June 2009 *Volume 3 Issue 1*

Trade Science Inc.

Research & Reviews in

BioSciences

Regular Paper

RRBS, 3(1), 2009 [47-50]

Comparsion of RT-PCR and nested PCR for the diagnosis of bluetongue viruse infection

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Received: 30th April, 2009 ; Accepted: 5th May, 2009

ABSTRACT

This study was preformed between 2003 and 2006. One hundred and sixty five blood samples were collected from BTV infected sheep of each district of Coimbatore, Dindigul, Erode, Karur, Namakkal and Salem. Out of 165 samples 13 are positive both RT-PCR and nested PCR. In nested PCR minimum RNA was amplified. Hence, the diagnosistic sensitivity of nested PCR greater than the RT-PCR. The accuracy in diagnosis, more sensitive and specific assays, such as those based on antigens produced by recombinant DNA technologies and the polymerase chain reaction should prove useful.

KEYWORDS

RT-PCR;
Nested PCR;
BTV.

INTRODUTION

Bluetongue (BT) is an infectious, non contagious arthropod-borne disease of ruminants caused by Bluetongue virus (BTV), prototype species of the genus *Orbivirus*, within the family *Reoviridae*. Twenty four serotypes of bluetongue virus have been identified[3].

In Tamil Nadu, 22 out of 24 districts were reported to be affected by the bluetongue virus. The reported case of bluetongue virus among sheep and goats occurs presumable in an epidemic form during the Southwest monsoon season which favours vector population of bluetongue virus[5]. Although the history of reporting was not continuous, the number of outbreaks, attacks and deaths among ruminants reported is of great concern that needs immediate attention for the protection of livestock and economic growth[12]. Outbreak of bluetongue in sheep and goats swept in an epizootic form during 1997-98 from 12 districts in Southern Tamil Nadu leaving alarmingly 5.23 lakhs infected and 2.98 lakhs dead.

Identification of BTV antibody is an essential part of the laboratory confirmation of bluetongue virus infections. This may be achieved in three different ways, namely (a) identification of antibody by serological assay, (b) identification of the virus antigen by virological assay, (c) identification of the specificity of nucleic acids by reverse transcriptase polymerase chain reaction (RT-PCR) and (d) sequence analysis[9].

Due to the complexity of the serotypes of bluetongue virus, current procedures for monitoring the prevalence of bluetongue infection are generally based on the determination of the serotype specific antibodies in animal serum samples. These procedures are cumbersome for highly serotype specific BT virus and time-consuming. Therefore, it is imperative to use simplified tests for the purpose of seromonitoring of bluetongue virus in a particular animal population in order to demonstrate that the population has been exposed to bluetongue virus infection. Until recently, tests such as agar

Regular Paper

gel immunodiffusion have been in use. However, apart from being less sensitive, these tests have the major drawback of being unable to consistently distinguish between antibodies against bluetongue virus and the closely related epizootic haemorrhagic disease virus. RT PCR and nested PCR have been used as highly specific and sensitive test for detection of bluetongue virus group.

Against the above backdrop, the aim these present investigations focus to compare sensitivity of bluetongue virus detection by RT PCR and Nested PCR.

MATERIALS AND METHODS

This study was preformed between 2003 and 2006. One hundred and sixty five blood samples were collected from BTV infected animals of each district of Coimbatore, Dindigul, Erode, Karur, Namakkal and Salem. The blood samples were collected from animals in their high febrile period by jugular vein puncture in a vaccutainer tubes containing EDTA[13]. The samples were kept in vaccine bath and transported to the laboratory. For long-term storage the blood samples were collected in a vial containing Oxalate-phenol-glycerine (OPG) and stored at -70^0C[9].

Extraction of viral nucleic acid

The viral nucleic acid[1] was used to extract and purify BTV RNA from blood samples. Extracted RNA was precipitated with phenol chloroform and purified by precipitation 4 mol/L lithium chloride. Finally the RNA pellet was washed with 75% ethanol and dissolved in nuclease free water.

