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Rapid Preclinical Detection of Sheeppox Virus by a Real-Time PCR Assay

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The Capripoxvirus (CaPV) genus of the Poxviridae family of viruses comprises sheeppox (SPPV), goatpox (GTPV), and lumpy skin disease (LSDV) viruses, which cause disease in sheep, goats, and cattle, respectively. These viruses are considered reportable agents to the World Organization for Animal Health due to their potential for significant economic impact. Members of the CaPV genus are closely related, with genomic identities ranging from 96% between viral species to 99% between isolates of the same species (27). They have complete open reading frame (ORF) colinearity (13, 26, 27) and are indistinguishable via serological methods (14–16). CaPVs tend to be host specific; however, incidences where SPPV and GTPV have crossed species, into goats and sheep, respectively. These viruses are considered reportable agents to the World Organization for Animal Health due to their potential for significant economic impact. Members of the CaPV genus are closely related, with genomic identities ranging from 96% between viral species to 99% between isolates of the same species (27). They have complete open reading frame (ORF) colinearity (13, 26, 27) and are indistinguishable via serological methods (14–16). CaPVs tend to be host specific; however, incidences where SPPV and GTPV have crossed species, into goats and sheep, respectively, have been documented (8, 20).

Transmission of SPPV is thought to occur through exposure to aerosols or respiratory droplets produced by acutely infected animals or by direct or indirect contact with lesions or oronasal secretions (8, 17). Virus can be detected in nasal secretions of animals infected by aerosol or contact exposure (17) as well as in lesions found in the upper and lower respiratory tracts of infected animals (1c, 11). Transmission may also occur through the dermis, after contact exposure with cuts or abrasions (1c, 21, 22, 25), or via mechanical transmission by arthropod vectors (19).

Clinical signs of SPPV infection include fever, anorexia, depression, conjunctivitis, rhinitis, respiratory distress, generalized skin lesions, and enlargement of lymph nodes (9, 22). A transient viremia develops but may not be detectable throughout the entire course of infection (14). Transient viremias have also been described for cattle infected with LSDV (7, 28). Internal lesions are often seen at necropsy, especially in the lungs, although lesions in the trachea, rumen, tongue, kidney, nasal turbinates, and reproductive organs have also been reported (1c, 11, 14).

SPPV is endemic throughout much of Africa, southwest and central Asia, and the Indian subcontinent (3, 4, 23). Young animals are most susceptible, where mortality rates can be as high as 50 to 70% (10, 12). Outbreaks are controlled by ring vaccination, quarantine, and slaughter and may result in substantial economic losses due to mortality, reduced productivity, and trade restrictions (6, 24; www.fao.org). Rapid identification of CaPV is key to curtailing outbreaks before major economic damage can be inflicted.

A rapid test for CaPV that can detect virus before the onset of clinical disease would be invaluable for controlling or managing outbreaks as well as for disease surveillance. Here we describe a fluorogenic probe hydrolysis (TaqMan) PCR assay designed for rapid detection of CaPV and tested on sheep experimentally infected with a virulent strain of SPPV. This assay can detect SPPV in buffy coats, nasal swabs, oral swabs, scabs, and skin lesions as well as in lung and lymph nodes collected at necropsy. This single-tube diagnostic assay can be performed in 2 h or less and can detect viral DNA in preclinical, clinical, and postmortem samples.

**Materials and Methods**

**Viruses and cell cultures.** The pathogenic field isolate SPPV strain A (SPPV-SA) was obtained from a sick sheep in the Almatinskaya region of Kazakhstan and passaged nine times in sheep at the Scientific Research Agricultural Institute, Kazakhstan (1987) (27). The pathogenic virus SPPV_RvKLP (RvKLP) was derived from SPPV-SA as previously described (2). Primary lamb testis (LT) cell cultures were obtained from the Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture, Plum Island Animal Disease Center, Greenport, NY.

