the loop-mediated isothermal amplification (LAMP) assay. Although the LAMP technique can be used for on-site detection, our previous protocol took about 90 min from sampling to detection. In this study, we report an on-site protocol that can be completed in 30 min for *C. sativa* identification based on a modified LAMP system. Under optimal conditions, the LAMP reaction started at approximately 10 min and was completed within 20 min at 63°C. It had high sensitivity (10 pg of purified DNA). Its specificity for *C. sativa* was confirmed by examining 20 strains of *C. sativa* and 50 other species samples. With a simple DNA extraction method, the entire procedure from DNA extraction to detection required only 30 min. Using the protocol, we were able to identify *C. sativa* from various plant parts, such as the leaf, stem, root, seed, and resin derived from *C. sativa* extracts. As the entire procedure was completed using a single portable device and the results could be evaluated by visual detection, the protocol could be used for on-site detection and is expected to contribute to the regulation of *C. sativa*.**

Key words *Cannabis sativa*; isothermal amplification; on-site detection; tetrahydrocannabinolic acid synthase gene; loop-mediated isothermal amplification (LAMP)

Cannabis sativa L. has been cultivated worldwide for thousands of years for use in medicine, food, cosmetics, and religious rituals. In many countries, including Japan, the possession and cultivation of *C. sativa* are strictly restricted by law. A number of countries have recently been moving toward the deregulation of *C. sativa* for medicinal, industrial, and recreational purposes.[1,2] Therefore, the identification of *C. sativa* has become increasingly important for forensic investigations and customs inspection in restricted countries. *C. sativa* is generally identified by chemical and morphological analyses. Chemical analyses are based on the detection of cannabinoids, such as tetrahydrocannabinol (THC), by GC-MS.[3] Morphological analyses are based on cystolithic hairs on leaves.[4] However, certain plant parts, such as the root and seed, contain little or no cannabinoids. DNA-based methods are useful for samples not identifiable by chemical or morphological analyses. Numerous studies have used DNA-based methods for *C. sativa* identification, including PCR or real-time PCR targeting chloroplast DNA and nuclear DNA, including the tetrahydrocannabinolic acid (THCA) synthase gene.[5–10] However, these analyses are time-consuming and are limited to well-equipped laboratories. Therefore, a rapid and convenient method is desirable for on-site application.

We previously developed a DNA-based method for *C. sativa* identification using the loop-mediated isothermal amplification (LAMP) assay.[10] Because the LAMP reaction proceeds at isothermal temperatures of 60–65°C and the results can be evaluated by visual detection, the assay can be used for on-site detection.[11] However, our previous protocol took about 90 min from sample preparation to detection. Further improvements for on-site application were therefore needed. In this study, we established a rapid on-site protocol based on the LAMP reaction that can be completed in 30 min for *C. sativa* identification and evaluated its reactivity for several samples.

MATERIALS AND METHODS

Materials Dried leaves, flowers, stems, and roots of drug-type (THC-dominant) *C. sativa* were obtained from Hokuriku University. Dried leaves of 20 strains of drug-type *C. sativa* were obtained from the Medicinal Plant Garden of Tokyo Metropolitan Institute of Public Health (Supplementary Table S1). Seeds of White Rhino, a drug-type *C. sativa* strain, were obtained from the Medicinal Plant Garden of Tokyo Metropolitan Institute of Public Health. Seeds of Tochigishiro, a fiber-type (Cannabidiol-dominant) strain, were obtained from Tochigi Prefectural Agricultural Experiment Station. The plant samples used as negative controls were collected from the Medicinal Plant Garden, Kanazawa University. DNA extracted from the *Papaver somniferum* extract and *C. sativa* resin was provided by the National Research Institute of Police Science.

