RT-PCR and its detection limit for cell culture grown bluetongue virus 1 using NS1 gene group specific primers

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RT-PCR was standardised for the detection of bluetongue viral RNA using highly expressed non structural protein 1 gene as the target gene with specific primers targeted to 274 bp of 5' end of NS1 gene. PCR product was consistently obtained in 30 PCR cycles. Further, detection limit of RT-PCR was estimated using serial 10 fold dilutions of BHK 21 cells grown BTV 1. The study suggested that RT-PCR can be used for detection of BTV in Indian conditions with the sensitivity limit of 10 infectious particles of the virus. The study suggested that this technique may be used as a tool for sensitive detection of BTV in carrier/reservoir animals, insect vectors and certification of animals and their germplasm for export and import purposes.

Bluetongue virus (BTV) is an economically very important vector (Culicoides) transmitted viral infection of susceptible domestic and wild ruminants in India. Outbreak of BTV have been reported from several states and 20 out of 24 known serotypes of BTV have been reported in the country1. BTV belongs to Orbivirus genus of the family Reoviridae and contains double stranded (ds) RNA genome having 10 segments packed inside the double layered protein capsid. In recent years, several countries have shown interest in the germplasm of Indian cattle and buffaloes for genetic and molecular studies. Presence of BTV infection in the country has resulted in trade barrier on movement of ruminant animals, their germplasm, embryos and other animal products thus disrupting animal related international trade. To prevent trade barrier, extensive and costly testing is required to assure BTV free animals.

Diagnosis of BTV infection is traditionally done by clinical signs and immunological assays2. The confirmatory diagnosis is done by isolating the virus from blood or insect vector following inoculation of embryonated chicken eggs3 or cell culture4. Dot immunobinding assay (DIA) and cELISA have been used in our laboratory for detection of the virus specific antibodies in field sera5,6. DIA and RNA-PAGE have detection limit of 10^3 TCID50/ml (Ref. 7). However, by employing these techniques, low level of BTV infection in carrier/reservoir, semen and insect vector will go unnoticed. Since couple of years, reverse-transcriptase-polymerase chain reaction (RT-PCR) has been used as the most sensitive method for detection of viruses in suspected animals8. RT-PCR has been successfully used for detection of BTV using VP7 gene specific primers. However, a slight homology between this and Tilligery virus VP7 gene has been reported9. As compared to VP7 gene, NS1 gene has been reported to be more specific to BTV serogroup and least likely to cross react with other Orbiviruses10. Therefore, RT-PCR has been evaluated for detection of BTV in BHK-21 cell culture using primers specific to NS1 gene.

Virus—BTV serotype 1 (Ref. 10) at 35 th passage level, was cultivated in BHK-21 cell line using BHK-21 medium supplemented with 7% new born calf serum (JRH Biosciences, Israel). After 36 hr of infection when about 90% of the cells showed cytopathic effect (CPE), the virus was harvested and titrated in microtitre culture plates using Read and Muench11 method.

Extraction of double stranded RNA—Extraction of the viral dsRNA was done as described earlier with slight modifications12. Neat BTV infected BHK-21 cell culture sample and its 10 fold serial dilutions up to 10^-7 were prepared in guanidine isothiocyanate (GITC) lysis buffer (Sigma, USA) containing 25 g. guanidine isothiocyanate in 29.3 ml diethyl pyrocarbonate (DEPC) treated water; 1.76 ml sodium citrate (0.75 M, pH 7.0); 2.64 ml 10% sarsosyl; and 0.36 ml β-mercaptoethanol and vortexed. Subsequently 0.1 ml of 2 M sodium acetate was added in each sample (0.9 ml) and vortexed again. An equal volume of phenol-
chloroform [(4:1); tris saturated phenol (pH 7.6) containing 0.01%, 8-hydroxyquinoline (w/v); 0.1%, β-mercaptoethanol; chloroform was constituted by mixing chloroform and isomyl alcohol in a ratio of 24:1] was added, vortexed and kept on ice for 15 min. The samples were then centrifuged at 12,000 rpm for 10 min and the clear aqueous phase was transferred to fresh eppendorf tubes and extracted once again with equal volume of chloroform: isomyl alcohol (24:1). The aqueous phase was again transferred to fresh eppendorf tubes and to this equal volume of isopropanol was added and kept at -20 ° C overnight. RNA was pelleted out by centrifuging at 12,000 rpm for 30 min at 4°C. The pellet was washed with prechilled 0.5ml of ethanol (70%) and centrifuged at 12,000 rpm for 20 min to remove extra salts. Finally, the pellet was air dried and suspended in 30 μl of DEPC treated water.

