Archives of Razi Institute, Vol. 64, No. 1, June (2009) 19-25

Capripoxvirus identification by PCR based on P32 gene

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Received 4 Jan 2009; accepted 20 June 2009

ABSTRACT

Sheeppox virus (SPV) and goatpox virus (GPV) belong to the capripoxvirus genus of Poxviridae family. Sheeppox and goatpox along with contagious ecthyma (CE) are endemic diseases in Iran. Capripox laboratory conformation based on virological and serological techniques are time consuming, laborious and most of them of low specificity, because of close antigenic relationship between capripoxvirus and parapoxvirus. The aim of this study was to develop a capripoxvirus specific PCR assay for SPV & GPV identification on the basis of 390 bp fragment of P32 gene encoding capripoxvirus immunodominant antigen. PCR reaction was optimized using two reference strains of SPV & GPV and four field isolates in tissue culture supernatants. The identity of PCR product was confirmed by sequence analysis and the sensitivity of PCR was performed with 10 fold serial dilutions genomic LK cell DNA infected with capripoxvirus. This PCR was carried out on Twenty-nine biopsy samples from different organs of sheep and goats suspected to SPV & GPV against six biopsy samples infected with CE. Twenty-five SPV & GPV samples were positive and all of CE samples were negative. This PCR assay showed high sensitivity in detection of capripoxvirus DNA and good specificity in differentiation of capripoxvirus from parapoxvirus.

Keywords: Capripoxvirus, PCR, P32 gene

INTRODUCTION

Sheeppox and goatpox diseases caused by members of the Capripoxvirus genus of the Poxviridae family (Murphy 1999, Esposito 2001). They are the most important poxviruses of animals, listed in group A diseases of OIE (Carn 1993, World Animal Health Organization 2004) which can cause significant economic losses. Capripox is endemic in Africa north of the Equator, the Middle East, India, Nepal, parts of the people's Republic of China. Recently, it has made frequent incursion into southern Europe (OIE, 2004).The diseases are characterized by fever, generalized skin and internal pox lesions, lymphadenopathy, and death. (Kitching & Taylor 1985, Carn 1993). Laboratory confirmation has been reliant upon classical virological techniques including virus isolation in cell cultures (LT, LK, …), electron microscopy(EM), histology, inoculation to susceptible animal; serological techniques such as Fluorescent antibody test(FAT), agar gel immunodiffusion test(AGID), Enzyme-linked immunosorbant assay(ELISA), virus neutralization test(VNT) and Western blot analysis (Murphy 1999,

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Chand *et al* 1994). These tests are time consuming and not readily available in everywhere. Most of them are low specificity and serologically indistinguishable, because of cross reactions between capripoxvirus and parapoxvirus (Murphy 1999, Subba Rao *et al* 1984). PCR is a simple, rapid, and accurate technique which has been routinely used for detection and characterization of many viruses in the world. The PCR-based test for capripoxvirus in biopsy samples has better analytical and diagnostic sensitivity than the antigen trapping ELISA (Carn 1995). Recently, PCR method was developed for identification of capripoxvirus virus (Ireland & Binepal 1998, Mangana-Vougiouka 1999, Hein *et al* 1999). In this study a PCR assay based on capripoxvirus P32 gene was developed to identify capripoxvirus DNA in cell culture and biopsy samples by detecting 390 bp band in agar gel electrophoresis.

MATERIALS AND METHODS

Viruses and cells. 1. The capripoxvirus strains SPV (RM/65 strain) and GPV (Gorgan strain) which were in, respectively, 34 and 63 passages in lamb kidney (LK) cells. 2. Four field isolates of SPV (Qom & Azerbayejan) and GPV (Yazd & Semnan) which confirmed by inoculation to susceptible animals grown on LK kidney cell cultures which were harvested when cells showed 90% CPE; frozed at -20 °C and thawed at room temperature,two times; clarified at 4000 rpm centrifugation for 45 'and stored at -20 °C until needed. 3. An Iranian CE strain 1783 isolated on lamb testis (LT) cell cultures and was in its 60 passage as negative control. 4. Uninfected LK cells as unrelated virus(Varshovi *et al* 2005).