† Present address: *Department of Pharmaceutical Sciences, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University; 1–1 Keyakidai, Sakado, Saitama 350–0295, Japan.*

* To whom correspondence should be addressed. e-mail: kitamura@josai.ac.jp

LAMP Reaction DNA was extracted from dried leaves obtained from Hokuriku University using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was quantified and diluted serially from 0.5 pg/μL to 5 ng/μL with sterile water. Purified DNA (2.5 ng/μL) was used for optimization of the LAMP reaction. Purified DNA was extracted from the dried leaves of 20 strains of *C. sativa* and 50 other plant samples using the DNeasy Plant Mini Kit. The DNA solution (2 μL) was used as the LAMP template. A *C. sativa*-specific primer set (FIP, BIP, F3, B3, loop primer 1, and loop primer 2) targeting the THCA synthase gene was used in accordance with our previous report, with minor modifications.[10] The primer sequences were as follows: FIP, 5′-CAA TGC TCC ATA GCC TCC TCC TGG TGG GTA TTG CCC TAC-3′, BIP, 5′-ATG CGA AAT TAT GGC CTT GCG CGA TCT AGA ACT TTT CCA TCA AC-3′, F3, 5′-TGG ATC AAT GAG AWG AAT GAG A-3′, B3, 5′-AGC CCA AAA YAG ATC TTC TCC-3′, loop primer 1, 5′-TGT CCA CCT ACG CCA ACA-3′, and loop primer 2, 5′-GCT GAT AAT ATC ATT GAT GCA CAC TTA G-3′. The LAMP reaction was performed with an Isothermal Master Mix (OptiGene, Horsham, U.K.). The 25-μL mixture consisted of 15 μL of Isothermal Master Mix, 0.2 μM F3 and B3 primers, 1.6 μM FIP and BIP primers, 0.8 μM loop primers, and 2 μL of DNA template. For real-time monitoring, the LAMP reaction was performed at 63°C for 20 min using a SmartCycler II System (Cepheid, Sunnyvale, CA, U.S.A.).

Simple DNA Extraction *C. sativa* samples were weighed and 1, 3 and 10 mg of leaves were used for simple DNA extraction. To evaluate on-site detection, 5 mg each of leaf, flower, stem, and root samples, 20 mg of seed samples, and 1 mg of resin samples were used. Approximately 3 mg of leaf samples from *Humulus lupulus, Humulus japonicus, Morus australis, Nicotiana tabacum*, and *Astragalus membranaceus* and a seed sample of *Hibiscus cannabinus* were used to evaluate specificity. Simple DNA extraction was performed with the alkalinization and neutralization method using the Kaneka Easy DNA Extraction Kit version 2 (Kaneka, Hyogo, Japan). A sample was mixed with 100 μL of alkaline solution (Reagent A) and heated at 98°C for 8 min, and 14 μL of neutralization solution (Reagent B) was added.

On-Site Application The colorimetric intercalating dye D-QUICK (Kaneka) was added to the LAMP reaction mixture before the LAMP reaction.[12] The LAMP reaction was performed using a portable device, MyAbscope (Kaneka). The LAMP reaction was monitored by measuring absorption at wavelengths of 575 to 660 nm at 63°C for 20 min and then at 70°C for 2 min to inactivate the DNA polymerase. After the LAMP reaction, the color of the reaction reagent was observed by the naked eye.

RESULTS AND DISCUSSION

Shortening the LAMP Reaction Time and Evaluations of Sensitivity and Specificity To reduce the reaction time, we modified the previously reported LAMP primers and LAMP reagent.[10] Two loop primers were added to accelerate the LAMP reaction, and a LAMP reagent containing pyrophosphatase was used because its substrate, pyrophosphate, is a byproduct of amplification and shows inhibitory effects.[12,13] The sensitivity and specificity of the optimized LAMP reaction were evaluated. Using purified DNA as the template, 10,

