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DETECTION OF SHEEP POXVIRUS BY NESTED-POLYMERASE CHAIN REACTION ASSAY

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SUMMARY

Sheep pox disease is one of the most important diseases caused by poxviruses in animals. This disease can pose an economic threat to animal husbandry in countries where the virus has not been eradicated. In this study, a rapid PCR assay was optimised and developed for the detection of sheep poxvirus. The target region was the inverted terminal repeats (ITRs) of the viral genome and the primers used were able to replicate a product of 289 bp. The primers detected the virus from skin biopsies of sheep suspected of poxvirus infection and the identity of PCR products was confirmed by sequencing which showed 97% homology to published sequences. The primers also work well in detecting a vaccine strain of goat poxvirus. The sensitivity of the assay improved 10^3 times by using nested-PCR assay. Detection of sheep poxvirus by PCR in clinical samples has many advantages compared with other methods that are currently being used. The assay proved to be sensitive, specific and rapid. Therefore it can be used as a robust diagnostic test in veterinary laboratories.

Keywords: Sheep poxvirus, goat poxvirus, sheep, nested polymerase chain reaction, rapid test

INTRODUCTION

Sheep poxvirus causes a severe and highly contagious disease in sheep, which is listed in Group A Diseases of the OIE (World Organisation for Animal Health, 2004). Sheep poxvirus and goat poxvirus, together with lumpy skin disease virus of cattle comprise the genus Capripoxvirus in the subfamily *Chordopoxvirinae*, family *Poxviridae* (Buller *et al.*, 2005). The Capripox virion is a brick-shaped particle with a linear double-stranded DNA and 270–290 nm in size (Damon, 2007). Transmissible diseases from poxvirus infections that tend to spread rapidly and cause pandemics have great importance for the public health and socio-economic status of any countries. These diseases cause great economic damage to international trade in animals and animal products (Gulbahar *et al.*, 2000; Markoulatos *et al.*, 2000). Capripoxvirus infections are endemic in Africa (north of the Equator), the Middle East, Central Asia and the Indian subcontinent (Bowden *et al.*, 2008). There are reports of outbreaks of sheep pox in Turkey (Oguzoglu *et al.*, 2006), Iran (Tafti and Namdari, 2001), Greece (Mangana-Vougiouka, 1999) and Kenya (Ireland and Binepal, 1998).

Diagnosis of the disease is made by identification of Capripox virus by virus isolation on cell culture and confirmation by virus neutralisation or immuno-fluorescence using hyperimmune anti-Capripox virus serum. Capripoxvirus isolation is a lengthy procedure due to the slow growth of the virus in cell culture, in which cytopathic effects may take up to 10 days to develop. Serology is limited in its application due to the low antibody response following infection. In addition, Capripoxvirus and Parapoxvirus are serologically closely related. Both viruses cause very similar diseases in sheep and goats and it is difficult to differentiate them clinically. Many serodiagnostic assays such as agar gel immunodiffusion, complement-fixation, counter immuno-electrophoresis or ELISA have low specificity, because they detect total antibody response including that from cross-reactive antibodies. Such tests are not reliable for differentiation between these infections (Markoulatos *et al.*, 2000). Electron microscopy can provide a rapid result but the technique is not able to distinguish the morphology of Capripoxvirus and Orthopoxvirus (Ireland and Binepal, 1998). The purpose of the present study was to optimise and develop a PCR assay for the detection of sheep poxvirus and to evaluate the sensitivity of PCR assay compared with nested PCR assay.

MATERIALS AND METHODS

Viruses

Two reference vaccine virus strains were used namely the Iranian sheep poxvirus vaccine strain, RM65 (Razi Institute), and the goat poxvirus vaccine strain, Gorgan (Razi Institute). Both vaccines were constituted of 10^5 $TCID_{50}$/ml. Skin specimens with crusted scab lesions were collected from two sheep that were suspected of sheep pox infection.

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DNA extraction

Viral DNA from both the vaccines and clinical samples were extracted using phenol-chloroform based method. Briefly, 1 ml of DNA extraction solution (NIGEB, Iran) was added to the sample and vaccine virus suspension in a 1.5 ml microfuge tube. This was followed by 0.2 ml chloroform extraction and precipitation of supernatant by adding equal volume of cold isopropanol and subsequent centrifugation at 10,000 rpm for 15 min. The resulting pellet was then washed with 75% ethanol, dried and resuspended in 20 μl of DEPC–dH2O. Extracted DNA was immediately used or stored at -70 °C until needed.

