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Detection of foot-and-mouth disease virus and identification of serotypes in East Azerbaijan province of Iran

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ABSTRACT

Three serotypes of foot-and-mouth disease virus (FMDV) including A, Asia1 and O, have been detected in Iran. Following the mass vaccination program which was implemented in all parts of the country in 2002, the number of outbreaks has been significantly reduced. Therefore, rapid detection of FMDV and its serotypes in clinical samples is essential for control of new outbreaks. In this study a reverse transcription-polymerase chain reaction (RT-PCR) was used for detection and serotyping of FMDV in clinical samples. Twelve tissue samples were collected from suspected outbreaks from East Azerbaijan province and tested by this method. Of 12 samples, 10 were found to be positive for FMDV. These samples were also tested in a multiplex-PCR for serotype identification. Four samples showed to be serotype O, 4 samples identified as serotype A and 2 samples detected to be serotype Asia 1. Multiplex-PCR products were sequenced and specificity of results was confirmed. Results indicate that in order to practice a good control measure, the RT-PCR and multiplex-PCR could be successfully used as a robust diagnostic method.

Key words: foot-and-mouth disease virus, polymerase chain reaction

Introduction

Foot and mouth disease (FMD) is a highly contagious viral disease of both domestic and wild cloven-hoofed animals. This acute and very fast spreading disease is characterized by vesicle formation in and around the mouth and on the feet, and has a very high morbidity but low mortality rate in adults (CALLENS and DE CLERCQ, 1997). There are direct losses due

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to deaths in young animals, loss of milk, meat and a decrease in productive performance. The costs due to eradication or control are high and there are major indirect losses due to the imposition of trade restriction (RWEYEMAMU and LEFORBAN, 1999). FMD virus belongs to the genus *Aphthovirus* of the family *Picornaviridae*. FMD viruses are divided into seven serotypes. Serotypes O, A, C are widely distributed, whereas serotypes SAT1, SAT2, SAT3 are normally restricted to Africa and serotype Asia1 to Asia (KNOWLES and SAMUEL, 2003). Three serotypes of virus, including A, O and Asia1, are identified in Iran (KITCHING, 1999). However, serotype A isolates from Iran have been genetically and antigenically different from other isolates in the World Reference Laboratory (KITCHING, 1999). There is considerable heterogeneity within serotypes and there is no cross-immunity between serotypes. The antigenic variation of Iranian type A isolates has been reported using monoclonal antibodies and molecular techniques (MARQUARDT and FREIBERG, 2000). Early detection is essential for effective control of the disease and requires a rapid and sensitive method of diagnosis. In addition to the classical techniques of virus isolation in tissue culture and antigen detection by enzyme-linked immunosorbent assay (ELISA), RT-PCR has become established as a reliable, fast and sensitive method of early FMD diagnosis (KITCHING, 1992). Rapid identification of the serotype of the virus that is responsible for an outbreak is essential to speed up diagnosis, for selection of an appropriate emergency vaccine. A rapid and sensitive RT-PCR method for FMDV detection, which differentiated FMDV from other genetically and/or symptomatically related viruses and, more importantly, comprised a multiplex-PCR method that differentiated between common serotypes, has been reported (VANGRYSPERRE and DE CLERCQ, 1996; GIRIDHARAN et al., 2005). In addition, the feasibility of using real-time RT-PCR as a diagnostic tool for detection of FMDV has recently been reported (REID et al., 2006; OEM et al., 2005). The first outbreak of FMD in Iran was reported in 1951. Presence of the disease in Iran limits its trade in animal and animal products due to international regulations designed to limit spread of the disease. In 2002, a mass vaccination of susceptible animals was implemented to cover all provinces simultaneously. Since then, the numbers of outbreaks have significantly decreased. In the East Azerbaijan province, 81 FMD outbreaks occurred in 2002, while only 8 outbreaks were reported in 2004 as a result of inclusive vaccination. Due to the rapid spread of FMD and the serious economic consequences that can arise from an outbreak, fast and reliable diagnosis of FMD is essential for effective disease control.

The aim of this study was to optimize a RT-PCR for the detection of FMDV in clinical samples obtained from suspect animals in East Azerbaijan province.

Materials and methods

FMDV samples. During a one-year period (September 2003 to September 2004) 12 clinical samples from suspect animals showing clinical symptoms were collected in

a transport buffer and stored at -20 °C until needed. Samples consisted of tongue and interdigital epithelia tissues and belonged to 10 different regions covering all parts of the province. Three viral vaccine strains (cell culture) of types A, O and Asia 1 donated by Razi Vaccine Institute (Karaj, Iran) were used as controls. Healthy tongue tissue sample was also used as a negative sample.

RNA extraction. Each tissue sample (50 -100 mg) was homogenized with a pestle and mortar in one mL DEPC-dH_2O. Total RNA was extracted using a phenol-chloroform-thiocyanate-based method described by CHOMCZYNSKI and SACCHI (1987). Briefly, 1 mL of RNA extraction solution was added to 300 μL of tissue suspension in a 1.5 mL tube. This was followed by adding 200 μL chloroform and subsequent centrifugation at 10000g for 5 min. Supernatant was transferred to a new tube and an equal volume of cold isopropanol was added. After centrifugation (10.000g for 15 min) the resulting pellet was washed with 70% ethanol, dried and resuspended in 20 μL of DEPC-dH_2O. Extracted RNA was immediately used in RT reaction, or stored at -70 °C until required.

