

Isolation and molecular diagnosis of Peste des petits ruminants (PPR) virus from contaminated areas in Iran

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Keywords

PPR, isolation, diagnosis, Iran

Abstract

Due to the numerous reports concerning the Peste des petits ruminants (PPR) in different regions of Iran, the isolation and genetic characterization of native isolates is very important. During 2013-2014, 168 samples were collected from whole blood, eye, nasal and oral swabs, and lymph nodes of sheep and goats with clinical signs in nine provinces with infected focal zones in Iran. Vero cell line and primary Lamb Kidney cells were inoculated with isolates and observed at least in 5 consecutive passages for cytopathic effects (CPE). The sheep samples from two provinces, created CPE in both kinds of cell cultures. Observation by electron microscopy and confirmation by RT-PCR was used to confirm PPR.

Abbreviations

PPR: Peste des Petits Ruminants
CPE: Cytopathic Effect
LK: Lamb Kidney
FBS: Fetal Bovine Serum
DMEM: Dulbecco's Modified Eagle Medium
EM: Electron Microscopy
RT: Reverse Transcription

Introduction

Peste des Petits Ruminants (PPR) is an acute and highly contagious viral disease of goats and sheep causing high morbidity and sometimes high mortality rates [1-4]. The disease is characterized by fever, necrotic stomatitis, gastroenteritis, pneumonia, and sometimes death [5]. The causative agent of the disease, PPR virus (PPRV), is classified as a member of the genus *Morbillivirus* in the family *Paramyxoviridae* [4, 6].

PPR was first reported in Cote d'Ivoire (the Ivory Coast) in 1942 and subsequently in other parts of west Africa [7, 8]. The disease occurs in a band that spreads across Africa between the equator and the Sahara, through the Arabian Peninsula, the Middle East, south-west Asia and India [9]. In China, the disease was reported in 2007. Following this report, in 2008, the disease was reported in North Africa, Morocco [5, 10]. In the last 15 yr, PPR has been reported in Turkey and Iran [3, 11]. In recent years, there are many reports of diagnosis of PPR in Iran but the causative virus has not been isolated [11]. In this study a specific cell culture condition and specific primers were used for virus isolation and molecular identification of PPRV from samples of sheep and goats in infected zones.

Results

Kits for rapid detection of morbillivirus in samples of eye and nasal swabs in two sheep farm from East Azarbaijan and Kerman provinces iden-



Figure 1
Test for rapid detection of morbillivirus. Positive (left), negative (right).

tified positive samples (Figure 1). In samples of whole blood and lymph nodes from two farms of East Azarbaijan and Kerman that were positive in the rapid detection method, after 4-5 days of inoculation onto Vero and LK cells, the CPE was observed and completed after 24-48 hours. In samples with cell lesions, the CPE observation were performed within 2-3 days after inoculation and completed within 12-24 hours (Figures 2 and 3).

In samples possessing cell lesions, the viral particles (150-300 nm) were similar to paramyxovirus particles in shape and size (Figure 4). These cells (containing lesions), were diagnosed positive by RT-PCR and nested PCR (Figure 5).

Discussion

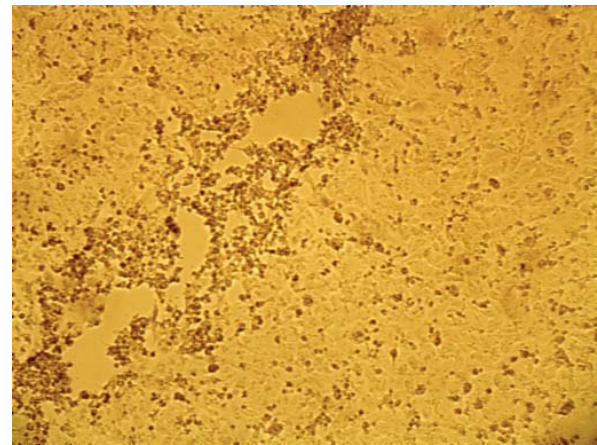


Figure 2
CPE observed in Vero cells, 3 days after inoculation of buffy coats of sheep, in third passage (magnification: x100).

