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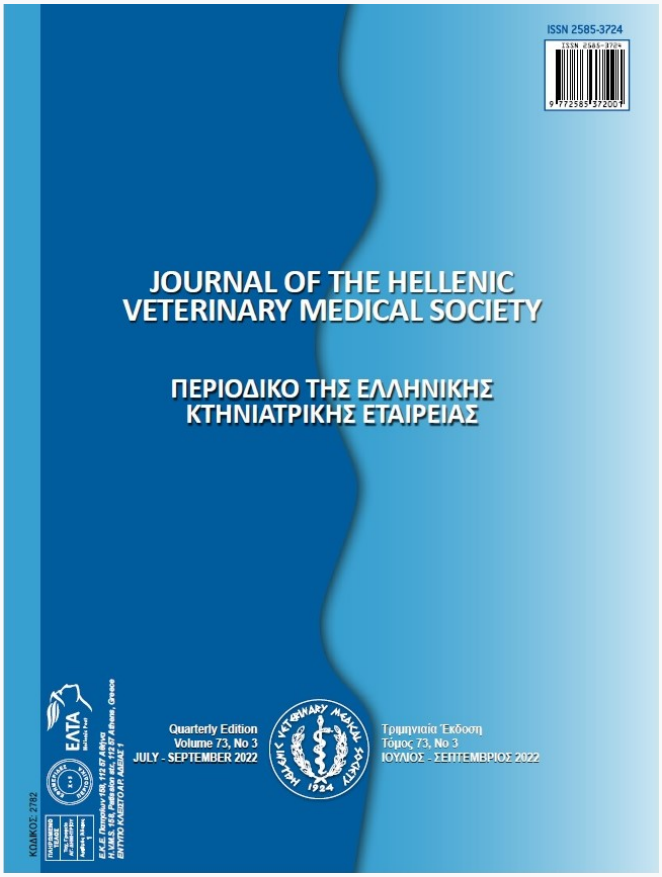

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Research article
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Molecular diagnosis of peste des petits ruminants virus (PPR) in goats and sheep populations

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ABSTRACT: Peste des petits ruminants (PPR) is an economically important viral disease of goats and sheep. The disease is confused clinically with other infections such as the mild strain of rinderpest in small ruminants. Effective control measures for PPR need that a proper and rapid diagnostic technique of disease. Therefore, the use of reverse transcriptase-polymerase chain reaction (RT-PCR) to detect suspected field samples collected from diseased goats and sheep in Dammam city, Kingdom of Saudia Arabia (KSA) has helped to give an effective diagnosis that was needed to control measure of the spread of the disease. This assay is based on the rapid purification of RNA on glass beads followed by the reverse transcription-polymerase chain reaction (RT-PCR). The primers (NP3/NP4) were used to amplify specifically a fragment of about 350 bp, that technique has a more specific and sensitive method for rapid diagnosis of disease.

***Keywords*:** Peste des petits ruminants virus; reverse transcriptase polymerase chain reaction; diagnosis; goats; sheep

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INTRODUCTION

Peste des petits ruminant (PPR) is a member of the genus Morbillivirus in the family Paramyxoviridae, a highly contagious viral disease of sheep and goats that leads to high morbidity and mortality rates in these species (Couacy Hymann et al., 2002. Clinically, the disease is characterized by severe pyrexia, catarrhal ocular and mucopurulent nasal discharges, erosive stomatitis in early stages followed by severe enteritis and pneumonia. The disease is leads to more economic losses, because of its high mortality rates, in young ages, costs of prevention and control (Nduaka and Ihemelandu, 1973; Taylor, 1984). The clinical disease is similar to rinderpest in cattle and characterized by clinical signs and lesions of respiratory and alimentary systems involvement (Obi et al., 1983). molecular biology techniques have permitted the development of specific and sensitive tests for rapid diagnosis. The structure and biology of peste des petits ruminants is now well known. As with other Morbilliviruses, it has an unsegmented negative stranded RNA genome encoding six structural and two non-structural proteins (Obi and Patrick, 1984). The causative agent is a morbillivirus, peste des petits ruminants virus (PPRV). It is closely related to rinderpest virus (RPV), another member of the morbillivirus genus which can also cause a disease in small ruminants (Anderson et al., 1990; Couacy-Hymann et al., 1995). PPR has an endemic disease in Nigeria and other parts of Africa, the Middle East, Bangladesh, Pakistan and India (Shaila, et al., 1989; Yamada, 1990; Choi et al., 2003), but up till now, the disease which is still widespread (Mcculloch, 1951). In Saudi Arabia, PPR was suspected clinically in sheep (Asmar et al., 1980) in gazelles and deer (Hafez et al., 1987). PPRV was successfully isolated during an outbreak in indigenous goats, Goats are usually more severely affected than sheep (Abu-Elzein et al. 1990). The diagnosis of PPR was based on conventional tests such as agar gel immunodiffusion test, counter immunoelectrophoresis, haemmagglutination test, immunocapture enzyme linked immunosorbent assay (ELISA) (Mathew, 1980; Libeau et al., 1994; Brindha et al., 2001; Dhar et al., 2002) or virus isolation (Diallo et al., 1995). These assays are mostly replaced by genome based detection techniques such as reverse transcription polymerase chain reaction (RT-PCR) (Mornet et al., 1956; Diallo et al., 1995; Couacy-Hymann et al., 2002). However, whatever the qualities of the new technique it is important to know though not necessarily used for all outbreaks, that virus isolation still remains the gold standard diagnostic technique (Govindarajan et al., 1997). The reverse transcription-polymerase chain reaction (RT-PCR) is described for specific detection of PPRV. In this test, we use a simplified RNA extraction technique instead of the classical phenol-chloroform extraction method (Gibbs et al., 1979; Barret et al., 1993).

