Isolation, Serological and Real time PCR diagnosis of Peste Des Petites Ruminants virus in naturally exposed Arabian Gazelle in Saudi Arabia

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Abstract

Oculo-nasal swab and serum samples collected from peste des petits ruminants (PPR)-suspected Arabian Gazelle (Gazella gazella) were tested . For the presence of peste des petite ruminants virus (PPRV) [pest of small ruminants virus] or it's RNA; Immune-capture enzyme linked immunosorbent assay (Ic-ELIZA), real time reverse transcription-PCR (rRT-PCR) assay using SYBR Green 1 chemistry as well as virus isolation (VI) were done. The serum was examined for the presence of the PPRV anti-bodies by competitive enzyme linked immunosorbent assay (C-ELIZA). The swab samples and harvested inoculated cells were positive by Immune-capture ELIZA and rRT-PCR. While the tested serum was negative for PPRV anti-bodies. The study indicated the scenario of probably virus circulation in these game animal population and prevalence in actual outbreaks situation, which may be kept in mind while deciding the vaccination strategy for the control of disease. For the authors this is the first report of PPRV isolation and detection among Arabian Gazelle in Saudi Arabia.

Keywords: Arabian Gazelle, enzyme linked immunosorbent assay, Peste des petite ruminants virus, Real time, Sybr Green.

Introduction

Peste des petits ruminants virus (PPRV); a member of the genus Morbillivirus in the family Paramyxoviridae (Gibbs et al., 1979, Murphy et al., 1995), Order Mononegavirales (Murphy et al, 1999); the causative agent of Peste des petits ruminants (PPR), or goat plague (Dhar et al., 2002; Asim et al., 2009); is an acute highly contagious disease of goats and sheep, characterized by fever, erosive stomatitis, conjunctivitis, gastroenteritis and pneumonia and morbidity and mortality can be as high as 100% and 90%, respectively (Abu-Elzein et al., 1990, Dhar et al., 2002). PPRV are enveloped, pleomorphic particles containing single stranded RNA, approximately 16 kb long with negative polarity genome (Barrett et al., 2005). Following the first report of the disease in sheep and goats (Gargadennec and Lalanne, 1942), for many years it was believed to have remained restricted to western part of the African continent.

However, in recent years, the disease has been recorded in several parts of the world, Southern Asia including India, Pakistan and Nepal; Near East and the Arabian Peninsula including Iran, Iraq, Jordan, Kuwait, Lebanon, Oman, the United Arab Emirates and Yemen (Shaila et al.,1989; Lefevre and Diallo, 1990; Taylor et al.,1990). PPR can be confused clinically with rinder pest, and hence the clinical observations for both the diseases should always be confirmed by a laboratory test. The diagnostic techniques used in the past viz. VNT, AGID and isolation of the virus in cell culture are time consuming and labour intensive. With the advent of molecular biological techniques like PCR, rapid and specific diagnosis of PPR has become possible (Forsyth and Barrett, 1995; Couacy-Hymann et al., 2002).

In Saudi Arabia, PPR was suspected clinically in sheep (Asmar et al., 1980) , gazelles and deer (Hafez et al., 1987), in both cases no virus could be isolated, antelope and other small wild ruminant species was also be severely affected (Abu-Elzein et al., 2004). PPRV was successfully isolated during an outbreak in indigenous goats (Abu-Elzein et al. 1990). However; there is presently no published data concerning the epidemiology of PPR among wild ruminants in the country. The present study was designed to gain an insight into the report , isolation and detection of PPRV

among Arabian gazelle for the first time in Saudi Arabia

Material and Methods

Animal: The materials (oculo-nasal swaps and serum) for the study were collected during submission of one Arabian mountain Gazelle (Gazella gazella) to clinical examination at Riyadh, Central Veterinary Laboratory (RCVL), Ministry of Agriculture KSA; from a flock raised in Riyadh and suspected to have PPR. In April 2009, the initial PPR case came from a flock containing 33 Arabian Gazelles . Some animals died within a 3 days after exhibiting clinical signs, such as fever, coughing and diarrhea. According to the history, the animals had not been vaccinated against PPRV infection as well as there are neighboring sheep and goat flock showed a disease like PPR infection. For laboratory analysis, the samples were stored at –20C° until assayed.

Bacteriological and fungal examination: Trails for isolation and identification of pathogenic bacteria or fungus from collected samples were done.

Cell culture and virus isolation (VI): The cell line of African green monkey kidney (Vero) cells was obtained from the Department of Virology section, (RCVL), where 0.2 ml of each sample was inoculated on confluent monolayer cell culture prepared previously in tissue culture flask (Nunc®) according to Burlson et. al. (1997). Harvested passage in each cell system was stored at – 65C° until used.

