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Full Article

Appling real time RT-PCR for bluetongue virus detection in Iran

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ABSTRACT

During 2009-10, real time RT-PCR and conventional RT-PCR techniques Used for detecting BTVs RNA in 310 blood samples. For real time and gel based RT-PCR segment-1 and segment-10 selected as conserve genes to search any BTV strains. Using these methods, 58 (%18.7) and 14 (%4.5) positive samples were detected among the clinically suspected sheep. Sensitivity of both molecular techniques evaluated by log-10 serial dilutions of BTV16 RNA, and determined $10^{1.8}$ and $10^{3.8}$ $TCID_{50}$/ml in rRT-PCR and conventional RT-PCR respectively. This report confirmed rRT-PCR assay could detect weak BTV positive samples even at end stage of infection. In this study Virus isolation from selected positive samples failed by inoculation to embryonated chicken egg, Vero and KC cell.

Keywords: Real time RT-PCR, Conventional RT-PCR, Bluetongue

INTRODUCTION*

Bluetongue disease (BTD) is a non-contagious, insect-transmitted disease of most domestic and wild ruminants (Verwoerd and Erasmus 2004). BTV infection occurs throughout much of the temperate and tropical regions of the world, coincident with the distribution of specific species of *Culicoides* biting midges that are biological competent vectors for the virus (Tabachnick 2004). BTV causes variable clinical signs depending on the strain of the virus and the breed of main host. Sheep are more sensitive to disease than cattle and European breeds are generally affected more severely than African ones. Bluetongue may cause high rates of abortion in ewes and high mortality in young animals. The huge economic losses by BTV justified its inclusion in the former list A of the World Organisation for Animal Health (OIE) (MacLachlan *et al* 1994).

BTV is prototype species of *Orbivirus* within the family Reoviridae. To date, twenty-four immunologically distinct serotypes of the virus and a lot of strains within each serotype have been determined (Roy 2005).

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Historically laboratory diagnosis of BTV has relied upon the detection of produced antibody against the virus by agar gel immunodiffusion test or enzyme-linked immunosorbent assays (ELISA). The Serological assays are useful in detecting animals that have prior exposure to BTV; however, they do not answer questions regarding the current state of viremia and they are insensitive to find the virus at first stage of infection (Batten *et al* 2007).

Recently molecular diagnosis based on different BTV conserved genome segments (L3, M6, S7 and S10) have been developed and accepted as a convenient and rapid method (Eaton & White 2004). The highly specific and sensitive nature of RT-PCR made it ideal for identification of BTV RNA directly in clinical samples without time consuming virus isolation process (Aradaib *et al* 2003).

Real time RT-PCR (rRT-PCR) technique has some advantages over the traditional nucleic acid assays. Among them, the higher sensitivity, specificity and reliability can be mentioned. Also requirement for examination of PCR product at the end of amplification by electrophoresis and staining with carcinogenic dye, like ethidium bromide, removes. Therefore final result will be gain faster and safer (Santis *et al* 2004, Jimenez-Clavero *et al* 2006).

The aims of the present study were set up and application of real time RT-PCR assay for rapid detection of any BTV strains in clinical samples and virus determination in late stage of infection. Finally sensitivity of rRT-PCR and conventional RT-PCR techniques were compared by a determined viral RNA. total numbers of 310 EDTA blood samples, gathered from suspected sheep to BTD in some provinces. These regions includs west Azerbaijan, East Azerbaijan, Ardabil, Kurdistan and Fars.

Viral RNA isolation. Viral RNA was extracted from EDTA treated blood samples. In each case 200 µl whole blood was used. The procedure continued by the QIAamp® viral RNA Mini Kit according to manufacturer's instructions. Finally RNA was eluted in 50 µl DEPC water and stored at -20 ºC. The RNA samples before using in PCR reaction denatured by heating for 5 min at 95 ºC and then rapid cooling on ice.

Real time RT-PCR primers and probe. In our study two sets of primers (Table 1) were used for detecting BTV segment-1(L1 or polymerase gene). These primers could amplify 97 and 91bp fragment in eastern and western genotype respectively. Primers and probe for the rRT-PCR reaction were designed according to Shaw et al. (2007).

Probe (Table 1) was synthesized based on TaqMan® PCR reaction and it can anneal to consensus sequence of segement-1 in different BTV isolates. The probe (supplied by Metabion International AG) was conjugated with 6-carboxyfluoroscein (FAM) at the 5' end and 6-carboxytetramethylrohdamin (TAMRA) at 3' end.

