



## Isolation and Identification of *Camelpox Virus* in Eastern Sudan

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### Abstract

In this study, skin biopsies were collected from camel herds in Eastern Sudan showed signs suggestive to *camelpox*, for histopathology, virus isolation and identification. The affected camels were febrile, depressed, prostrated and anorexic. Also edema of face and head and lesions involved the whole head, neck, buttock, abdomen, chest, legs, ears, udder and tail. Histopathology results of the skin biopsies revealed marked degree of acanthosis and ballooning (hydropic) degeneration and vacuolation of acanthocytes. Both Hyperkeratosis and the dermal layer infiltrated with inflammatory cells, with dermal fibroplasia. Virus isolation succeeds on CAM and Vero cells. The isolates were identified as camel pox by AGPT and VNT and PCR. PCR was considered the rapid and sensitive for the diagnosis of *Camelpox*.

**Key words:** *Camelpox*, Histopathology, VNT, PCR

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### Introduction

*Camelpox* is one of the common contagious skin diseases of Camelids (Bhanuprakash *et al.*, 2010 a). *Camelpox* is caused by *Camel pox* virus belonging to Family Poxviridae, subfamily Chordopoxvirinae, genus Orthopoxvirus. Viruses belonging to genus Orthopoxvirus (OPV) are antigenically closely related (Panning *et al.*, 2004; Essbauer *et al.*, 2010). The disease is enzootic in almost all regions where camel husbandry is practiced, and is responsible for severe economic losses (Wernery and Kaaden., 1995). *Camelpox*, which was known to exist for years in Sudan under the name Al Geddari, was first reported in 1953 (Anonymous, 1954). The disease is characterized by fever, enlarged lymph nodes and skin lesions. Skin lesions appear 1-3 days

after the onset of fever, starting as erythematous macules, developing into papules and vesicles and later turning into pustules. Crusts develop on the ruptured pustules. Skin lesions may take up to 4-6 weeks to heal. (Wernery and Kaaden, 2002). The disease has been reported in all camel rearing countries (Bhanuprakash *et al.*, 2010a, b). Although it is genetically the closest known virus to Variola virus, the etiologic agent of smallpox, virus remains poorly studied. Genus Orthopoxvirus are among the best and longest known viruses to mankind and they are among the most feared viruses of livestock animals and humans.

Sudan is the second most densely populated country in the world by camels, following Somalia. In the literature there is only a little information on camel diseases compared to

other species of animals. This may be due mainly to the fact that camel production is usually practiced on a migratory system in remote areas with harsh living conditions that make such studies difficult and expensive to execute. Despite the general reputation for hardiness and resilience, camels are however, vulnerable to many infections (Wernery *et al.*, 2004). *Camelpox* is the most common infectious disease, potentially occurring wherever the existence of large herds and the movement of infected animals between herds makes the continuous circulation of the virus possible (Fowler, 2010). The disease was also reported in the late seventies in Kassala province of Eastern Sudan. However, the identification of its causative agent has not been made (Shommein and Osman, 1987). The biological physico-chemical properties of the virus were reported by (Khalafalla *et al.*, 1998). The clinical diagnosis is relies on the identification of classical signs. However, confirmatory diagnosis is required to avoid confusion with other disease conditions like contagious ecthyma (orf), papilloma virus, and reaction to insect bites (OIE Manual, 2008), and from other Orthopoxvirus (OPXV) and parapoxvirus infections. Due to the drawbacks of the conventional methods, PCR has become the method of choice for detection and differentiation of *Camelpox* virus from other related pox viruses. Earlier, ATI (A type inclusion), haemagglutinin (HA) and ankyrin repeat protein (C18L)-gene-based PCR assays have been reported for specific diagnosis of *Camelpox* (Balamurugan *et al.*, 2009). The objectives of this study was; isolation, identification and characterization of *Camelpox* virus in Eastern Sudan by using conventional and molecular techniques methods. The reason for selecting this area based on camel population about 25.7% of the country camel stock is found in the Eastern region of Sudan and recently *Camelpox* the effect on ship's cargo rejection of export of camel from country.

## Materials and Methods

### Study area:

This study was conducted during the period from January to May 2013, in Kassala and El Gedaref states, Eastern Sudan.

