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CASE REPORT
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Monkeypoxvirus (MPXV) in a Baby Monkey - Molecular Investigation

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ABSTRACT

***Background*:** Monkeypox is a zoonotic virus that transmits from animal to human and human to human. Despite the fact that the Monkeypox virus (MPXV) was initially isolated in 1958 and the first human case was reported in 1970, thousands of cases have been documented in European countries, the United States, and Turkey in recent years. The goal of this work was to present the results of the first molecular diagnostic analysis of MPXV-induced skin lesions in a baby monkey in Turkey.
***Case*:** In 2019, a baby monkey in Antalya Zoo developed skin lesions after being brought from Africa by a female monkey. The baby monkey died within a few weeks due to postnatal respiratory problems. Skin lesions were taken from this case and analyzed molecularly and virologically at the Department of Pathology, Burdur Mehmet Akif Ersoy University Faculty of Veterinary Medicine, and the Department of Virology. To perform molecular diagnosis, skin and pock lesions developed after inoculation on the chorioallantoic membrane (CAM) of the embryonated chicken eggs (ECE) were extracted, and 25 parapoxvirus (orf virus) type-specific primers and 2 MPXV type-specific primers were examined. The inoculum was prepared from the skin lesions for virus isolation, which was carried out in Vero cell culture. Then, the virus titer was determined using the microtitration method. The polymerase chain reaction (PCR) test with 25 parapoxvirus (orf virus) type-specific primers on the extracted skin lesion samples did not detect the presence of viral genome. The presence of viral genome was detected in 1 of the 2 MPXV type-specific primers acidophilic-type inclusion body (ATI gene) in the skin lesion extracts. However, the presence of viral genome could not be determined by the Gabon (1/2) primers. Cytopathological effects (CPE) were observed 72 h. after inoculating the skin lesion inoculum in Vero cells. The virus titer was determined to be 10^{22} $TCID_{50}$ /mL. During immunohistochemical examination with orf virus antibodies, positive reaction was observed in the epidermal cells.
***Discussion*:** The researchers have been investigated the reservoir or natural hosts of MPXV. The virus has been found in squirrels, rodents, monkeys and chimpanzees. Multiple examinations have revealed that a number of animal species, primarily rodents and nonhuman primates, are susceptible to the virus. The presence of MPXV was detected in the skin lesion of a baby monkey in the study. Capripoxvirus, Cervidpoxvirus, Avipoxvirus, Molluscipoxvirus, Orthopoxvirus, Leporipoxvirus, Suipoxvirus, Yatapoxvirus, and Parapoxvirus are all members of the Chordopoxvirinae subgroup. However, no relationship between MPXV and parapoxvirus (25 type-specific primers) was found in the extracted skin lesion samples. Virus isolation, electron microscopy, (PCR), IgM and IgG ELISA, immunofluorescence assays, and histopathological examination are all laboratory diagnostic procedures that can be used to diagnose monkeypox infections. To detect the MPXV agent and/or specific viral DNA sequences, real-time or traditional PCR techniques should be utilized. Hemagglutinin, ATI gene, and the crmB gene are all MPXV genes which are commonly used for conventional PCR testing. A MPXV type-specific primer (ATI gene) observed the presence of viral genome in the skin lesion extract. After inoculating the skin lesion inoculum in Vero cells, (CPE) were observed. The virus titer was found to be extremely high. Positive reactions were seen in epidermal cells during immunohistochemical testing with orf virus antibodies.

Keywords: Monkeypox virus, molecular diagnosis, virological diagnosis, immunohistochemistry, Parapoxvirus.

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INTRODUCTION

Monkeypox virus (MPXV) is an enveloped, double-stranded DNA virus that belongs to the Orthopoxvirus genus in the *Poxviridae* family [6,18]. Variola, cowpox, and vaccinia viruses are also included in this group [33]. MPXV has 2 genetic groups, Western and Central Africa [13,27]. Various animal species susceptible to MPXV have been identified, including squirrels, Gambian pouched rats, dormice, non-human primates, and rope squirrels [2,17]. However, detailed research is needed to identify the exact reservoir and natural circulation of the virus [35]. MPXV can be transmitted from animals to humans through blood, body fluids, skin and mucosal lesions (especially rodents), consumption of undercooked infected animal meat, contact with wild animals (sick/dead), human-to-human transmission through respiratory secretions, skin lesions, contaminated objects, prolonged face-to-face contact, transmission from mother to offspring through the placenta, venereal route, and individual susceptibility due to the loss of efficacy of smallpox vaccine [4,17]. Furthermore, physical contact is considered the most effective mode of transmission [22]. Serological diagnosis (antigen/antibody) is not reliable for orthopoxvirus infections due to their cross-reactivity and false positivity in individuals who have been vaccinated [21]. Therefore, it has been suggested that a good diagnostic method for MPXV cases is the detection of viral genome by polymerase chain reaction (PCR) from skin lesion fluids or crusts obtained using sterile swabs [5].