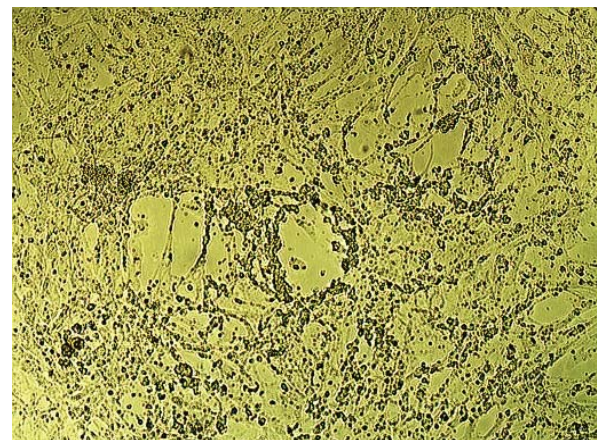


Figure 3
CPE observed in LK cells, in the first passage of sheep buffy coats (magnification: 100x).

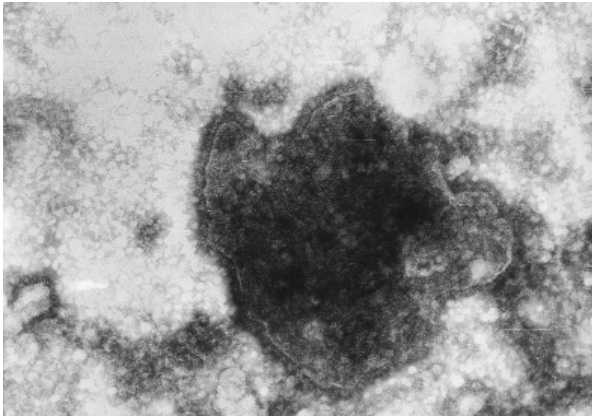


Figure 4
Viral particles resembling paramyxovirus particles observed in cells with lesions (magnification: x74100).

This study was performed to isolate PPR virus circulating in sheep and goats population in contaminated areas of Iran. In this study, the presence of PPR virus was demonstrated by clinical signs, virus isolation and molecular diagnosis. The RT-PCR is an effective test for diagnosis of PPR from field samples and identification of PPRV in cell culture supernatant. The specific primers based RT-PCR is now accepted as an alternative for virus isolation and diagnosis of PPR by Office Internationale des Epizooties for being simple, rapid, highly specific and sensitive [12, 13].

Initially, primary LK cells were employed for the isolation of PPRV from the field samples to increase the sensitivity of this technique. Later, Vero cells were preferentially used because of their continuity and having lesser chances of contamination. The Vero cells also proved suitable for the isolation of PPRV. CPEs observed in this study due to PPRV on LK and Vero cells were initial cell rounding, detachment from the surface, retraction, vacuolation and multi-nucleate syncytia formation. Other studies have also reported similar findings [13-16].

The two PPRV isolates obtained on primary LK and Vero cells were confirmed using RT-PCR. This low success rate for the isolation of PPRV may be attributed to the fact that the samples were collected at the time of necropsy after disease had run its full course. Successful isolation of PPRV depends on various factors including the phase of the disease during which samples were collected from donor animals [13, 17]. The samples collected during the infectious period i.e. in febrile phase are ideal for the isolation of PPRV. Following the regression of the fever the titers of infectious vi-

