

Full Length Research Paper

Comparisons of competitive enzyme-linked immunosorbent assay and one step RT-PCR tests for the detection of Bluetongue virus in south west of Iran

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Bluetongue is a noncontagious, arthropod-borne viral disease of both domestic and wild ruminants. Bluetongue virus (BTV) is the type of species of the genus Orbivirus within the family Reoviridae. BTV is endemic in some areas with cattle and wild ruminants serving as reservoirs for the virus. Clinical symptoms are often seen in sheep. There are several methods for the detection of Bluetongue virus, among them the molecular technique like RT-PCR is considered as the most sensitive and reliable one. The aim of this study was to comprise competitive enzyme-linked immunosorbent assay (C-ELISA) with one step RT-PCR test for the detection of BTV in sheep. A total of 770 blood samples were obtained from sheep (265 serum positive samples and 505 serum negative samples in C-ELISA). According to our data, out of the 265 serum positive samples in ELISA test, 234 were positive in RT-PCR assay whereas all serum negative samples were negative in RT-PCR experiment. According to the results, the PCR assay was more sensitive and reliable than ELISA technique for the diagnosis of Bluetongue virus.

Key words: Bluetongue virus, C-ELISA, RT- PCR, Sheep, Iran.

INTRODUCTION

Bluetongue virus (BTV) belongs to the genus Orbivirus of the family Reoviridae. The virus particle is nonenveloped and icosahedral in shape and consists of a double-layered protein coat. Nowadays, at least 24 serotypes of this virus have been identified (Davies et al., 1992; Lee et al., 2010; Roy, 1992; Schwartz-Cornil et al., 2008). BTV has been found where suitable vectors are present (Tabachnick, 1996). Bluetongue disease is on the multi-

species list of notifiable diseases by the office of international epizootics (OIE) because of its substantial economic impact and potential for rapid spread. Infection with BTV can cause serious hemorrhagic disease with high mortality rates in sheep and deer (Howerth et al., 1988; Osburn, 1994; Rodriguez-Sanchez et al., 2010). BTV is a small icosahedral virus with a ten-segmented, double stranded RNA (dsRNA) genome. Each of the ten segments codes for at least one of ten distinct viral proteins, seven of which are constructed components of the virus particle and three of which are nonstructural. The inner capsid of BTV is composed of five poly-peptides: three minor proteins (VP1, VP4 and VP6) and two major proteins (VP3 and VP7) (Roy, 1992). The outer capsid is composed of two major viral proteins, VP2 and VP5, which determine the antigenic variability of the BTV. The core proteins and nonstructural proteins NS1, NS2,

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Abbreviations: C-ELISA, Competitive enzyme-linked immunosorbent assay; RT-PCR, reverse transcriptase-polymerase chain reaction; BTV, Bluetongue virus.

NS3/3A (encoded by seg. 5, 8 and 10, respectively) are thought to be relatively conserved and are antigenically cross-reactive between different strains and serotypes of BTV. However, cross-hybridization and sequencing studies have shown that these genome segments can vary in manner that reflects the geographical origin of the virus strains (Pritchard et al., 2004). Efficiency of PCR in diagnosis of BTV in several parts of the world, verified this technique as a rapid, reliable and sensitive diagnosis methods. The highly specific and sensitive nature of RT-PCR based assay makes it ideal for rapid detection of BTV genome segments in clinical samples without requirement to virus isolation (Anthony et al., 2007). BTV infection previously has been determined in several parts of Middle East in Iran (Shoorijeh et al., 2010). The history of BTV detection in this part of the world is more than 50 years. Serological evidence indicates that the virus has been present in Iran, at least since 1974 (Afshar and Keyvanfar, 1974). The purpose of this study was to use a valid RT-PCR assay to detect any BTV isolates in South West of Iran, from sheep blood specimen.

MATERIALS AND METHODS

Samples

Blood and serum samples were collected randomly from male and female sheep of 3 different geographic regions in Iran between May 2009 and October 2010.