RT-PCR

Single step RT-PCR for VP7 gene using primers which amplify a region of 1156 bp was carrying out as per the procedure[10]. The PCR mix was prepared according to manufactures instructions. The reverse transcription was carried out in a 25 µl reaction mixture containing approximately 100 ng of heat denatured, purified BTV RNA, 20 pmol of each primer, 40µl RNAse inhibitor, 400 µmol/L dNTPs, 8 mmol/L double distilled water and 200 U of MMLV reverse transcriptase. After initial incubation at 37^0C for 45µl, the enzyme was inactivated by holding it at 70^0C for 15 min. The same protocol was followed for cDNA synthesis by the total RNA extracted from clinical samples. In order to determine the sensitivity of RT-PCR, the RNA was serially 10 fold diluted in nuclease free water before subjecting it to reverse transcription. Subsequently, cDNA was amplified in the same tube, using denaturation step at 94^0C for 30 sec., followed by 40 cycles of annealing at 39^0C for 1 min., extension at 72^0C for 2 min by 40 cycles. In order to determine the sensitivity of RT-PCR, the RNA was serially 10 fold diluted in nuclease free water before subjecting it to reverse transcription. The primers used in the RT-PCR/nested PCR were as follows:

Primer 1

Forward Primer : 5' GTTAAAAATCTATAGAG-3' (1-17)
Reverse Primer : 5' GTAAGTGTAATCTAAGAGA – 3' (1156-1138)

Primer 2

Forward Primer : 5' ACACTGATG CTG CGA ATG A-3' (321-340)
Reverse Primer : 5' AAC CCA CAC CCG TGC TAA GTG G-3' (1090 – 1069)

Nested PCR

Nested PCR of primary product of BTV genome segment 7 was carried out as described by Tiwari et al.[10] to amplify a 770 product. The nested PCR were carried out using, denaturation step at 94^0C for 30 sec., followed by 35 cycles of annealing at 55^0C for 1 min., extension at 72^0C for 2 min by 35 cycles and final elongation was done at 72^0C for 7 min.

PCR amplicons were electrophoresed on a 1.5% agarose gel containing ethidium bromide and the gel was documented by Alfa Digital documentation imaging system (Alfa Innotech Corporation, USA).

RESULTS

Bluetongue viral RNA extracted from the infected blood samples and it was purified by differential lithium chloride precipitation method, yielded pure RNA, without any visible cellular RNA contamination. In the RT-PCR, with the set 1 primer, a product of 1156 bp was amplified, whereas 770 bp products was observed in agarose gel when set 2 primer were used (Figures 1 and 2). In both the set of primers used, similar pattern of bands were observed for all the 13 isolates (Figures 1 and 2). When RT-PCR was carried out on serially diluted purified viral RNA using set 1 primers, RNA of

Regular Paper

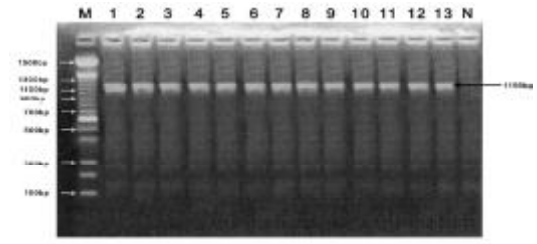

Figure 1 : Agarose get electrophoresis of BTV isolates 156 bp PCR product of VP7 gene using primer set 1

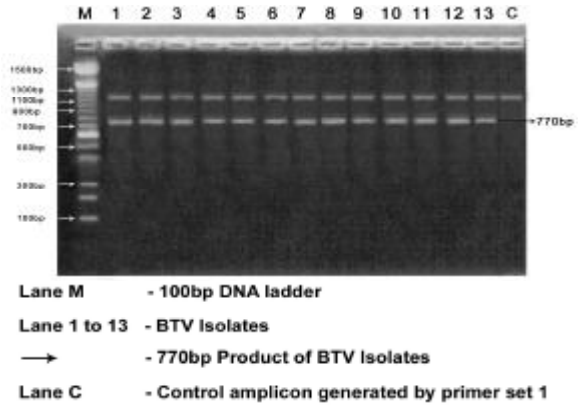

Figure 2 : Agarose gel electrophoresis of BTV isolates 1158, 770bp product of VP7 gene using primer set 1 and 2

40 pg or more were detected, whereas with the primer set 2, the sensitivity was increased 10 folds and as little as 4 pg RNA could be detected. With the nested PCR, performed on the amplicons generated using the external primers, the sensitivity was 100 fold greater than that achieved with the first PCR with primer set 1, since as little as 0.4 pg of purified viral RNA could be detected. The sensitivity of nested PCR was higher then the RTPCR.

DISCUSSION

In the present study RT-PCR using VP7 gene external primers successfully identified 13 field isolates producing desired amplification. This result was in concurrence with Tiwari et al.[10] where they successfully amplified an 1156 bp product by using VP7 gene external primers for Indian isolate of serotype 23, but failed to get the amplification in clinically suspected BTV blood samples. In the present study, the RT-PCR performed using VP7 external primers could detect field isolates.

