Quantitative PCR: A quality control assay for estimation of viable virus content in live attenuated goat pox vaccine

D J Kallesh¹, M Hosamani²*, V Balamurugan¹, V Bhanuprakash¹, V Yadav¹ & R K Singh¹
¹Division of Virology, Indian Veterinary Research Institute, Mukteswar 263 138, Nainital, India
²Indian Veterinary Research Institute, Hebbal Campus, Bangalore 560 024, India

Received 26 August 2008; revised 29 July 2009

The efficacy of live viral vaccine and vaccine-induced sero-conversion depends on the optimum number of live virus particles in a vaccine dose, which is one of the important aspects of quality control. In the present study, TaqMan® probe quantitative polymerase chain reaction (QPCR) based on conserved DNA pol gene of capripoxvirus was developed for the quality control of attenuated monovalent goatpox and/or combined attenuated goatpox and peste des petits ruminants (PPR) vaccines. Cells infected with vaccine virus were harvested at critical time point and subjected to QPCR. A critical time point for harvest of Vero cells infected with various log10 dilutions of reference virus was determined to be 36 h (highest slope 3.062), by comparison of slopes of standard curves established with harvests at different time intervals. The assay method was evaluated using different batches of goatpox vaccine, and bivalent goatpox and PPR vaccine. The titers estimated by QPCR and TCID50 method were comparable to each other. The QPCR assay thus, could be used as an alternate method or supplementary tool for estimation of live GTPV particles in monovalent goatpox or bivalent goatpox and PPR vaccines.

Keywords: Goat pox vaccine, PPR vaccine, TaqMan QPCR.

Goatpox is Office International des Epizooties (OIE) notifiable disease owing to its severity and economic loss in enzootic countries. The causative agent goatpox virus (GTPV), belongs to the genus Capripoxvirus, subfamily Chordopoxvirinae, family Poxviridae. Control of the disease can contribute to improved goat productivity and also international trade. Vaccination has been considered to be the cheapest and sustainable means of disease control in the enzootic situation like India. Vero cell based live attenuated goatpox (GTP) vaccine¹ is presently under field-testing in many parts of India.

Efficacy of attenuated vaccine depends on the optimum number of viable virus particles in the vaccine dose. To estimate the number of infective viral particles in the vaccine, TCID₅₀ or PFU in cell culture or egg infective dose (EID₅₀) in embryonated eggs assays are commonly used. However, these assays are time-consuming, laborious, require extensive standardization and are subjective. Though these assays are gold standard, there is a possibility of variations in the estimated virus titer due to experimental conditions⁴⁻⁶. Furthermore, these assays are not preferred for quality control of multivalent or bivalent vaccines such as goatpox and peste des petits ruminants (PPR) vaccine⁶, where the cytopathic effect (CPE) produced by either of these vaccine viruses (PPRV and GTPV) is hard to distinguish. Recently, QPCR based method has been successfully employed to estimate the viable virus load in various multivalent attenuated vaccines, viz., pentavalent rotavirus, adenovirus and trivalent measles, mumps and rubella vaccines⁷⁻⁹. Estimation of live virus content by QPCR is based on the fact that the quantity of the viral nucleic acid harvested at a particular time point is proportional to the amount of the initial viable virus used for cell culture infection. In the present study, this approach has been evaluated for quality control of live goatpox vaccine or goatpox vaccine component of the bivalent vaccine.

Materials and Methods

Cells and viruses — Vero cells (African green monkey kidney, CCL81 from ATCC) were used for virus growth and quantitation. The cells were propagated in Eagle’s minimum essential medium
(EMEM)\(^1\) supplemented with 10\% new born bovine calf serum (M/s Hyclone, USA). For maintenance of the cells and titration experiments, EMEM with 1-2\% bovine calf serum (BCS) was used. Both attenuated goatpox vaccine and bivalent PPR and GTP vaccines were produced in different batches. Mean virus titre of the reference batch of the goatpox vaccine sample was determined based on end point assay (TCID\(_{50}\))\(^2\) in quadruplicate wells, after three experiments. Goatpox vaccine virus, sheeppox virus (SPPV) (Srinagar-38/00 isolate, passage 26), Orf virus (ORFV) (Mukteswar 59/05 isolate, passage 15) and camel poxvirus (CMLV) (Hyd-06 isolate, passage 2) were used for assessing the specificity of QPCR assay.