Immune- capture ELISA (Ic-ELISA) : A commercial Ic-ELISA was used to detect PPR viral antigen in both clinical samples and harvested inoculated Vero cells. It was applied according to manufacturer's instructions [Biological Diagnostic Supplies Ltd. (BDSL)®, Flow laboratories and Institute for Animal Health Pirbright, Surrey, England]. Ic-ELISA was developed according to (Libeau et al., 1994) where the nucleocapsid (N) protein, is captured using a virus specific mouse monoclonal antibody (Mab). Then biotinylated Mabs directed against specific antigen domains on the N are used to detect the virus in supernatant from the pathologic samples. Tested samples demonstrating percentage positivity (PP) values of 15% or greater are considered positive. PP = OD of test sample / OD of reference PPR Ag x100.

Competitive ELISA (C-ELISA): A commercial C-ELISA kit ; used to detect sero-positive animal ; was applied according to manufacturer's instructions [Biological Diagnostic Supplies Ltd. (BDSL)®, Flow laboratories and Institute for Animal Health Pirbright, Surrey, England]. C-ELISA were developed according to (Libeau et. al., 1995) using a virus neutralizing monoclonal antibody directed against the nucleoprotein (N) specific for PPRV. All serum samples tested in the present study were processed in duplicates. Samples with a % colour inhibition (% inhibition of the enzymatic colour reaction) of equal to or greater than 50%, when compared to wells containing the Mab control (no serum), were considered positive. % inhibition (PI) = 100 - (OD of the sample/ OD of the control) X %.

RNA extraction: Naso-ocular swabs ,tissue culture harvests, and Saudi field isolate 406/29/08 [(Abdel Baky; et al.2008) used as positive control] were extracted using QIAamp® Viral RNA Mini Kit (Qiagen®, Germany), following manufacturer's instructions. In brief, the 560µl of the lyses buffer (Buffer AVL® containing Carrier RNA) was mixed with 140µl of clinical sample or cell culture supernatant and incubated at room temperature for 10 minutes then 560 ul of absolute ethanol was added to the sample and mixed. Micro centrifuge tube was short centrifuged to remove drops from inside of the lid and each 630 µl of the solution was applied to the QIAamp® spin column and centrifuged at 8000 rpm for 1 minute. Viral RNA was washed with 500µl of washing buffer 1(Buffer AW1®) & washing buffer2 (Buffer AW2®) sequencelly by short centrifugation. High quality RNA was eluted in a 60 µl of RNase- free buffer (Buffer AVE®).

Reverse Transcription (RT) : Synthesis of cDNA was performed in 20 µl reaction using transcriptor first strand cDNA synthesis kit (Roche®, cat no. 04 374 012 001) according to the manufactures procedures. Reverse transcription was carried out on dry block at 55C° for 30 minutes followed by 85C° for 5 minutes. RT products were cooled on ice and stored at -20°C until use.

SYBR Green assay: Two specific PPR virus oligonucleotide primers b1(5\-AGTACAAAAGATTGCT GATCACAGT- 3\) and d2 (5\- GGGTCTCGAAGGCTA GGCCCGAATA - 3\) were used , based on sequences from highly conserved regions within the PPR virus genome (Dhar, et al.,2002), manufactured by TIB-MOL BIOL syntheselabor GumbHm Berlin, Germany. PCR was performed on a capillary system of LightCycler TM (Roche Diagnostics). Each reaction had a volume of 20 µl including 15 µl of reaction mixture containing Faststart DNA Master plus SYBR Green 1(Roach) and 10 pmol/µl concentration of each primer and 5 µl of cDNA. Cycling was performed as follows: initial denaturation step at 95°C for 10 min, 40 cycles of 10 seconds at 95°C to denature and 5 seconds at 62°C for annealing and 15 seconds at 72°C for extension. Fluorescence data were acquired at the end of each cycle in a single step. Once the plateau phase of the PCR had been reached, amplification was stopped and a standard melting curve analysis was performed (95°C for 0 second, 65°C for one minute, and a 0 second rise to 95°C then cooling cycle of 40 °C for 30 seconds) with continual fluorescence measuring. PCR data were analyzed using Light-Cycler2 software version 4.05.

Results

Clinical Findings: Catarrhal nasal discharge and crusts were present around the nostrils. Erosions of the oral cavity were localized to the lips, soft and hard palates and consisted of, gray to yellow pseudo membranous foci. Prominent erosions and ulcerations were detected on the gum, inner surfaces of the lips and mechanical papillae. Nasal conchae was diffusely hyperemic. Dehydration, severe lachrymal and ocular discharge were seen (Figs. 1&2).