Biopsy samples. Twenty-nine biopsy samples included Skin, tongue, lung, abomasom, kidney and lymph node suspected to sheeppox or goatpox and six biopsy samples suspected to CE with relevant results of virus isolation and inoculation to susceptible animal tests were obtained from Razi institute, reference laboratory. As negative control, skin tissue from normal sheep was prepared.

Preparation of samples. Biopsy samples washed two times in wash buffer included phosphate buffer saline (PBS) with 2000 IU/ml penicillin G Potassium, 2000 µg/ml Streptomycin and 200 U/ml Nystatin. One gram of biopsy sample was ground up in approximately 5 ml PBS containing antibiotics in a sterile glass tissue grinder. The medium freezed and thawed two times. The suspension was clarified by centrifugation at 4000 rpm for 45'. Then, it was aliquoted and stored at -20 °C until needed.

DNA extraction. Infected and uninfected cells suspensions and prepared biopsy samples were subjected to DNA extraction using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH), according to the manufacturer's instructions, DNAs were exracted via specific binding on glass fibers in presence of guanidium HCl .

Primers. The primers used in PCR assays were B68 and B69 (Hein *et al* 1999). The PCR primers had the following sequences and were synthesized by MWG-biotech, Germany:

B68: 5' - CTA AAA TTA GAG AGC TAT ACT TCT T- 3'

B69: 5' - CGA TTT CCA TAA ACT AAA GTA-3'

The primers amplified 390 bp fragment of P32 gene

Polymerase chain reaction (PCR). One µl sample of each prepared genomic DNA were placed in 25 µl of the final volume of a reaction mixture contained 2.5 µl of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP(cinnagen,Iran) , 0.2 µM of each oligonucleotide primer and 1 U Taq-DNA

Polymerase(Fermentas).The PCR had an initial denaturation of 94 °c for 3 minutes, was followed by for 35 cycle of denaturation at 94°c for 30", annealing at 48 °c for 30" and extension at 72 °c for 30". Final extension step was at 72 °c for 10 minutes. Each amplified product were analysed by gel electrophoresis on 1.5% low electroendosmosis agarose (TopVision LEGQ, Fermentas), in 1% TBE buffer for 80 minutes. The gel put in 0.5 µl/ml ethidium bromide for 30 minutes, washed by D.D.W for 2 minutes and visualized on an UV transilluminator (Uvitec). The PCR results were compared to that of virus isolation and inoculation to susceptible animal tests on biopsy samples.

Sequence analysis. Amplicon (390 bp) of SPV (RM/65 strain) was excised from the gel and purified using PCR product purification kit (Roch Diagnostics GmbH), according to the manufacturer's instructions. The purified PCR product was sequenced using DNA sequencing system (MWG Company, Germany). Sequence was analyzed by comparison with sequences of different capripoxviruses available in GenBank database using online BLAST server

Sensitivity and specificity. To evaluate the sensitivity of the PCR was carried out with 10 fold serial dilutions of genomic LK cell DNA were amplified from cells infected with the SPV (RM/65 strain) in $10^{5.5}$ $TCID_{50}$/ml and GPV (Gorgan strain) in 10^{6} $TCID_{50}$/ml titers. The specificity of the PCR reaction was tested with minimum detectable amount of genomic LK cell DNA infected with the SPV (RM/65 strain) and GPV (Gorgan strain) in the presence of maximum amount of uninfected LK cell DNA and infected LK cell DNA with the CE 1783.

RESULTS

PCR. The virus yield of infected cell culture supernatant subjected to PCR for the SPV (RM/65 strain) was $10^{5.5}$ $TCID_{50}$/ml, for GPV (Gorgan strain) was 10^{6} $TCID_{50}$/ml, and for CE was10^{7} $TCID_{50}$/ml. It was varied between $10^{4.5}$ and 10^{6} $TCID_{50}$/ml for field isolates adapted to LK cell cultures. Amount of DNA templates, primers, Taq-DNA Polymerase, $MgCl_2$, annealing temperature and duration of PCR steps were optimized as mentioned in above PCR reaction. This PCR assay successfully amplified the DNA of both reference strains and four field isolates by showing the expected band of 390 bp in gel electrophoresis. No amplified product was observed when DNA of negative control (uninfected LK cell) and infected cell with CE strain 1783 were examined in PCR (Figure 1).

