Ovine herpesvirus 2 can cause skin lesions in susceptible animals

Iman, M. Bastawecy[1]; Hend, M. El-Damaty[2]; Elsahima, F.[2] and El-Said, H[3].

[1]. Dept. of virology, Animal Health Research Institute, Dokki, Giza
[2]. Dept. of Veterinary Medicine, Faculty of Vet. Med. Zagazig university
[3]. Dept. of Int. Medicine and Infectious Diseases, Faculty of vet. Med. Cairo University
iman_baz@yahoo.com

Abstract: Skin lesions of Holestein cattle, native breeds of sheep and dromedary camels suspected to be caused by ovine herpesvirus 2 (OvHV-2) was investigated by isolation, identification and confirmation. Isolation was performed in Madden Derby bovine kidney (MDBK) cell culture and chrioallantoic membrane (CAM) of specific pathogen free-embroynated chicken eggs (SPF-ECE). Identification was carried out by negative and positive staining electron microscopy (EM) with agar gel precipitation test (AGPT) as herpesvirus. Confirmation was achieved using virus neutralization test (VNT) after isolation in the MDBK cell cultures. Our results denoted that the tested animals were infected with OvHV-2. On conclusion, the current study illustrated that OvHV-2 can cause skin lesions only or skin lesions with other symptoms in susceptible animals. EM is recommended as front line to give " open view" along with AGPT for identification and confirmation with VNT when different viral causes were suspected. Epidemiological studies to know factors control form or symptoms of malignant catarrhal fever (MCF) in susceptible animals are also recommended with further researches for the possibility of OvHV-2 to infect humans because there is strong suspicion for infection of contact persons (specially children) to infected animals, material or samples.

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1. Introduction:

Malignant catarrhal fever (MCF) is a lymphoproliferative syndrome of large ruminants caused by bovid γ-Herpesviruses, alcelaphine herpesvirus[1] (ALHV-1), ovine herpesvirus 2 (OvHV-2) and possibly others, such as hipptragine herpesvirus[1] (HiPHV-1) (**schock and Reid, 1996**).

Two distinct epidemiological forms of MCF were described based on the geographic distribution, etiologic agent, and reservoir host. The wildebeest associated MCF (WA-MCF) occurs in Africa and also elsewhere in zoologic gardens and collections containing wildebeests. This form of MCF is caused by AIHV-1. it is transmitted to susceptible species from wildebeest. The sheep associated form of MCF (SA-MCF) has a world-wide distribution caused by OvHV-2. it is transmitted to cattle and other susceptible hosts by sheep and goats (**Zemljic et al, 2012**) through nasal secretion (**Li et al, 2004**) The excreted infectious virus can be transmitted from carriers to clinically susceptible hosts through nasal and ocular secretions by direct contact or by poorly defined air borne routes. Mechanical vector or contaminated feed or water plays a role in transmission (**Brown and Torres, 2008**). Epidemiologically, the occurrence of SA-MCF has occasionally been observed in susceptible hosts without close contact with sheep, which may suggest that OvHV-2 can be carried mechanically by an intermediate host (**Barnard et al., 1989**). The slow rate of spread in most instances and the seasonal incidence in the wormer months suggests the spread by an insect vector of an infection that is available from the donor for a short period only. However, the occurrence of outbreaks in which large numbers of cattle become affected within a short period and during the winter months suggests that infection can occur by other routes. Cattle – to – sheep – to – cattle transmission has also been effected on a number of occasions (**Blood et al., 1983**). Case reports suggest that some non-reservoir hosts might be able to transmit MCF viruses to their offspring (**OIE,2008**). In cattle, MCF usually occurs sporadically in one or few animals, but outbreaks may also be seen. Morbidity rates of 28% to 45% have been reported in some outbreaks and although many animals die, chronic infection or recovery is possible (**OIE, 2008**). MCF is an almost invariably fatal disease in cattle, characterized by fever, depression, profuse nasal, ocular discharge and encrustation, drooling of saliva, photophobia, keratitis, erosions and diphtheresis of oral membranes, generalized lymphoadenopathy, skin lesions, and occasionally, cystitis and central nervous involvement (**Selman, 1981**). OvHV-2 should be considered as possible aetiology of ovine ulcerative

vulvitis, balanoposthitis (**Rutten, 2012**) and abortions in susceptible animals (**Bastawecy et al., 2014**).Subclinical, possible latent, infections have been reported in some incident hosts. Recrudescence might be possible in these animals (OIE, 2008).

