Establishment of an on-site diagnostic procedure for detection of orf virus from oral lesions of Japanese serows (*Capricornis crispus*) by loop-mediated isothermal amplification

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ABSTRACT.   Orf virus infection has been prevalent continuously in the population of wild Japanese serows (*Capricornis crispus*), goat-like grazing cloven-hoofed mammal species that live mainly in mountainous areas of Japan. Currently, definitive diagnosis of infection requires time-consuming laboratory work. To diagnose rapidly on-site, we developed a field-friendly procedure for the detection of orf virus from oral cavity lesions. DNA was extracted from goat saliva spiked with orf virus as a proxy for Japanese serows by a commercial kit without the use of electricity, and the quality of the extracted DNA was evaluated by conventional polymerase chain reaction (PCR). Extracted DNA was amenable to DNA amplification, the same as when extracted in a laboratory. Next, to find optimal conditions for DNA amplification by loop-mediated isothermal amplification (LAMP), *Bst* and *Csa* DNA polymerases and 3 colorimetric indicators for visual diagnosis, hydroxy naphthol blue (HNB), malachite green and D-QUICK, were compared using a portable cordless incubator. The combination of *Bst* or *Csa* DNA polymerase with HNB was found to be easiest for visual diagnosis by the naked eye, and viral DNA was successfully amplified from all orf virus strains used. These results suggest that the procedure established here can work completely on-site and can be useful for definitive diagnosis and differentiation of orf virus infection in Japanese serows in remote mountainous areas.
KEY WORDS: DNA extraction, Japanese serow, LAMP, on-site diagnosis, orf virus

Orf virus, a member of the genus *Parapoxvirus* in the family *Poxviridae*, causes contagious pustular dermatitis (also referred to as scabby mouth, contagious ecthyma and orf) in sheep and goats, and is occasionally transmitted to humans from infected animals and virus-contaminated materials, causing human orf that usually presents as inflammatory papules/nodules on the fingers and hands [17]. In sheep and goats, clinical signs of oral lesions of orf virus infection can be confused with those of foot-and-mouth disease (FMD), a highly infectious and economically significant viral disease in cloven-hoofed mammal species [3]. Clinical signs of FMD in sheep and goats are frequently transient and not definitive [16], and therefore, the differentiation of orf virus infection from FMD is difficult and laboratory confirmation is necessity. In wild animals in Japan, orf virus infections have been prevalent continuously in the population of Japanese serows (*Capricornis crispus*), goat-like grazing cloven-hoofed mammal species that live mainly in mountainous

areas all over Japan, except Hokkaido and Okinawa islands. The Japanese serow is one of the protected wild animal species as the special natural monument in Japan. When dead (or sometimes debilitated) serows were found, general information including external measurements, age, sex and so on are recorded. Clinical signs including orf virus infection are also recorded by on-site visual observations in public works as various levels. These information are gathered by local governments and then by the Agency for Cultural Affairs of Japan. Clinically affected serows are widely spread given the expanse of their habitat [4]. Previous serological surveys revealed seroprevalence in Japanese serows from 1981 to 1985 of 76/579 (13%) [30], but then, it increased to 40/167 (24%) from 1984 to 1999 [10, 11]. Wild Japanese serows sometimes appear in cattle and sheep pastures in certain areas [4, 13]. These overlapping areas could increase the risk of pathogen transmission from domestic animals to free-ranging serows, and *vice versa*. It could be also possible to result in host switching of microorganisms including viruses, as previously reported for nematodes in wild animals [2]. Although there has never been an FMD outbreak in Japanese serows so far and clinical signs of FMD in serows are unknown, it is possible that clinical signs of FMD in serows in some cases may resemble those of orf virus infection. In that case, it would require definitive diagnosis.

Diagnosis of orf virus infection in Japanese serows as well as domestic animals and humans is currently based

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on laboratory examinations, such as electron microscopic observation of viral particles [26], immunohistochemical detection of viral antigen [25, 27], virus isolation [7, 9, 12, 30], detection of viral DNA by polymerase chain reaction (PCR) [8], and serological tests by agar gel immunodiffusion test and enzyme-linked immunosorbent assay [10, 11]. Since free-ranging Japanese serows mainly live in mountainous areas, samples for diagnosis must be moved from these areas to laboratories in towns, and it is a time-consuming process. Recently, we produced a portable cordless incubator for genetic diagnosis of orf virus infection by a loop-mediated isothermal amplification (LAMP) assay, which consists of a battery, an aluminum heat block and a thermoregulator [5]. It was designed to maintain a stable temperature for more than 1 hr in the field, and DNA was successfully amplified by this method. However, DNA extraction from test samples required the use of laboratory equipment, meaning that LAMP could not be used for complete on-site diagnosis in mountainous areas. Therefore, in the present study, we established an on-site and field-friendly procedure for the detection of orf virus from oral cavity lesions of affected animals.