Serological test

Sera collected from 770 sheep of 120 flocks in 10 different regions of Chaharmahal va Bakhtiari, Khuzestan and Isfahan provinces (166 samples from male sheep and 604 samples from female sheep) were screened for group specific antibodies to BTV, using a commercial competitive enzyme linked immunosorbent assay (C-ELISA; ID Screen1, ID VET Co., Montpellier, France) and an ELISA micro plate reader (model Stat Fax 2100, Awareness Technology Inc., USA). The sera were collected from 284 aborted and 320 non-aborted sheep during 2009 to 2010. The clinical signs of BTV disease were not described in any of these animals.

Extraction of viral RNA

The dsRNA extractions were carried out using the RNeasy[®] mini kit (Qiagen) from whole blood samples according to the manufacturer's instructions.

Oligonucleotide primers

Two pairs of primers: (TF-1: 5'-GTAAAAATCTATAGAG-3'; TR-1: 5'-GTAAGTGAATCTAAGAG-3') and (TF-2: 5'- GTAAAAATC TATAGAGATG-3'; TR-2: 5'- GTAAGTGAATCTAAGAGA-3') which amplify full length of BTV serogroup S7 gene (1156 bp), were used. For nested PCR, internal primers (IF-1: 5'-ACAACTGATGCTGCC AATGA-3'; IR-1: 5'-AACCCACACCCGTGCTAAGTGG-3' were applied (Bréard et al., 2003; Wade-Evans et al., 1990). The second

primer set amplified internal part of S7 segment in length of 769 bp. All oligonucleotide primers were synthesized commercially (Cinnagen Co., Iran).

RNA extracted from reference strain BTV1 (RSAvvv/01, which was received from Institution of Animal Health, Pirbright, UK) was used as positive control and sterile distilled water was used as the negative control in RT-PCR assay.

One step RT-PCR

The one step RT-PCR kit (QIAGEN[®] OneStep RT-PCR Kit) was used for the detection of S7 BTV gene in blood samples. The master mix was made as follows: 10 µl of 5X Qiagen RT-PCR buffer, 2 µl dNTPs mixture (0.2 mM each), 0.5 µl (20 pmol) of each of four primers (TF-1, TR-1, TF-2, TR-2), 2 µl Qiagen enzyme mix and 28 µl of RNase free water. Then 6 µl of denatured RNA was added to master mix. In RT-PCR, the RNA was initially reverse-transcribed at 45°C for 30 min; then, followed by a step at 95°C for 15 min to simultaneous activation of DNA polymerase and inactivation of reverse transcriptase. Forty amplification cycles were performed at 95°C for 1 min, 45°C for 1 min and 72°C for 2 min. The PCR cycles was terminated by final extension step at 72°C for 10 min (Anthony et al., 2004).

Nested PCR

PCR products of first amplification (RT-PCR) were used as template in nested PCR. The mixture of master mix contained, 100 µM dNTPs, 1.5 mM MgCl₂, 1 µM of each primer (IF-1 and IR-2), 1 U of Taq DNA polymerase and 35 µl of RNase free water, at the end, 5 µl of template was added to the reaction in a final volume of 50 µl. The Mastercycler gradient PCR machine (Eppendorf, Mastercycler[®] 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) was set to amplify the nested fragment as follow: first step was at 95°C for 1 min, then 30 cycles were performed at 95°C for 1 min, 59°C for 1 min and 72°C for 1 min. The reaction was stopped by extension at 72°C for 10 min (Anthony et al., 2004).

Analysis of PCR products

20 µl of the final PCR products were run on a 1% agarose gel containing ethidium bromide in 1X TBE buffer along with 1 Kb DNA ladder (Fermentas). The gels were analysed using gel documentation system (Uvidoc imaging system).