MCF has an important economic impact on beef and dairy as well as in bison, deer farming (**Russel et al, 2009**), equine (**Costa et. al, 2009**) and camel laedae **(Goerigk and Merbach, 2012 and Bastawecy et al., 2014).**

The disease is characterized histologically by a generalized necrotizing vasculitis and multisystemic lymphoreticular proliferation and infiltration with fibrinoid and/ or mononuclear cells in many tissues (**Reid et al., 1984 and Ploweright, 1990**). Deposition of immunoglobulin and complement has been described in the glomeruli of affected cattle, suggesting an immune mediated disease (**McVey et al., 2013**).

A diagnosis of MCF is based on the combination of a history of exposure, clinical signs and histopathological findings. Histopathology, detection of virus specific antibodies in blood and detection of viral DNA sequences in blood or tissue samples are considered definitive diagnostic tests (**Zemljic et al., 2012**).Polymerase chain reaction (PCR) and sequencing sometimes are not available. PCR is only capable of identifying the genomic material for previously identified agents. Further mutations in the primer target region may negate the effectiveness of primers. Also PCR will not indentify subviral components such as empty virions, which may be produced late in an infection (**Hazelton and Gelderblom, 2003**). However, the identification of viral nucleic acid does not prove that an infectious virus was present, so PCR-positive samples often should be subjected to traditional virus isolation procedures (**McVey et al., 2013**). Previously, there was difficulty for understanding the pathogenesis of sheep associated MCF due to lack of tools to study replication and otherbiological properties of ovine gammaherpesvirus type 2 (**Achermann, 2005**). OvHV-2 was isolated for the frist time from Egypt by **Bastawecy and Abd El-Samee (2012).** It was confirmed by sequencing. The aim of our study is the diagnosis of the cause of skin lesions in cattle, sheep and dromedary camels where OvHV-2 were suspected to be the etiology of these symptoms. We use Maden Derby bovine kidney (MDBK) cell culture and specific pathogen free embryonated chicken eggs (SPE-ECE) for isolation. Identification and confirmation of the isolates with the classical virological methods are attempted using positive and negative staining electrom microscopy (EM), agar gel precipitation test (AGPT) and virus neutralization test (VNT).

2. Material and Methods:

Animals:

Holestein breed of cattle, native breeds of sheep and dromedary camels of both sex and of different ages were subjected for this study. They belonged to Sharkia, Giza and Gharbia governorates. These animals were with skin lesions only or skin lesions with other symptoms as for example corneal coned opacity, nasal and ocular discharges.

Samples:

Skin lesions:

Skin lesions were collected for virological examination samples were submitted to the laboratory without delay. They were prepared and stored at – 20^{o}C for examination.

Blood samples:

Five ml of heparinized blood were collected from the tested animals during fever and the buffy coat were separated for positive staining EM. Blood was layered over ficoll-histopaque (sigma) and centrifuged at 3000 g for 15 minutes. Leukocytes at the interphase were collected and washed three times with RPMI / 1640 medium (Gibco) according to **Talwar (1983)**. Leukocytes wre subjected for positive staining EM.

Positive serum:

Positive serum against OvHV-2 (isolated and confirmed with sequencing by **(Bastawecy and Abd El-Samee,2012**) was prepared in rabbits according to method described by **Taus et al. (2010)** at Animal Health Research Institute (AHRI) Dokki, Giza, Egypt.

