Recent isolation of ovine herpesvirus 2 from unusual symptomatic infection of sheep in Egypt

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Abstract: Ovine herpesvirus 2(OvHV-2) was recently isolated from unusual symptomatically infected native sheep in Egypt, 2013. The isolated virus was characterized as herpesvirus with negative staining and positive staining electron microscopy (EM). Further identification using agar gel precipitation test (AGPT) and finally confirmation with virus neutralization test (VNT). Based on clinical signs, epidemiological data and laboratory diagnosis with virological methods, our results denoted that the tested sheep were infected with OvHV-2. On conclusion, the current study illustrated that OvHV-2 could be isolated *in vitro* using specific pathogen free-embryonated chicken eggs (SPF-ECE) and Madden Derby Bovine Kidney (MDBK) cell culture. Further studies as sequencing of OvHV-2 had been isolated from sheep in this study is recommended to know if this virus diverges over time or during circulation among susceptible hosts and comparing its sequence to that had been isolated before from cattle and buffalos in Egypt, 2012.
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1.Introduction

Ovine herpesvirus 2 (OvHV-2), a gamma herpesvirus in the genus Macavirus, establishes a lifelong subclinical infection in domestic sheep (Davison et al., 2009).However, transmission of OvHV-2 from sheep to susceptible members of the order Artiodactyla, can result in the disease called malignant catarrhal fever (MCF); MCF caused by infection with OvHV-2 is referred to as sheep – associated malignant catarrhal fever (SA-MCF) (Plowright, 1990).

MCF is a fatal vasculitis and lymphoproliferative syndrome (Luvizotto et al., 2009). It was caused by alcelaphine herpesvirus 1 (AIHV-1), alcelaphine herpesvirus 2 (AIHV-2), OvHV-2, caprine herpesvirus 2 (CpHV-2) as well as other MCF viruses (OIE, 2008).

OvHV-2 is endemic in most sheep and it is the major cause of MCF worldwide (OIE,2008). SA-MCF normally occurs sporadically although epizootics, representing substantial economic losses have been described (Collery and Foley, 1996) and high morbidity rates of MCF infection in cattle have been reported worldwide (Li et al., 2004 and Taus et al., 2006). It occurs year –round in cattle with a modestly increased incidence during the lambing season (Maclachlan and Dubovi, 2011).

OvHV-2 appears to be transmitted mainly by the respiratory route in aerosols. This virus is shed intermittently in nasal secretions (OIE,2008). It predominatly replicates in cells in nasal turbinate when naturally infected sheep experience intensive shedding episodes (Cunha et al., 2008). However OvHV-2 productive replication sites in sheep are different for the entry and shedding events and the virus probably changes its cell tropism at three different stages during the complete life cycle: turbinate (Sheding), lung (entry) and lymphocytes (Latency) (Li et al., 2008).

Usually, latent infection in cattle can lead to clinical disease even in the absence of further sheep contact (Luvizotto et al., 2010).

Rapid drop of OvHV-2 DNA in the lung at 9days post infection suggest the OvHV-2 replication may be controlled by a host-defence mechanism (Li et al., 2008).

OvHV-2 infection has been demonstrated based on polymerase chain reaction (PRC) and blocking enzyme linked immunosorbent assay (ELISA) (Dunowska et al., 2001). The blocking ELISA test is based on an American cell culture isolate of AIHV-1 (Li et al., 1994 and 2001). Although this test does not cross react with other common sheep or bovine herpesviruses, it does not discriminate between AIHV-1, OvHV-2 and newly discovered MCF-like viruses in goats and deer. Thus the establishment of more specific diagnostic reagents is needed for OvHV-2 or related viruses (Dunowska et al., 2001).

Electron microscopy (EM) of infected cells may be used to morphologically indentify typical herpsviruses (Anthony and Werner, 1992).

The virus neutralization test (VNT) has been the only test available for the presumptive diagnosis of MCF and its use was limited to the detection of the African (wildebeest – associated) virus; AIHV-1(Blood et al., 1983).It does not show any evidence of cross- reaction with antibodies to any other herpesviruses (Anthony and Werner, 1992), but paired serum samples, collected 3 to 4 weeks a part, should be taken for serology.Single samples are of limited value as some asymptomatic animals carry antibodies to these viruses (OIE, 2008).

Serological tests other than VNT as indirect immunofluorescent (IIF) and agar gel precipitation test (AGPT) used for detection of OvHV-2 antibodies has the problem of detecting cross reacting antibodies to other herpesviruses specially gamma herpesviruses (Anthony and werner,1992).

Continued progress in SA-MCF studies required the development of an in vitro system to propagate and manipulate OvHV-2 (Taus et al., 2010).