Bacteriological examination: Neither significant pathogenic bacteria nor fungi were isolated

VI: The inoculated Vero cell culture mono layers showed cell rounding four days post inoculation. The cytopathic effect progressed to form cell aggregates. Syncytium cell formation was seen at low level in cultured systems. One week after inoculation, destruction of the cell mono layers was evident (Figs. 3).

Ic-ELISA and C-ELISA: Examined naso-ocular swabs and harvested tissue cultures were positive for detection of PPRV antigen using Ic- ELISA , while the examined serum sample was found negative for PPR antibodies with C-ELISA .

Molecular detection (PCR): Real-time PCR assay revealed positive amplification and melting curve analysis for All examined samples (2 oculo-nasal & 2 harvested tissue culture) like that detect for positive control PPRV at a dilution of 1:10, corresponding, None of the PPRV negative controls tested was positive by real time PCR assay. Reference PPRV, Saudia field isolate 406/29/08 was detected by the15 cycle of amplification with temperature of melting (Tm) score of 87.38 Co, whereas the samples were detected between the 27th and 30th cycle of amplification with one peak of Tm (87.77 Co ~ 87.87Co).

Discussion

Three methods may be used to diagnose and monitor the distribution and prevalence of PPR: Case recording of PPR outbreaks, detection of the virus or its nucleic acid (RNA) and serological detection of PPR specific antibodies . Although; case recording of PPR outbreaks could give some clues in the areas where the disease is endemic, laboratory diagnosis is essential for confirmation.

The present study reported suspected severe of PPR in one Arabian Gazelle during winter 2009. Clinically, the affected animal showed fever, anorexia, Catarrhal nasal discharge, conjunctively encrustation in the medial canthus , sever lachrymal and ocular discharge, ulcerative stomatitis, profuse diarrhoea and bronchopneumonia. These symptoms agree with those described by (Taylor, 1984; Lefevre and Diallo, 1990 ; OIE, 2000) in sheep or goat PPRV infection . The same clinical signs have been reported in wildlife resulting in deaths of gazelle, ibex, gemsback and larsian sheep (Furley et al., 1987 and Abu-Elzein et al., 1990). Nonetheless, PPRV was isolated from an outbreak of Rinderpest like disease in Indian buffaloes in 1995 (Govindrajan et al., 1997). PPRV was also suspected to be involved in the epizootic disease that affected single humped camels in Ethiopia in 1995–1996 (Roger et al., 2000, Roger et al., 2001). Experimental infection of camels in Saudi Arabia with PPR virus resulted in only subclinical infection or mild respiratory disease although; PPRV infection was transmitted to other camels and goats but not to sheep (El-Hakim, 2006). PPRV transmission from live stock remains a potential threat to the gazelle and other wild ungulates of the region (Bhatnagar et al., 2006).

Only two PPRVs were isolated from nasal swab samples of two sheep flocks from Sakarya Province Turkey . The reason for the poor success in isolating virus could be the nature of the samples (Özkul et al., 2002) since; the PPRV would not be expected to survive for a long time outside the host (Diallo et al., 2003). In previous studies, virus isolations were made from spleen, mesenteric lymph nodes (Furley et al.,1987), or intestinal epithelial smears (Taylor and Abegunde, 1979) collected during necropsy of affected animals by inoculation onto Fetal Lamb Kidney (Furley et al.,1987,) or Vero (Taylor and Abegunde, 1979) cells. In our study PPRV isolation were successfully done from all inoculated oculo-nasal swabs, however; all samples were taken from surviving animal that shows the clinical phase of the disease when the virus is secreted, and so were likely to yield more virus particles from swabs. Detection PPR virus in cultured cells can be very valuable method for diagnosis as well it provides live virus for biological characterization studies. If facilities are available, it should always be attempted and isolated viruses stored for later studies.

The C-ELISA was developed for detection of antibodies to PPR virus in serum samples of goats and sheep. The test used monoclonal antibody to a neutralizing epitope of hemagglutinin protein of the virus. Efficacy of C-ELISA compared very well with VNT, having high relative specificity (98.4%) and sensitivity (92.4%).The sensitivity of C-ELISA for PPR sero-surveillance was more (95.4%), if the target population was non-vaccinated. It was opined that the C-ELISA developed could easily replace VNT for sero-surveillance, seromonitoring, diagnosis from paired sera samples and end-point titration of PPRV antibodies (Singh et al., 2004).

In the present study, the collected serum samples were screened for the presence of antibodies against PPRV using C- ELISA; With regards to sensitivity of C-ELISA for PPR, all examined serum samples were negative. The apparent absence of

PPRV antibodies could attributed to the fact that usually the antibody titers are low and undetectable at the beginning of the infection and increase to reach high level in recovered and clinically healthy animals in which previous infection were more likely to be occurred specially in endemic area and this may be explain the drastic melody recoded specially PPR is immunosuppressive virus in nature as revealed by Dhar et al.,(2002) and can occasionally overcome the resistance of large ruminants and lead to the development of clinical signs similar to rinder pest (Diallo et al., 2007).