The objective of this work was conducted to demonstrate the sensitive and specific technique for virus detection and the possibility of its use as a curette diagnosis of PPR in the field.

MATERIALS AND METHODS

Samples Required for study

From live animals suffered from signs of PPR virus: swabs of the conjunctival discharges (Lachrymal swab), and from the nostrils (nasal Swab), should be taken using sterile absorbent cotton-wool swabs, then the samples were suspended in 200 µl of phosphate buffer saline (PBS, pH 7.4). The fluid was expressed from the swab within few hours after collection, and centrifuged at 10.000 rpm for 5 min and then stored at -20ºC. The tissue samples were collected from a goats and sheep dying of a PPRV-like disease. Fragments of organs collected for virus isolation placed in sterile PBS. The samples for analysis were transported to branch of the Virology, in the Veterinary Diagnostic laboratory, in Dammam. During the early stage of the disease, whole blood samples is also collected in -tubes contain EDTA as anticoagulant for virus isolation**.** Samples should be taken from animals with suffer to high body temperatures preferably before diarrhea starts. Samples should also be collected aseptically, chilled on ice and transported under refrigeration to laboratory **(**Diallo et al., 1989; Diallo et al., 1994**)**.

RNA-isolation

RNA was extracted from all the samples using RNeasy kit (Qiagen GmbH, Hilden, Germany). the samples were put into a sterile microcentrifuge tube containing 250 µl extract mixture (20 % Chelex 100® Resin, 250 mg proteinase-K and 40 mM Dithiothreitol). The samples were then incubated at 37ºC overnight. The mixture was boiled for 8 min to inactivate proteinase-K. The samples were centrifuged at 13.000 g for 3 min to pellet any undigested hair, skin and the Chelex 100® Resin. The supernatant, which contained RNA, was transferred into a clean sterile tube and subjected to purification with Qiagen Tissue Kit®

(Qiagen GmbH, Hilden, Germany). The mixture was transferred into a RNeasy® spin column placed in a 2 ml collection tube and centrifuged at 6.000 g for 1 min. The RNeasy® spin column was placed in a new 2 ml collection tube. Then, 500 µl buffer AW1 was added and centrifuged for 1 min at 6.000 g and the RNeasy® spin column placed in a new 2 ml collection tube. In addition, 500 µl buffer AW2 was added and centrifuged for 3 min at 13.000 g until the RNeasy® membrane dried. The RNeasy® spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl buffer AE was pipetted directly onto the RNeasy® membrane. Consequently, the tube containing buffer and RNA samples was incubated at room temperature for 1 min. The mixture was centrifuged for 1 min at 6.000 g. The supernatant containing the extracted and purified RNA was stored at -20ºC until use. The DNA concentration was estimated by means of a spectrophotometer (Gene Quant Calculator®, Amersham Pharmacia Biotech, Freiburg, Germany).

First strand cDNA synthesis

In briefly, 7 µl of extracted RNA was mixed in a 0.2 ml sterile tube with 2 µl of mix primers (15 pmol/ µl of each), 1 µl of RNase inhibitor (10 U/ µl), 5 µl ll of cDNA synthesis bulk (first strand cDNA synthesis kit, Healthcare - Austria). The mixture was briefly spun at 2000 rpm for 1 min

Amplification PPR virus

RT-PCR were carried out in 25 µl reaction volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl*2*, 100 µM each of the four dNTP´s (dATP, dCTP, dGTP, dTTP), 1 unit of Ampli Taq Gold® polymerase (Applied Biosystems, GmbH, Darmstadt, Germany), approximately 5 ng cDNA and 0.5 µM of each of the oligonucleotide primers, Foeward NP3: (5'-TCT CGG AAA TCG CCT CAC AGA CTG-3' and Reverse NP4: (5' -CCT CCT CCT GGT CCT CCA GAA TCT -3').