The purpose of the work reported here is the trial to isolate OvHV-2 onto Madden Derby Bovine kidney (MDBK) cell culture and specific pathogen free – embryonated chicken egg (SPF-ECE), chorioallantioc membrane (CAM) route. Then identification of the isolates to offer virological methods for accurate diagnosis of OvHV-2.

2. Material and Methods:

Animals:

Native breeds of new born and adult sheep of both sex were subjected for this study. They belonged to Gharbia, Fayoum, Cairo, Alexandria, Sharkia and New Valley governorates. These animals suffering from fever, anorexia and showing different clinical signs such as nasal and ocular discharges, stomatitis, erosions in oral mucosa, swollen tongue which is bluish in some cases, edematous eye lid, lameness, skin of the muzzle showed discrete patches of necrosis which coaless to make scabs covering parts of the muzzle, skin lesions on the body which may result in crust formation specially beneath the fatty tail, on the perineum and inside the thigh with or without diarrhea. These animals did not respond to antibiotics and they had mortalities of 10% especially in new born lambs.

Samples:

Tissue samples:

Skin lesions on the body and muzzle were collected from living animals and heart muscle, lung, liver, kidney, spleen and mesenteric lymph node of dead animals. Samples were submitted to the laboratory on ice without delay.

Blood samples:

Five ml of heparinized blood was taken from the living animals in the current study, layered over ficoll – Histopague (Sigma) and centrifuged at 3000 g for 15 minutes. Lymphocytes at the interphase were collected and washed three times with PRMI 1640 medium (Gibco) according to Talwar (1983). Lymphocytes were subjected for transmission electron microscopy (TEM).

Positive serum:

Positive serum against OvHV-2 (previously 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, Dokki, Giza, Egypt.

Cell culture:

Madden Derby Bovine kidney (MDBK) cell culture was provided by Virology Department, Animal Health Research Institute, Dokki, Giza, and used for isolation.

Specific Pathogen Free – Embryonated Chicken Eggs (SPF-ECE):

SPF-ECE,9-11 days old were obtained from Poultry Department, Animals Health Research Institute, Dokki, Giza.

It was inoculated by the prepared samples via CAM route 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 maintained in Eagle's essential medium containing 2% fetal calf serum, 100 IU of penicillin per ml and 100 mg of streptomycin per ml. The inoculated cultures were incubated at 37°C. Cell cultures should be examined for cytopathic effect (CPE) for 5 to 10 days. If no CPE is detected, cultures 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 (9-11) 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.

Identification:

Positive staining electron microscopy:

Blood lymphocytes were harvested and processed for positive staining electron microscopy and ultra thin sections were examined with EM according to method described by Payment and Trudel (1993) and Vasconcelos and Lam (1994).

Negative staining electron microscopy:

Negative staining EM was conducted according to Payment and Trudel (1993). Supernatants of tissue samples and suspentions of inoculated MDBK showing CPE and oedematous CAM with bock

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 tissue samples and the suspensions of the (MDBK cell culture and SPF-ECE) isolates 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 preimmune serum as a negative control.

3. Results:

Result of isolation in MDBK cell culture:

The inoculated MDBK cell cultures revealed CPE characterized by formation of multinucleated syncytial giant cells. The CPE began after 3 to 5 days (Fig.1A) and it became more clear after 5 to 10 days. Syncytia degenerate rapidly by contraction and rounding (Fig.1B) followed by detachment leaving large bare cell – free areas.

Results of isolation on CAM of SPF-ECE:

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

Result of electron microscopy (EM):

Result of negative staining EM:

Herpesviral particles were detected in supernatants of tissue samples and suspensions of isolates (MDBK cell culture and SPF-ECE isolates). Several intact negatively stained herpesvirions occurred (Fig.3A) where the intact envelope is not permeable to negative stain.

Result of positive staining EM:

Cross section of herpesvirions was detected on thin sectioning of the blood lymphocytes. The electron micrograph showed the DNA inside the icosahedral capsid. An irregular tugment lies between the icosahedral capsid and the envelope (Fig. 3B).

Result of AGPT:

A clear precipitation lines (positive results) appear between the supernatants of tissue samples or the suspensions of (MDBK cell culture and SPF-ECE) isolates and the positive control serum (OvHV-2 antiserum) and not for the negative control serum (preinmune serum collected prior to immunization of the rabbits).

Result of VNT:

MDBK cell culture isolates were identified as OvHV-2 with VNT as a confirmatory test after their identification as herpesvirus with negative staining EM and AGPT. All isolates were being neutralized against OvHV-2 positive serum. where complete neutralization of all isolates were occurred (no CPE) after the inoculation of the virus-positive serum mixture on MDBK cells.