Primers

The oligonucleotide primers were chosen from the inverted terminal repeats (ITRs) of InS-1 strain as previously published (Mangana-Vougiouka *et al.* 2000). The sequence of the Forward primer (5' AGA AAC GAG GTC TCG AAG CA 3') and for the Reverse primer (5' GGA GGT TGC TGG AAA TGT GT 3') would produce an expected product of 289 bp. The primers were synthesised by MWG-BIOTECH (Ebersberg, Germany). The specificity of these primers to amplify only poxviruses, as well as the sensitivity of the PCR assay have been reported. Nested primers were designed from the above sequence with the Forward primer (5' CAA TAT TCT GCT GCT CTT GC 3') and Reverse primer (5' GGA TGC CTC ACT TGT ATT GT 3') with the expected product of 177 bp.

Polymerase Chain Reaction (PCR) assay

A reaction mixture containing 5 μl of each DNA sample, 5 μl of 10×reaction buffer, 1.5 μl $MgCl_2$ (50 mM), 1 μl of mix dNTPs (10 mM), 1 μl of each oligonucleotide primer (250 ng/μl) and 0.5 μl of Taq-DNA Polymerase (5 U/μl) was set up, making a total final volume of 50 μl. PCR had an initial pre-denaturing step of 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30s, 72°C for 30 s and a final elongation step of 72°C for 10 min in the thermocycler (TECHNE, England). Negative control contains reaction mixture without DNA template. Results were observed after 7 μl of PCR product were mixed with 2 μl of gel loading buffer and visualised after electrophoresis on an ethidium bromide stained 1% agarose gel using a UV transilluminator. The resulting amplicon was purified by high pure PCR product purification Kit (Roche, Germany) and sequenced. The sequence was aligned and compared with published sheep poxvirus data. For determining the sensitivity of PCR, 10-fold dilutions of the virus stock (2×10^4 $TCID_{50}$ to 2×10^{-3} $TCID_{50}$) were prepared in distilled water. DNA was extracted from diluted virus suspensions and examined by PCR assay.

Nested-PCR assay

In nested-PCR assay, 1 μl of first round PCR product was used as the template DNA and the annealing temperature was increased to 57°C. The materials and procedure were similar to the PCR protocol as described above. All procedures were carried out under conditions that minimised the risk of contamination with exogenous viral DNA during the PCR procedure. Separate rooms and sets of pipettes with plugged pipette tips were allocated for each step of the PCR. To compare the sensitivity between PCR and nested-PCR, each amplicon of first round PCR from serial 10-fold dilutions of virus stock were tested in the nested-PCR.

RESULTS

The primer set was able to amplify the sheep poxvirus by producing a DNA product with a distinct expected size of 289 bp (Figure 1). The nucleotide sequence of PCR product was compared with the published sequences of sheep poxvirus. Analysis showed that there was a high identity with 97% homology to different strains of sheep poxvirus (data not shown). To evaluate the sensitivity of the PCR, 10-fold serial dilutions from the sheep poxvirus strain RM65 were amplified. The PCR product was detected at the highest dilution of 2×10^2 $TCID_{50}$ (Figure 2). This test was able to amplify the virus of the goat poxvirus vaccine (Figure 3). The PCR assay was subjected to the pool samples of two clinical biopsy samples suspected of sheep poxvirus. There were PCR products with the expected band size of 289 bp (Figure 4). Nested-PCR was developed and tested on the serial DNA dilution of sheep poxvirus strain RM65. The expected size of 177 bp was observed. The PCR products were detected at the highest dilution of 0.2 $TCID_{50}$ (Figure 5).