RESULTS AND DISCUSSION

Out of 770 samples, 265 specimens had antiviral antibodies with C-ELISA test. All the samples were tested with PCR assay too. The results are shown in Table 1. According to these results, the 769 bp segment relates to bluetongue positive samples in Nested-PCR assay.

In this study, PCR technique was employed as a quick, sensitive and specific method for diagnosis of Bluetongue virus from the cases suspected to the disease. This research is the first report on BTV identification based on molecular method in South West of Iran. It is worth mentioning that the situation of diagnosis of this virus in neighboring countries and the Middle-East (except

Table 1. Comparison of competitive enzyme linked immunosorbent assay (C-ELISA) with RT-PCR for the detection of Bluetongue virus.

Test	RT-PCR positive	RT-PCR negative	Total
C-ELISA positive	234	31	265
C-ELISA negative	0	240	505
Total	234	271	770

Turkey and Occupied Palestine) is not better than our country. In such countries as Saudi Arabia, Syria, Yemen, Oman and Pakistan, the presence of the virus has been documented only relying on serological tests (Akhtar et al., 1997; Lundervold et al., 2003; Shoorijeh et al., 2010).

According to recent studies, there is an evidence of occurrence of BT disease in tropical and subtropical countries (such as Iran). In such areas generally, the disease appears subclinically and does not attract attention. In such circumstances, the presence of the virus often confirms via serological evidences. It should be mentioned that in such foci, in spite of unrevealed disease and manifestations, sometimes sudden incidence of acute forms of the disease sustain a loss (Basak et al., 1997; Nikolakaki et al., 2005).

In Iran, identification of BTV in suspected cattle and sheep based on clinical manifestations was performed. However, there are some limitations and problems. Firstly, it should be considered that clinical expression of BTV regarding strain and virus intensity, cattle race and environmental condition varies from peracute to subclinical. Secondly, symptoms of disease in sheep can be mistaken with those of other viral much diseases and even some of the non-viral diseases.

For the first time, Afshar and Kayvanfar (1974) reported the existence of antibody against this virus via AGID test in Iran. In recent years, based on serological methods, infection with this virus has been proved in various provinces of Iran (based on veterinary organization reports). It should be mentioned that some suspected cases have been reported by veterinarians but unfortunately because of the lack of reliable and quick laboratory techniques, we could not accept such cases.

During the BTV epidemics in Europe in 2008, Williamson et al. (2008) considered clinical signs for diagnosis of the disease. The results showed low specificity of this method. These researchers believe that sometimes clinical signs of BTV in sheep are mistaken with those of such diseases as FMD, PPR, contagious ecthyma and haemonchosis (Elbers et al., 2008; Tan et al., 2001).

So far, many methods have been employed in order to detect BTV. The most important of them are isolation of virus via embryonic egg or cell culture, serological and molecular methods.

Among these laboratory diagnostic methods, molecular

techniques enjoy the highest level of sensitivity and specificity for diagnosis of arboviruses including BTV. This method is so sensitive that even 6 molecules of the virus genome in blood can be traced (Anthony et al., 2004).

Biteau- Coroller et al. (2006) and Afshar, (1994) in their studies introduced the PCR method as a 'golden test' for BTV diagnosis compared with other procedures. In sheep, being infected by vector anopheles, PCR readings from 5 days to 38 up to 54 days the onset of infection becomes positive. This period in infected cattle is up to 100 days following onset of infection (Afshar, 1994; Biteau-Coroller et al., 2006; Koumbati et al., 1999).

In a study conducted in 2005 by Bréard et al. (2005) from the 46 feverish sheep blood and suspected to BTV, only one cast of virus isolation via embryonated chicken egg (ECE) injection was possible, while a great number of them were PCR-positive (Bréard et al., 2003). Also, through a study conducted by MacLachlan et al. (1994), the sensitivity of various diagnostic methods like injection to ECE and PCR and feeding colicoides mosquitoes were compared. Their study revealed that it is possible to diagnose the virus in cattle blood, with infecting mosquito method for about 2 weeks, injecting to ECE about 2 to 8 weeks and in PCR procedure between 16 to 20 weeks following the onset of infection.