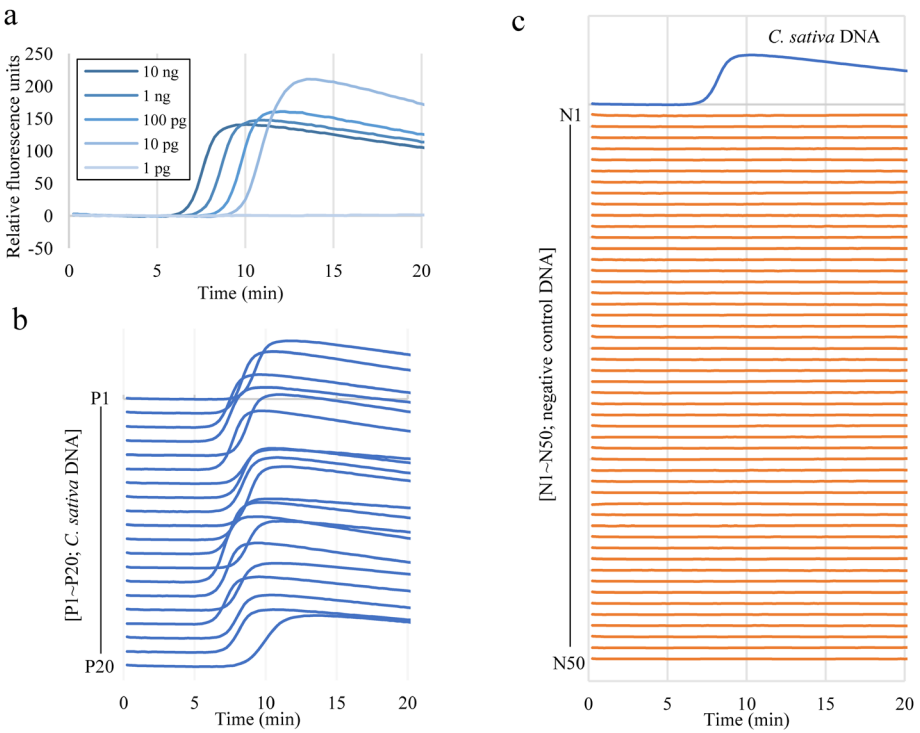

Fig. 1. Sensitivity and Specificity of the LAMP Reaction

LAMP sensitivity and specificity were evaluated using purified DNA under the optimized LAMP conditions. (**a**) The LAMP reaction was performed using 10-fold serial dilutions of purified DNA (10 ng, 1 ng, 100 pg, 10 pg, and 1 pg). (**b**) Amplification was observed with purified DNA from 20 *C. sativa* strains (P1–P20). (**c**) No amplification was detected with purified DNA from 50 other plant species (N1–N50). Sample names and species names are listed in Supplementary Table S1. (Color figure can be accessed in the online version.)

1 ng, 100, and 10 pg DNAs were amplified in 7.2±0.2, 8.1±0.2, 9.0±0.2, and 10.1±0.1 min (means±standard deviation), respectively, but no amplification was detected for 1 pg of DNA, even after 20 min (Fig. 1a). The sensitivity was 10-fold higher and the reaction time was approximately four times shorter than those in our previous report.[10] The specificity of the LAMP reaction was examined with purified DNA from 20 strains of *C. sativa* and 50 other plant species (Figs. 1b and c). All strains of *C. sativa* showed a positive reaction, whereas no amplification was detected for the other species after 20 min. These results indicated that the optimized LAMP reaction started at approximately 10 min and was completed in 20 min, with high specificity.