### Skin Samples:

Twenty samples of Skin biopsies and/or scabs were collected from free- ranging camels showed different stages of skin lesions suggestive of *Camelpox*. Samples were kept in sterile vials containing 50% buffered glycerol saline with antibiotics (One gram of Streptomycin powder and 2 million IU Penicillin were dissolved in 10 ml of sterile DDW) and antifungal (One gram of Neomycin powder (700 000 IU) was dissolved in 13.3 ml of sterile DDW to give a concentration of 52500 IU/ ml. The content of one vial of Fungizone (500 000IU) was dissolved in 10 ml of sterile DDW) were crushed, mixed and used for virus isolation attempts and PCR detection.

### Histopathology:

Ten skin biopsies were collected in 10% formal saline for histopathology examination according to Bancroft *et al.*, (1996).

### Virus isolation:

#### Embryonated eggs:

Eleven to 13days old Embryonated chicken eggs were inoculated onto Chorioallantoic membrane (CAM) with 0.2 ml of prepared sample and incubated at 37C°. The harvested CAMs were examined for pock lesions up to 4-5<sup>th</sup> passages.

#### Cell culture:

Monolayer's of Vero was kindly provided by the CVRI, Soba. Sudan. Cells were adapted to growth (MEM - Cultilab®) with 10% of bovine fetal serum (Cultilab®). One ml of CAMs suspension from samples with characteristic pock lesions was filtrated through 0.45-µm filter according to (Kaaaden *et al.*, 1992; Abdel Bakey *et al.*, 2006), were inoculated on to 25cm<sup>2</sup> tissue culture flask (Nunc®) of 90% confluent monolayers of Vero cells. The flasks were examined daily

for up to 6 days for Cytopathiceffect (CPE).Harvested tissue cultures isolated virus were titrated following the procedure described by Villegas and Purchase (1983) and 50% infective dose end point was estimated in accordance with the method of Reed and Muench (1938).

#### **Sterility test:**

Cell culture suspensions were subjected to bacterial, Mycoplasma and fungal sterility according to the revised requirements for biological substances N.6 (WHO, 1992).

#### **Virus Identification:**

##### **Agar gel diffusion test (AGID):**

AGID was carried out according to Kitching *et al.*, (1986); the harvested (CAMs and cell culture) were examined against known hyper immune serum of *Camelpox* virus (kindly supplied by CVRI, Soba Sudan).

##### **Virus neutralization test (VN):**

The isolated field viruses were examined by VN using *Camelpox* virus hyper immune serum and Reference virus (kindly supplied by CVRI, Soba Sudan) according to the method described by Beard (1983). The neutralization index (NI) was calculated according to Reed and Muench (1938).

#### **PCR:**

##### **DNA Extraction:**

A commercial Nucleic Acid Extraction Kit (*GF-TD-100; VIVANTIS, Malaysia*), was used for extraction of viral DNA from reference Camel pox virus (Jouf-78 Strain), CAMs, and samples from camels with skin lesions and of suspected field virus suspension. Extractions were performed according to the manufacturer's instructions. DNA was stored at  $-20^{\circ}\text{C}$  until it was used.

##### **Primer selection:**

Specific primers targeting ankyrin repeat protein (C18L) - gene as described by (Balamurugan *et al.*, (2009) were purchased (VIVANTIS, Malaysia).

#### **DNA amplification:**

Extracted DNA from the reference Camel pox virus, CAMs, and samples from camels with skin lesions and of suspected field virus suspension of cell culture were amplified using Maxime PCR preMix kit (*i-Taq*, for 20 $\mu\text{l}$  rxn), 1 $\mu\text{l}$  from each of the DNA of the samples, 10 pmol/ $\mu\text{l}$  of each primer, and 16  $\mu\text{l}$  of DDW were included in each test. The amplification was performed in a TP3 Thermocycler (G-STORM) as described by Balamurugan *et al.*, (2009).

#### **DNA electrophoresis**

Amplicons were separated electrophoretically in 1.5% Agarose gels containing safe stain (3 $\mu\text{l}$ /40 ml Agarose), then analyzed by Electrophoresis in a Mini gel electrophoresis (*BIO-RAD*) using 75 volt for 45min after the gel was submerged in TAE buffer.

#### **Results**

##### **Histopathology:**

Histopathology revealed marked degree of acanthosis with degeneration of acanthocytes as well as ballooning (hydropic) degeneration and with vacuolation of acanthocytes (Fig.1 A and B).Some sections revealed both hyperkeratosis and dermal layer infiltrated with inflammatory cells mainly neutrophils with dermal fibroplasia (Fig.1C).

