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Specific qPCR assays for the detection of orf virus, pseudocowpox virus and bovine papular stomatitis virus

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The genus Parapoxvirus (PAPV) is comprised traditionally of orf virus (ORFV), pseudocowpox virus (PCPV) and bovine papular stomatitis virus (BPSV), which cause infections of ruminants and their handlers in the U.S. and worldwide. Unlike orthopoxvirus infections, which can cause systemic or localized infections, PAPV infections present normally as benign, self-limited and localized skin lesions; infections do not confer lifelong immunity. In recent years, related potentially to enhanced awareness and the availability of diagnostic methods, there has been an observed increase in reported cases of PAPV in animals and humans. This study describes TaqMan based real-time PCR assays for both generic and specific detection of PAPV species for surveillance and outbreak investigations. These assays target highly conserved PAPV RNA polymerase gene sequences and are capable of detecting three known species of PAPVs (ORFV, PCPV, and BPSV). The assays were evaluated using a panel of PAPV DNA derived from human infections or animal specimen remainders. The sensitivities of all four assays were determined using droplet digital PCR; fewer than 10 copies of clinical PAPV DNA can be detected consistently. These assays provide a reliable and sensitive method for rapid confirmation and characterization PAPV infections with varying clinical presentations.

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1. Introduction

Parapoxviruses are zoonotic and distributed widely in the Q3 United States and worldwide (Dal Pozzo et al., 2011; Damon, 2007; Gallina et al., 2006a,b; Leavell et al., 1968). Parapoxvirus infections were recognized initially in domestic cattle and small ruminants. ORFV is maintained in sheep and goats – causes "ecthyma contagiosum", a vesiculoulcerative disease of both keratinized skin and mucosal surfaces; PCPV and BPSV are maintained in cattle and are called commonly milker's nodule and bovine papular stomatitis respectively (Leavell et al., 1968; Mercer et al., 1997). Human parapoxvirus infections usually result from contact with infected animals. Humans with normal immune systems who had contact with PAPV infected animals or fomites developed self-resolving cutaneous ulcers. The infections normally have a vigorous and short-lived cell-mediated immune response and a relative poor and short-lived humoral response, about 8–12% of individuals had second infections (Damon, 2007).

Parapoxvirus infections are common in ruminants and their handlers (Lederman et al., 2013); however, the handlers are usually familiar with the infections and often do not seek medical attention (Gill et al., 1990). Human parapoxvirus infection can resemble skin lesions associated with potentially life-threatening zoonotic infections such as tularemia or cutaneous anthrax (Lederman et al., 2013). Parapoxvirus infection in an immunocompromised or immunosuppressed individual can lead to the development of more severe infections or lesions that are confused with tumors (Damon, 2007; Lederman et al., 2007b). Recently, an increased number of suspected human parapoxvirus infections were reported to, and confirmed in, the CDC poxvirus laboratory (Lederman et al., 2007a,b, 2013; MacNeil et al., 2010; Roess et al., 2010). PCR positive parapoxvirus samples were identified from both symptomatic and asymptomatic ruminants: 100% (7/7) of sheep and goats with symptomatic disease, as well as 21% (3/14) of asymptomatic sheep and goats were positive for orf virus by real-time PCR from community farms in a recent investigation (Lederman et al., 2013). Oral swab surveillance samples obtained from asymptomatic cattle in Virginia, United States, revealed 31 out of 45 swab samples to be positive for BPSV DNA (Roess et al., 2013). ORFV-like virus and PCPV-like viruses were also discovered in other animal populations, including North America white-tailed deer (Roess et al.,

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2010), European reindeer (Tikkanen et al., 2004) and other deer populations (Scagliarini et al., 2011).

The laboratory diagnosis of parapoxvirus virus is traditionally based on the negative stain electron microscopy; the virion of parapoxvirus is ovoid shape with a distinctive criss-cross filament pattern and is smaller in size than that of orthopoxviruses (Damon, 2007). It is difficult to grow parapoxviruses in typical transformed cell lines used in diagnostic laboratories, which limits the types of techniques that can be used to identify these viruses. PCR technology provides a valuable approach to identify parapoxvirus infections. One traditional PCR assay (B2L) for parapoxvirus diagnosis targets a conserved EEV protein B2L gene (Sullivan et al., 1994), a homolog of F13L of vaccinia genome sequences or ORF 11 in orf virus genome sequences (Delhon et al., 2004). Sequencing of the amplicon derived from the B2L assay has been used for the typing of parapoxviruses (Inoshima et al., 2000). Recently a new pan_poxvirus high GC PCR assay (Panpox_hGC) was developed to amplify high GC content chordopoxviruses including parapoxvirus, molluscipoxvirus and unclassified crocodile poxvirus (Li et al., 2010). The Panpox_hGC targets the conserved RNA polymerase gene (J6R in vaccinia genome and ORF 056 in orf virus genome) and the sequence comparison of amplicons derived from the Panpox_hGC assay can be used for the diagnosis and typing of parapoxviruses.

Real-time quantitive PCR (qPCR) assays further augment the diagnosis of parapoxvirus infections, or presence of virus, due to the high efficiency and sensitivity of these assays. This is especially important for human clinical samples which often have a low viral load and/or poor DNA quality. Such specimens are encountered commonly with parapoxvirus samples as the diagnosis is thought of in latter stages of clinical disease when the amount of virus present in a lesion is low. Currently, an ORFV specific qPCR assay has been reported, however this assay demonstrated cross reaction with PCPV (Gallina et al., 2006a,b); a parapoxvirus generic qPCR assay with B2L as the assay target was published which detects all three commonly seen ruminant parapoxvirus species and the related sealpox virus (Gallina et al., 2006a,b; Nitsche et al., 2006). There are no reported qPCR assays for the specific detection of PCPV and BPSV.

