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LETTER TO THE EDITOR

Rapid detection and quantification of Orf virus from infected scab materials of sheep and goats

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Orf (contagious ecthyma) is a local proliferative skin disease of sheep and goats caused by the orf virus (ORFV). This zoonotic disease occurs worldwide and is enzootic in India, causing high morbidity in sheep and goats (*7*). Though it is easy to diagnose, the lesions and symptoms confounding with other skin diseases require laboratory confirmation, which includes serological and nucleic acid-based techniques (*6, 10*). In this study, a rapid, sensitive SYBR green-based real-time PCR (rt-PCR) targeting the *DNA polymerase* gene (*DNA Pol*), a highly conserved non-structural gene of the ORFV, has been developed for the detection and quantification of ORFV particles in skin lesions of sheep and goats. Orf virus propagated in primary lamb testes cells and other viruses, namely sheeppox virus (n = 4), goatpox virus (n = 3), buffalopox virus (n = 4) and camelpox virus (n = 7), available at Poxvirus Disease Laboratory were used in this study. Viral genomic DNA was extracted using commercial DNA extraction kit (AuPrep, Life technologies Pvt. Ltd., New Delhi, India) and used to assess the analytical specificity of the assay. The clinical samples in the form of scab materials from sheep and goats (Table 1) were used to evaluate the assay. Initially, rt-PCR conditions [primer concentration and annealing temperature (Ta)] were optimized by conventional PCR using specific primers [Ov RT-F: 5′-TACACGGAGTTGGCCGTGATCTTGTA-3′ (24894–24920); Ov RT-R: 5′-CGCCAAGTACAAGAAGC TGATGA-3′ (24973–24997)]. The amplicon was gel-purified and cloned in to pGEM-T Easy vector (Promega, Madison, USA) and the recombinant plasmid DNA was used in rt-PCR. Ten-fold serial dilutions of plasmid DNA ranging from 10^6 to 1 copy in 2 µl of TE buffer were used in SYBR Green rt-PCR in order to generate a standard curve for absolute quantification as well as to determine the PCR efficiency of the assay. The reaction was carried out in a 25µl volume using 10 pm of each primer and QuantiTect™ SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) containing HotStar*Taq* DNA polymerase in Mx3000p qPCR machine (Stratagene, La Jolla, CA, USA). The PCR conditions were: initial denaturation at 95°C for 10 min, 40 cycles of 94°C for 30 sec, 63°C for 30 sec, and 72°C for 30 sec and melting curve analysis with ramping down (95°–55° C) at a rate of 0.2°C/sec for fluorescence data collection. Mean threshold cycle (*Ct*) values in triplicate samples were used for analysis. The analytical specificity of the assay was evaluated using ORFV and other non-ORFV isolates by melting curve analysis to discriminate from non-specific products. The reproducibility, repeatability and linearity of the developed assay were determined using serial 10-fold dilutions of standard plasmid DNA as described earlier (*3*). The diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of the assay were obtained using ORFV (n = 16) and non-ORFV (n = 29) isolates/samples as true positive and true negative

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Abbreviations: diagnostic specificity = DSp; diagnostic sensitivity = DSn; DNA Pol = DNA polymerase; *Ct* = mean threshold cycle; orf = contagious ecthyma; ORFV = orf virus; rt-PCR = real-time PCR

Table 1. Results of clinical specimens tested in different assays

Clinical specimens from different geographical locations (States of India)	Species		Total samples	Counterimmune electrophoresis		B2L gene conventional PCR		SYBR Green rt-PCR	
	Sheep	Goat		Sheep	Goat	Sheep	Goat	Sheep	Goat
Jammu and Kashmir	–	6	6	–	–	–	1	–	6
Uttar Pradesh	1	9	10	–	1	1	1	1	7
Andhra Pradesh	1	8	9	1	–	1	4	1	8
Maharashtra	–	6	6	–	1	–	1	–	6
Punjab	–	11	11	–	7	–	11	–	11
Orissa	–	4	4	–	–	–	2	–	4
Himachal Pradesh	–	3	3	–	–	–	1	–	2
Rajasthan	–	7	7	–	1	–	4	–	5
Uttarakhand	9	1	10	2	1	6	1	9	1
Tamil Nadu	1	3	4	–	1	–	2	1	3
Total	12	58	70	3	12	8	28	12	53
Percent positivity				21.4		51.4		92.9	

samples, respectively, which was based on B2L gene-based semi-nested PCR (*8*). Further, clinical samples (n = 70) suspected of orf were subjected to SYBR green rt-PCR, counterimmunoelectrophoresis (*4)* and conventional B2L gene- based semi-nested PCR (*8)* to compare the diagnostic efficacy of the developed assay.

The developed assay was found to be optimum at Ta of 63°C using standard plasmid DNA with a PCR amplification efficiency [Y = 3.362 Log X + 45.53] of 98.4% and R^2 value of 0.995 from the standard curve. The specific amplification signal was observed only with ORFV DNA with a melting curve peak at 84.2°C. No such signal was detected with other related poxviruses. The lower detection limit (or cut off) of 6 copies per 25 µl reaction was corresponding to a *Ct* of 38.9 and was applied to test samples. The developed assay had repeatability and reproducibility with an acceptable range of intra-assay (0.58–1.54%) and inter-assay (0.84–2.37%) variations. Further, the DSp and DSn of the assay were 93.5 and 100%, respectively, in relation to B2L gene-based semi-nested PCR. The generated standard curve (figure not shown) was found to be linear from 6.0×10^7 to 6.0 copies of plasmid range with a slope = -3.314 and was used to derive the virus load of unknown samples based on the *Ct* values obtained. The virus load of samples ranged from 2×10^1 to 2×10^7 per mg of scab materials. TaqMan hydrolysis probe- and SYBR Green- based rt-PCR assays have been reported for rapid detection and quantification of viral load in infected materials for buffalopox virus (*11*), camelpox virus (*1*), parapoxviruses (*5, 6, 10*) and capripoxviruses (*2*) and other viral diseases (*3, 9*). The testing of 70 clinical samples by counterimmunoelectrophoresis, conventional PCR, and rt-PCR (Table 1) showed percent positivity of 21.4, 51.4, and 92.9, respectively, which implies the high sensitivity of rt-PCR. Moreover, the rt-PCR assay was found simpler and more rapid (only 1 hr and 40 min) in comparison with conventional PCR (> 4 hr) or B2L gene-based semi-nested PCR, as the latter requires two rounds of PCR amplification (*8*). The assay could be used as a routine diagnostic tool for bulk processing and analysis of ORFV-suspected clinical samples as it holds better DSp and DSn when compared to the conventional PCR that is currently being routinely used for rapid diagnosis in our laboratory.

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