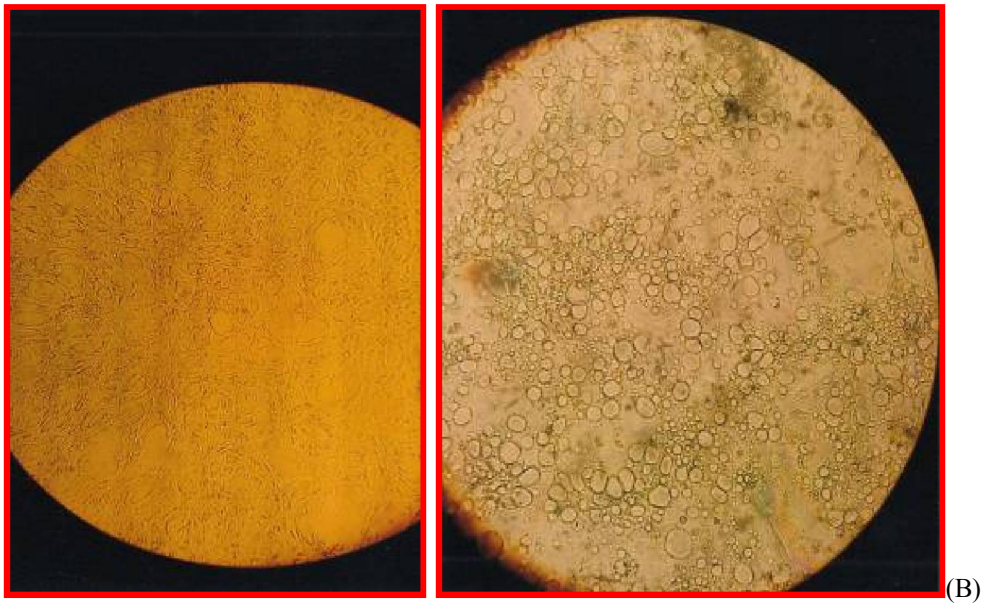
(A)  (B)

Fig. (1): Inoculated MDBK cell culture revealed CPE characterized by:
(A): Syncytia at the beginning of CPE. (Mag.40×); (B): Contraction and rounding of syncytia before detachment (Mag.40×).

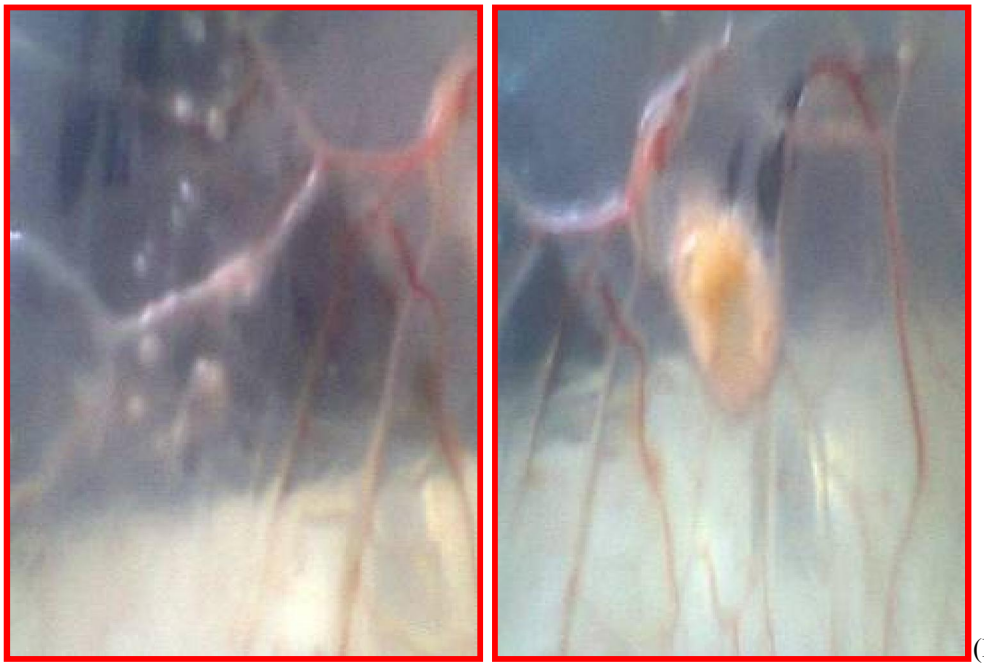
(A)  (B)

Fig. (2): Inoculated CAM of SPF-ECE revealed lesions which are numerous white and circumscribed (A), and enlarged considerebly on prolonged incubation (B) and CAM became edematous.