Sequence analysis. 390 bp of PCR product was sequenced and the data submitted to NCBI GeneBank (Accession No. FJ917518). BLAST program showing 100 % and 97 % maximum identity of this nucleotide sequence with sheeppox and goatpox P32 genes available in GenBank, respectively.

Sensitivity and Specificity. The sensitivity of the reaction for various amount of infected LK cell DNA with the SPV (RM/65 strain) based on 10 fold serial dilutions showed that 0.3 ng of that is detectable in the gel electrophoresis in expected amplimer size 390 bp (Figure 2). The specificity of the PCR reaction was achieved to detect 0.3 ng of genomic LK cell DNA infected with the SPV (RM/65 strain) in the presence of 0.3 µg of uninfected LK cell DNA (Figure 3). The same results were obtained on GPV (Gorgan strain), not shown.

Tests of clinical material. Twenty-five samples suspected to sheeppox and goatpox infections were positive for viral DNA by PCR primers amplifying 390 bp of P32 gene(table 1). An individual sharp band of the predicted size near 400 bp (390 bp) was indicated in gel electrophoresis for positive samples. In contrast, none of negative controls including

CE (1783/P60) and skin biopsies from normal sheep or DNA from uninfected LK cell gave any amplified product by the specific capripox primers (Figure 4).

Table 1. The comparison PCR assay, inoculation to susceptible animals and virus isolation results.

No.	Biopsy Samples	PCR	Inoculatin to Suspected Animal	Virus Isolation following VNT		
				1st	2nd	3rd
1	Tongue (sheep)	+	+		+	
2	lymph node (sheep)	+	-	-	-	-
3	lymph node (sheep)	+	-	-	-	-
4	Lymph node (goat)	+	+		+	
5	Cotyledon (sheep)	+	+	+		
6	Lung (sheep)	+	-	-	-	-
7	Abomasum (sheep)	+	+	-	-	-
8	Kidney (sheep)	+	-	-	-	-
9	Skin (sheep)	+	+	+		
10	Skin (sheep)	+	+		+	
11	Skin (sheep)	+	+	+		
12	Skin (goat)	-	-	-	-	-
13	Skin (goat)	+	+	+		
14	Skin (sheep)	+	+	-	-	-
15	Skin (sheep)	+	+	-	-	-
16	Skin (goat)	+	-	-	-	-
17	Skin (goat)	+	+	+		
18	Skin (sheep)	+	+	+		
19	Skin (goat)	-	-	-	-	-
20	Skin (goat)	+	+	+		
21	Skin (sheep)	+	+	-	-	-
22	Skin (sheep)	-	-	-	-	-
23	Skin (sheep)	+	+		+	
24	Skin (sheep)	+	+		+	
25	Skin (sheep)	+	-	-	-	-
26	Skin (goat)	+	+	-	-	-
27	Skin (goat)	-	-	-	-	-
28	Skin (sheep)	+	+	-	-	-
29	Skin (sheep)	+	+			+

DISCUSSION

In the present study a rapid and accurate PCR assay was described for capripoxvirus detection and differentiation from CE virus in biopsy samples. However CE is a rather mild disease, it may be confused with sheeppox and goatpox in differentiation based on clinical sings. Therefore, sheeppox and goatpox diagnosis needs an urgent and accurate laboratory conformation, once occurred in a herd.

