Quantitative polymerase chain reaction: another tool to evaluate viable virus content in live attenuated orf vaccine

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Summary

A probe-based real-time polymerase chain reaction (PCR) assay based on the highly conserved *DNA* polymerase gene of orf virus (ORFV) for the quality control of attenuated orf vaccine is reported. Primary lamb testis (PLT) cells were infected with orf vaccine virus and harvested at a critical time point to obtain maximum viable virus content as determined by real-time PCR. DNA extracted from these harvests was subjected to real-time PCR. A critical time point for the harvesting of PLT cells infected with various log_{10} dilutions of vaccine virus was found to be 42 h (highest slope of 3.335), which was obtained by comparing the slopes of standard curves of different time intervals. The assay was employed to evaluate viable virus content in different batches of orf vaccine. The titres estimated by real-time PCR and conventional $TCID_{50}$ were comparable with a correlation of 0.8169. Thus, the real-time PCR assay could provide an alternative method or supplementary tool to estimate live ORFV particles in attenuated orf vaccine.

Keywords

Contagious ecthyma, DNA polymerase gene, India, Orf, PCR, Polymerase chain reaction, Potency, Real-time PCR, TaqMan, Vaccine, Virus.

PCR quantitativa: una valida alternativa per quantificare le particelle virali infettanti di un vaccino ORF vivo attenuato

Riassunto

In questo studio, è descritta una metodica di real-time PCR per valutare la qualità di un vaccino attenuato del virus ORF (ORFV), agente eziologico dell'ectima contagioso. Il protocollo descritto prevede una sonda specifica per il gene altamente conservato codificante per la DNA polimerasi virale. Monostrati confluenti di cellule primarie testicolari di agnello sono stati infettati con diversi lotti e diluizioni di vaccino attenuato. Il surnatante, raccolto a vari intervalli, è stato sottoposto ad estrazione e quantificazione del DNA tramite real-time PCR. E' stato quindi possibile determinare come 42 ore post infezione rappresenti il momento ottimale per la raccolta del virus vaccinale dai monostrati infetti. E' stata riscontrata una correlazione pari a 0.8169 tra i titoli ottenuti in real-time PCR e quelli ottenuti tramite titolazione convenzionale. In conclusione, questo metodo fornisce una valida alternativa a supporto delle metodiche classiche per quantificare le particelle virali infettanti di un vaccino ORF vivo attenuato.

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Parole chiave
Ectma contagioso, DNA *polymerase gene*, India, ORF, PCR, *Polymerase chain reaction*, Potenza, *Real-time PCR*, TaqMan, Vaccino, Virus.

Introduction

Orf (synonyms: contagious ecthyma, contagious pustular dermatitis), is a non-systemic eruptive skin disease of sheep and goats that occurs worldwide (7). Orf virus (ORFV) is a DNA virus that belongs to the genus *Parapoxvirus*, subfamily *Chordopoxvirinae*, family *Poxviridae*. The disease is of economic importance as it is associated with high morbidity, low mortality and reduced productivity and is endemic across the globe. Furthermore, it is a zoonotic disease that can cause skin lesions in humans.

Control of the disease contributes to improved productivity of sheep and goats. Vaccination is a reliable method of control. A primary lamb testis (PLT) cell-adapted live attenuated orf vaccine has been developed recently by the Indian Veterinary Research Institute (IVRI) in Mukteswar and was found to be safe, potent and efficacious, both in-house and in field conditions (V. Bhanuprakash, unpublished data).

The number of live virus particles in a vaccine dose (potency) determines the efficacy of vaccine and seroconversion in a host. Conventional methods used to estimate potency of live viral vaccines are plaque-forming unit (pfu), cell culture infective dose ($CCID_{50}$) (13) and egg infective dose (EID_{50}), that are based on virus-induced cytopathology (5). However, these assays are subjective and can lead to inconsistent results, depending on the laboratory, personnel and time (2, 3, 4). In addition, the preparation, handling and propagation of PLT cell cultures used for orf vaccine are associated with many shortfalls when compared to the use of continuous cell lines, namely: Vero and BHK-21 cells. These drawbacks affect them from being implemented for the estimation of potency of live vaccines. In addition, they are labour-intensive, time-consuming and are not highly accurate. Furthermore, they are not preferred in assigning the titres to individual components of bivalent or multivalent vaccines as they require highly specific neutralising antibody against each component of these vaccines to determine viral titres. Moreover, ORFV, like some of the other poxviruses, does not induce strong antibody response which makes it difficult to acquire specific neutralising antibody that is required to measure viral titres in bivalent vaccines. To overcome these issues, real-time polymerase chain reaction (PCR) has been employed recently to estimate the number of live virus particles in different veterinary and human live viral vaccine formulations (6, 8, 9, 12, 13, 15). Thus, in our study, orf vaccine was evaluated for its potency in terms of the number of live viral particles based on a *DNA* polymerase gene-specific TaqMan® real-time PCR assay. This gene was targeted because the gene is a highly conserved non-structural gene of ORFV (1). To the best of authors' knowledge, this appears to be the first report on estimation of live virus particles in an orf vaccine using the quantitative PCR (qPCR) assay, while comparing this to the routine conventional cell culture method.