Evaluation of the Simple DNA Extraction Method Several protocols for simple DNA extraction from plant samples have been reported.[10,11,14,15] We employed a simple DNA extraction method based on alkalinization and neutralization, which took only 10 min and ensures a sufficient quality to amplify DNA from a *C. sativa* seed.[14] To evaluate the sensitivity of the protocol, we performed the LAMP reaction using DNA

Table 1. LAMP Reactivity of Simply Extracted DNA from the *C. sativa* Leaf

Leaf weight	Dilution rate				
	Crude	10-fold	100-fold	1000-fold	10000-fold
1 mg	13.1±0.6 min	9.2±0.1 min	10.4±0.4 min	11.3±1.1 min	ND
3 mg	17.3±2.2 min	9.7±0.3 min	9.4±0.2 min	13.4±3.4 min	ND
10 mg	ND*	12.8±0.3 min	9.5±0.2 min	10.8±0.2 min	ND

*ND: not detected. *C. sativa* leaf samples (1, 3, and 10 mg) were simply extracted and the crude sample and serial dilutions (10-, 100-, 1000- and 10000-fold) were used as DNA templates for the LAMP reaction.

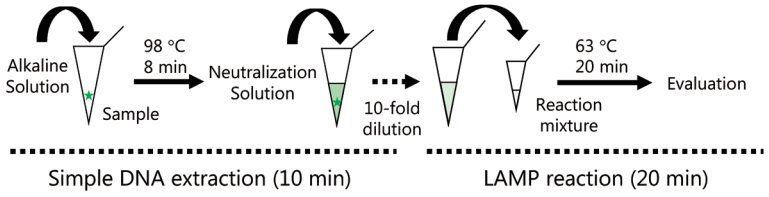

Fig. 2. On-Site Protocol for *C. sativa* Identification

Summary of the protocol. The protocol is divided into two processes: simple DNA extraction and the LAMP reaction. Simple DNA extraction was performed as follows. Samples were lysed with alkaline solution and heated at 98°C for 8 min, and neutralization solution was added. A 10-fold diluted DNA solution was used as a DNA template. LAMP amplification was conducted at 63°C for 20 min.

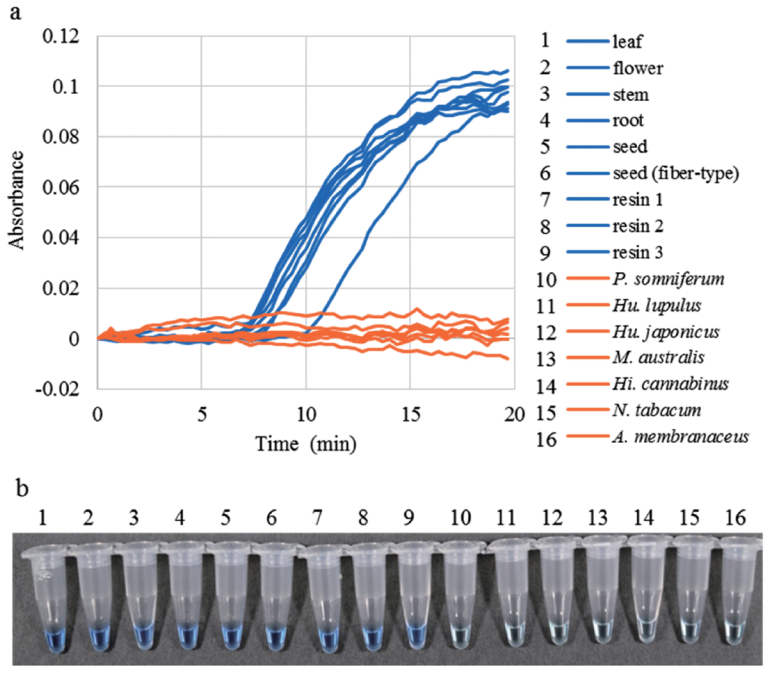

Fig. 3. Reactivity of the On-Site Protocol Using Samples from *C. sativa* and Other Species