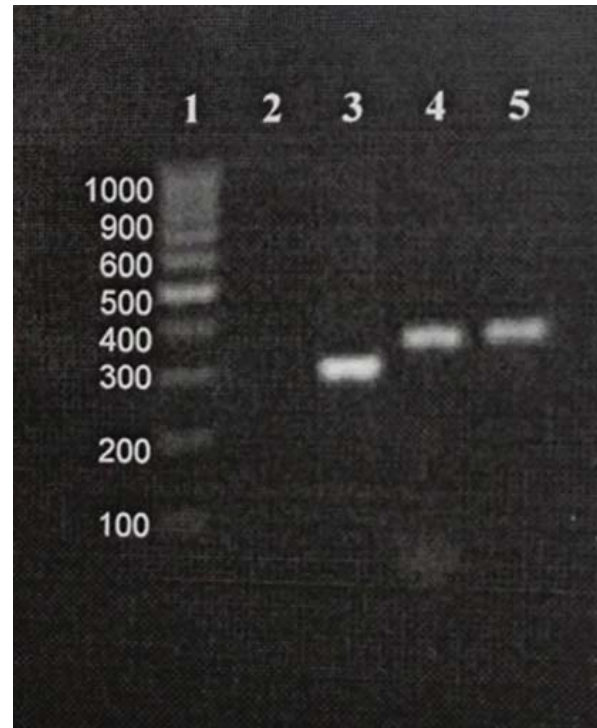


Figure 5
The electrophoresis of the RT-PCR and nested PCR of the PPRV F gene. 1) DNA size marker (GeneRuler 100 bp DNA Ladder, Fermentas), 2) Negative control, 3) Amplicon of F gene in nested PCR; 309bp, 4) Amplicon of F gene in RT-PCR; 370bp, 5) Positive control.

rus decline rapidly [13, 14]. The isolation of PPRV using Vero cells is reported to be difficult since it requires one or more blind passages to become tissue culture adapted, even from very fresh clinical samples or isolated virus [13, 14, 18].

The RT-PCR using primers for highly conserved sequences within F gene of PPRV proved suitable for diagnosis and/or confirmation of the PPRV isolates obtained on cell culture and effectively tracked the changes in virulence of PPR virus. The assay described by Forsyth and Barrett, (1995) has extensively been used for the specific diagnosis and molecular epidemiological studies of PPR virus [3, 13, 17]. The results of the present study are in agreement with the report of Bahadar et al. (2009), who reported that detection of PPR in buccal mucosa, nasal and ocular discharges was applicable by virus isolation using Vero cell cultures [19]. Also the present study is closely related to the previous findings by Housawi et al., (2004); Libeau et al., (1995); Forsyth and Barret, (1995) regarding isolation of PPR virus using Vero cell line [20, 21]. We applied the methodology which has been reported before to confirm the outbreaks

caused by PPRV in India, using Vero cell line for isolation of PPR virus, and amplification of PPRV F gene by RT-PCR [22]. Many researchers recommend the RT-PCR method for confirmation of PPRV[4].

Materials and Methods

Sampling

During 2013-2014, 168 samples were collected from whole blood, eye, nasal and oral swabs, and lymph nodes (bronchial, mediastinal, and mesenteric) of sheep and goats with clinical signs in Ghom, Fars, Ardebil, Ghazvin, East/West Azarbaijan, Lorestan, Tehran and Kerman provinces. The samples transferred to the virology laboratory under standard conditions (in less than 12-24 hours and on ice) and kept in -70°C until use. In animals with clinical symptoms the samples were taken from nasal and eye discharge. Then the samples were evaluated with rapid morbillivirus detection kit (Svanodip-Sweden).

Sample preparation and inoculation

Following the centrifugation at 117g for a period of 10-15 minutes the buffy coat layer was removed from blood samples. Also, 10% suspension was prepared from the homogenized lymph nodes. The prepared samples (buffy coat and homogenized lymph nodes) were inoculated (50 µl/cm²) on Vero cell line and Lamb Kidney (LK) primary cells. Following an hour of inoculation, DMEM (GIBCO) containing 2% FBS (GIBCO) was added to cell culture. A period of 14 days was considered to investigate each sample for cytopathic effects (CPE). In the absence of CPE, the blind passages were performed after two freeze-thawing steps and this was continued for at least 5 times.