Materials and methods

The seed ORFV (ORF MUK 59/05, P-49) maintained in PLT cells at the Division of Virology of the IVRI was used for the preparation of vaccine batches. Orf vaccine batch (ORFV-06/09) with a mean titre of $10^{5.79 \pm 0.42}TCID_{50}$/ml was used as a reference. PLT cells maintained at the Division of Virology were used for ORFV growth and quantitation. The cells were propagated in Eagle minimal essential medium (EMEM) supplemented with 10% bovine calf serum (Hyclone, Logan, Utah); for maintenance of the cells and titration of virus, EMEM supplemented with 2% serum was used. Different live orf vaccine batches were produced and used for qPCR-based potency estimation.

The oligonucleotide primers and probe used in this study were orf virus (OV) real-time-F: 5′-TACACGGAGTTGGCCGTGATCTTGTA-3′,

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OV real-time-R: 5′-CGCCAAGTACAAGAAG CTGATGA-3′ and TaqMan hydrolysis probe: 5′Hex TGCATCGA GTTGTAGATCTCGCGGT BHQ-1-3′ amplify-ing a conserved 103 bp DNA fragment of *DNA* polymerase gene sequence of ORFV. The real-time PCR was performed as described by Bora *et al.* (1). The real-time PCR cycling conditions were as follows:

- initial denaturation at 95°C for 4 min
- 40 cycles of denaturation at 94°C for 30 sec
- annealing and extension at 64°C for 45 sec.

The assay developed had an efficiency of 98.7% with high specificity and sensitivity.

The reference orf vaccine batch (ORFV 06/09) was reconstituted in EMEM and serially diluted tenfold, ranging from neat to 10^{-4}. A total of 100 µl of each dilution was inoculated onto six-well plates with confluent PLT cell monolayers and a cell control and maintained in an incubator at 37°C under 5.0% CO_2. Each plate well was harvested at six-hourly intervals for 48 h using 300 µl of sterile phosphate buffered saline (PBS) (pH 7.4) and freeze-thawed three times before employing real-time PCR. Total genomic DNA (gDNA) was extracted from all harvests using AuPrep GEN DNA extraction kit (Life Technologies India (Pvt) Ltd, New Delhi) and TaqMan real-time PCR was performed to assess the *Ct* (cycle threshold) values. To establish the standard curve, the *Ct* values were plotted against the $\log_{10}$ dilutions of virus titre followed by linear regression analysis. The slopes of standard curves at different time interval harvests were compared and a standard curve of particular time interval harvest with maximum slope was selected as the critical time of harvest for test batches. The critical time point harvest is determined by three consecutive experiments and the mean of the *Ct* values was used to plot the standard curve. The *Ct* values for the test batches of orf vaccine were determined and the titre for each batch was calculated by plotting *Ct* values on the standard curve.

The potency of the live orf vaccine batches using the $TCID_{50}$ method was also determined (10). The real-time PCR results obtained for each vaccine batch were compared with those of virus titres estimated with the $TCID_{50}$ method and a correlation was drawn between these methods using Student's *t*-test.

Results and discussion

Vaccines must be evaluated for their safety and efficacy prior to application in the field. The efficacy of any attenuated live vaccine (ALV) depends on the number of live virus particles in a vaccine dose. The potency of live vaccines is determined *in vivo*, either in the target host/laboratory animal or *in vitro* titration of the virus in a suitable cell culture system/embryonated egg (14). These gold standard assays are time-consuming, laborious, require extensive standardisation, are prone to variations (2, 3, 4) and are subjective.

Recently, real-time PCR has been applied to assess the potency of a monovalent, bivalent and multivalent vaccines. Using the standard nucleic acid of known concentration, the template nucleic acid concentration (of both infectious and non-infectious virus) of an unknown sample can be quantified. To overcome the drawbacks of conventional assays described earlier, cells were initially infected with vaccine virus and it was presumed that if the live virus was first propagated in cell culture, the amount of genomic DNA produced corresponded to the amount of viable particles in the vaccine preparation. Furthermore, an estimation of potency using real-time PCR is particularly useful in cases of delayed cytopathic effect (CPE) producing/non-cytopathic viruses as it can estimate the titre of the vaccine prior the appearance of CPE.