##### **Embryonated egg**

After 4-5 successive passages CAMS inoculated by skin samples, result in dense, grayish-white, enlarged pock lesion (Fig. 2). No death of the embryo was recorded.

##### **Cell culture**

The virus isolated on CAMs replicate on Vero cells after 2 blind passages, producing CPE 1-2 days post inoculation. The CPE started as focal areas, characterized by cell rounding, multinucleated giant cell formation, aggregation and detachment of the cell sheet from the monolayer, resulting in a plaque type of CPE (Fig. 3 B). The mean

tissue culture infective dose<sub>50</sub>% (TCID<sub>50</sub>/ml) was found to be 10<sup>6.9</sup> /ml. for the isolated viruses.

#### AGID

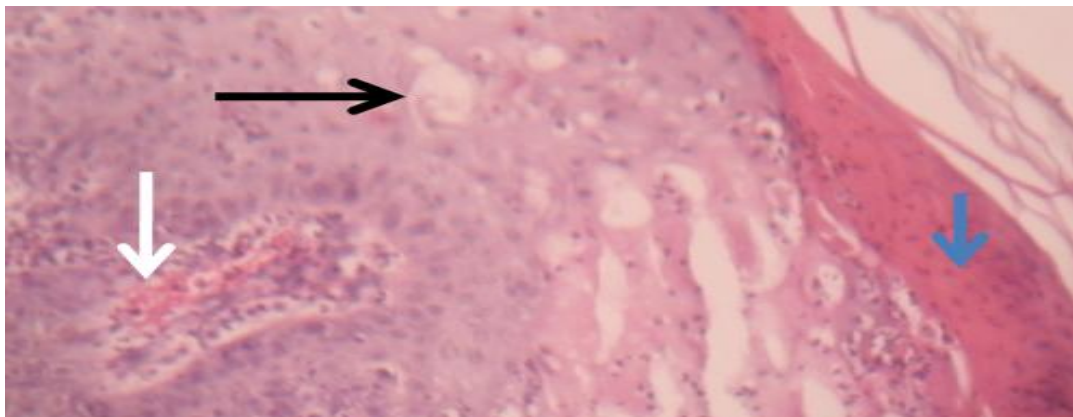
Clear precipitation lines appeared between the virus isolates and the hyper immune serum.

#### VNT

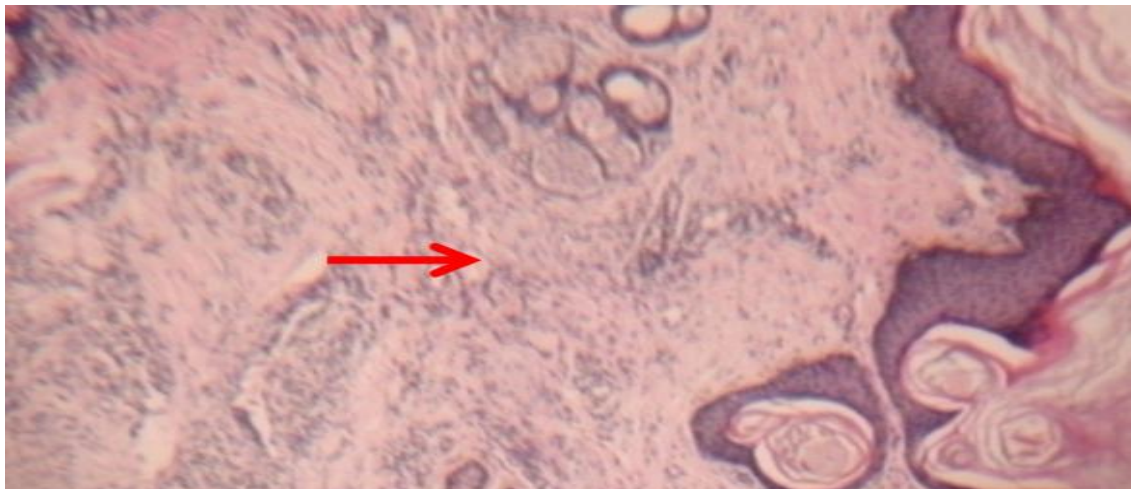
The mean Neutralization Index (NI) for the isolated viruses was found to be 1.85