Reverse transcription. Oligonucleotide primers for RT reaction and subsequent PCR amplification were used as previously published (VANGRYSPERRE and DE CLERCQ, 1996). Synthesis of first strand cDNA was performed in a 40 μL reaction mixture. Reaction mixture consisting of the following reagents: 4 μL extracted RNA, 0.2 μg reverse primer (P33) and 10 μL DEPC dH_2O. This mixture was incubated for 5 min at 70 °C and quickly transferred on ice. Then, 5 μL 5XRT buffer (Fermantas, Lithuania), 2 μL 10 mM dNTP mix, 40 U RNase Inhibitor (Roche, Germany) was added. The reaction was incubated at 37 °C for 5 min. Finally, 40 U reverse transcriptase (M-Mulv) was added and the mixture was further incubated at 37 °C for 60 min, followed by 70 °C for 10 min.

Polymerase chain reaction for FMDV detection. A PCR reaction mix consisted of the following reagents: 5 μL of 10X PCR buffer, 0.2 μg of each primer (P33, P32) (VANGRYSPERRE and DE CLERCQ, 1996), 1 unit Taq DNA polymerase, 1 μL of 10 mM dNTPs and dH_2O to a final volume of 45 μL. To this mixture was added 5 μL of cDNA. The reaction was subjected to one cycle of 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 74 °C for 2 min, and finally, one cycle of 74 °C for 10 min, using a Techne thermocycler (UK).

Multiplex-PCR. A multiplex-PCR was optimized with the same conditions as previously mentioned for PCR, using degenerative primers for O, A and Asia1 serotypes based on published oligonucleotides (VANGRYSPERRE and DE CLERCQ, 1996).

Electrophoresis. PCR and multiplex-PCR products were electrophoresed in a 1% agarose gel followed by ethidium bromide staining. A UV transilluminator then visualized results.

Sequencing the PCR products. The multiplex-PCR products were purified using High Pure PCR purification Kit (Roche, Germany) and cloned into a T-vector (pTZ57R),

using Inst/A Clone Kit (Fermentas, Lithuania), based on manufacturer's instructions. Recombinant plasmids were sequenced and data were compared to GenBank database.

Results

Of 12 clinical samples, 10 were found to be positive for FMDV in PCR. Positive and control samples produced a 131 bp fragment in the first round of PCR, while the negative sample failed to produce any DNA band (Fig. 1).

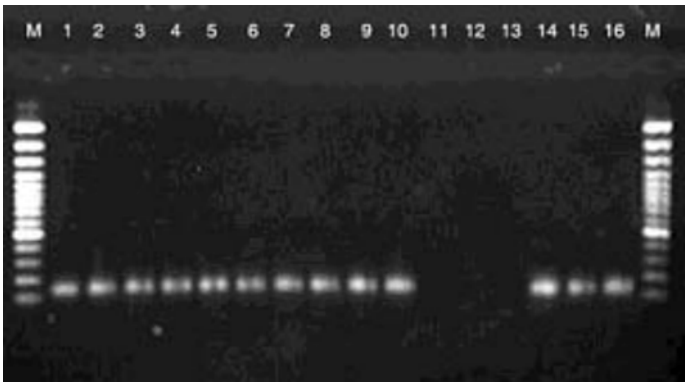

Fig. 1. A 131 bp PCR product resulting from amplification of FMDV RNA extracted from clinical samples and from cell culture. M: DNA size marker, ladder 100, Lane 1-10: Positive clinical samples Lane 11-12: Negative clinical samples Lane 13: Negative control tissue sample, Lane 14: Serotype Asia1 (positive control cell culture), Lane 15: Serotype O (positive control cell culture), Lane 16: Serotype A (positive control cell culture).

In multiplex-PCR, DNA Fragments of 292, 402 and 732 bp were amplified for the Asia1, O and A serotypes, respectively (Fig. 2). The clinical samples examined in multiplex PCR revealed 4 samples of serotype O, 4 samples of serotype A and 2 samples of serotype Asia1. The FMDV RNAs of three positive control samples were amplified by the multiplex-PCR and produced the expected size of DNA amplicon (Fig. 2). The nucleotide sequence of multiplex-PCR amplicons was determined by sequencing. Comparison of sequence data for each sample with GenBank database revealed that the test is specific and confirmed the identified serotypes.