RT-PCR by single tube method - Extracted viral RNA (5 μl) from each dilution and cell culture control was mixed with 3.5 μl of DMSO (Sigma, USA) and denatured at 97°C for 5 min in thermal cycler. Samples were subsequently chilled on ice for 5 min. In each tube, 10 μl, 5xRT buffer (Promega); 1 μl, dNTPs (10 mM) (Promega); 2 μl, MgSO4 (25 mM); 1 μl each primer (1 μM, Gibco-BRL); 1 μl, (5 units) reverse transcriptase (AMV); and 1 μl, (5 units) Taq (Promega) were added on ice and final volume of 50 μl was made by nuclease free water (Sigma). These were mixed gently and spun for 10 sec in microfuge and kept at 46°C for 60 min for reverse transcription (RT). After completion of the reaction, RT enzyme was inactivated at 97°C for 5 min. Thermal cycler was programmed for 30 cycles of amplification using 94°C denaturation temperature for 1 min, 45°C annealing temperature for 2 min, 72°C extension temperature for 2 min and final extension at 72°C for 7 min. The following primer sequences for amplification of NS1 gene (segment 6) were used:

Primer 1—5’ to 3’ GTT CTC TAG TTG GCA ACC ACC
Primer 2—5’ to 3’ AAG CCA GAC TGT TTC CCG AT

The 5' end of NS1 gene (segment 6) containing highly conserved sequences was used for RT-PCR target. Primer 1 extended from position 11 through 31. Primer 2 was located between position 284 through 265 of the complementary strand. These primers produce 274 bp PCR product.

Agarose gel electrophoresis—RT-PCR products (10%) were analysed by agarose gel electrophoresis using 2% of agarose (Sigma) gel containing 0.5 μg, ethidium bromide /ml (Sigma) in 1x tris-borate-EDTA (TBE) buffer along with 100 bp DNA ladder (Promega). Electrophoresis was carried out at 101 volt in 1x TBE running buffer till the dye reached 2/3rd of the gel. The gel was visualised under UV transilluminator (UVP’S GDS 5000) and photographed.

RNA extracted from BTV 1 infected BHK 21 cells was subjected to reverse transcription reaction for amplification. These reactions yielded an expected product of 274 bp when electrophoresed in 2% of agarose and observed under UV transilluminator (Fig. 1). This amplification product was not present in RNA sample from non-infected BHK 21 cell culture. To assess threshold of sensitivity for detection of BTV ds RNA, neat BHK 21 grown BTV 1 was serially diluted in PBS and subjected to viral RNA extraction followed by RT-PCR protocol. The neat preparation of the virus contained 10⁶ TCID 50 ml. Observation of agarose gel after electrophoresis under UV transilluminator exhibited PCR products of expected 274 bp in lanes representing RNA from 10⁻¹ to 10⁻⁴ dilutions while no
PCR product was visible in 10^-7 dilution and BHK 21 cell culture control sample (Fig. 2). These observations indicated that the primers used to target amplification of NS1 gene were effective in amplification of the desired sequence and sensitivity threshold limit was 10 infectious virus particles of BTV serotype 1. Using the same primer pair for amplification of BTV serotypes 2, 10, 11, 13 and 17, earlier workers have suggested a detection limit of 0.01 TCID_{50} of BTV.\(^5\) The difference in the sensitivity limit may be due to the difference of BTV serotypes used by earlier workers.\(^4\) Using nested PCR utilising NS1 gene specific primers different than the ones used in the present study, a sensitivity limit of one plaque forming unit of BTV in RNA extracted from Culicoides variipennis vector has also been reported earlier.\(^3\) Such sensitivity limits may be accounted for by a particle to infectivity ratio of 10^-50 for BTV\(^4\) and also due to presence of the virus specific mRNA in RNA preparation subjected to RT-PCR which are not accounted in infectious virus titration assay.

RT-PCR described here combines the amplification and detection which can be completed in a few hr. RT-PCR procedure is an improvement over RNA-PAGE and DIA techniques which have been used earlier in the authors laboratory for detection of BTV.\(^7\) In our earlier study, it has been suggested that both DIA and RNA-PAGE have a minimum detection limit of 10^4 TCID_{50}/ml of the virus.\(^7\) The virus titre below this could not be detected by both of these assays efficiently. Other workers have reported that a minimum of 1×10^3 plaque forming units of the virus are required for optimal detection of BTV RNA in blood and other biological specimens by in situ and dot blot hybridisation.\(^5\) Mecham\(^6\) has described a theoretical limit of 2×10^3 TCID_{50}/ml BTV particles for detection by antigen-capture ELISA. Therefore, RT-PCR is a superior assay as compared to previously described procedures.

Genome segment 6 of BTV 1 encodes the serogroup specific antigen NS1. The results obtained with PCR suggested that primers complementary to 5' terminus of segment 6 might be suitable for development of a BTV serogroup specific diagnostic PCR assay. The result of our preliminary experiments further suggested that RT-PCR would amplify approximately 10 infectious virus particles of BTV serotype 1 to a concentration detectable by agarose gel electrophoresis. Further work is necessary to determine not only the sensitivity of the procedure through the course of BT disease in sheep, but also the most reliable clinical sample for detection of the virus. Our laboratory is currently engaged in evaluating RT-PCR for detection of BTV in spiked blood and semen of cattle and buffalo. Availability of such a sensitive diagnostic test will be very useful in epizootiological studies and biosafety of ruminant livestock and their germplasm for export and import purposes. The speed, specificity and sensitivity of RT-PCR would facilitate application of this method to diagnosis of viral diseases in animals.

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