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Short communication

TaqMan real-time PCR assay based on DNA polymerase gene for rapid detection of Orf infection

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A B S T R A C T

Both conventional and real time PCR (rt-PCR) assays based on the amplification of a 103 bp fragment from the *DNA polymerase* (*DNA pol*) gene (conserved, non-structural) of Orf virus (ORFV) were developed for detection and semi-quantitation of ORFV DNA from infected cell culture and clinical samples. The latter technique was based on TaqMan chemistry. The rt-PCR assay was specific and sensitive as it could detect as low as 3.5 fg or 15 copies of ORFV genomic DNA. Both intra- (0.38–1.0%) and inter-assay (0.53–2.87%) variabilities of rt-PCR were within the acceptable range meaning the high efficiency and reproducibility of the assay. The rt-PCR was applied successfully to detect ORFV DNA from suspected clinical samples. Further, the assay has shown a relative diagnostic sensitivity and specificity of 100% and 93.5%, respectively, when compared to *B2L* gene based semi-nested PCR implying a wide potential of this rt-PCR for rapid field diagnosis of Orf in sheep and goats.

Orf (*Synonym: contagious ecthyma or contagious pustular dermamitis*) is a non-systemic eruptive skin disease of sheep and goats and it is globally prevalent (Mondal et al., 2006). Orf virus (ORFV) is a DNA virus in the genus *Parapoxvirus* of sub family *Chordopoxvirinae* and the family *Poxviridae*. Bovine papular stomatitis virus (BPSV) and pseudocowpox virus (PCPV), the other members of the genus affect bovines. Orf is zoonotic and affects humans (Lederman et al., 2007). The disease is enzootic in India and outbreaks have been reported in sheep and goats (Mondal et al., 2006; Hosamani et al., 2006, 2007; Dey and Kundu, 2009). Orf is characterized by proliferative and often self-limiting lesions including papules, vesicles and scabs on the skin of lips, oral mucosa and around the nostrils of target species (Housawi and Abu Elzein, 2000). A mortality rate up to 10% and 93% in lambs and kids, respectively, has been reported in some cases, although the disease is associated in mild form in sheep and goats (Hosamani et al., 2007). The main differential diagnosis of Orf is foot and mouth disease, bluetongue, capripox as well as staphylococcal infection, therefore laboratory diagnosis is essential. Laboratory tests such as virus isolation, electron microscopy, counterimmunoelectrophoresis, serum neutralization test, enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and real-time PCR (rt-PCR) assays have been applied for diagnosis (Hosamani et al., 2009). Among these, real time PCR is essential for rapid diagnosis of virus infections. Likewise, pan-parapox specific PCR (Inoshima et al., 2000) has been employed widely and has been extended further for developing the rt-PCR (Gallina et al., 2006) based on the major envelope gene (B2L) (a structural protein of ORFV), for diagnosis of Orf infection. The present study was undertaken, with an objective to develop a TaqMan probe based rt-PCR targeting the *DNA polymerase* gene (*DNA Pol*), a highly conserved non-structural gene of the ORFV for rapid diagnosis of Orf infection in the target species using the clinical samples.

The following vaccine strains of virus/isolates available at the Division of Virology, Indian Veterinary Research Institute (IVRI), Mukteswar, Uttarakhand, India were used in the study. (i) Orf virus (ORFV) Mukteswar 59/05 (Passage-50), ORFV 79/04, ORFV-67/04, and ORFV-Muk/09; (ii) Sheeppox virus (SPPV) Srinagar 38/00 (Passage-40), SPPV-Ranipet, SPPV-RF and SPPV-Pune/08; (iii) Goatpox virus (GTPV)-Uttarkashi (Passage-60), GTPV-Muk and GTPV-Akola; (iv) Camelpox virus 1 (CMLV-I, P-50) and CMLV-II, CMLV-Hyd, CMLV-CADRAD-I & II, CMLV 108/06, 109/06, 110/07 isolates; (v) Buffalopox virus (BPXV)-Vijayawada 96 (Passage-50), BPXV-Vij/97, Pune/08, Pune/09, Hyd/04 and Bang/09 isolates. The vaccine strains of virus and other isolates were used as true positive/negative samples in analyzing diagnostic sensitivity (DSn) and specificity (DSp) of the rt-PCR. ORFV was propagated in primary lamb testes (PLT) cells, while the remaining afore listed viruses were in Vero cells using Eagle's minimum essential medium (EMEM) with 2% bovine calf serum. The infected cells were

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Table 1
Comparative evaluation of TaqMan rt-PCR assays with CIE and conventional PCR assays.

Clinical specimens different states	Species		Total samples tested	CIE [positive]		Conventional PCR [positive]		TaqMan rt-PCR [positive]	
	Sheep	Goat		Sheep	Goat	Sheep	Goat	Sheep	Goat
Andhra Pradesh	1	8	9	0	0	1	4	1	6
Himachal Pradesh	0	3	3	0	0	0	1	0	2
Jammu and Kashmir	0	6	6	0	0	0	1	0	4
Madhya Pradesh	0	4	4	0	0	0	3	0	4
Maharashtra and Gujarat	0	6	6	0	1	0	1	0	6
Orissa	0	4	4	0	0	0	2	0	2
Punjab	0	11	11	0	7	0	11	0	11
Rajasthan	0	7	7	0	1	0	4	0	5
Uttar Pradesh	1	9	10	0	1	1	1	1	5
Uttarakhand	9	1	10	2	1	6	1	9	1
Other states	1	3	4	0	1	0	2	1	3
Total	12	62	74	2	12	8	31	12	49
Positivity (%)				**2.7**	**16.2**	**10.8**	**41.9**	**16.2**	**66.2**
				18.9		**52.7**		**82.4**	

Note: CIE, counterimmunoelectrophoresis; PCR, polymerase chain reaction; RT-PCR, real-time PCR.

harvested after 80% cytopathic effect (CPE) using phosphate buffered saline (PBS, pH 7.2) and was used for extraction of viral genomic DNA.