The aim of this study was to investigate the presence of MPXV in skin lesions developed in a baby monkey by molecular methods.

CASE

A female monkey brought from Africa to Antalya Zoo gave birth to a female baby monkey at the end of the normal gestation period. Skin lesions, on the other hand, were observed developing within a few weeks. Because the mother did not care, the baby monkey was fed by the caretakers, and subsequently respiratory problems developed, resulting in the baby monkey's death when it was around 1 month old. The skin lesions obtained from this case brought to the Pathology Department of Burdur Mehmet Akif Ersoy University Faculty of Veterinary Medicine were examined, immunohistochemically in the Department of Pathology and molecularly and virologically in the Department of Virology. After the extraction (DNeasy Blood & Tissue Kit)[1] of the skin lesion taken for molecular diagnosis, the extract obtained was inoculated on the chorioallantoic membrane (CAM) of the embryonated chicken eggs (ECE), and 25 parapoxvirus (orf virus) type-specific primers [GIF/IL-2, PPP1 and PPP4, PPP3 and PPP4, ATI gene primer, Orf 1 Orf 2, ORFV-B2L, ORFV-A32, vIL-10-3 vIL-10-4, OVA32 LF1 OVA32 LR1, VR1 VR2, PPV JGR, ORFV011-A, ORFV059-A, ORFV011-B, ORFV059-B, ORFV109, ORFV110, ORFV127, 045 gene, B2L gene primer, F1L gene primer, Alpha-tubulin, ORFV011-C (B2L), ORFV059-C (F1L)] were examined [1,3,8,10,11,14,16,20,25,26,29,34,36]. In addition, the skin lesion extract and extract of pock lesions developed after inoculated on the CAM of the ECE were also analyzed for 2 types of MPXV-specific primers (Table 1). Inoculation was performed on Vero cell culture (25T flask, CCL-81™)[2] from the inoculum prepared only from the skin lesions of the baby monkey. Control checks were performed under a tissue culture microscope every day until strong cytopathological effects (CPE) was observed at 72 h. Then, MPXV was stored properly. MPXV was inoculated onto the cells produced in the microplate in Vero cell culture base and the plate wells were examined daily under an inverted microscope for CPE. MPXV titer was calculated using the Spearman-Kaerber method. Sections with a thickness of 5 microns were taken from paraffin blocks onto polylysine-coated slides[3]. After routine deparaffinization and rehydration, staining was

Table 1. MPXV-specific primers.

Primers	Sequencing	Size of fragment (bp)
Gabon-1 (100 nanogram)	5'-GAG AGA ATC TCT TGA TAT-3'	601 bp*
Gabon-2 (100 nanogram)	5'-ATT CTA GAT TGT AAT C-3'	
ATI up-1 (100 nanogram)	5'-AAT ACA AGG AGG ATC T-3'	396 bp**
ATI low-1 (100 nanogram)	5'-CTT AAC TTT TTC TTT CTC-3'	298 bp**

*Neubauer *et al.* 1998 [30]; **Meyer *et al.* 1997 [28].

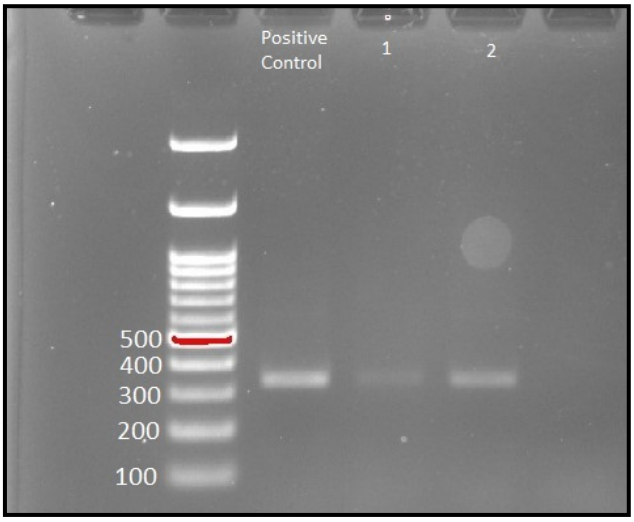

Figure 1. Agarose gel electrophoresis of PCR products of MPXV (ATI gene): Pock lesions on the CAM of the ECE, 396bp (1), Skin lesions, 396bp (2).

completed using a ready-to-use secondary antibody (Mouse and Rabbit Specific HRP/DAB IHC Detection Kit - Micro-polymer (ab236466)[4]. Sheep orf antibody (positive control)[5] was used as the primary antibody.