To observe the viral particles using electron microscopy (EM), the affected cells were subjected to two times freeze-thawing procedure, and centrifugation at 6000 and 53000g. Then, the precipitated pellet was removed to be evaluated under a Philips 400 electron microscope.

RNA extraction and RT-PCR

Cell cultures with cell lesions were used for extraction of RNA by RNA extraction solution (Cinnagen) according to manufacturer's instructions. Reverse transcription (RT) reaction was performed using Revert Aid First Strand cDNA synthesis kit (Fermentas). The RNA and random hexamer primers were heated at 65°C for 5 minute and the thermal procedure was performed as follows: 25°C (5 minutes), 42°C (60 minutes) and 70°C (5 minutes). PCR reactions were performed using 2x PCR Master Mix (Cinnagen). The following primers were used [17, 23]:

PPRV F, 5' -ATCACAgTgTTAAAgCCTgTAgAgg- 3'
 PPRV R, 5' -gAgACTgAgTTTgTgACCTACAAGC- 3'
 PPRV F(Nested), 5' -ATgCTCTgTCAgTgATAACC- 3'
 PPRV R(Nested), 5' -TTATggACAgAAgggACAAG- 3'

Thermal program for amplification of PPR F was: 94°C (45 seconds), 60°C (30 seconds) and 72°C (20 seconds) for 40 cycles, starting with 95°C (4 minutes) and ending with a final extension of 72°C (5 minutes). For nested PCR of the F gene, the thermal program included 94°C (45 seconds), 52°C (30

seconds) and 72°C (20 seconds) for 22 cycles, starting with an initial denaturation of 94°C (4 minutes) and a final extension of 72°C (5 minutes).

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Author contributions

RF participated in the design of the study, writing the manuscript, final approval of the version to be published and managing the research of Animal Viral Diseases laboratory.

Conflict of Interest

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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جداسازی و تشخیص مولکولی ویروس طاعون نشخوارکنندگان کوچک (PPR) از مناطق آلوده ایران

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چکیده

با توجه به گزارش های متعدد در مورد طاعون نشخوارکنندگان کوچک (PPR) در مناطق مختلف ایران، پرداختن در موضوع تحقیقات گوناگون بویژه در مورد جداسازی و بررسی خصوصیات ژنتیکی ویروس های بومی جدا شده جهت استفاده برای تولید واکسن اهمیت زیادی دارد. در طی سال های ۲۰۱۴-۲۰۱۳، تعداد ۱۶۸ نمونه خون کامل، سواب چشمی، بینی و دهانی و غدد لنفاوی گوسفندان و بزهای با علائم بالینی در نه استان (قم، فارس، اردبیل، قزوین، آذربایجان شرقی، آذربایجان غربی، لرستان، تهران و کرمان) از کانونهای آلوده ایران جمع آوری شد. نمونه ها پس از آماده سازی، بر روی تیره سلولی Vero و سلول های پرایمری کلیه بره (LK) تلقیح و جهت مشاهده ضایعات سلولی (CPE) حداقل ۵ پاساژ متوالی از آنها تهیه شد. در نمونه های گوسفندان از دو استان (آذربایجان شرقی و کرمان)، CPE در هر دو نوع سلول مشاهده شد. در کشت های سلولی واجد CPE، مشاهده با میکروسکوپ الکترونی و تأیید تشخیص توسط روش RT-PCR انجام شد. در میکروسکوپ الکترونی، ذرات ویروسی در شکل و اندازه شبیه پارامیکسوویروس ها مشاهده شد. نتایج حاصل از روش RT-PCR مثبت بود. با این نتایج، PPR در برخی از کانون های مورد مطالعه تأیید می گردد.

واژگان کلیدی: طاعون نشخوارکنندگان کوچک، جداسازی، تشخیص، ایران