**QPCR primers and probes**— The primers and probes used for the development of QPCR specific for capripoxvirus were designed targeting DNA polymerase gene, which is conserved among capripoxviruses. The sequences of the primers and probes were —FP3: 5' GGAATGATGCCRTCTARATTCC-TATC 3'; RP3: 5' CCCTGAAACATTAG-TATCTGTATTTGTTGC3'; TaqMan probe (P3): CATCRCATCTAGGTTCRCAATGGATT. The oligonucleotide probe was designed with a fluorescent molecule, carbocyanin (Cy5), at 5' end and a quencher (TAMRA) at 3' end (Integrated DNA Technologies Inc., Coralville, IA).

**Optimization of conventional PCR and QPCR**— PCR conditions were initially optimized using template DNA of GTPV and SPPV, and specificity of the assay was assessed using DNA samples of unrelated poxviruses such as ORFV and CMLV. The test was then applied on the template isolated from infected cell culture harvest. For this, harvests were subjected to three cycles of freeze-thawing and DNA isolated into 50 µl volume using the AuPrep genomic DNA isolation kit (M/s Life Technologies India (Pvt) Ltd, New Delhi) as per manufacturer’s protocols.

TaqMan QPCR was carried out to establish the standard curve using ten-fold serially diluted DNA isolated from purified GTPV. A typical reaction mixture (25µl) consisted of 2µl of template DNA, 2.5 µl of 10X buffer (pH, 8.2), 10 µmol/l of each dNTP, 1.5mM of MgCl\(_2\), 10pmole of each primer, 5 pmol of probe and 0.25 IU of Tag DNA polymerase (M/s Invitrogen) in Stratagene Mx3000P system. Specificity of the test was assessed using DNA from *Capripoxivirus* (SPPV and GTPV), *Parapoxvirus*, and *Orthopoxvirus*. The sensitivity of the real time PCR was checked at annealing temperature (Ta) 57°C with the ten-fold serially diluted DNA extracted from purified GTPV.

**Infection of Vero cells with vaccine virus and estimation of critical time of harvest** —The freeze-dried live attenuated goatpox vaccine (1609/06 batch) virus was reconstituted in EMEM and serially diluted in ten-folds ranging from neat to 10\(^{-2}\) dilutions. A 100 µl of each dilution was inoculated onto six-well plates having confluent Vero cell monolayer including uninfected cell control. Virus from each plate was harvested by replacing supernatant with 500 µl of sterile PBS (pH 7.4) before freezing the samples. Harvests thus, collected at 24, 36, 42, 48, 60, and 72 h of post-infection (hpi) were freeze-thawed twice before employing in QPCR. The DNA was isolated from each plate and real time PCR was carried out to establish the standard curves. The slopes of standard curves at different interval harvests were compared. Similarly, samples of vaccine test batches were processed for determining their Ct values, which were plotted on the standard curve to assign the titre of each. The results obtained were compared with titer estimated by end point dilution (TCID\(_{50}\)) and correlation was analyzed by Student’s t-test.

**Results**

**Optimization of QPCR for capripox viruses** — For QPCR, at 57°C with MgCl\(_2\) (2mM) was found optimum. The standard curves at these conditions were found to have efficiency of 100.2\%, slope 3.318 and Rseq 0.995. The assay was found specific to capripoxvirus as there was no amplification observed in any of the related poxviruses, Vero cells and non-template control (NTC; Fig.1). It was found that

![Fig. 1 — TaqMan probe based QPCR assay showing specificity of the assay for capripoxviruses viz. sheeppox (SPPV) and goatpox viruses (GTPV). Negative control plots are represented by DNA isolated from ORFV (A), CMLV (D), Vero cells (B) and non-template DNA (C).](image-url)
even with capripoxvirus specific primers having two base mis-matches relative to GTPV sequence, the assay showed high specificity to capripoxvirus only (not shown). The sensitivity of the test was 0.042 pg of viral DNA as determined on purified DNA preparations from GTPV (Fig. 2).

**Estimation of critical time for harvest** — The standard curves for the vaccine were established in TaqMan probe based QPCR for each time interval harvest at 24, 36, 42, 48, 60 and 72 hpi. Further, the experiment was repeated thrice and average slope of standard curves from each time interval of harvest was calculated and compared each other. Among the slopes compared (Fig. 3), the slope of standard curve at 36 hpi was optimum (3.062) followed by gradual flattening of slope with the increase of duration of infection. Hence, 36 hpi was considered as the critical time (Ct) for harvest.