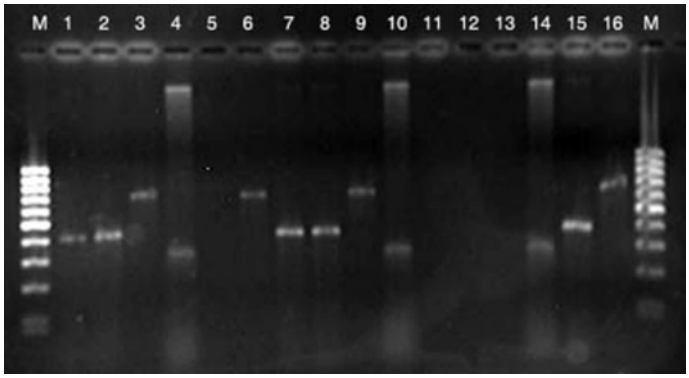

Fig. 2. Multiplex-PCR for identification of FMDV serotypes. M: DNA size marker, ladder 100; Lane: 1, 2, 7 and 8: 402 bp DNA fragment representing serotype O (clinical tissue samples); Lane 3, 5, 6 and 9: 732 bp DNA fragment representing serotype A (clinical tissue samples); Lane 4 and 10: 292 bp DNA fragment representing serotype Asia1 (clinical tissue samples); Lane 11 and 12: Negative clinical tissue samples; Lane 13: Negative control tissue sample; Lane 14: Serotype Asia1 (positive control cell culture); Lane 15: Serotype O (positive control cell culture); Lane 16: Serotype A (positive control cell culture)

Discussion

Rapid detection and identification of FMDV and its serotypes is both important and essential in animal health and vaccination programs. In general, serotyping of FMDV is done using the antigen capture ELISA that has replaced the complement fixation test as the routine method of choice (FERRIS and DAWSON, 1988). Samples sometimes have to be inoculated into cell cultures for the production of sufficient antigen, which may require several days, before typing can be undertaken. Molecular biology is providing extremely sensitive and specific tools for identifying and characterizing FMDV strains in clinical samples (KITCHING, 1992). The molecular biological technique is rapid, accurate, highly sensitive and only small quantities of material are needed to do the test. In this study a RT-PCR was optimized in order to detect FMD viral RNA in clinical samples, regardless of their serotypes. The first step was a PCR for detection of viral genome in clinical samples and a 131 bp DNA fragment was amplified for all three serotypes. The specific primers were selected from the 2B region of FMDV genome as previously explained (VANGRYSPERRE and DE CLERCQ, 1996). The next step, which can be achieved within 24 hours of receipt of the sample, is to carry out a multiplex PCR in order to determine the serotype of FMDV. Multiplex PCR differentiated serotypes A, O, Asia 1 and consisted of P33, as a consensus downstream primer that hybridizes to the highly conserved 2B region, and type-specific upstream primers selected from the neighbouring and more variable 1D gene. An FMDV strain can be identified as A, O or Asia1 if there are PCR fragments of 732, 402 or 292 bp, respectively. In this study 12 clinical bovine tissue samples were tested

with these two assays. Results indicated that three FMDV serotypes (A, O and Asia1) are present in East Azerbaijan province and outbreaks are due to circulation of these viruses among susceptible animals. Therefore, a suitable vaccine consisting of all these serotypes is required to reduce the risk of FMD infection. These molecular diagnostic methods are sensitive and specific for detection of FMDV and its serotypes in clinical samples in less than 24 hours. Therefore, they are useful in rapid diagnoses of FMD outbreaks. Since a vast vaccination program is carried out three times a year in the country and bearing in mind that that most of susceptible animals are vaccinated in a short period of time, clinical FMD is significantly decreased. Therefore, detection of new outbreaks would be important for controlling the disease. Using the above described multiplex-PCR, it would be possible to detect FMDV serotypes, especially in border areas where illegal animal movements occur. In addition, proper samples from FMD carriers and sub-clinically infected animals, which are always a threat to susceptible herds, could be tested by this method.

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SAŽETAK

Serotipovi A, Azija 1 i O virusa slinavke i šapa dokazani su u Iranu. Broj pojava slinavke i šapa znatno se smanjio nakon što je 2002. uvedeno masovno cijepljenje protiv te bolesti u svim dijelovima zemlje. Brzi dokaz virusa slinavke i šapa i identifikacija serotipa u kliničkim uzorcima ključan je za kontrolu novih pojava. U radu je rabljena lančana reakcija polimerazom uz prethodnu reverznu transkripciju (RT-PCR) za dokaz i serotipizaciju virusa slinavke i šapa u kliničkim uzorcima. Dvanaest uzoraka tkiva prikupljeno je iz sumnjivih životinja na području Istočnog Azerbaijana te je pretraženo spomenutom metodom. Deset od njih bilo je pozitivno na virus slinavke i šapa. Za identifikaciju serotipa rabljen je multipleks-PCR. Serotip O identificiran je u četiri uzorka, serotip A također u četiri uzorka, a serotip Azija 1 u dva pretražena uzorka. Proizvodi dobiveni metodom multipleks-PCR bili su sekvencionirani te je potvrđena specifičnost rezultata. Rezultati su pokazali da se u donošenju dobrih kontrolnih mjera uspješno mogu upotrijebiti RT-PCR i multipleks-PCR kao priznate dijagnostičke metode.

Ključne riječi: virus slinavke i šapa, lančana reakcija polimerazom