DEVELOPMENT OF MULTIPLEX RT-PCR FOR THE DETERMINATION OF BLUETONGUE VIRUS SEROTYPES

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ABSTRACT

Multiplex reverse transcriptase polymerase chain reaction (mRT-PCR) was standardized by designing the primers for the amplification of VP5 gene of bluetongue virus (BTV) serotypes 1, 2, 10, 16 and 23 by using primer-BLAST software of NCBI. mRT-PCR can be routinely used for the rapid determination of BTV serotype from clinical specimens from BT suspected sheep if 4 or 5 BTV serotypes are in circulation in a geographical area. Sequencing of mRT-PCR product is also useful in molecular epidemiological studies.

Key words: Multiplex RT-PCR, Bluetongue viruses serotypes

INTRODUCTION

In an area (e.g. Europe since 1998) where multiple BTV strains of several distinct serotypes are co-circulating with repeated new introductions, a full epidemiological understanding inevitably depends on assay systems that can rapidly detect and identify the different viruses involved (Davies et al., 1992; Mertens et al., 2005). Serotype identification of BTV isolates is also important for the epidemiology of the virus. With the development of serotype specific RTPCR based typing assays and by generating sequence data from the amplified cDNA, it is possible to confirm BTV serotype within 30h of sample receipt. (Maan et al., 2004). Development of mRT-PCR was taken up with the objective of rapid detection of BTV serotype from the samples collected from bluetongue suspected sheep.

MATERIALS AND METHODS

BTV serotypes 1, 2, 10, 16 and 23, which were available at TANUVAS centre of All India Network Programme on Bluetongue, were used in the presence study. mRT-PCR for the detection of BTV VP5 gene was carried as per the method of Johnson et al. (2000) and de Arce et al. (2009) with modifications. By using BTV VP5 gene sequences available from National Centre for Biotechnology information (NCBI) and by using Primer-BLAST software of NCBI, the primer pairs specific to BTV VP5 gene of BTV serotypes 1, 2, 10, 16 and 23 were generated and custom synthesized.

Accession numbers for VP5 gene sequences used for generating mRT-PCR primer pairs for BTV serotypes 1, 2, 10, 16 and 23 were AJ783903, AJ783906, AJ586709, FM179951 and AJ586728 respectively.

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Preparation of primer mix for mRT-PCR

1. Using RNase-free sterile water, a stock solution of each of the 10 primers (Genei, Bangalore) to contain 50 pmol/µl of primer was prepared and stored at -70 ºC.

2. Primer mix for mRT-PCR was prepared by pooling together equal volume of each of the 10 primer stock solutions which resulted in a final concentration of 5 pmol/µl of each primer.

3. Primer mix for mRT-PCR was aliquoted to 20 µl and stored at -20 ºC.

Standardization of mRT-PCR

mRT-PCR mix was prepared as follows:

- BTV RNA (BTV1/2/10/16/23) 2 µl
- RNAase free water 15 µl
- Multiplex Primer Mix 3.0 µl
- Heated at Thermal cycler for 80ºC for 2 mins. Kept on ice for 1 min. Then immediately the following superscript was added which was kept on ice.
  - 5x first strand synthesis buffer 7 µl
  - dNTP (2.5 mM) 3 µl
  - 0.1M DTT 3 µl
  - RNasin 1 µl
  - Superscript 1.5 µl
- Incubate @ 42ºC for 2 hours

Then for the amplification of cDNA, the mix was prepared as follows:

- 10 x Taq buffer 10 µl
- Mgcl2 (50mM) 3 µl
- dNTP (2mM) 5 µl
- Multiplex Primer Mix 3.0 µl
- Template cDNA 1 µl
- Taq polymerase 1 µl
- H2O 77 µl

PCR Programme for mRT-PCR was as follows:

- Hold 95ºC 2 mins
- 28 cycles: Hold 95ºC 20 seconds
- Hold 58ºC 20 seconds
- Hold 72ºC 2 mins
- Hold 72ºC 5 mins

PCR product was checked on 1% AGE for BTV serotype specific band.

In the same way mRT-PCR using BTV dsRNA from the mixture of all the five BTV serotypes (1,2,10,16 and 23) was also standardized. For this purpose, pooled BTV RNA contained 500ng/µl of each BTV serotype. PCR product was checked on 1% AGE for BTV serotype specific band.

RESULTS AND DISCUSSION

In mRT-PCR, dsRNA from BTV serotypes 1/2/10/16/23 produced PCR product sizes 371 bp, 410 bp, 128 bp, 532 bp and 210 bp respectively in 1% AGE. In mRT-PCR, dsRNA from the mixture of all the five BTV serotypes produced PCR products of sizes 371 bp, 410 bp, 128 bp, 532 bp and 210 bp in
The laboratory diagnosis of BTV is a laborious procedure that requires well-trained personnel having a good understanding of the overall isolation procedure. The approximate time required to confirm BTV from one sample is: 2hrs for initial sample preparation, 2hrs for ECE, preparation and inoculation, 3-7 days for ECE incubation, 2hrs for tissue culture preparation and inoculation, 4-6 days for tissue culture incubation, 2hrs for immunoperoxidase test preparation, 2-5 days incubation of tissue culture plates and 32 h for immunoperoxidase assay including fixation and staining of the cells. In summary, the total time required is between 10 and 20 days (Clavijo et al., 2000). It takes another 3-4 weeks for serotyping of the isolate at All India Network Programme on Bluetongue, Hisar or 3 months incase of BTV serotyping of BTV isolates at International Reference laboratories. With the development of serotype specific RT-PCR based typing assays and by generating sequence data from the amplified cDNA, it is possible to confirm BTV serotype within 30h of sample receipt (Maan et al., 2004).

Six BTV serotypes viz. BTV 1, 2, 9, 10, 16 and 23 have been isolated from BT outbreaks in different parts of India (Rishendra Verma and Pandey, 2010).

In conclusion, the present study has indicated that the mRT-PCR is an accurate and reliable technique for the detection of BTV. The assay is easy and quick to perform and the results are available within one day. Therefore, this assay can be useful to detect the infection in a situation where 4 or 5 BTV serotypes are in circulation in a geographical area.

REFERENCES