Cell culture:

MDBK cell culture was provided by Virology Department, AHRI, Dokki, Giza, Egypt and used for isolation

SPF-ECE:

SPF-ECE, 10 – 12 days old were obtained from Poultry Department, AHRI, Dokki, Giza, Egypt. It was inoculated by the prepared samples via chorioallanotic sac route (CAM) according to **Burleson et al. (1992).**

Diagnostic methods:

Isolation: Isolation in MDBK cell culture:

Samples were subjected for inoculation in MDBK cell culture according to **Bastawecy and Abd El-Samee (2012).** The cells were mentained in Eagle's essential medium containing 2% fetal calf serum, 100 Iv of penicillin per ml and 100mg of streptomycin per ml. The inoculated cultures were incubated at 37^{o}C. Cell cultures should be examined for cytopathic effect (CPE) for 5 to 10 days. If no CPE is detected, cuitures should be frozen and thawed 3 times and used for inoculation up to 3 blind passages.

Isolation in SPF-ECE: Samples were subjected for inoculation of SPF-ECE, 10-12 days old via the CAM route according to **Versteeg (1990)** and eggs were

examined daily for bock lesions and thickened (oedematous) CAM from 3 to 7 days. CAM of controls and those showing lesions stored in formalin 10% for hitopathological examination.

Identification:

Positive staining EM:

Blood leukocytes were harvested and processed for positive staining EM and ultra thin sections were examined with EM according to method described by **Payment and Trudel (1993)** and **vasconcelos and Lam (1994).**

Negative staining EM:

Negative staining EM was conducted according to **Payment and Trudel (1993).** Supernatants of skin lesions were mixed with a droplets of 3% phosphotungestic acid (PTA). A copper grid coated with carbon formvar was dipped into the mixture and after drying, the grid was examined by EM

A gar gel precipitation test (AGPT):

It was carried out according to **Payment and Trudel (1993)** using the supernatants of skin lesions, supernatants of CAM showing bock lesions and suspensions of MDBK cell cultures showing CPE against positive OvHV-2 antiserum. Pre immune serum of the rabbits used for the preparation of OvHV-2 antiserum was used as a negative control.

Virus neutralization test (VNT):

The test was carried out with the MDBK cell culture isolates only according to **Payment and Trudel (1993)** using OvHV-2 antiserum prepared in rabbits as a positive control and the preimmune serum as a negative control.

3- Results:

Results of isolation in MDBK cell culture:

The inoculated MDBK cell cultures revealed CPE characterized by formation of multinucleated syncytial giant cells after 5 to 10 days. Syncytia degenerate rapidly by contraction and rounding followed by detachment leaving large bare cell- free areas. **(Fig.1).**

Results of isolation on CAM of SPF-ECE:

Typical appearance of bock lesions in the CAM after the third day of inoculation. The numerous lesions were small, white and circumscribed **(Fig. 2A).** The lesions enlarged considerably on prolonged incubation for 7 days **(Fig.2B)** and the CAM became edematous.

Results of histopathological examination of the CAM of SPF-ECE used for isolation:

A section of CAM shows necrosis of ectoderm with oedema and infilamatory cellular infiltration of the mesoderm **(Fig.3A).** Blood vessels show thrombosis and perivascular coughing with inflammatory cells (**Gig.3B).**

Results of electron microscopy (EM):Results of negative staining EM:

Enveloped herpesviral particles could be seen in the supernatant of skin lesions **(Fig.4).**

Results of positive staining EM:

Cross section of herpesvirions were detected on thin sectioning of the blood leukocytes. The electron micrograph shows irregular tugment lies between the icosahedral capsid and the envelope **(Fig.5).**

Results of AGPT:

A clear precipitation lines (positive results) appear between the supernatants of skin lesions, supernatants of SPF-ECE or suspensions of MDBK cell culture isolates and the positive control serum (OvHV-2 antiserum) and not for the negative control serum (preimmune serum collected prior to immunization of the rabbits). **Results of VNT:** MDBK cell culture isolates were identified as OvHV-2 with VNT as a confirmatory test after their identification as herpesvirus with AGPT. All isolates were neutralized by OvHV-2 positive serum, where no CPE occurred after the inoculation of the virus-positive serum mixture on MDBK cells.