DISCUSSION

Sheep pox is a highly contagious disease which needs an urgent and accurate laboratory diagnosis once it is suspected in a herd. At present, the diagnosis is based on the clinical signs, serological tests, virus isolation and identification of its morphology under electron microscopy. These assays are time consuming, laborious and sometimes the interpretation of results is rendered difficult. The most specific test which involves virus isolation on susceptible cell cultures followed by confirmation test using hyperimmune serum is a lengthy procedure and may take up to 2 weeks (Mangana-Vougiouka, 2000). These diagnostic problems could be overcome by the use of PCR assay. The present PCR assay requires only biopsy material that is easily obtained. Tissue culture facilities are not needed. Results can be obtained within one working day.

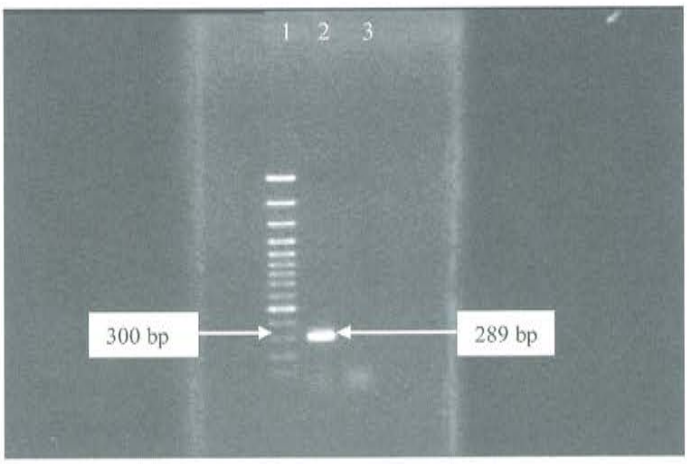

Figure 1: Agarose gel electrophoresis of the 289 bp PCR product obtained with the oligonucleotide pair specific for inverted terminal repeats (ITRs) of sheep poxvirus. 100 bp ladder was used as a marker.

Lane 1: 100 bp DNA ladder
Lane 2: Sheep poxvirus strain RM65
Lane 3: Negative control

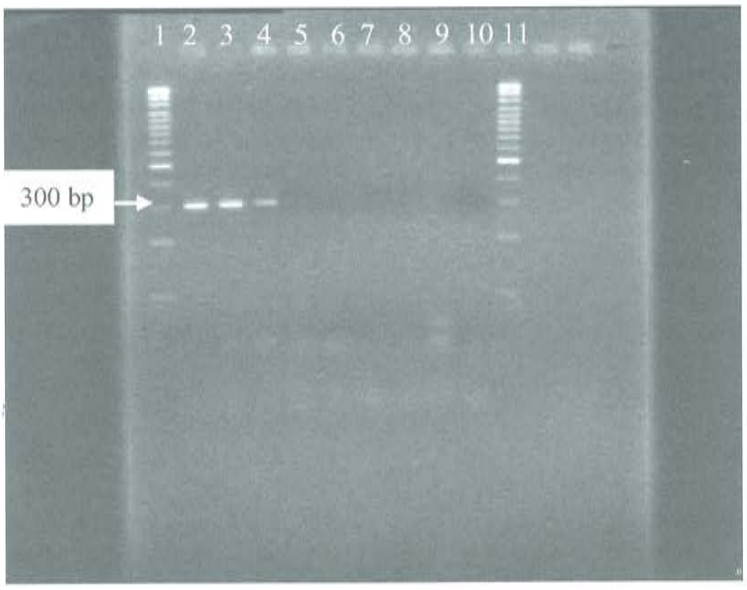

Figure 2: Sensitivity of the PCR assay for detection of sheep poxvirus strain RM65. The virus was diluted into 10-fold serial dilutions with the detection limit being $2x10^2$ $TCID_{50}$.

Lanes 1 and 11: 100 bp DNA ladder
Lanes 2 to 9: correspond to $10^4\times 2$ $TCID_{50}$, $10^3\times 2$ $TCID_{50}$, $10^2\times 2$ $TCID_{50}$, 10×2 $TCID_{50}$, 2 $TCID_{50}$, 0.2 $TCID_{50}$, 0.02 $TCID_{50}$ and 0.002 $TCID_{50}$, respectively
Lane 10: Negative control