Real time RT-PCR. The rRT-PCR set up and optimized as a single step procedure combining the reverse transcription and the quantitative PCR. The assay was preformed by the eastern (EST) and western

Table 1. primers and probe that used for BTV detection

Name	Sequence (5'-3')	Size of amplicon	Target gene segment	Detecting topotype
EST forward	GCGTTCGAAGTTTACATCAAT	97bp	Segment-1	Eastern
EST reverse	CAGTCATCTCTCTAGACACTCTA AATTACG			
WES forward	GCTTTTGAGGTGTACGTGAAC	91bp	Segment-1	western
WES reverse	TCTCCCTTGAAACTCTA AATTACG			
Probe	FAM-CGGATCAAGTTCAC CCACGGT-TAMRA	--	Segment-1	Both
S10 forward	GTTAAAAAG TGTCGCTGCCATG	705bp	Segment-10	Both
S10 reverse	GGTTAAGGYAGTTCTAAACCTTC			

(WST) topotype specific primers in a single tube. The One Step PrimeScript™ RT-PCR Kit (supplied by TaKaRa, Japan, cat no. RR064A) by TaqMan® probe technique was used for all rRT-PCR tests. Each 25 µl final reaction containing, 12.5 µl 2X buffer III, each primers 20pmol, probe 2.5pmol, TaKaRa Ex Taq™ HS (5U/µl) 0.5 µl, PrimeScript™ RT enzyme Mix II 0.5 µl. The remaining volume was substituted with DEPC water. Prepared mastermix distributed in 0.2 µl tube and then 6µl denatured RNA was added to the reaction. The amplification was carried out in a Corbett Rotor-Gene™ 6 PCR machine.

The rRT-PCR steps set as follow programme: 55ºC for 30 min, one cycle (reverse transcriptase), 95 ºC for 2 min, one cycle (inactivation of PrimeScript™ RT enzyme), and 50 cycle of 95 ºC for 15s and 60 ºC for 45s. The fluorescence acquired at the end of the 60 ºC annealing/extension step.

Cycle threshold (Ct) value for each sample was determined from the point at which fluorescence breached a threshold fluorescence line. Standard curve, threshold line and R^2-value (correlation coefficient) was determined by Rotor-Gene™ software (version 6.0.1) by including four standard dilutions of BTV16 RNA ($10^{4.8}$ – $10^{1.8}$ $TCID_{50}$/ml) in each reaction.

The sensitivity of rRT-PCR checked by a log-10 serial dilution of BTV16 purified RNA. The RNA extracted from a viral stock with $10^{4.8}$ $TCID_{50}$/ml viral loads. Also to evaluate whether RNA extraction affecte the efficiency of virus detection, 10 fold dilution of viral stock were extracted and checked by real time and conventional RT-PCR.

Conventional RT-PCR. This technique used for evaluating 310 blood samples and comparing the sensitivity of rRT-PCR by 10 fold serial dilution of BTV16 RNA. S10 Oligonucleotide primers (Table 1) were synthesized commercially (Cinnagen Co., Iran) and used to amplify 705bp of S10 segment as a conserve gene in all BTV strains and serotypes.

The RT-PCR reaction and primers' sequences were optimized as previously described (Nikolakaki & Nomikou 2005). In order to amplify cDNA, one step RT-PCR kit (QIAGEN® OneStep RT-PCR Kit cat. no. 210210) was applied. The final reaction master mix adjusted to 25 µl with RNase free water and contained 10 µl of 5x Qiagen RT-PCR buffer, 2 µl dNTPs mixture (0.2 mM each), 0.5 µl (20 pmol) each of primers, 2 µl Qiagen Enzyme Mix. Six microliters of denatured RNA was then added to master mix. The RNA initially reverse-transcribed at 45 ºC for 30 min. Then heated to 95 ºC for 15 min, to simultaneously activation of DNA polymerase and inactivation of reverse transcriptase. Forty amplification cycles were carried out at 95 ºC for 1 min, 55 ºC for 1 min and 72ºC for 1 min. The PCR cycles terminated by final extension at 72 ºC for 10 min. The PCR products were analyzed by %1.3 agarose gel electrophoresis and visualized in UV transilluminator after staining with ethidium bromide (1µg/ml).