#### PCR:

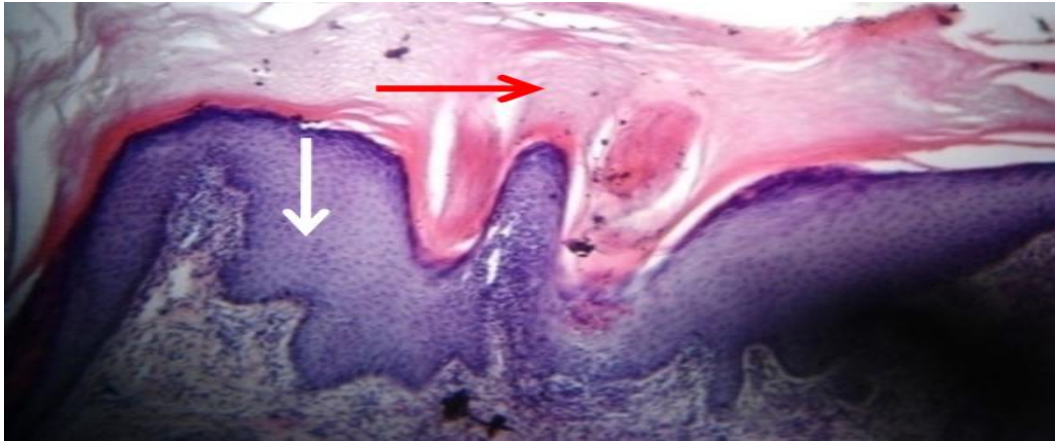
Specific PCR led to amplification of amplicons with the expected size of 477bp (Fig 4). No product was detected when control negative (DDW) was used as template.



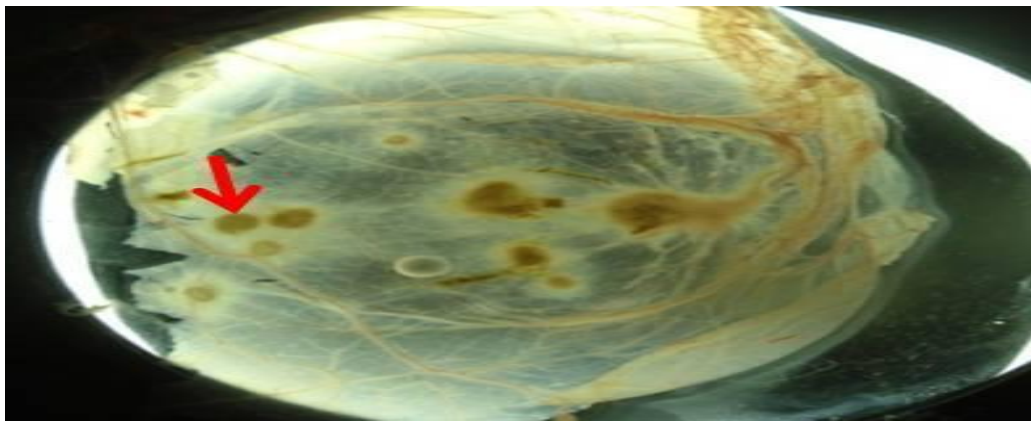
**Fig. 1: Histopathology of the Skin; A(400X), showing ballooning and necrosis of acanthosis (Black arrows). Note the dermal infiltration of inflammatory cells (White arrow) and parakeratosis. (Blue arrow):**



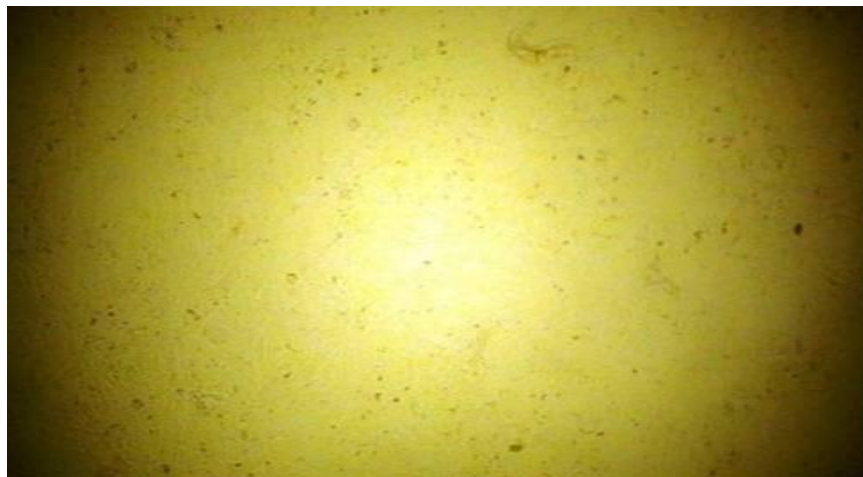
**Histopathology of the Skin: B(40X), showing slightly thickening of the epiderm and dermal fibroplasia (Red arrow)**