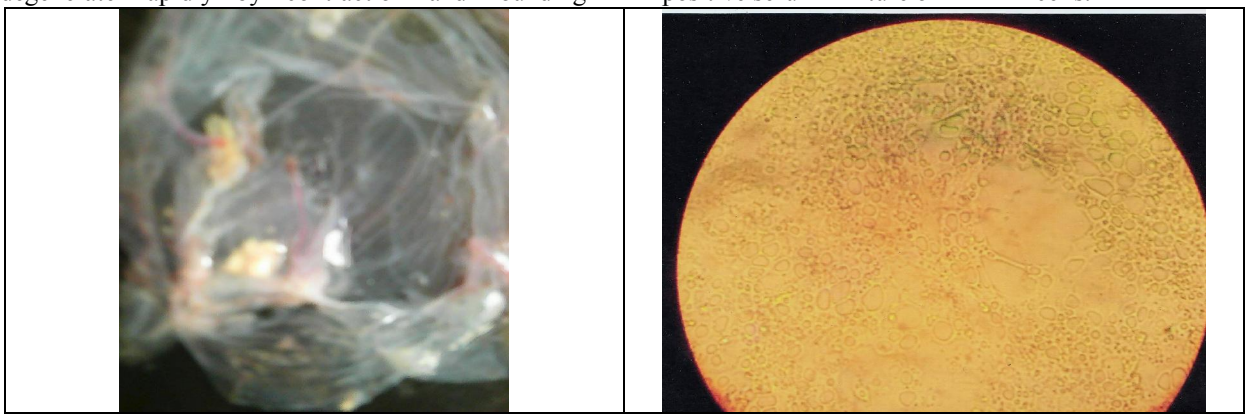
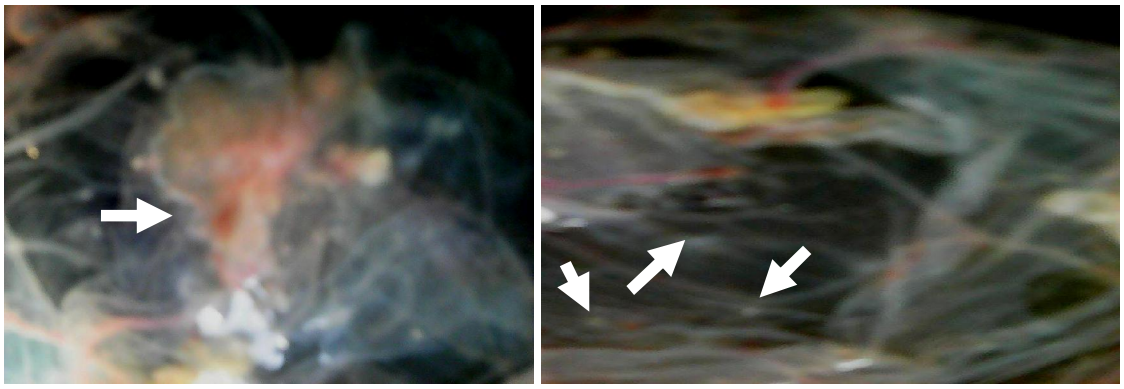
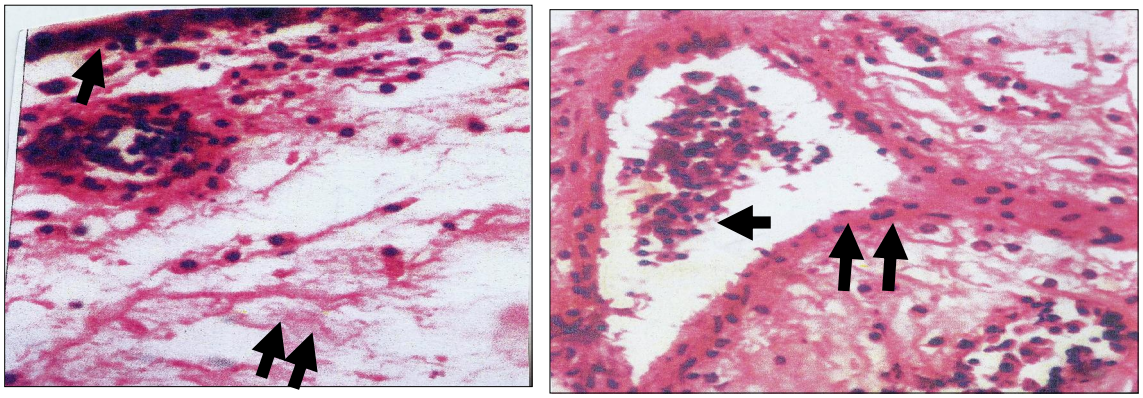