Virus Isolation. Virus isolation from eleven positive samples, attempt to make simultaneously, by injection to ECE and sensitive cell line, including Vero and Culicoides (KC) as previously described **(**Afshar 1994, White *et al* 2005**)**. These samples were selected, based on quality of blood samples and rRT-PCR results. They had Ct value less than thirty, also they showed specific S10 gene band after running the product of conventional RT-PCR on agarose gel. For evaluating virus isolation process, Injected ECEs and cell lines were analyzed by rRT-PCR protocol seven day post inoculation.

RESULTS

In this study, after evaluating 310 field samples from clinically suspected sheep to BTD, 58 (%18.7) and 14 (%4.5) positive samples were detected by real time and gel based RT-PCR, respectively. The Ct value for positive samples in rRT-PCR considered fewer than forty. Viral load in samples from different provinces showed variation. For example the highest and lowest positive samples found in East Azerbaijan (%17) and Kurdistan(%3) respectively. Sensitivity of rRT-PCR and conventional RT-PCR was assessed by log-10

serial dilutions of BTV16 RNA. The result indicated that in rRT-PCR and conventional RT-PCR, the minimum detectable load of BTV RNA was $10^{1.8}$ and $10^{3.8}$ $TCID_{50}$/ml respectively (figure 1). As indicated in figure 3, there was linear and indirect relationship between Ct values and concentration of RNA dilutions. Also for analyzing the specificity of BTV real time and conventional RT-PCR, Foot and Mouth Disease Virus (O_{manisa}) RNA was checked by BTV primers and probe, and no amplification detected.

Injection of selected PCR positive samples to ECE and cell line didn't induce hemorrhage/CPE in chicken embryon and Vero, respectively. Also evaluating ECE, Vero and KC cells by rRT-PCR didn't cause any detectable BTV amplification.

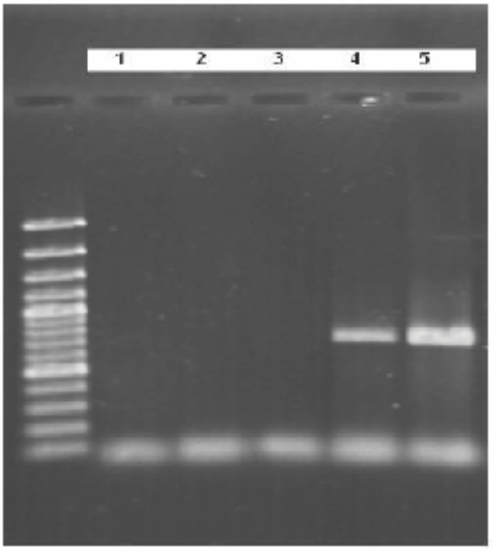

Figure 1. for comparing real time and conventional RT-PCR sensitivity, electrophoresis result of the same RNA dilutions by S10 RT-PCR was demonstrated. Lanes 1, 2, 3, 4 and 5 show the result of RT-PCR reaction from 1+ E 0.8, 1+ E 1.8, 1+ E 2.8, 1+ E 3.8 and 1+ E 4.8 TCID50/ml BTV16 viral RNA titers, respectively.

DISCUSSION

Today real time RT-PCR techniques, especially fluorogenic probe hybridization, such as TaqMan probes, become a routine detection method for animal viral diseases (Santis *et al* 2004**).**

The development of a universal rRT-PCR assay detecting all bluetongue serotypes and strains has been challenged by the high diversity of this virus (Pritchard *et al* 2004). Therefore the recognized target footprints by rRT-PCR primers and probe should be as highly conserved as possible across the whole BTV viruses, to ensure that they can detect any BTV strains **(**Shaw *et al* 2007**)**. Alignment of segment-1 from different BTV strains confirmed the presence of two distinct groups, representing two geographically different clusters, eastern topotype including the viruses from Middle-east, Asia, Australia and western topotype including isolates from Africa and Americas (Shaw *et al* 2007**)**. Therefore in order to detect all BTVs in field samples, segment-1 is selected as a best candidate and two sets of primers were used. On the other hand, sequence evaluation of segment-1, determined the presence of consensus nucleotides for designing a common probe for both topotypes (Table 1). This experiment showed that combination of two pairs of primers (ESTs & WSTs, Table 1) can detect both of topotypes that probably exist in our country **(**Azimi *et al* 2008).