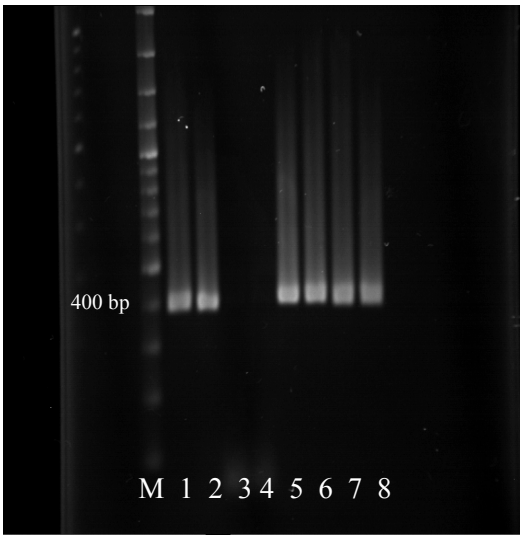

Figure 1. Identification of sheep & goat poxvirus in cell cultures. Lane M: 100 bp marker. Lane 1. amplification performed on genomic Lk cell DNA infected with SPV (RM/65 strain). Lane 2. amplification performed on genomic LK cell DNA infected with GPV (Gorgan strain). Lane 3. amplification performed on uninfected LK cell DNA. Lane 4. amplification performed on genomic Lk cell DNA infected with CE virus strain 1783/P60. Lanes 5. amplification performed on genomic Lk cell DNA infected with SPV (Qom) Lanes 6. amplification performed on genomic Lk cell DNA infected with SPV (Azerbayejan). Lanes 7. amplification performed on genomic Lk cell DNA infected with GPV (Yazd). Lanes 8. amplification performed on genomic Lk cell DNA infected with GPV (Semnan).

Many serodiagnostic assays and antigen detection are available for differentiation between capripox virus and parapoxvirus but most of them are not reliable, because of the existence of a common antigen between these viruses (Kitching *et al* 1986, Subba Rao *et al* 1984). In recent studies, PCR technique was developed for identification of GPV and SPV based on capripoxvirus fusion and attachment protein genes in biopsy samples (Ireland and Binepal 1998) and DNA fragment from inverted terminal repeats (ITR_S) of the genome in cell cultures (Mangana-Vougiouka *et al* 1999). In

this study, a PCR assay based on amplifying of 390 bp fragment of P32 gene were developed to identify capripoxvirus in different samples of internal organs besides skin samples using the specific primers described by Heine *et al* (1999).

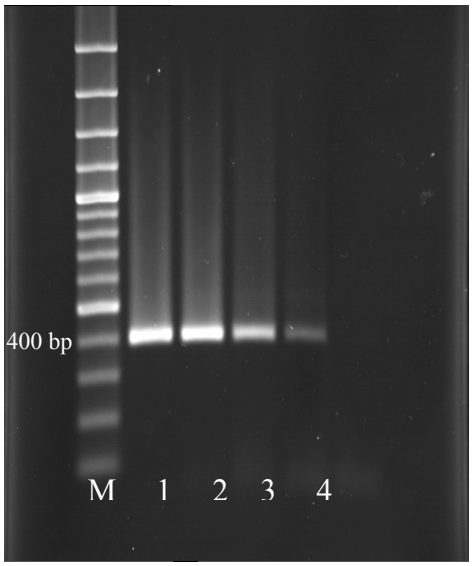

Figure 2. Sensitivity of the PCR applied to 10 fold serial dilutions of the genomic LK cell DNA infected with the sheeppox strain RM/65. Lane M: 100 bp marker. Lane1: amplification from 300 ng of genomic LK cell DNA infected with the SPV. Lane 2: amplification from 30 ng of genomic LK cell DNA infected with the SPV. Lane 3: amplification from 3 ng of genomic LK cell DNA infected with the SPV. Lane 4: amplification from 0.3 ng of genomic LK cell DNA infected with the SPV

The full-length P32 protein contained a transmembrane region close to the carboxy terminus and was membrane associated (Heine *et al* 1999). It is a structural protein present in all strains of capripoxvirus and contains a major antigenic determinant (Chand 1992) .The gene encoding this protein is located in highly conserve region of capripoxvirus genome (Tulman 2002). Our sequence analysis of PCR products showing high homology of 100 % and 97 % with sheeppox and goatpox P32 genes available in GenBank, respectively. However the following PCR was previously described for detection of capripox viruses (Heine *et al* 1999), so far the sensitivity, specificity and reproducibility of this PCR were not shown. Here we reported higher sensitivity in amplifying the detectable band for 0.3 ng/µl of genomic LK cells infected with the SPV or GPV, while Mangana-Vougiouka et al (1999) and Markoulatos et al (2000) reported that their PCR assays based on different target genes (above mentioned) showed the limit of detectable band for up to 30 and 10 ng/µl of genomic LK cells infected with the sheeppox virus, respectively.