In this report, four TaqMan-based parapoxvirus specific qPCR assays which target the conserved *parapoxvirus* RNA polymerase subunit RPO147 gene sequence (ORF56 in ORFV IA82), the homolog of J6R gene of orthopoxvirus vaccinia virus were described: parapox generic assay (PARV_J6R) detects three species of parapoxvirus: ORFV, PCPV and BPSV; ORFV specific (Orf_J6R), PCPV specific (PCPV_J6R) and BPSV specific (BPSV_J6R) assays detect specifically the strains of ORFV, PCPV and BPSV viruses respectively. All assays were validated using collected panels of parapoxvirus human clinical DNA and animal samples for the specificities and sensitivities. The droplet digital PCR (ddPCR) methods are used to determine the sensitivities of those new qPCR assays and avoid the traditional steps of using purified viral DNA as reference for the quantifications.

2. Materials and methods

2.1. Clinical samples and DNA preparation

ORFV2012_014, ORFV2011_0563, PCPV2011_027, BPSV2010_186, BPSV2010_096, MOCV2009_029, Sealpox2006_054, are clinical samples and some of those samples have been reported recently (Roess et al., 2010). ORFV_vaccine is purchased commercially, PCPV_VR634 was purchased from ATCC (www.atcc.org), Deerpox_NA, VARV_Congo, VACV_WR, MPXV1979_005, CPXV_BRT, Tanapox DNA are CDC poxvirus laboratory collections and have been reported elsewhere (Li et al., 2006).

Table 1
The primers and probe sequences of Parapoxviruses generic and specific qPCR assays.

Orf virus specific assay (ORFV_J6R)	
Forward primer	GAGTTCGAGGAGATGATCTTGA
Reverse primer	GCCGAGGAGCAGGTC A
Probe	CTCGATCACGGCGCGCT
Pseudocowpox virus specific assay (PCPV_J6R)	
Forward primer	CCGACTACATCCGGAACA
Reverse primer	CGCACGCGCTTGCT
Probe	CTCACGCAGAAGATCTTCGTGAACTAC
Bovine papular stomatitis virus specific assay (BPSV_J6R)	
Forward primer	GAGATGATCTTGATGTTGTCGTACT
Reverse primer	TGGGCATGATCGTGAAGTAC
Probe	ATCATCGCGCGCTGGATCAC
Parapoxvirus generic assay (PAPV_J6R)	
Forward primer	CGCGGTCTGGTCCTTG
Reverse primer	CAGCATCAACCTCTCCTACATCA
Probe	CCACGAAGCTGCGCAGCAT

Notes: the oligo sequences are in 5′ to 3′ direction; all probes are labeled of FAM at 5′ and BHQ1 at 3′.

The parapoxvirus DNAs used in the assay validations were extracted using the Qiagen BioRobot EZ1 Workstation (Qiagen, Valencia, CA), and stored at −20 °C. The EZ1 Advanced DNA Tissue Card in which the extraction protocol is storied was used for all the clinical DNA extractions.

2.2. Taqman real-time PCR assays

The species specific, or species generic, design of each new assay was determined after sequence alignment among parapoxvirus genome sequences and other high G + C content poxvirus genome sequences (Fig. 1). The detection probe of all four assays contains a 5′ reporter molecule (FAM) (Glen Research, Sterling, VA) and a 3′ quencher molecule (BHQ1) (Molecular Probes, Eugene, OR). The details of the primers and probe sequences of four assays are listed in Table 1.

2.3. Validation of parapoxvirus generic and specific qPCR assays with clinical samples

The ORFV, PCPV, and BPSV specific and generic assays were optimized and validated using parapoxvirus clinical samples which had been previously confirmed by sequencing of the amplicon. qPCR validations were run in triplicate. The reactions were carried out in 25-μL volumes for analysis on the ABI7900 instrument (Applied Biosystems, Foster City, CA). Each reaction mixture contained 1X FastStart TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.4 μmol/L each primer, 200 nmol/L TaqMan probe, and 2 μL of template DNA. Thermal cycling for the ABI7900 was: one cycle 95 °C for 2 min; 45 cycles of 95 °C for 5 s, and 62.5 °C for 20 s for Parapoxvirus virus generic (PAPV_J6R) assay, PCPV specific (PCPV_J6R) and ORFV specific (ORFV_J6R) assay; one cycle 95 °C for 2 min; 45 cycles of 95 °C for 5 s, and 60 °C for 20 s for BPSV virus specific (BPSV_J6R) assay. The measurement of PCR amplification is based on the recording of fluorescent emission after the annealing/elongation step.

2.4. Measurement of the sensitivities of parapoxvirus generic and specific qPCR assays using ddPCR

The ddPCR validations of parapoxvirus qPCR assays were performed on the QX100 system (Bio-Rad, CA). A master mix of 6.3 μL PCR grade water, 12.5 μL Bio-Rad ddPCR Supermix, 0.5 μL each of forward and reverse primers (20 μM), 0.5 μL probe (FAM-10 μM), and 5 μL of DNA template was prepared. In the center wells of a DG8 droplet generation cartridge, 20 μL of each

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