However, other factors which determine strain virulence remain essentially unknown and those identified so far have not been related to a single event. The capability of cells to be infected and support active virus replication has important implications on the pathogenesis and epidemiology of the disease. Therefore, we interested to test the cell susceptibility of this PPRV isolate latter on. The Ic- ELISA was found suitable for routine diagnosis of PPRV in field samples such as ocular and nasal swabs (Diallo et al., 1995). Of the five swab samples tested for PPRV parallel to blood samples from the same animals with Ic-ELISA, four (80.00%) including three nasal swab and one oral swab yielded positive results, while all the four blood samples from the same goats were negative with the same test. Comparable reports regarding suitability of swab or blood sample have not been found in literature. However, difference in tissue distribution of PPRV has been observed (Pawiya et al. 2004).), although; The suitability of the samples for dot ELISA revealed that tissues were preferred than blood (Obi and Ojeh 1989). Thus, from present work, it appears that oculo-nasal swab samples (crude or harvested inoculated Vero cell) are better for detection of PPRV antigen. In a comparative study; Ic-ELISA was positive for 71.9 per cent PPR suspected samples, while single passage virus isolation was positive for 65.2 per cent samples (Saliki, et al. 1994). This also appears logical as the virus excretion from the nasal and ocular openings is high during and after the active infection, which may remain for a longer period of time than the comparative time duration of the viraemic phase when the virus can be detected from blood or tissues. Therefore, the success of detecting the virus in blood depends on the time of sampling and there are more chances of missing the presence of PPRV in the blood (Tiwari.2004). Traditionally, scientists and clinicians have used assays that involve growing cultures to screen samples for the presence of pathogenic micro-organisms.

Now-a-days, culture- based methods for pathogen detection are rapidly being replaced by faster and more specific Real Time PCR assays that discriminate between micro-organisms based on a signal from specific nucleic acid sequences (Johnson, et al. 2005). Detection of PPRV genetic material is performed by the reverse transcriptase polymerase chain reaction (RT-PCR) which requires special facilities and expertise, despite its high cost, it is now one of the tests used most frequently in reference centers, together with enzyme linked immunosorbent assay, because it is rapid, accurate, highly sensitive and can discriminate between PPR and rinder pest, combining this test with nucleotide sequencing provides virus characterization information that is useful in epidemiological studies (FAO,1999). In this our study, SYBR Green Real-time RT-PCR (rRT-PCR) assay for identifying infection caused by PPRV was applied with the primer set that has been used in application of traditional RT-PCR for amplification of the (F) protein gene of PPRV (Dhar, et al.,2002). Very high specificity and sensitivity for the detection of PPRV were obtained by the present rRT-PCR where all examined samples (crude or harvested inoculated Vero cell) and reference PPRV strain were detected.

These all results in conclusion could be suggest that the described real-time PCR assay has the potential to be used for the rapid detection of PPRV isolates and qualitative/quantitative measurement of the virus load. The assay offers an attractive alternative

Figure-1. Lachrymal secretion, dehydration and ruff coat of Arabic Gazelle

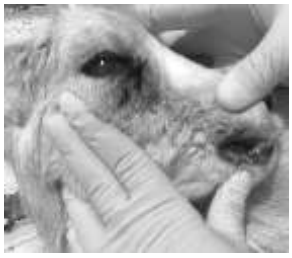

Figure-2. Erosion and ulceration of gum of affected Gazelle

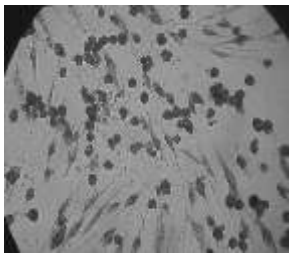

Figure-3. Rounding, aggregation, Syncytium formation and destruction of the cell monolayer

method for the diagnosis of PPRV. Although; PPRV had been earlier confirmed serologically in KSA in Gazelle, and except for one report publishing of PPRV isolation, no documentation with regards to detection of PPR virus from clinical cases could be traced in literature. Thus, the present study may be appears to be the first such report of isolation, serologically and real - time PCR detection of peste des petites ruminants virus in naturally exposed Arabian gazelle in Saudia Arabia.

The concrete of PPRV infection in gazelle accompanied by PPR like infection in neighboring sheep and goat flock strongly suggests that these flock was the source of the PPRV infection . This, in turn, indicated the scenario of probably virus circulation in these game animal population and prevalence in actual outbreaks situation, which may be kept in mind while deciding the vaccination strategy for the control of disease. The results could also stimulate research to identify the gene sequences and lineage of the PPR isolated virus.

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