Fig.(1): Inoculated MDBK cell cultures revealed CPE characterized by formation of multinucleated giant cells. Syncytia degenerate rapidly by contraction and rounding followed by detachment leaving large bare cell-free areas. (Mag.40x).



A B

Fig. (2): inoculated CAM of SPF-ECE revealed small, white and circumscribed lesions (A). The lesions enlarged considerably on prolonged incubation and the CAM became oedematous (B).



A B

Fig. (3): A section of CAM shows necrosis ( ) of ectoderm with oedema and inflammatory cellular infiltration of the mesoderm (A) (H&Ex400). blood vessels shows thrombosis and perivascular coughing with inflammatory cells (B) (H & E x400).

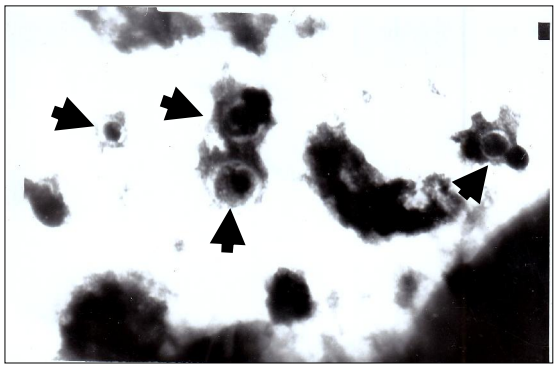


Fig. (4): Negative staining EM for supernatant of skin lesions revealed enveloped herpesviral partieles (70.000 x).

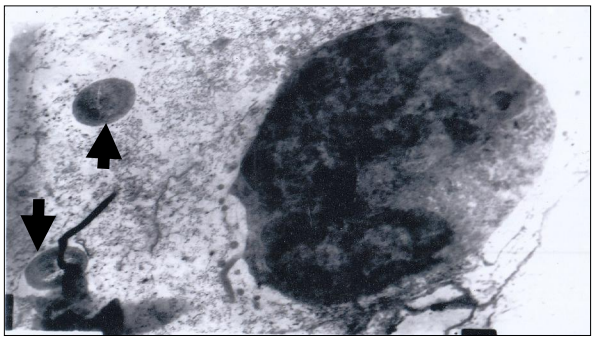


Fig. (5): Positive staining EM showed cross section of herpesvirions on thin sectioning of blood leukocytes where irregular tugment lies between the icosahedral capsid and the envelope (21.000x).

4- Discussion:

OvHV-2 continued to cause sporadically skin lesions among cattle, sheep and dromedary camels where lumpy skin disease (LSD), sheeppox (SP) and camelpox (CP) is suspected respectively. However, these diseases were exculded by laboratory diagnosis with PCR.OvHV-2 was suspected to be the cause of skin lesions in the tested animals due to in case of longer duration of MCF infection, skin changes, including local papule formation with clumping of the