**Animal infections.** Merino lambs (3 to 4 months old) were inoculated intranasally with SPPV-SA (n = 3) or RvKLP (n = 5) at 10^6 PFU. Clinical signs of

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Footnotes:
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enized tissue samples were filtered through a 0.45-
formed on LT cells as described in World Organization for Animal Health
ately frozen at
isolation (VI) or DNA extraction. Tissues collected at necropsy were immedi-
at the end of the 30-day experiment. Tissue samples were collected for virus
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Virus isolation. Isolation of SPPV in clinical or necropsy samples was per-
formed on LT cells as described in World Organization for Animal Health

DNA extraction. DNAs were extracted from 200 μl of cell culture supernatant, transport medium containing nasal or buccal swabs, buffy coat samples, or hom-
genized tissue samples by using a DNeasy blood mini kit (Qiagen, Stanford,
and stored at 4°C until use. Blood samples were collected into BD
Vacutainer cell preparation tubes with sodium heparin (BD, Franklin Lakes,
NJ). Buffy coats were separated by centrifugation in a swinging rotor at 1,500
rpm (800 x g), collected, spun down, resuspended in phosphate-buffered saline,
and stored at 4°C until use. Lambs were euthanized when they were moribund or

Real-time PCR assay for SPPV. Reagents from an EZ-RT PCR kit (Applied Biosys-
tems, Branchburg, NJ) were used to prepare the reaction mix according to the
manufacturer's instructions. DNA from purified virus was extracted as previously described (29).

RESULTS AND DISCUSSION

Assay design and optimization. Complete sequences available
for the genomes of eight CaPVs were used for primer and
probe selection (13, 26, 27). These sequences included three
strains of SPPV (SPPV-SA, SPPV_Turky, and SPPV_Niskhi),
two strains of GTPV (GTPV_Pellor and GTPV_G20-LKV), and
three strains of LSDV (LSDV_Neethling 2490, LSDV_Neethling
Warmbaths, and LSDV_Neethling Warmbaths vaccine). Additionally, a
partial sequence was available for the KS-1 strain of SPPV (5).

Primers and probes were selected using ABI Prism Primer Ex-
press primer design software (Applied Biosystems, Foster City,
CA) from central regions of the genome (ORFs 24 though 123),
where the least genomic variability was observed (26, 27). Primer-
probe sets were searched against the NCBI nucleotide database
and selected based on 100% nucleotide identity to all members of
the CaPV genus. The target region falls within ORF 068 [poly(A)
.sdker (small subunit)] gene. Although this gene is present in
other poxviruses, only members of the CaPV genus were detect-
able with the selected primer-probe system (see below).

Assay analytical sensitivity and specificity. The analytical
sensitivity of the CaPV real-time PCR assay was determined based on
viral DNA extracted from purified SPPV-SA grown in LK cells. Virus was diluted in
log_{10} steps in Dulbecco's modified Eagle's medium, and DNA was extracted and
tested by real-time PCR to determine the limit of detection. Sensi-
tivity was based on four independent experiments. After ad-
justment for extraction (100 μl) and sample (2.5 μl) volume,
the sensitivity of the assay ranged between 1.6 and 15.8 50%
tissue culture infective dose of SPPV. The efficiency of ampli-
ification [10^{1-ΔCT}] was 1.99, with an R^2 value of 0.99 (Fig.
1), indicating an approximate doubling of product after
each cycle. In four independent experiments, the mean standard
deviation was determined to be 1.0 cycle threshold (C_{T}) among
all dilutions.

The specificity of the CaPV real-time PCR assay was determined for
members of the CaPV genus. DNAs were extracted from
five strains of SPPV, two strains of GTPV, and four strains of
LSDV and tested by real-time PCR (Table 1). All samples
were positive, with C_{T} values ranging from 16 to 23. Members of
the Orthopoxviruses, Parapoxviruses, and Suipoxviruses
genera of the Poxviridae tested negative by the CaPV real-time PCR
assay (Table 1).