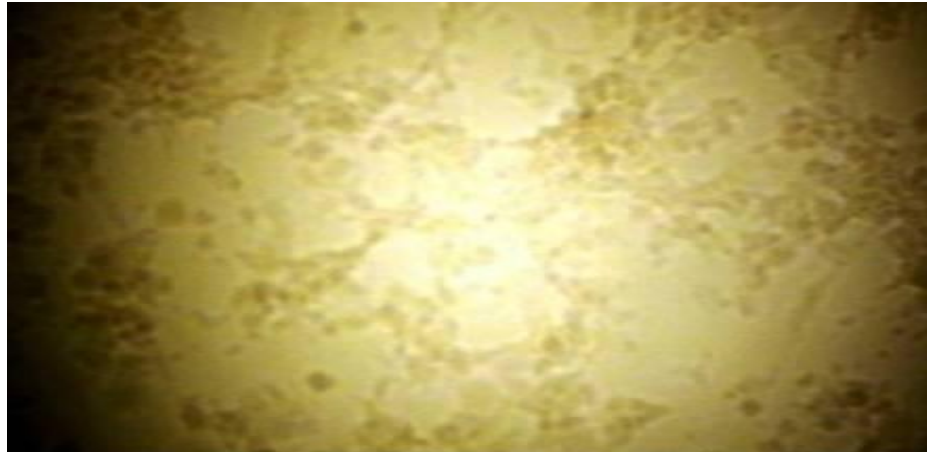
**Histopathology of Skin: C (X40), showing hyperkeratosis (Red arrow) and dermal infiltration of inflammatory cells. (White arrow) (H&E).**



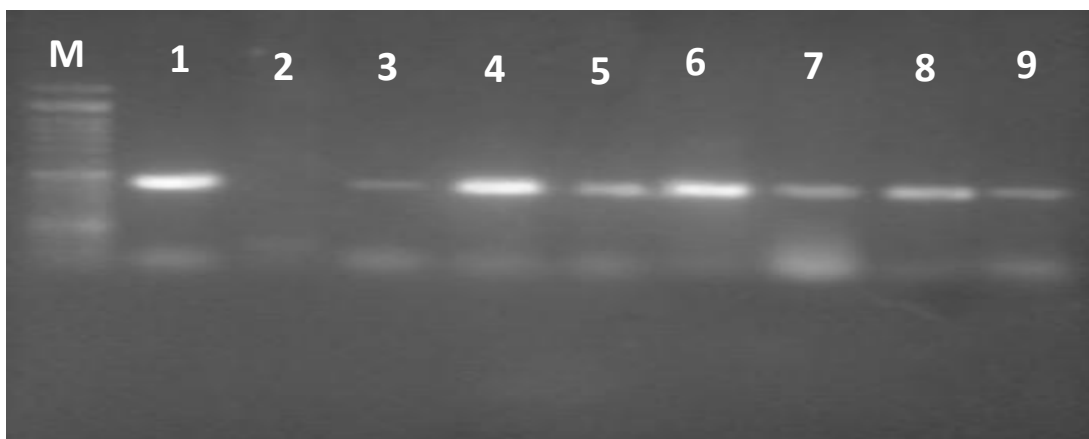
**Figure 2: Pock lesions on CAM inoculated with skin scab suspension.**



**Figure 3: A: control: none infected *Vero* Cells:**



**Fig. 3: B: Cytopathic effect on *Vero* cells inoculated with clinical samples (field isolates) after 48-hour post infection, note accumulation of rounded cells, plaque and Syncytia.**



**Fig. 4: Agarose gel electrophoresis amplicons targeting C18L-gene (~477bp), Lane M: 100bp DNA ladder. Lane 1: positive control. Lane 2: DDW. Lane 3-5: DNA from cell culture. Lane 6-7: DNA from CAMs. Lane 8-9: DNA from skin biopsies**