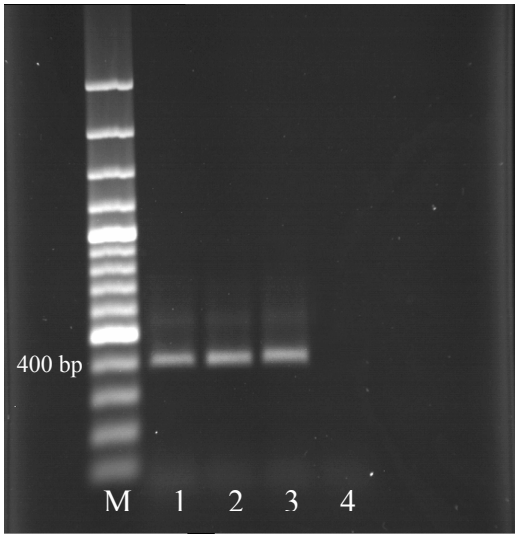

Figure 3. Specificity of the PCR to detect sheeppox strain RM/65. Lane M: 100 bp marker. Lane 1: amplification from 0.3 ng of genomic LK cell DNA infected with the SPV. Lane 2: amplification from 0.3 ng of genomic LK cell DNA infected with the SPV in the presence of 0.3 µg of uninfected LK cell DNA. Lane 3: amplification from 0.3 ng of genomic LK cell DNA infected with the SPV in the presence of 0.3 µg genomic LT cell DNA infected with the CE virus (1783/P60). Lane 4: amplification from 0.3 µg of uninfected LK cell DNA.

According to the present results, this PCR assay showed excellent sensitivity, specificity and reproducibility on cell cultures and biopsy samples of different organs. Furthermore, the preliminary comparison results of the PCR assay to that of virus isolation and inoculation to susceptible animal tests on biopsy samples (available in reference laboratory, Razi institute) showed higher sensitivity of this PCR in detection of sheeppox and goatpox viruses (table 1). In addition, Two latter tests are time consuming and laborious. Sheeppox and goatpox viruses isolation is difficult; they grow slowly or require additional several passages, even

if cultured in the most sensitive lamb testis or lamb kidney cells (Plowright 1958).

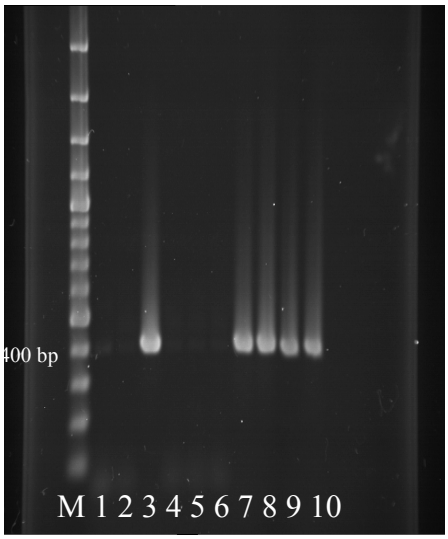

Figure 4. Identification and differentiation of sheep & goat poxvirus from CE virus in clinical samples. Lane M: 100 bp marker. Lane 1. amplification performed on genomic Lk cell DNA infected with CE virus strain 1783/P60. Lane 2. amplification performed on uninfected LK cell DNA. Lane 3. amplification performed on genomic LK cell DNA infected with the SPV (RM/65 strain). Lanes 4-5. amplification performed on skin biopsies with CE virus infection. Lane 6. amplification performed on normal skin biopsy. Lanes 7-8. amplification performed on skin biopsies with sheeppox virus infection. Lanes 9-10. amplification performed on skin biopsies with goatpox virus infection.

Virus isolation should be followed by confirmation (virus neutralization or immunofluorescence using hyperimmune anti- capripox serum) is a lengthy procedure and may take up at least 2 weeks. Virus isolation in cell cultures fails to detect virus particles that are bound to neutralizing antibody (Ireland & Binepal 1998). As it could be seen in table 1 susceptible animal inoculation is not always accurate since the live virus in the sample might be insufficient or inactivated by neutralizing antibody or other factors. Anyway, inadequate sample due to incorrect time in sampling or poor transportation may result in false negative in live-virus detection methods. In conclusion, the present PCR assay has considerable potential as a rapid and accurate diagnostic method for capripox virus detection and distinction from CE virus. It should be mentioned that PCR method is not yet capable in differentiating SPV from GPV. In the future, in order to establish a molecular epidemiological survey of capripox in Iran, it is needed to develop rapid and reliable molecular techniques such as PCR-RFLP in differentiation of SPV from GPV.

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