Cycling parameters

PCR was performed by incubating the samples at three temperatures corresponding to three steps (denaturation, annealing and extention) in a cycle of amplification. The polymerase chain reaction was carried out in a Thermocycler Trio-Thermoblock*TM* (Whatman Biometra, Göttingen, Germany), programmed to perform a denaturation step at 5ºC for 10 min (to activate the Taq Gold® DNA Polymerase), followed by 35 cycles consisting of 30 sec at 94ºC, 30 sec at 55ºC and 45 sec at 72ºC. The last extension step was 5 min longer. The samples were stored at 4ºC until use

Gel electrophoresis

Two percent agarose gel containing 2.5 µl ethidium bromide in 1 x electrophoresis buffer was prepared. The electrophoresis chamber was filled with buffer solution (1 x electrophoresis buffer) until the top surface of the gel submerged by approximately 1 mm (about 400 ml). Two microlitres of the sample buffer was mixed with 10 µl PCR product and centrifuged at 10.000 g for 20 sec. The DNA ladder (100 bp) was prepared. The samples (10 µl) and DNA ladder (10 µl) were placed into the agarose gel wells. The chamber was connected to a 60 Volt power supply and

Table 1: The results of examined samples by RT-PCR

species	Type of samples	No. of examined samples From each species	No. of Positive samples (%)
sheep	Whole Blood	13	13 (100%)
goat	Whole Blood	13	13 (100%)
sheep	Lachrymal swab	8	8 (100%)
goat	Lachrymal swab	8	8 (100%)
sheep	Nasal swab	6	6 (100%)
goat	Nasal swab	6	6 (100%)
sheep	Lung tissue	5	5 (100%)
goat	Lung tissue	5	5 (100%)
sheep	Liver tissue	3	2 (66.6%)
goat	Liver tissue	3	3 (100%)
sheep	Spleen tissue	3	3 (100%)
goat	Spleen tissue	3	3 (100%)
sheep	Lymph node	2	1 (50%)
goat	Lymph node	2	2 (100%)
Total		80	78 (97.5%)

the run was initiated. The separated DNA products were detected using UV transillumination. The PCR products containing a RNA sequence of 350 bp were amplified and later photographed using a Polaroid® camera **(**Ularamu et al., 2012**)**.

RESULTS

From our results RNA extracted from various samples as, blood, swabs and tissues of goats and sheep suffering to fever and lesions was successfully amplified using NP3/NP4 primers. A single PCR amplicon, corresponding in size to the predicted 350 bp fragment was observed (Figure 1). The results revealed that 78 samples positive out of 80 samples. The samples type and number positive by RT-PCR was described in the **(Table 1)**. A total of 4 organs from goats and sheep (lung, liver, spleen and lymph node), nasal swabs and lachrymal swabs, and whole blood samples. The results were obtained the high sensitivity and specificity of RT-PCR and its more accurate for diagnosis of PPR virus in the outbreaks of disease.

DISCUSSION

RT-PCR, rapid and specific diagnostic technique for detection of PPR virus has become possible (Raj et al., 2008; Mornet et al., 1956; Mccaustland et al., 1991; Nanda et al, 1996)**.** RT-PCR is a sensitive method for the detection PPRV Shaila et al. (1996). PCR is carried out with two primers that detect one virus strain (Couacy-Hymann et al., 2002). The result of the investigation was the finding that virus-specific RNA was detected in lachrymal and nasal swabs Forsyth and Barrett (1995). The swabs taken from the infected animals in the field not only provided a suitable source of viral RNA, but also was not subject to the same storage and transport problem associated with postmortem tissue samples which is in agreement with Forsyth and Barrett (1995). RT-PCR to detect PPR RNA in pathological tissues Shaila et al. (1996). These conditions are sometimes difficult to meet in many regions due to high environmental temperature. With the immunocapture (Libeau et al., 1994, 1995) or the RT-PCR tests, PPR infection could still be confirmed on samples where there is no more viable virus. RT-PCR used for the identification of PPRV of different samples origins as shown in figure 1. They allow the specific amplification of a target of 350 nucleotides long from all PPR tested samples.

CONCLUSION

RT-PCR, a sensitive and specific method for diagnosis of PPRV. And can be used to confirm of the disease. Assessment, Control and Eradication of disease.

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CONFLICT OF INTEREST

The author declares no conflict of interests.

Figure 1. The amplified 350 pb products were analyzed by electrophoresis on 1.5% agarose gel stained by ethidium bromide. **Lane M**, 100 bp molecular weight marker; **lane 1**, Goat Blood; **lane 2**, Sheep Blood; **lane 3**, Goat Nasal swab; **lane 4**, Sheep nasal swab; **lane 5**, Goat liver tissue; **lane 6**, Goat Lymph tissue; **lane 7**, Goat lung tissue; **lane 8**, Sheep Lymph tissue; **lane 9**, Sheep liver tissue; **lane 10**, negative control

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