### Discussion

The clinical signs of *Camelpox* virus observed in this study coincides with several reports from other investigators, who reported that a typical *Camelpox* infections showed four usual stages : papules, vesicles, pustules and crusts (Munz., 1992; Murphy *et al.*, 1999 and Tarek *et al.*, (2012) where affected camels showed one form with varying degrees of severity. Further evidence of *Camelpox* infection was provided by the histopathology comparable to those observed

in other pox group virus infection Khalafalla, (1998). Inoculation of samples from suspected *Camelpox* lesions into Chorioallantoic membrane of embryonating chicken eggs (CAMs) and Vero cell culture were done successfully where distinct pock lesions and characteristic cytopathic effects were observed on CAMs and Vero cells, respectively. Pock lesions appeared after the fourth passage as dense, grayish-white, enlarged areas, embryos were live 5 days post inoculation. The results were similar to those

obtained by Al Zi'abi *et al.*, (2007). Ausama *et al.*, (2011) reported pock lesion after the second passage as dense grayish- white, with elevated centre. The Cytopathic effect (CPE) produced by the field isolates characterized by cell rounding, multinucleated giant cell formation, aggregation and detachment of the cell sheet from the monolayer then followed with a plaque formation which is similar to results described in many others countries. (Al Zi'abi *et al.*, 2007). Serological identification of the field isolates viruses by AGIDT against reference hyper-immune serum of *Camelpox* showed clear precipitin lines. The virus neutralization test was used in this study as a confirmatory test for the identity of *Camelpox* isolates and a neutralization index of 1.85 was obtained. *Camelpox* diagnosed by isolation of agent in CAMs of Embryonated chicken eggs, tissue culture propagation and subsequent identification by serological method, however these methods are tedious and require days for diagnostic results. Therefore, a rapid, specific, sensitive and an economical diagnostic tool is highly imperative for the diagnosis. Three cell culture suspensions, two CAMs suspensions as well as two samples extracted from clinical samples. The PCR-based test is preferable to virus neutralization test (VNT) for additional reasons than its greater sensitivity. It does not require any reagents that cannot be obtained commercially. Many firms sell custom primers for PCR, and all the other reagents are common to all PCR reactions. The neutralization test reagents, on the other hand, include hyper immune serum (HIS) which is commercially not available. Therefore, the PCR test for *Camelpox* virus directly in skin biopsy samples and CAMs and tissue culture supernatants described in this study considered the faster and sensitive molecular technique for diagnosis of *Camelpox* in comparison to conventional methods.

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### العزل والتحقيق الجزيئي لفيروس جدري الابل في شرق السودان

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#### المستخلص:

في هذه الدراسة جمعت الخذعات الجلدية من قطعان ابل من شرق السودان ظهرت عليها أعراض نعتقد إنها أعراض لجدري الجمال بغرض العزل والتعريف، تميزت الأعراض السريرية للحيوانات التي تم فحصها بوجود حمى ، دبرسة، الانهك و فقدان الشهية. وذمة في الراس والوجه مع وجود آفات الجدري المنتشرة في الراس والرقبة و الارداق والبطن ، الصدر، الارجل، الاذان و الضرع والذيل. أيضا ظهور آفات الجدري المنتشرة في الراس والرقبة و أجزاء كثيرة من الجسم. الفحص النسيجي المرضي للخذعات الجلدية أظهر الشواك مع وجود تنكس في الخلايا الشوكية، وتنكس إستسقاوي وتجويف في الخلايا الشوكية. أيضا أظهر الفحص النسيجي وجود فرط تقرن و نظير التقرن مع إرتشاح الخلايا الالتهابية في الادمة ووجود فرط تنسج فبريني في طبقة الأدمة. تم عزل الفيروس في الغشاء السقائي المشيمي لجنين البيض وخلايا الزرع الخلوي لخلايا القرد الافريقي، تم التعرف على الفيروسات المعزولة بإنها فيروسات جدري الابل بواسطة إختبار الترسيب على هلام الأجار و إختبار التعادل الفيروسي و تم تأكيدها بواسطة تقنية الاحياء الجزيئية. تقنية تفاعل البلمرة التسلسلي، المستخدمة في هذه الدراسة تعتبر أسرع وأكثر حساسية وتعتبر تقنية متطورة يمكن أن تطبق بنجاح في تشخيص مرض جدري الإبل مقارنة بالطرق التقليدية.