hair into tufts and eczematous weeping may result in crust formation. Skin of the teats, vulva and scrotum in acute cases may slough off entirely on touching or become covered with dry, tenacious scabs (**Blood et al,1983**). Necrotic skin lesions are loosed or sloughed specially at hoof or horn junction (**Anthony and Werner, 1992**). The skin is sometimes erythematous or ulcerated, and hardened scabs may develop. Skin lesions have been reported in cattle without other clinical signs which may resolve spontaneously (**OIE, 2008**).OvHV-2 was successfully isolated in some cell cultures, embryonated chicken eggs (**Bastawecy et al., 2014**) and rabbits (unpublished data).In, the current study, MDBK cell cultures and SPF-ECE (CAM route) were used for isolation with results agreed with those obtained by **Bastawecy et al. (2013).** It was attempted parallel to negative staining EM of skin lesions and positive staining EM of blood leukoctyes of the studied animals with the skin lesions where herpes viral particles were detected and these results in agreement with **Anthony and Werner (1992).**Negative staining EM has the advantages of ease for sample preparation and rapid analysis (same day result) and the undirected (open view) of EM allows rapid morphologic identification and differential diagnosis of different agent which may be present in the sample (**Hazelton and Gelderblom, 2003**). We found that enveloped herpesvirions cloud be seen in the supernatant of skin lesions as mentioned by **versteeg (1990).**Positive staining EM for blood leukocytes supports the method of differential diagnosis which excludes infection of cattle, sheep and dromedary camels with LSD, SP and CP respectively which were suspected from clinical symptoms. Because of this capability, EM must be a fronline method (**Green et. al., 2002**) when EM is available.Irregular tugment lies between the icosahedral capsid and the envelop could be seen on cross sectioning of the blood leukocytes as stated by **Bastawecy et al (2013).** The isolates were detected as herpesvirus in AGPT which is group specific. VNT identifies MDBK cell culture isolates as OvHV-2. VNT is the most reliable and specific test and gives no cross reaction with other herpesviruses so it is the most accurate test although it is time consuming (**Anthony and Werner, 1992 and Bastawecy et al, 2013**).AGPT could be used as group specific test to exclude viral infections suspected (other than herpesviral infection) when their reference antisera are used if EM is not available. Because of its less reliability than VNT although it gives result after 24 hours, VNT was recommended for positive reactors in AGPT as stated by **Anthony and Werner (1992).**OvHV-2 infection of cattle and other ruminant results in T-lymphocyte proliferation and transformation **(Baxter et al,1993)** which helps in givining detailed characterization of the nature of this virus where the pathological features, which affect all organs and tissues are necrotizing vasculitis, marked cellular infiltration and superfaical necrosis of both epithelial and mucous surfaces (**Selman, 1981**) These data explains our results of histopathological examination of CAM inoculated with skin lesions which are necrosis of ectoderm with oedema and infiltration of the mesoderm. Moreover, blood vessels shows thrombosis and perivascular coughing with inflammatory cells. These results agreed with **Plowright (1953)** who stated that the mononuclear cell proliferation is mainly found perivascularly giving rise to necrotizing polyvasculitis which is pathognomonic for MCF. Also, **Swa et al (2001)** mentioned that MCF is caused by the autodestruction of tissues by indiscriminately cytotoxic lymphocytes, produced as a consequence of MCF virus infection. This is supported by **Thonur et al. (2006)** who mentioned that the arrangement of OvHV-2 genes is co-llinear with respect to other γ-herpesviruses. Morevoer there are 12 genes (termed Ov-2Ov10) that have no obvious sequence homologue in other γ-herpesviruses has shown that the functions of genes unique to each virus are often involved in host-specific pathogenesis. So, it seems likely that OvHV-2 unique genes will be involved in the distinct pathology of MCF.Based on the clinical signs, epidemiological data, and laboratory diagnosis with virological methods (isolation, identification and confirmation) our results denoted that the tested animals were infected with OvHV-2 which was isolated and fully sequenced by **Bastawecy and Abd El-Samee (2012).**On conclusion, the current study illustrated that OvHV-2 can cause skin lesions only or skin lesions with other symptoms in susceptible animals. EM is recommended as frontline to give "openview " along with isolation, identification with AGPT and confirmation with VNT when different viral causes were suspected. Epidemiological studies to know factors affecting form or symptoms of MCF in susceptible animals are also recommended. Finally, further studies is recommended for the possibility of OvHV-2 to infect humans because there is strong suspicion for infection of contact persons with infected animals, materials or samples.

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