There have been no previous reports on the molecular characterization of an Indian isolate of BTV using RT-PCR followed by restriction enzyme digestion. So a diagnosis of bluetongue still largely relies on conventional tests. Bluetongue virus has 11 polypeptides, of which VP-3 and VP-7 present on the inner core are responsible for group specificity. VP-7, encoded by segment 7 of the BTV genome, is the major group specific protein and detection of this gene would help in the diagnosis of BTV[4]. As expected, the two sets of primers used in the present study amplified the VP 7 gene sequences of isolates, yielding specific product of 1156 bp and 770 bp. $MgCl_2$ concentration of 1.5 mmol/L was found to be optimal for amplification of the VP -7 gene sequences. The sensitivity with which BTV RNA could be detected was less than that reported by other workers[11]. The reason for this may be heat treatment that led to denaturation of the double stranded RNA before reverse transcription without using any further denaturant Wade-Evans et al.[11]; McColl and Gould[7] reported that the addition of denaturants such as, formamide and methyl mercuric hydroxide, as well as the use of random hexamer primers, enhanced the sensitivity of RT PCR Wilson et al.[14] reported that the sensitivity of RT PCR may also change with the serotype of BTV. In our experiments, although the RNA seemed to be pure in agarose gel, the possibility of some cellular nucleic acid contaminants affecting the actual concentration of BTV RNA could not be ruled out.

The sensitivity of RT-PCR was 10 fold higher with the internal primers amplifying a smaller portion (770bp) of the VP 7 gene than with the external primers amplifying 1156 bp sequence. Primer dependent sensitivity of PCR for bovine herpes virus 1 has been reported by Kataria et al.[6]. As reported by Belak and Pordany[2], nested PCR is found to be 100 times more sensitive than the single PCR using external primers, and this was also evident from our experiments on clinical samples, where BTV could only be detected after a nested PCR. Hence, applying these techniques to genes encoding the outer capsid proteins responsible for serotype specificity. VP2 and VP5 will help in the identification of the most suitable serotype/genotype prevalent in a particular region as a candidate strain for vaccination to control diseases.

For accuracy in diagnosis, more sensitive and specific assays, such as those based on antigens produced by recombinant DNA technologies and the polymerase

chain reaction should prove useful. The potential for application of new sophisticated technologies could greatly enhance diagnostic capabilities for virus identification and differentiation in the near future.

ACKNOWLEDGMENTS

We take our pleasure in expressing sincere thanks to Lion. Dr. K.S.Rangasamy, MJF, President, Smt. Kavitha Srinivaasan, Executive Director, Principal, K.S.Rangasamy College of Arts and Science, Tiruchengode, for providing the required facilities, encouragement and support for carried the work.

REFERENCES

[1] S.K.Bandopadhyaya, R.S.Katria, A.K.Tiwari; Ind.J. Exp.Biol., **36**, 1034-1037 **(1998)**.

[2] S.Belak, A.Ballagi-Pordany; Vet.Res.Com., **17**, 55-72 **(1993)**.

[3] F.G.Davis, J.N.Mungai, A.Pini; Vet.Microbial., **31**, 25-32 **(1992)**.

[4] I.D.Gum, J.F.Newman; Arch.Virol., **72**, 83-93 **(1982)**.

[5] K.Ilango; Curr.Sci., **90(2)**,163-167 **(2006)**.

[6] R.S.Kataria, A.K.Tiwari, P.K.Gupta, M.L.Mehrotra, A.Rai, S.K.Bandyopadhyaya; Acta Virologica., **41**, 311-315 **(1997)**.

[7] K.A.McColl, A.R.Gould; Aust.Vet.J., **71**, 97-101 **(1994)**.

[8] OIE; Manual of standards, Diagnostic tests and vaccines, Chapter 2.1.9. Office International des Epizooties, **(2000)**.

[9] J.J.Reddington, G.M.Reddington, N.J.MacLachlan; J.Wild L.Dis., **30(1)**, 99-102 **(1994)**.

[10] A.K.Tiwari, R.S.Kataria, G.Desai, G.Butchaiah, S.K.Bandyopadhyaya; J.Vet.Res., **62(4)**, 217-22 **(2000)**.

[11] A.M.Wade-Evans, P.P.C.Mertens, C.J.Bostock; J. Virol.Methods., **30**, 15-24 **(1990)**.

[12] A.Wilson Aruni, N.D.J.Chandran, W.Manohar Paul, T.G.Prabakar, R.A.Venkatesan; Ind.Vet.J., **74**, 899-900 **(1997)**.

[13] A.Wilson, M.Parthiban, K.Kumaran, N.D.J. Chandran, A.Koteeswaran; J.Vet.Anim. Sci., **1 (3-4)**, 63-67 **(2000)**.

[14] W.C.Wilson, C.C.L.Chase; J.Virol.Meth., **45**, 39-47 **(1993)**.