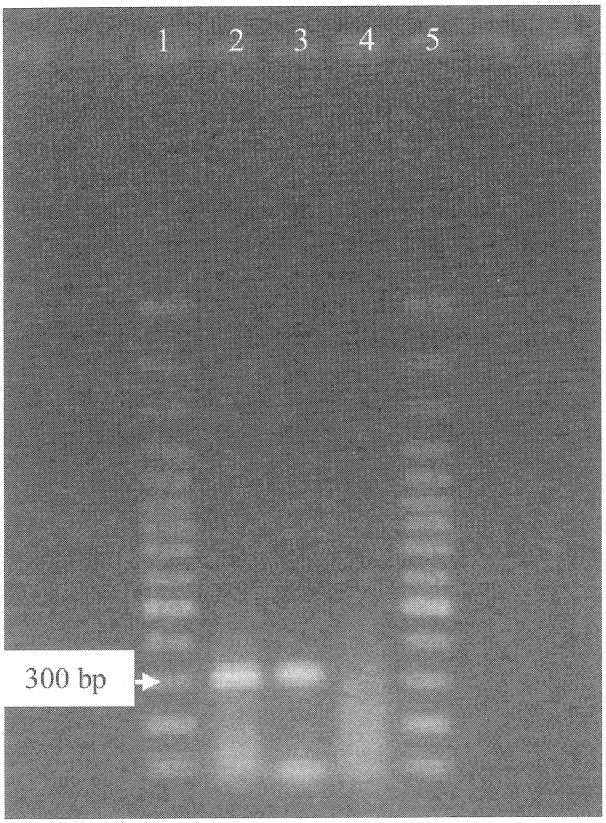

Figure 3: Specificity of the PCR assay for detection of sheep poxvirus. The assay is able to detect both sheep and goat poxviruses. 100 bp ladder was used as a marker.

Lane 1 and 5: DNA size marker, ladder 100
Lane 2: Sheep poxvirus (strain RM65)
Lane 3: Goat poxvirus (strain Gorgan)
Lane 4: Negative control

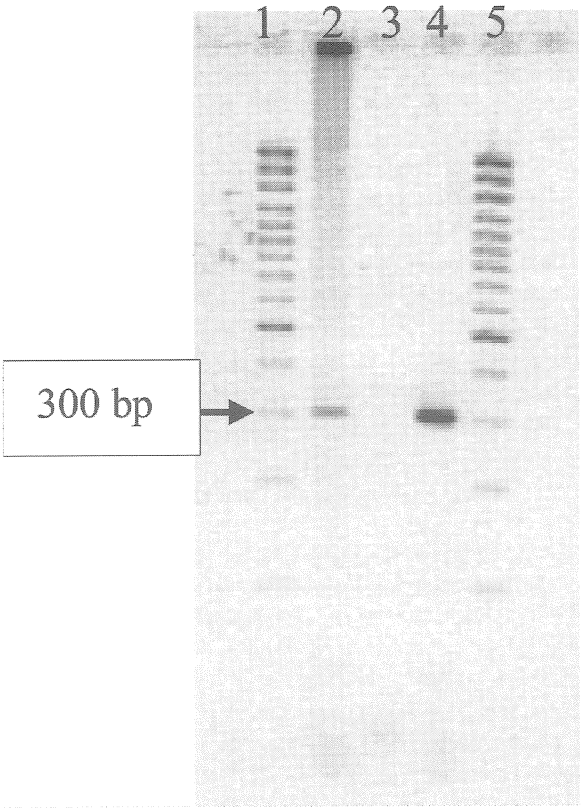

Figure 4: Detection of sheep poxvirus in clinical sample. The target band of PCR products are obtained from clinical sample suspected of sheep poxvirus and positive control. 100 bp ladder was used as a marker.

Lane 1 and 5: 100 bp ladder DNA marker
Lane 2: Clinical sample suspected of sheep poxvirus
Lane 3: Negative control.
Lane 4: Positive control (sheep poxvirus strain RM65)