The presence of viral genome targeting 25 specific parapoxvirus (orf virus) type primers was not detected by PCR testing in the examined skin lesion extract. However, MPXV type-specific primers were used to examine the skin lesion and pock lesions developed after inoculation on the CAM of the ECE, and the presence of viral genome was detected with ATI gene (up-1/low-1) primer (Figure 1), while no viral genome presence was detected with Gabon (1/2) primer. As a result of the inoculation from the skin lesions to Vero cell culture, CPEs were observed at 72 h (Figure 2), and the virus titer was determined as 10^{22} $TCID_{50}$/mL in the microtitration test. Since the results of histopathological examination were given in the previous article [31] they are not mentioned here. According to the results of immunohistochemical staining, a varying degree of positive reaction was observed in the cytoplasmic accumulations of epidermal cells in the lesioned areas. Degenerative changes were also detected in poxvirus positive cells (Figure 3). No positive reaction was observed in dermal cells.

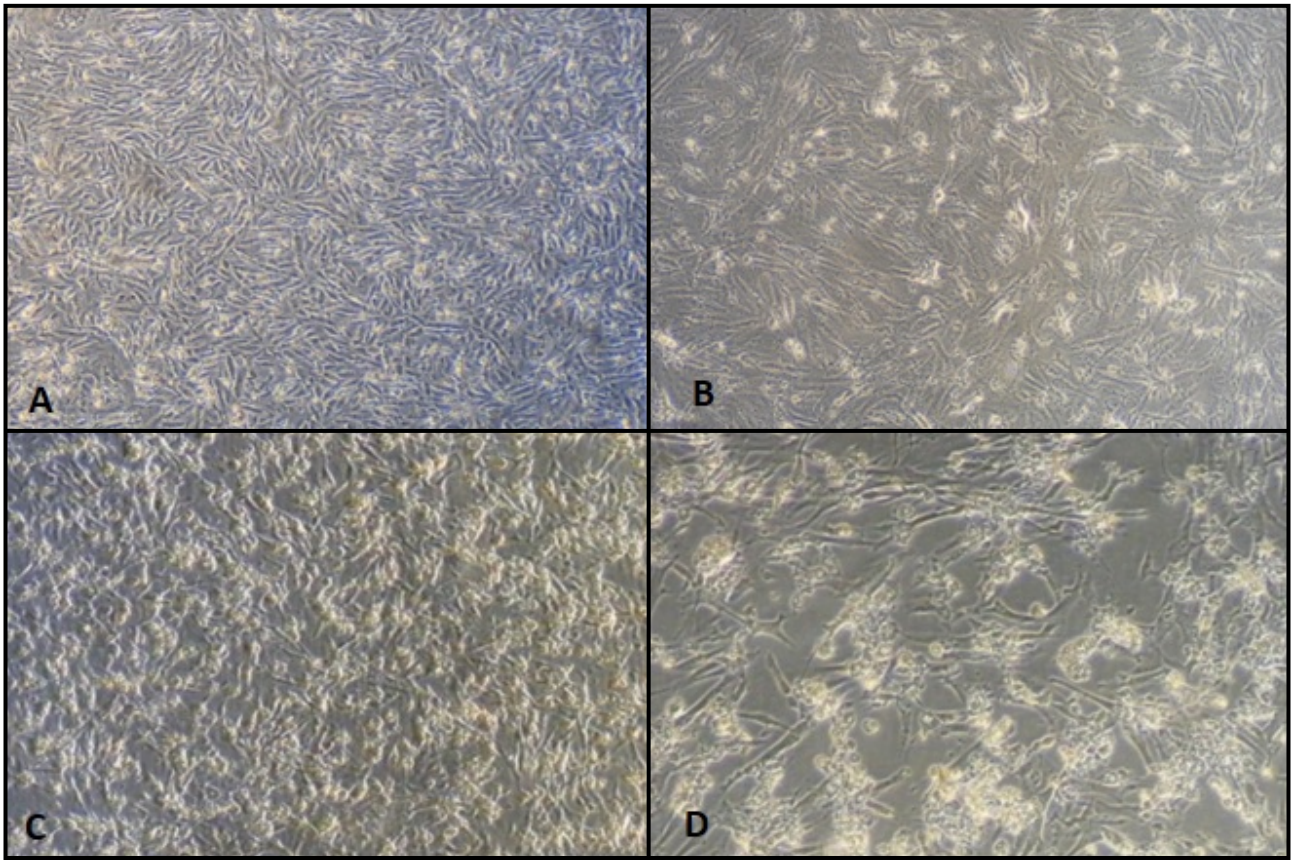

Figure 2. Evaluation of MPXV isolation in Vero cells using inverted microscopy. A- Vero cells (x4). B- Vero cells were infected with sample and CPE were examined by microscopy at 24th h postinfection (x4). C- Vero cells were infected with sample and CPE were examined by microscopy at 48th h postinfection (x10). D- Vero cells were infected with sample and CPE were examined by microscopy at 72nd h postinfection (x4).

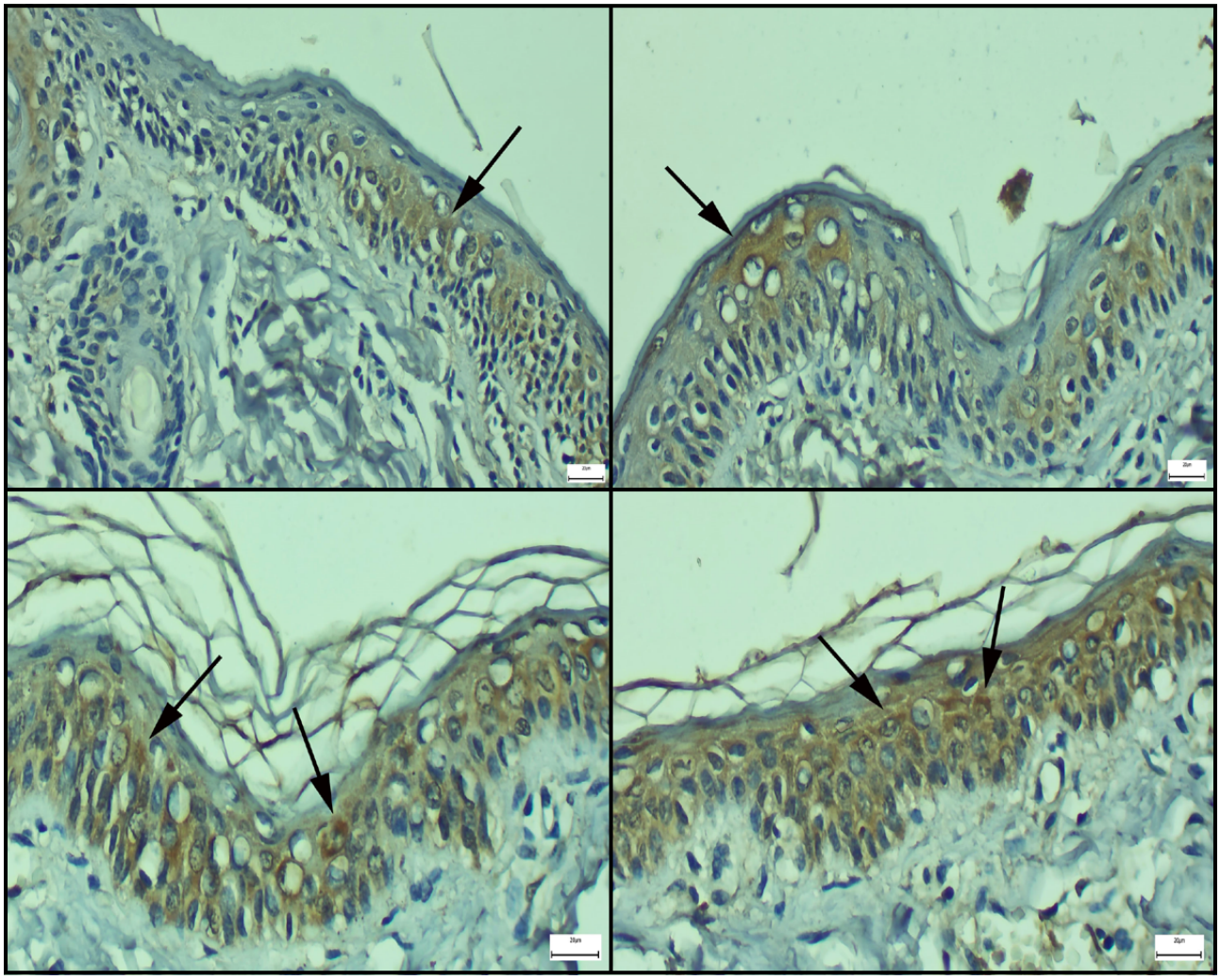

Figure 3. Immunohistochemical appearance of skin of monkey infected with monkeypox, poxvirus positive immunoreaction of the epidermal cells [Streptavidine biotin peroxidase method; scale bars= 20 µm].