Agents included in differential diagnosis of CaPV as well as
samples of other viral vesicular diseases affecting sheep, goats,
or cattle were obtained from the Foreign Animal Disease Di-
agnostic Laboratory (FADDL), APHIS (U.S. Department of
Agriculture, Plum Island Animal Disease Center, Greenport,
NY), and tested using virus-specific primers in order to verify
the presence of viral nucleic acid. Samples were then examined
using the CaPV real-time PCR assay. Bovine herpesvirus 2, blue-
tongue virus, rinderpest virus, peste des petits ruminants virus,

FIG. 1. Sensitivity of the CaPV real-time PCR assay, calculated
using SPPV samples obtained from infected cell culture supernatants and
run in quadruplicate. An inverse linear relationship exists between
C_{T} values and log_{10} 50% tissue culture infective dose (TCID_{50})/ml of
virus in the samples. The efficiency of amplification was 1.99, with an
R^2 value of 0.99, indicating an approximate doubling of product after
each cycle.
foot-and-mouth disease virus, and vesicular stomatitis virus all tested negative by the CaPV real-time PCR assay (not shown).

**Determination of cycle cutoff values.** The optimum cutoff value for the real-time PCR assay was determined using clinical samples (buffy coats and nasal swabs) collected from eight SPPV-inoculated animals and tested by real-time PCR and VI, with conventional PCR used as a confirmatory test (18). The Describe program of the WINPEPI statistical analysis program was used to calculate an optimum cutoff value of 45.7 cycles. Further analysis showed that while increasing the cutoff value to 50 cycles resulted in a sensitivity of 100% for both sample types, there was a resulting loss of specificity (90% buffy coats and 80% nasal swabs) due to the appearance of false-positive results. All subsequent results were based on a cutoff value of 45 cycles.

**Assay clinical sensitivity and specificity.** Infected lambs developed signs of clinical disease, including fever, loss of appetite, depression, conjunctivitis, and nasal discharge. All lambs developed fever by day 5 p.i. Lesions developed by day 7 and began to scab over by day 14 p.i. All lambs developed neutralizing antibodies by day 14, and all but one lamb were euthanized by day 21 or when they were found moribund. There was no significant difference in disease onset or severity between animals inoculated with the SPPV-SA and RvKLP viruses.

Oral swabs, conjunctival swabs, nasal swabs, buffy coats, and lesion scabs were evaluated as clinical samples for early SPPV detection. Oral and conjunctival swabs were not considered good candidates for early detection of SPPV, since positive results were not obtained until after the onset of clinical signs (not shown). Nasal swabs were found to be positive by real-time PCR starting on day 2 p.i., with all samples being positive by day 4 (Fig. 2). The real-time assay was able to detect viral DNA in nasal swabs 1 to 5 days prior to the onset of clinical disease, as defined by fever (temperature of ≤39.7°C) and the presence of skin lesions, and up to 2 days before VI-positive results were seen. Virus was detected in nasal secretions of infected animals until the day of death or day 20 for the one surviving animal. The clinical sensitivity for the real-time assay on nasal swabs was 100%, with a specificity of 95.2% (Table 2). In buffy coat samples, virus was first detected by the CaPV real-time assay on day 3, 1 to 3 days prior to the onset of clinical disease. However, comparison to VI results showed a few instances (4/42 samples) where samples were positive by VI but negative by real-time PCR. The VI results were confirmed by conventional PCR, indicating that for buffy coat samples, VI was more sensitive than real-time PCR (Table 2). This may reflect the presence of inhibitors in blood or the relatively low viremias associated with SPPV infections. Virus was not detected in buffy coat samples by real-time PCR or VI after day 12, even though virus was present in nasal secretions of infected animals until day 20.

Scabs from pox lesions collected throughout the course of the experiment and skin lesions samples collected at necropsy were tested by VI and real-time PCR. Skin samples taken from noninfected lambs and scabs collected from sheep, goats, and cows infected with parapoxvirus (provided by APHIS/FADDL) were used as negative controls. The results showed a clinical sensitivity of 95.5% and a specificity of 100% (Table 2). We tested the ability of our assay to detect SPPV in various tissues obtained from acutely infected sheep during postmortem examination. The CaPV real-time assay was able to detect virus in the lungs, periapical lymph nodes, and pharyngeal tonsils of all eight lambs tested.