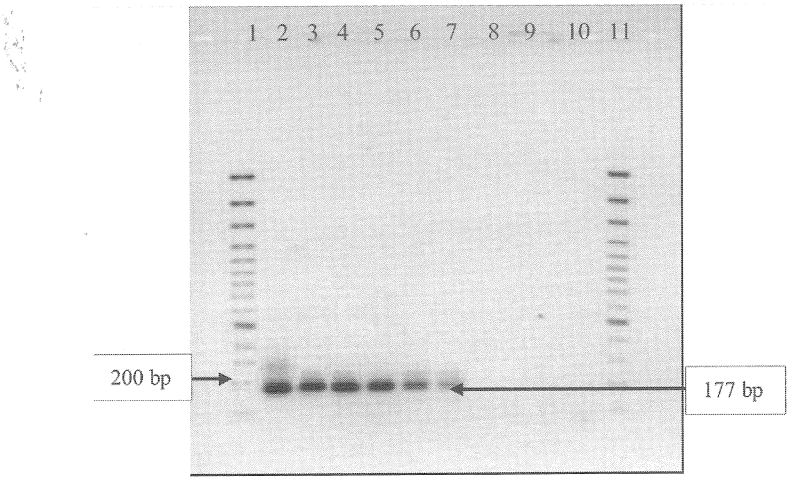

Figure 5: Sensitivity of the nested-PCR for detection of sheep poxvirus in 10-fold serial dilutions. The results show that the detection limit is reached to $2x10^{-1}$ $TCID_{50}$.

Lanes 1 and 11: DNA size marker, ladder 100
Lanes 2-9: correspond to $10^4 \times 2$ $TCID_{50}$, $10^3 \times 2$ $TCID_{50}$, $10^2 \times 2$ $TCID_{50}$, 10×2 $TCID_{50}$, 2 $TCID_{50}$, 0.2 $TCID_{50}$, 0.02 $TCID_{50}$ and 0.002 $TCID_{50}$, respectively
Lane 10: Negative control

Comparing between the conventional PCR and nested-PCR assay, the latter assay was 1000 times more sensitive. The sensitivity of nested PCR test has been reported widely and careful handling of the test material and reagents are important to prevent false-positive results. In other biopsy studies, PCR was able to detect 10^{-1} $TCID_{50}$ compared to ELISA which requires $10^{1.5}$ TCID50 to give a positive result. The diagnostic sensitivity of the ELISA is low probably due to the presence of Capripoxvirus-specific antibody in the PCR-positive biopsy samples (Ireland and Binepal, 1998).

A multiplex PCR for detection of sheep poxvirus was able to detect virus up to 10^{-4} $TCID_{50}$ (Markoulatos *et al.,* 2000). Similar primer sets were used for this study, but the maximum sensitivity obtained was 2×10^{-1} $TCID_{50}$. Different levels of sensitivity obtained in this study compared to an earlier study may due to the quality of extraction solution and also probably due to the presence of PCR-inhibitors such as the vaccine's adjuvant and phenol. Although the sensitivity of this test was lower than earlier reports, the test was able to detect virus in clinical samples. The viral titre in skin lesions of infected animals which ranged from $10^{2.7}$ $TCID_{50}$ to $10^{7.2}$ $TCID_{50}$ (Bowden *et al.,* 2008) was high enough for the test to detect the virus. The identity of PCR products is specific to sheep poxvirus and showed 97% homology to the published sequence. Since there is an average of no less than 96% nucleotide identity between strains of sheep poxvirus and goat poxvirus (Bowden *et al.,* 2008), this could be the reason as to why the PCR assay in this study was able to detect goat poxvirus. It is a norm in many countries for sheep and goats to be kept together. As this PCR assay has a set of primers that are able to detect both species of poxviruses, it could be a valuable diagnostic tool for detection of Capripoxviruses.

Vaccination is the major strategy to prevent Capripoxvirus infections in affected countries, especially in enzootic and outbreak areas (Kitching *et al.*, 1987; Carn, 1993; Carn *et al.*, 1994). In Iran, since 1935, the disease is being controlled by the use of live-attenuated vaccine. However, there have been occasional outbreaks in partly immune flocks. The common practice of herding sheep and goats into an enclosure at night provides adequate exposure to maintain endemic infection. During an outbreak, the virus is probably transmitted between sheep by respiratory droplets and biting arthropods. In addition, the delay in notifying to appropriate authorities and inappropriate disposal of infected animals play an important role in the maintenance of the disease in Iran. The PCR test and its derivative nested-PCR test are valuable tests to detect and diagnose Capripoxviruses. These tests were able to provide a rapid, sensitive and easy testing and enabled control measures to be taken immediately by isolating and disposing infected animals.

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