In case of ORFV, the appearance of CPE commences 24 h post infection (hpi) and is completed by 72 hpi. In our study, the titre of orf vaccine was determined by qPCR as early as 42 hpi. The method required a standard reference vaccine batch (ORFV-06/09) of known titre ($10^{5.79+0.42} TCID_{50}$/ml). The reference vaccine batch was selected in such a way that the titres/*Ct* values of the test batches might fall within the wide range of reference vaccine dilutions. This minimises the number of

dilutions of test vaccine batches. In addition, the critical time point harvest for the reference vaccine batch was determined and it corresponded to the minimum time required for the appearance of sufficient viral particles in all the dilutions of the virus infected cells. Accordingly, the samples were harvested at 6, 12, 18, 24, 30, 36, 42 and 48 hpi and subjected to *DNA pol* gene based TaqMan® real-time PCR and slopes of standard curves for each interval of harvest were compared. The slope of the curve was greatest at the 42 hpi ($R = 3.335$) harvest than that of other intervals (Fig. 1); this was considered the critical time point to harvest the reference as well as test vaccine batches so as to get maximum live viral particles.

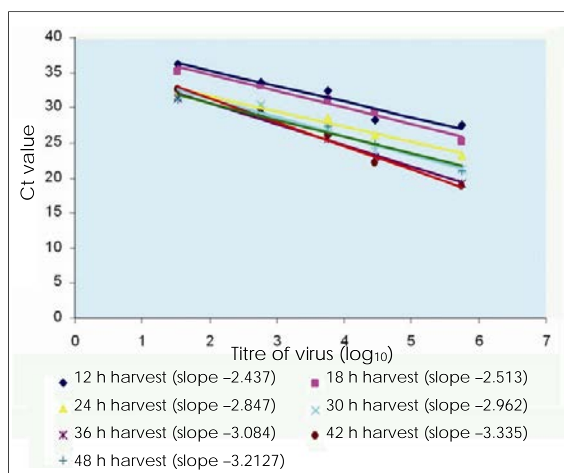

Figure 1
Standard curves established for different time interval harvest using *DNA* polymerase gene-based real time-polymerase chain reaction

A total of seven different batches of live attenuated orf vaccine, including a reference batch, were selected to estimate their titres using the TaqMan® real-time PCR and end-point dilution assays. The titres obtained were satisfactory and were within the acceptable range of accuracy (Table I) as reported earlier for goatpox vaccine (6). A high degree of correlation (0.8169) between assays with no significant difference ($p>0.005$) was observed. The assay is simple, rapid and easy to perform and does not require expertise compared to the $TCID_{50}$ method. Furthermore, the estimating using real-time PCR overcomes variations between laboratories due to discrepancies in conventional assay procedures as the potency of an unknown vaccine batch was estimated relative to a reference batch (2). However, for accuracy, the virus in the test vaccine batches should have the same replication rate as that of the virus in the reference batch. As the difference in the sample/vaccine composition might affect the replication rate of the virus (11, 12), vaccine batches (reference and test) of similar composition were used in this study.

Conclusions

The potency of live attenuated orf vaccine was evaluated using *DNA pol* gene based TaqMan PCR. The critical time-point for harvest was

Table I
Comparison of potency estimation of orf vaccine by real-time polymerase chain reaction and end-point dilution ($TCID_{50}$) assays

Vaccine batch	*Ct* values for different dilutions of vaccine					Virus titres	
	Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Real-time PCR	$TCID_{50}$
ORFV 06/09	21.12	24.24	27.18	30.12	33.25	5.68 ±0.20	5.79+ 0.42
A-01/060909	19.96	22.54	26.03	29.28	33.12	5.48 ± 0.11	5.40± 0.04
B-02/311209	20.69	24.06	27.65	30.93	32.68	5.38 ±0.11	5.35± 0.03
C-03/311209	21.02	24.34	27.08	30.02	33.24	5.57 ±0.22	5.75±0.04
D-01/010610	22.56	25.58	28.42	30.76	34.12	5.34 ± 0.32	5.45±0.04
E-01/011209	21.05	23.98	27.45	30.56	32.83	5.64 ±0.21	5.56±0.04
F-02/060909	19.07	22.42	25.61	28.23	32.16	5.68 ± 0.44	5.70±0.06

Ct cycle threshold
ORFV orf virus
PCR polymerase chain reaction
TCID tissue culture infective dose

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42 hpi to estimate the maximal virus load. The titres obtained in qPCR assay and the conventional end-point dilution methods ($TCID_{50}$) were comparable with each other without significant differences. Therefore, as for other vaccines, real-time PCR is a better approach for quantification of live orf vaccine. Moreover, this assay could provide a potent alternative test. Nevertheless, to obtain greater repeatability and reproducibility of this novel qPCR for potency estimation, large number of vaccine batches should be tested.

Acknowledgments

The authors would like to thank the Director of the IVRI for providing the necessary facilities to conduct this study and the staff of the Pox Virus Laboratory for providing technical assistance.

Grant support

The first author acknowledges the IVRI for financial support in terms of the Fellowship to conduct his PhD programme. The financial support provided by the Indian Council of Agricultural Research in New Delhi as part of the 'Niche Area of Excellence: Production and Quality control of Veterinary Immuno-diagnostics and immunoprophylactics' is also gratefully acknowledged.

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