The clinical samples consisting of infected scab material from sheep and goats collected from different geographical locations of the country were used for evaluation of the assay (Table 1). Clinical samples were homogenized in PBS and a 10% (w/v) suspension was prepared. Tissue homogenates were evaluated using counter-immunoelectrophoresis to ascertain the presence of Orf virus specific antigen (Housawi et al., 2008) as well as the extraction of viral genomic DNA using a commercial DNA extraction kit (AuPrep, Life technologies Pvt. Ltd., New Delhi, India). The conserved region of the *DNA pol* gene coding sequences of retrieved genomes of ORFV/BPSV/PCPV strains/isolates were identified using Clustal W method in the MegAlign program of Lasergene 6.0 (DNASTAR, Inc., Madison, WI, USA) for designing oligonucleotide primers and probes. The primers and probe with their location in respect of the genome of ORFV strain NZ2 (Acc # DQ184476) are shown in the parenthesis. The primers were [Ov RT-F, Forward: 5′-TACACGGAGTTGGCCGTGATCTTGTA-3′ (24894–24920); Ov RT-R, Reverse: 5′-CGCCAAGTACAAGAAGCTGATGA-3′ (24973–24997); the TaqMan hydrolysis probe was (P): 5′Hex-TGCATCGAGTTGTAGATCTCGCGGT-BHQ-1 3′ (24928–24953)] synthesized commercially (M/s Metabion GmBH, Germany). The primers amplify a conserved 103 bp DNA fragment of *DNA Pol* gene sequence of ORFV. The probe was labeled with 6-carboxyfluorescein (HEX) as reporter at the 5′ end and Black hole quencher (BHQ-1) at the 3′ end as non-fluorescent quencher. Primers and probe would react with BPSV/PCPV/seal parapox virus genome based on alignment of published gene sequences of these isolates. However, these infections are not reported from India.

The rt-PCR conditions such as annealing temperature (T_a) and primer concentration were first optimized by conventional gel based PCR using designed primers (OvRT-F and OvRT-R primers) and purified ORFV genomic DNA. PCR was carried out in a 50 μl reaction volume containing 2 μl template DNA, 10 pmol of each primer, 5 μl of the 10× buffer, 10 mmol/l dNTPs, and 0.25–0.5 IU of *Taq* DNA polymerase (M/s Invitrogen, Carlsbad, CA, USA) in a Thermal cycler (MyCycler, BioRad, Hercules, CA, USA). The PCR cycling conditions were, initial denaturation at 95 °C for 4 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s and extension at 72 °C for 30 s, with a final extension for 5 min at 72 °C. The amplicons were analyzed on a 3% agarose gel after staining with ethidium bromide. For analyzing the detection limit of conventional PCR assay and evaluation of clinical samples, the reaction was carried out for up to 40 cycles.

Then rt-PCR was optimized using viral genomic DNA and ORFV *DNA pol* gene-specific primers with probe. In brief, 50 μl reaction

Table 2
Diagnostic sensitivity and specificity of TaqMan rt-PCR.

True positive (vaccine viruses/isolates/clinical scab samples)	C_t value	Diagnostic sensitivity (DSn %)	True negative (vaccine viruses/isolates/clinical samples)	C_t value	Diagnostic specificity (DSp %)
ORFV isolates = 4	12.2–15.5		SPPV isolates = 4	39.5–39.9	
1. SP 45 (M), Goat, 22/03	19.26		GTPV isolates = 3	38.9–39.8	
2. 82/04, Goat, 10.08.04	19.25		BPXV isolates = 5	No C_t	
3. 143/05, Sheep, 23.05.05	25.20		CMLV isolates = 8	No C_t	
4. 59/05, Goat, 05/02/05	16.16		1. 22/04, Goat, 2003–2004	No C_t	
5. 55/06, Goat, 17.03.06	25.20		2. 9/06, Goat, 19.01.06	No C_t	
6. 41/06, Goat, 22.03.06	23.63		3. 21/06, Goat, 23.02.06	No C_t	
7. SP26, Goat, 07.08.06	22.29	Positive by rt-PCR/total true positive samples = 18/18 = 100	4. 24/06, Goat, 03.03.06	No C_t	Negative by rt-PCR/total true negative samples = 29/31 = 93.5
8. 56/06, Goat, 17.03.06	27.21		5. 43/06, Goat, 22.03.06	30.04	
9. 51/06, Goat, 17.03.06	29.11		6. VSP5M/14, Goat, 11.04.08	No C_t	
10. 52/06, Goat, 17.03.06	26.17		7. VSP5M/15, Goat, 11.04.08	No C_t	
11. 612/09, Sheep, 11.01.09	29.57		8. AJM08/2, Goat, 13.10.08	No C_t	
12. 47/09, Sheep, 11.01.09	28.88		9. VSP29/614, Goat, 10.11.08	No C_t	
13. 66/09, Sheep, 11.01.09	24.46		10. VSP8/09/5, Goat, 22.04.08	25.59	
14. 83/09, Sheep, 11.01.09	17.07		11. AJM08/1, Goat, 13.10.08	No C_t	
Total = 18			**Total = 31**		

True positive and negative samples for ORFV were selected by published semi-nested PCR based on B2L gene (Inoshima et al., 2000).