MATERIALS AND METHODS

DNA extraction: Instead of oral swabs from Japanese serows, saliva was collected and gathered from 3 healthy goats (2 Shiba and 1 crossbreed) in the Yanagido Field Science Center, Gifu University, under anesthesia by xylazine. This study was approved by the Gifu University Animal Care and Use Committee (Approval no. 14094). To mimic DNA extraction from oral swab samples from lesions, stock of the orf virus HIS strain [14] was added to goat saliva and was serially diluted from 10^5 to 1 tissue culture infectious dose 50% ($TCID_{50}$) /ml. DNA was extracted from each of the diluted saliva samples by a Loopamp PURE DNA Extraction Kit (PURE Kit, Eiken Chemical, Tochigi, Japan) according to the manufacturer's instructions. The kit requires neither electricity, heating, repeated micropipetting nor centrifugation [20], and it can be performed almost within 5 min. For comparison of quality and applicability to DNA amplification, DNA was also extracted from the same samples by a PureLink Genomic DNA Mini Kit (PureLink Kit, Invitrogen, Carlsbad, CA, U.S.A.), a common laboratory kit, according to the manufacturer's instructions. Moreover, six orf virus strains isolated from sheep, NZ2 [28], Iwate [18] and HIS [14], and from wild Japanese serows, S-1 [30], IJS081 [12] and GE [7], were also added to goat saliva, and DNA was extracted by a PURE Kit from each. As a negative control, DNA was also extracted from goat saliva without adding virus.

Semi-nested polymerase chain reaction (PCR): The amenability of the extracted DNA for DNA amplification was evaluated by conventional semi-nested PCR for the detection of parapoxvirus DNA as described previously [8].

LAMP: Extracted DNA from saliva was used as template DNA for the LAMP assay. Six LAMP primers sensitive and specific for detecting orf virus DNA were used as described previously [32]. Five microliters of extracted DNA were added to a total volume of 25 μl in a LAMP reaction mixture containing 0.2 μM F3 and B3, 1.6 μM FIP and BIP, and 0.8 μM LF and LB primers; 1× reaction mixture; and 8U of *Bst* DNA polymerase (Loopamp DNA Amplification Kit, Eiken Chemical). To find optimal conditions for extracted DNA in the LAMP assay, 1 ×*Csa* Reaction Buffer, 8U of *Csa* DNA polymerase (319–07281, Nippon Gene, Toyama, Japan) and 1.4 mM of each dNTP were also used instead of *Bst* DNA polymerase. For easy visual identification of the results after the LAMP reaction, three colorimetric indicators were also compared. In the LAMP assay, 1 μl of colorimetric indicator, either 3 mM hydroxy naphthol blue (HNB) (final 120 μM, 349–01461, Dojindo Laboratories, Kumamoto, Japan) or 0.1% malachite green (MG) (final 0.004%, 940–00674, Kishida Chemical, Osaka, Japan), was added to the mixture. D-QUICK DNA amplification checker tube type (Kaneka, Osaka, Japan) [21] containing dried color and reducing reagents at the bottom of the tube was also used for visual identification. These three colorimetric indicators are stable for several days in storage at room temperature [6, 24] as are the DNA polymerases for LAMP [6, 31]. The reaction mixture in each tube was covered with one drop of mineral oil (M5904, Sigma-Aldrich, St. Louis, MO, U.S.A.) to prevent evaporation. DNA amplification by LAMP was done at 65°C for 1 hr with the cordless incubator previously developed for on-site LAMP [5]. To evaluate the specificity of LAMP reaction, LAMP assay was also carried out by using the same reaction mixture and a LoopampEXIA real-time turbidimeter (Teramecs, Kyoto, Japan), and turbidity was measured.

RESULTS

Semi-nested PCR was carried out using the extracted DNA from goat saliva containing serially diluted virus by both kits. Amenability to amplification of DNA extracted by the PURE Kit, which was developed for DNA extraction from human bodily fluids [20], was almost the same with that by the PureLink Kit, which is regularly used in laboratories (Fig. 1). These results indicated that DNA extracted from goat saliva by the PURE Kit was of high enough quality to be used for enzymatic DNA amplification with the same efficacy as that extracted by the PureLink Kit for laboratory use.

Next, LAMP assay was carried out. All six orf virus DNA samples were amplified from goat saliva, and no amplifications were observed from negative controls (Fig. 2). From DNA extraction to visual diagnosis, this procedure can be performed on-site within 70 min. As for visual diagnosis by the naked eye, the combination of *Bst* or *Csa* DNA polymerase with HNB was best (Fig. 2). Using *Csa* DNA polymerase, but not *Bst* DNA polymerase, detectability by the naked eye was similar with HNB and D-QUICK, and determining whether the sample tested positive or negative was straightforward. Positive reactions with the combination of *Csa* DNA polymerase and D-QUICK were easier to identify than those with *Bst* DNA polymerase and D-QUICK (Fig. 2). Among the three colorimetric indicators, MG could not clearly differentiate positive and negative results with
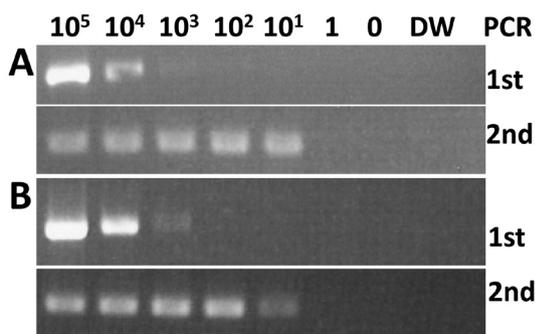
Fig. 1. Comparison of quality and amenability of DNA extracted from goat saliva for conventional semi-nested PCR. (A) PCR using DNA extracted by a PureLink Genome DNA Mini Kit for laboratory use. (B) PCR using DNA extracted by a Loopamp PURE DNA Extraction Kit. 1st, conventional PCR. 2nd, semi-nested PCR. The orf virus HIS strain was spiked and serially diluted with goat saliva from 10^5 to 1 $TCID_{50}$/ml. 0, goat saliva without virus as a negative control. DW, distilled water.