The on-site protocol was applied to drug-type *C. sativa*-derived samples (1: leaf, 2: flower, 3: stem, 4: root, 5: seed), a fiber-type *C. sativa* sample (6: seed), resin samples (7–9: resin), and samples derived from other species (10: *Papaver somniferum* extract, 11: *Humulus lupulus* leaf, 12: *Humulus japonicus* leaf, 13: *Morus australis* leaf, 14: *Hibiscus cannabinus* seed, 15: *Nicotiana tabacum* leaf, and 16: *Astragalus membranaceus* leaf). (**a**) *C. sativa*-derived samples showed amplification in 20 min. In contrast, samples derived from other species did not show amplification, even after 20 min. (**b**) Reaction tubes after the LAMP reaction. The LAMP reagent for all *C. sativa*-derived samples turned blue, whereas that for the other species remained colorless.

simply extracted from 1, 3 and 10 mg of leaves (Table 1). For DNA extracted from 1 and 3 mg of leaves, crude DNA and DNA in 10-, 100-, and 1000-fold serial dilutions were amplified in 20 min. The LAMP reaction with 10-fold diluted DNA was more rapid for DNA extracted from 1 and 3 mg of leaves (1 mg: 9.2±0.1 min, 3 mg: 9.7±0.3 min) than for crude DNA (1 mg: 13.1±0.6 min, 3 mg: 17.3±2.2 min). Crude DNA from 10 mg of leaves was not amplified in 20 min, but DNA in 10- to 1000-fold diluted solutions from 10 mg of leaves was amplified in 20 min. The amplification of 10000-fold diluted DNA did not occur, even after 20 min. The serial dilution of crude DNA samples did not show a DNA concentration-dependent LAMP reactivity because the LAMP reactions of crude DNA could be affected by inhibitory substances such as polysaccharides and secondary metabolites. These results indicated that the LAMP reaction using simply extracted DNA had high sensitivity, although inhibitory effects on the LAMP reaction were observed using crude DNA.

On-Site Protocol for *C. sativa* Identification Based on the LAMP reaction and simplified DNA extraction step, a simple protocol was established (Fig. 2). The LAMP reaction was monitored based on the increase in absorbance that resulted from the binding of colorimetric intercalating dye with double-stranded DNA (dsDNA) and also by visual detection. In terms of reaction time and sensitivity, there were no differences between real-time detection using a real-time PCR device and real-time or visual detection using a portable device (data not shown). Figure 3a shows an amplification plot of the LAMP reaction with simply extracted DNA from the drug-type *C. sativa* leaf, flower, stem, root, and seed, and the fiber-type *C. sativa* seed. All *C. sativa*-derived samples were amplified within 20 min. Resin samples, which are extracts of *C. sativa*, were also amplified within 20 min. In contrast, no amplification was detected for the LAMP reaction with the extracts of *Papaver somniferum*, leaves of species related to *C. sativa* (*Humulus lupulus*, *Humulus japonicus*, and *Morus australis*), seeds of a morphologically similar species (*Hibiscus cannabinus*), leaves of *Nicotiana tabacum*, and leaves of *Astragalus membranaceus*. Figure 3b shows representative results of the LAMP reaction. *C. sativa*-derived samples turned blue, indicating a positive reaction, whereas samples that yielded a negative reaction remained colorless.

Plant samples contain substances that inhibit DNA amplification, such as secondary metabolites and polysaccharides. In addition, resin samples require specific DNA extraction using the modified cetyltrimethylammonium bromide method.[9] Our protocol was sufficient for detection from various samples, including resin samples. Because the detection of *C. sativa* may result in legal punishment, species detection has to be performed with care. Our method is highly sensitive and specific for *C. sativa*. At the same time, DNA contamination is possible owing to the high DNA amplification efficiency of the LAMP reaction. Therefore, a quality check for a false-positive reaction is necessary for each test, and the standards for evaluation should be set by each institute or laboratory.

CONCLUSION

In summary, we established an on-site DNA-based protocol for *C. sativa* identification using the LAMP reaction. The entire procedure was accomplished in 30 min with high sensitivity and specificity for *C. sativa*. Various *C. sativa* samples, such as seed and resin samples, were successfully identified. Our protocol shows promise for on-site investigations, including criminal investigations and customs inspection.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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