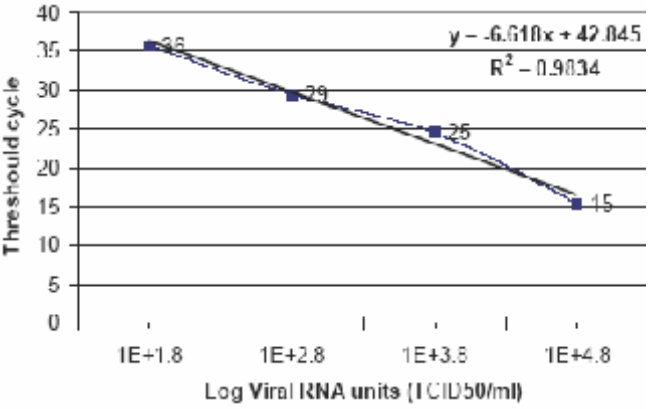

Figure 2. In this figure linearity and efficiency of rRT-PCR for BTV detection was depicted. As shown there is a reverse relationship between viral RNA dilutions (equivalent to TCID50/ml on logarithmic scale) and cycle threshold values.

In our investigation by conventional RT-PCR, only 14 positive samples were detected, but rRT-PCR, could determine 58 positive samples. It should be mentioned that every detected positive sample by conventional RT-PCR, also find as positive case in rRT-PCR. In rRT-PCR we taken into account Ct value less than 40 as a positive result, because the Ct value of $10^{1.8}$ $TCID_{50}$/ml viral RNA was equal to 63 viral particle/ml.

Next dilution ($10^{0.8}$ $TCID_{50}$/ml) didn't show any detectable Ct and considered as a negative sample. Also in pervious reports, clinical samples that had Ct value less than 40 selected as a positive case. **(**Jimenez-Clavero *et al* 2006, Shaw *et al* 2007**)**

Assessing the 10 fold dilution of BTV RNA by both methods showed that end point RT-PCR, had sensitivity threshold less than $10^{3.8}$ $TCID_{50}$/ml (equal to 6309 particle/ml) but in rRT-PCR this border was near to $10^{1.8}$ $TCID_{50}$/ml equal 63 viral particles in each ml of blood sample. The pervious researcher reported that the sensitivity of real time assay is comparable to nested PCR, which can detect 0.1 fg of BTV RNA (equal to 10 copy number of viral RNA) in clinical samples **(**Jimenez-Clavero *et al* 2006**).** Therefore the sensitivity of rRT-PCR in our investigation was near to nested PCR. But usage of nested PCR has some restriction, including risk of false positive after cross contamination, and time consuming to get final result.

Unfortunately in our experiment, virus isolation from selected positive samples was failed. Blood samples from far regions sometimes arrived to lab in undesirable condition; usually these kinds of samples completely or partially lysed. In this case the virus will be degraded or neutralized and subsequently loss of its infectivity. The other golden key of successful BTV isolation is sampling at the fever stage. Elapsing pick of viremia (after fever subsided) will cause dreadfully diminishing the viral load. In this condition the cycle threshold value will be increase significantly. It should be mentioned in our experiment, near %75 of positive samples (44 samples), showed Ct value more than thirty. It can be concluded that, these samples collected from the late stage of infection. As described previously, non contagious BTV can be circulated in blood stream of sheep and calves for 30 up to 90 days after biting by infected midges **(**Katz *et al* 1994**)** and cause PCR positive result. These kinds of samples are never suitable for virus isolation. The same problem has been reported in other works (MacLachlan 1994).

Comparing the Ct values between RNA 10 fold dilution and extracted RNA from 10 fold diluted viral stock, didn't show significant different results (data not shown). But totally RNA dilutions indicate more linearity of Ct value than viral dilution.

In our study we used 36-well rotor-Gene but it could be increase to 72-well by GeneDisc (72 or 100) rotor. Thus BTV diagnostic assay that developed here is suitable for screening large numbers of samples in each PCR run and it is suitable for monitoring the suspected animal populations in a high throughput format.

In conclusion, the present study is first report about application of real-time RT-PCR for BTV detection in our country. Both molecular methods are able to detect all strains of BTV serogroup. But rRT-PCR comparing to conventional RT-PCR can be used as a quick, sensitive and powerful laboratory tool for diagnosis. But it is necessary to plan a complementary study for analyzing the variation of Ct value during an experimental BTV inoculation in animals and determining a cut off for successful virus isolation.

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