Steinrigl et al. (2010) from Australia injected an inactivated vaccine of the BTV 8 serotype to the understudy sheep and showed that RT-PCR is the best method for the detection of trace amount of BTV in samples.

Yin et al. (2010) used RT-PCR method for the detection of NS1 gene. They succeeded in tracing all 24 serotypes of BTV in China. Vandebussche et al. (2010) employed multiplex RT-PCR as a routine method for BTV identification in their study. They also used this method in order to find other genes simultaneously in BTV and showed that this method enjoys extraordinary sensitivity for the identification of some different genes together. So far, various methods of RT-PCR have been employed for BTV diagnosis. Usually, the genes used to identify BTV serogroups are retained in all serotypes. These genes produce such core-proteins as (VP7), (VP1) L1, (VP3) L3 and (NSI) M6 and/or nonstructural proteins such as NS10 (NS3/NS3A) (Bréard et al., 2004; Dangler et al., 1990; Hwang et al., 1992; MacLachlan et al., 1994; Orrù et al., 2006; Parsonson and McColl, 1995).

Among these genes, S7 segment is the best candidate for the identification of this virus because it is a stable protein in all BTV serotypes and topotypes and it's highly sensitive in PCR (Anthony et al., 2007; Billinis et al., 2001; Zientara et al., 2004). Therefore in this study, we used S7 segment as a marker for the identification of infected samples. Moreover, we do not have enough information about the prevalent strain of BTV in Iran, so we used RT-PCR-duplex: two supplementary primer pairs at 3' and 5', for performing the reaction simultaneously. In addition, recently, it has been identified that noncoding ends of the S7 segment are similar in the 7, 10 and 19 serotypes and also vary a little in other serotypes (Anthony et al., 2007, 2004). Therefore, by using two primer pairs, all serotypes of BTV, apart from their serotypes would be identified.

In this study, we applied one step RT-PCR too. The advantage of this procedure is decreasing the test time in comparison with two-step RT-PCR method and to decrease the number of false positive cases by omitting additional manipulations at cDNA generation and reducing number of pipette used.

In this study, we gathered suspected sheep blood. Firstly, we applied RT-PCR then we used nested PCR assay to verify the product of the primary PCR and to increase sensitivity of virus identification in clinical samples. As the matter of fact, this method is a simple, fast and sensitive assay for confirming of PCR products. The sensitivity of nested PCR is at the rate of 10 copies of the target gene in the clinical samples (equal to sensitivity of real time PCR) since in this method, we use four specific primers (Biteau-Coroller et al., 2006).

According to many studies, the second PCR (nested PCR) in comparison with the first PCR is 10 to 100 times more sensitive. So, when the amount of RNA is less than 100 fg, the nested PCR method is so valuable. It was shown that this method is able to identify even 0.1 fg of the BTV genome (= 5 molecules of the BTV genome) in the cell culture (Aradaib et al., 2005, 2003, 1998).

Eaton and White (2004) also introduced nested PCR of the S7 gene as a sensitive and appropriate method for identification of BTV in suspicious cases. This point on employing RT-PCR in BTV diagnosis is that most of the researchers have designed and optimized this method based on laboratory specimens obtained from cell cultures inoculated with virus and/or experimental samples supplied through injecting the acute virus to sheep. However, they have not evaluated efficiency of their procedure in clinical cases (Aradaib et al., 2003).

In this study, the results are in agreement with other investigators; so the first PCR products were unclear or weak. This was probably due to the low level of virus, quality of samples and/or low quantity or quality of the extracted RNA. Anyway, by using nested PCR assay, we obtained specific and clear bands and the earlier mentioned problem were solved. Meanwhile, some negative specimens in the first PCR assay were positive

in this procedure, so that it is obvious that the first bands were weak.