DISCUSSION

Cases have been reported extensively from the European region, most commonly Spain, Germany, France, and the UK. By 8 August 2022, over 28.000 confirmed cases and 12 deaths have been reported worldwide. Until 9 August 2022, 5 cases were reported from Turkey [7]. Scientists have reported concerns that the spread of MPXV cases in human populations could be due to the transmission of the disease to wildlife or rodent populations, and that this transmission could also occur in countries where the disease is not currently present [12,17]. Interestingly, in the 2003 cases, humans were infected with MPXV after being exposed to infected animals, whereas in the current global outbreak, it has been reported that the virus spreads through close physical contact among humans in large urban areas in the USA, Europe, and Brazil [24]. In this case that occurred in 2019, we suspect that lesions due to MPXV developed in the offspring of a foreign mother monkey and the virus was transmitted transplacentally from the mother to the offspring. Lesions were not detected in either the mother or other monkeys living in the same environment. In this case, skin lesions developed in the offspring of a female monkey imported from Africa a few weeks after giving birth at the Antalya Zoo. It was reported in personal interviews that the animal caretakers and the veterinarian who took care of the animal also developed skin lesions and sought treatment at the hospital, but the treatment was unsuccessful due to misdiagnosis. After the diagnosis was made in this animal, the zoo officials were informed, and the treatment procedure for the sick caretakers was changed by medical doctors, and they reported that they recovered. The animal caretakers who were taking care of this group of animals reported that they

were using cloth gloves, not wearing masks, washing their hands with soap and water, and rarely using alcohol-based disinfectants. Scientific studies have emphasized the importance of using hand sanitizers containing at least 60% alcohol, in addition to soap and water [9]. Experimental studies have demonstrated that a suspension of $4\log_{10}$ of the virus (at a temperature of 20-25°C) can be inactivated by the following disinfectants: 70% ethanol, 0.2% peracetic acid, 1-10 % prebiotic cleanser, 0.25-2.5% sodium hypochlorite, 2% glutaraldehyde, 0.55% ortho-phthalaldehyde, and 99.9% copper [23]. In the study, none of the 25 type--specific primers for parapoxvirus (orf virus) showed the presence of viral genomes. No relationship was established with MPXV, which belongs to the *Poxviridae* family and Orthopoxvirus genus. However, viral genomes (396 bp) were detected in both the dermal lesion extract and extract of pock lesions on CAM of ECE using 1 of the 2 MPXV type-specific primers (ATI gene). Indeed, Meyer *et al.* [28] determined the presence of viral genomes using MPXV ATI gene primers within the range of 298-396 bp. The MPXV ATI gene is the most common secondary target protein/gene of MPXV (also known as A-type inclusion or A27L) [19,28,30,32]. The MPXV ATI gene shows 72%-95.3% identification with other Orthopoxvirus species, such as variola, vaccinia, cowpox, ectromelia, and camelpox viruses. In a detection study using more than 90 primer/probe sets for poxviruses, HA and ATI were also identified as the most common genes [15].

Orf virus and monkeypox are 2 different viral infections that clinically show similarities. Orf virus is a virus that mostly causes spotted and blistered skin lesions on the mouth and nose area of sheep and goats, rarely in humans. Monkeypox, on the other hand, is a disease with similar symptoms initially seen in monkeys but can also cause infection in humans, caused by a virus from the Orthopoxvirus family. At the immunohistochemical examination revealed that positive immunoreaction with orf antibody. Both Orf virus and MPXV belong to the Poxvirus family [6]. This study has demonstrated the similarity in antigenic structures between these two viruses.

It was suspect that the lesions caused by MPXV in the offspring of a monkey imported from abroad were transmitted transplacentally from the mother to the offspring. High titers of MPXV were detected in the skin lesions. In 2019, the first molecular diagnosis of an MPXV case in a monkey was made in our Turkey. It is recommended the use of ATI gene primers in addition to virological diagnostic methods for molecular diagnosis. It is also emphasized the need for collaboration between veterinary and medical doctors in MPXV cases in animals and humans.

MANUFACTURERS

[1]QIAGEN Sciences Inc. Germantown, MD, USA.
[2]ATCC - American Type Culture Collection. Manassas, VA, USA.
[3]Sigma-Aldrich Chemicals P Ltd. St. Louis, MO, USA.
[4]Abcam. Cambridge, UK.
[5]Sunlong Biotech Co. Ltd. HangZhou, China.

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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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