either DNA polymerase. Overall, the combination of *Bst* or *Csa* DNA polymerase with HNB was much clearer for visual identification of the reaction results compared to the other combinations. The specificity of DNA amplification was confirmed using the same reaction mixtures by measurement of turbidity by real-time turbidimeter (data not shown).

DISCUSSION

In this study, we established an on-site and field-friendly diagnostic procedure for the detection of orf virus from oral lesions of Japanese serows by LAMP assay using a cordless incubator. The LAMP assay can amplify DNA with high specificity and rapidity under isothermal conditions, unlike PCR that needs two or three different temperatures for enzymatic reaction [23], and it has been used for the detection of many kinds of pathogens [1, 22]. Moreover, it has been reported that DNA polymerases and reagents for LAMP are stable at irregular storage temperatures, such as

room temperature, for several days [6, 31]. Therefore, the LAMP assay is useful especially for field research, including diagnosis of infectious diseases in wild animals. Furthermore, nucleic acid amplification kits in which the reaction mixture for LAMP is premixed, dried and fixed in tubes are now commercially available (Eiken Chemical). The kits can be stored at room temperature, and only the template nucleic acid and LAMP primers are required, making for easier handling of the LAMP procedure in the field. However, because the 8-strip tubes of the kit are not suitable for tandem-setting in the heat-block of our cordless incubator, we did not use the kit in this study.

Even though the enzymatic LAMP reaction and visual diagnosis can be done outdoors, the extraction of template DNA is laborious and thus using the LAMP assay in the field is still difficult. To resolve this problem, we used a Loopamp PURE DNA Extraction Kit that does not need electricity, centrifugation, repeated micropipetting or heating [20], and a cordless incubator for the enzymatic reaction that is portable and can be maintained at a constant temperature [5]. Extracted DNA was applicable to the LAMP reaction, and viral DNAs were successfully amplified with easy visual identification of positivity. These results suggest that the procedure established here can be carried to completion in the field and could be a powerful tool for on-site diagnosis in remote mountainous areas where Japanese serows are living. Because orf virus is maintained mainly in sheep and goats [17, 29], this procedure could also be useful for on-site diagnosis of infection in domestic animals on farms and pastures.

Visual identification of positivity by combinations of *Csa* DNA polymerase and HNB or D-QUICK was easier than with *Bst* DNA polymerase, which is consistent with previous results using DNA extracted from virus-infected cells [5]. However, in some cases, we have found that *Csa* DNA polymerase is disadvantageous for use in the LAMP assay. Certain LAMP primers with *Csa* DNA polymerase will amplify non-specific DNA even from distilled water, whereas non-specific amplification is not observed in with *Bst* DNA polymerase under the same conditions (data not shown). Moreover, it has been reported that in cases with low pathogen quantity, the sensitivity of LAMP using DNA extracted

Fig. 2. Detection of orf virus strains in goat saliva and comparison of DNA polymerases and colorimetric indicators in the LAMP assay. 1, NZ2; 2, Iwate; 3, HIS; 4, S-1; 5, IJS081; 6, GE strains; 7, saliva (negative control); 8, distilled water. HNB, hydroxy naphthol blue. DNAs from goat saliva spiked with orf virus strains were positively identified by observation with the naked eye (sky blue for HNB, light green for malachite green and dark violet for D-QUICK).

by the PURE Kit is less than that with DNA extracted by other kits for laboratory use [15, 19]. These findings together with our current results suggest that repeat testing is necessary to assess the optimal combination of extraction method, DNA polymerase, LAMP primers, reaction solution and colorimetric indicator.

In conclusion, we established an on-site and field-friendly visual diagnostic procedure for the detection of orf virus from oral lesions of Japanese serows. All steps of the procedure can be performed completely in the field (DNA extraction, amplification and diagnosis), and therefore, it should be useful for on-site definitive diagnosis and differentiation of orf virus infection from other diseases showing similar clinical signs in domestic and wild animals at farms, pastures or mountainous areas where electricity is not available. Additionally, by using different LAMP primers, this procedure could be used for on-site diagnosis of other infectious diseases in animals and humans, not only in outdoor environments but also in rural areas and developing countries where electric power and laboratory equipment are not widely available.

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