**Evaluation of vaccines** — Three batches each of attenuated monovalent goatpox (#0304/07, 2508/05, 0511/06) and bivalent goatpox and PPR vaccine (#3005/07/I, 3005/07/II, 3005/07/III) along with a reference goatpox vaccine (#1609/06) were chosen for validation of QPCR. Vero cell monolayer (24h confluent) in six-well plates was infected with inoculum (100 μl) from each dilution of the test and reference vaccine batches diluted ten-folds serially and harvested at 36 hpi. Standard curves were established for the reference by plotting Ct values against log_{10} virus titres in TaqMan probe based QPCR. The slope data of the reference sample was only presented as this data is more important than several batches of test vaccine. Further, corresponding titer of the test vaccine batches were determined by interpolating Ct values of test vaccine in the standard curve of the reference (Table 1). The results of both TaqMan probe based QPCR and end point assays were found to be within the acceptable limits (P ≤ 0.05) and high correlation (0.9648) was observed between titers estimated by QPCR and conventional infectivity assay.

**Discussion**

In a live virus vaccine preparation, all the genome quantified by QPCR is not necessarily infectious hence, PCR measure does not correspond to infectious load. To overcome this, cell cultures are infected with vaccine sample initially and is presumed that, if the content of the live virus is first propagated in cell culture, the amount of genomic DNA produced in the cell culture corresponds to amount of viable virus particles present initially in the preparation. This approach has been successfully demonstrated with measles vaccine, and also applied for quantifying the live individual virus component in pentavalent rotavirus vaccine, Rotarix® multivalent measles, mumps and rubella vaccine and to assign infectious potencies to adenovirus based vaccine, vectors for gene therapy and also to quantify the gene therapy vectors.

Conditions for QPCR were initially optimized using purified viral DNA preparations. Sequence of the amplicon (GenBank accession EU344752) confirmed the fidelity of the reaction. TaqMan probe based QPCR was 100-times more sensitive than the conventional PCR with high specificity as observed in the present study. The assay was intended to estimate
virus titres of both GTPV and SPPV on a common platform using a common set of primers.

QPCR based quality control of vaccine necessitates a standard reference vaccine the titer of which is already known. We determined the critical time point of harvest post-infection for the reference sample. This critical time point for harvest corresponded to the minimum time required for appearance of sufficient viral particles in different dilutions of GTPV infected cells. Standard curve obtained by TaqMan probe based QPCR showed that there was gradual decrease in slope of standard curve with increased duration of infection, which implied the unavailability of the healthy Vero cells for freshly replicating GTPV in those wells, that received less diluted virus. The slope of standard curve for 36, 42 and 48 hpi were almost comparable with minor differences. However, 36 hpi was found to have maximum slope (3.062) with least incubation period.

Two most important advantages of QPCR based estimation of vaccine titer was that the titer could be estimated earlier than the appearance of CPE, hence most suitable for viruses which produce delayed CPE. By this method, titer of GTPV in a given batch of vaccine could be determined at 36 hpi. Furthermore, QPCR was particularly valuable to estimate the viable GTPV load in combined goatpox and PPR vaccine. We successfully evaluated this approach to estimate the titer of GTPV in 3 batches each of monovalent and combined goatpox and PPR vaccines. Results obtained were found to be satisfactory and within the acceptable range of accuracy. High degree of correlation was observed between the titer estimated by QPCR and conventional infectivity assay with no significant difference ($P \leq 0.05$) noticed between the methods. Additional QPCR methods available now\textsuperscript{12,13} can be further used to compare the assay for its reproducibility.

During production of combined vaccine, the titres of the individual vaccine viruses are estimated by conventional method and on blending and lyophilization, the titres can then be validated by this QPCR, when conventional method of virus titration cannot be employed. Our attempt to develop QPCR for quantitation of live GTPV is a better approach in the quality control of both monovalent goatpox and bivalent goatpox and PPR vaccines. Further validation of the QPCR is warranted to obtain more accuracy and repeatability of this rapid method of potency estimation for its wider application.

### Acknowledgement

This study was supported by grants from Ministry of Forest and Environment, Government of India under the All India Coordinated Project on Taxonomy capacity building of poxviruses. The authors thank the Director, IVRI, India for facilities to carry out this work. The author (DJK) acknowledges the ICAR-JRF for financial assistance.

### References