The clinical sensitivity presented here was determined by utilizing a limited number of experimentally inoculated animals. Further validation of this test will require larger numbers of both infected and noninfected animals, ideally in a natural setting.

### TABLE 1. CaPVs and other poxviruses tested by the CaPV real-time PCR assay

<table>
<thead>
<tr>
<th>Genus</th>
<th>Virus</th>
<th>Strain</th>
<th>Species of origin</th>
<th>6-Carboxyfluorescein C&lt;sub&gt;r&lt;/sub&gt;</th>
<th>Reference or source</th>
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<tr>
<td>Capripoxvirus</td>
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<td>Almatinskaya (SA)</td>
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<td>Niskhi</td>
<td>Sheep</td>
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<td>27</td>
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<td>SPPV</td>
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<td>Sheep</td>
<td>17.28</td>
<td>USDA, FADDL</td>
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<tr>
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<td>Sheep</td>
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<td>Goat</td>
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<td>Neethling</td>
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<td>26</td>
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<td>Cow</td>
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<td>Cow</td>
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<td>Orf virus</td>
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<td>Goat</td>
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<tr>
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<td>Goat</td>
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<tr>
<td></td>
<td>Orf virus</td>
<td>Indiana</td>
<td>Goat</td>
<td>0</td>
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<tr>
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<td>Kansas</td>
<td>Cow</td>
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<td>Cow</td>
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<td>Mangistauskiy</td>
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<td>Swinepox virus</td>
<td>Nebraska</td>
<td>Pig</td>
<td>0</td>
<td>1a</td>
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</tbody>
</table>

* Samples were first tested by PCR or reverse transcription-PCR, using virus-specific primers, and then by real-time PCR using the CaPV real-time assay. Positive (SA) and no-template controls were included with each set of reactions.
Here we describe a TaqMan-based assay designed to detect CaPV in sheep, goats, and cattle and tested on lambs experimentally infected with SPPV (the type species of the genus CaPV). As designed, this assay was capable of detecting LSDV and GTPV in tissue culture samples. However, the clinical effectiveness of this assay for LSDV in cattle and GTPV in goats remains to be determined. Further validation of this assay under field conditions will be necessary to ascertain its viability as a diagnostic tool.

The CaPV real-time PCR assay provides a rapid, sensitive test for CaPV that is able to detect SPPV prior to the onset of clinical disease. For preclinical detection of SPPV, the sample of choice was nasal swabs, which provided better sensitivity than buffy coat samples. For clinical identification of SPPV, scabs or skin lesion biopsies provided high sensitivity and specificity and were easy to collect without the need for euthanizing suspect animals. Postmortem samples included those most likely to be submitted for diagnosis, i.e., lung and regional lymph nodes. These are all likely samples to be submitted for testing during a field investigation.

In conclusion, our results indicate that the CaPV real-time PCR assay is comparable to or exceeds the established method of VI for preclinical detection of SPPV in sheep. Based on our preliminary results, this assay should also be a useful tool for

FIG. 2. Detection of SPPV in nasal secretions of lambs at 0 to 8 days postinoculation. Bars represent body temperature (°C), and the solid horizontal line indicates the cutoff temperature of 39.7°C, above which lambs were considered febrile. Arrows indicate the first day that clinical signs were evident. Empty bars represent samples that were negative both by real-time PCR and VI. Hatched bars represent samples that were positive by real-time PCR but negative by VI. Filled bars represent samples that were positive by both real-time PCR and VI.
early detection and control of infections by other CaPV viruses, including GTPV and LSDV. This assay can differentiate between CaPV and other viruses causing vesicular disease in ruminants and has a high sensitivity in clinical samples. The CaPV real-time PCR assay represents a significant improvement over other established methods of CaPV detection due to its speed, simplicity, and ability to be carried out in laboratories without the need for tissue culture facilities.

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REFERENCES