As it was mentioned earlier, one of the exclusive applications of PCR, which has made it superior to other diagnostic techniques, is the possibility of molecular epidemiologic surveys, study of genetic modifications and determination of origin of virus transfer to other foci (Gould and Pritchard, 1990; Mecham and Johnson, 2005; Pritchard et al., 1995; Ritter and Roy, 1988).

Another important method of BTV identification is the competitive ELISA. In this method, anti-VP7 antibodies are traced. Nowadays, this method due to high specificity, rapid action and ease of application is considered as a standard method such that in all the international reference laboratories in addition to molecular methods, this procedure is also used for evaluating blood samples.

In fact, the competitive ELISA test is considered as a powerful tool in seroepidemiologic studies of BTV (Batten et al., 2008). The only weak point of C-ELISA is its limited sensitivity which is due to delayed antibody formation. This time, it is about 7 to 28 days post-infection. During this period, the test sensitivity is low and the test will show false negative responses. To remove this defect, employment of PCR is recommended. Because of this reason, many of the investigators recommend that in order to study the status of disease in endemic foci and/or in foci where the status of disease is not clear, both procedures could be employed concurrently (Biteau-Coroller et al., 2006; Singer et al., 1998).

Those investigators, who have used molecular and serologic method in identifying BTV, unanimously believe that PCR and ELISA results complement each other and that one of them being positive necessarily does not influence the result of the other. Bréard et al. (2005) and Singer et al. (1998) in their study fields found that PCR and ELISA results have no direct relations to each other.

In this study, a number of samples were positive both in PCR and ELISA tests and this is in agreement with other investigator's reports. To explain this matter, we can say the positivity of the cattle's serum does not have any incompatibility with the present of virus in its blood. This can be due to the reinfection of serum-positive cattle with heterogenous serotype of the virus (Billinis et al., 2001; Bréard et al., 2005; Singer et al., 1998).

On the other hand, it should be noted that in C-ELISA, anti-VP7 antibodies are traced. As it was mentioned earlier, this type of antibody does not have any role in neutralizing the BTV in vertebrate hosts. Thus, in spite of formation of such antibodies, virus can continue its activity.

In this study, a number of samples were positive only in ELISA or in PCR assays. The reason for inconsistency of PCR results with those of ELISA goes back to the difference between the viremia times (incubation period) and sero-conversion. Viremia in sheep starts between 4 and 8 days following the onset of infection and continues till the next 54 days. With seroconversion, however, on

average starts 14 days post induced infection but continues for long periods (Biteau-Coroller et al., 2006; Maan et al., 2004). In fact, PCR is very valuable in new infected cattle when no serum response has been produced.

The reason for increased serum-positive cases to PCR is that following sero-conversion unlimitedly (occasionally for years) blood antibody level remains high. Clavijo et al. (2000) and Dadhich (2004) believes that one of the reasons for long-term serologic response in BTV infection as the continuous stimulation of the cattle's immunity system is due to the attachment of virus to the blood cell membranes and remaining with them up to the end of the cell's life. Definitely, such cattle are also PCR-positive and as soon as infected red blood cells are removed from their blood, they will become negative from this point of view. From the serological point of view however, they will remain positive thus, accordingly. Therefore, one of the suitable tools for BTV diagnosis in foci where the disease is found in quiescent (latent) state is to use the serum ELISA (Biteau-Coroller et al., 2006).

In order to know the BTV status in South West of Iran, first it is necessary to isolate circulating viruses in foci. Then, we have to analyze at least 2 to 3 segments of their genome. This is important since genotyping of a gene only reveals part of genomic characteristics of the virus. Regarding independence of various segments of BTV in transferring to subsequent generations, it is necessary to study a greater number of them. Accordingly, which increasing precision of topotyping, the possibility of study of probable rearrangements between the viruses prevalent in the country will be provided?

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