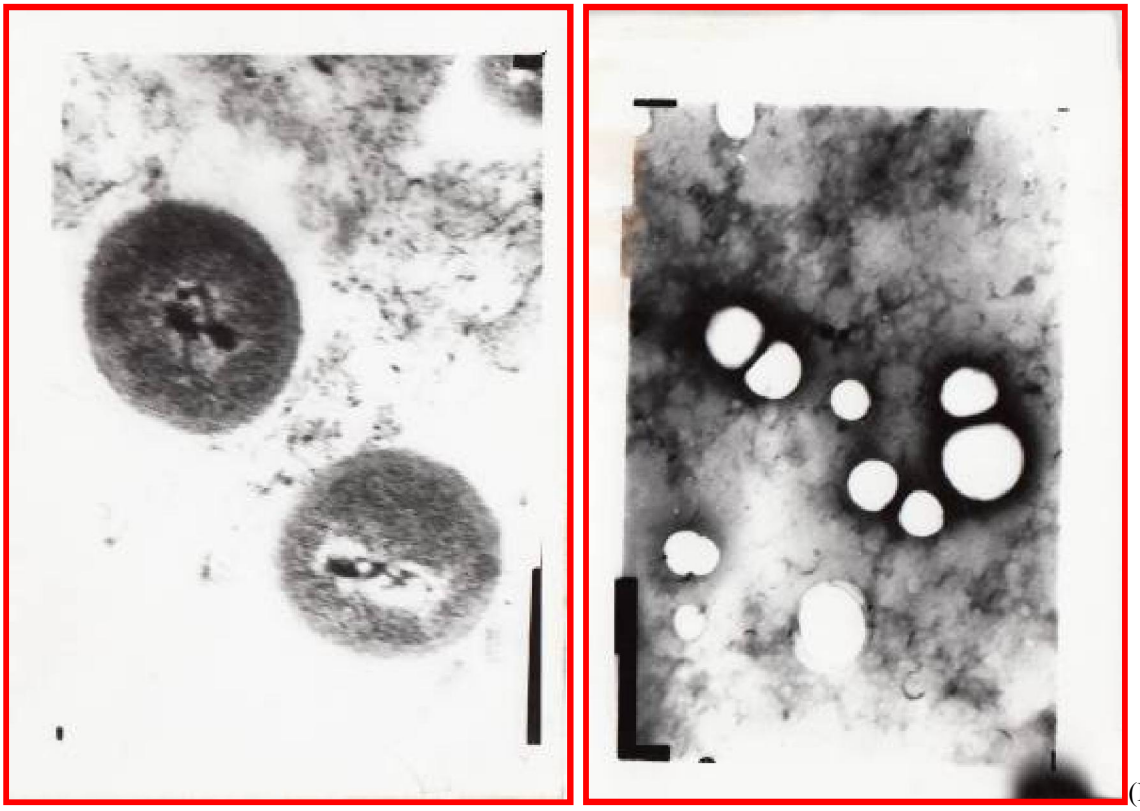
(A)  (B)

Fig. (3): Electron micrographs showing:
(A) An intact, negatively stained herpesviral particles; the intact envelop is not permeable to negative stain. (28.000×); (B) Cross section of positively stained herpesvirions; the DNA appears inside the icosahedral capsid and the irregular tugument lies between the icosahedral capsid and the envelop. (70.000×)

4. Discussion:

Ovine herpesvirus 2 was isolated from acutely infected cattle and buffalos in Egypt (Bastawecy and Abd El-samee, 2012) although previous studies mentioned that its isolation is unsuccessful depending on that, members of the gamma herpesvirinae in general can not be propagated easily onto cell culture. Therefore a molecular approach was the method of choice to verify histopathological findings (Taus et al.,2006 and Taus et al., 2007).

Natural transmission of OvHV-2 from latently infected sheep to naïve animal is predominatly through the shedding of virus in nasal secretions (Li et al., 2004) specially during lambing (Maclachlan and Dubovi, 2011). Amongst domestic animals the clinical disease occurs only in cattle and buffalo, but sheep and goats develop inapparent infections (Blood et al., 1983 and Anthony and Werner, 1992). However, the current study represented that some of the native breed of sheep in Egypt are symptomatic host showing clinical disease. These data may be due to host responses to the virus (which may be due to immune status of the animal or breed susceptibility), rather than differences between infecting viruses. The most susceptible animals were new born lambs and those which were about one year old. These results in agreement with those obtained by Taus et al. (2005) who found that newborn lambs are equally susceptible to infection as adults via aerosol transmission and Li et al. (2008) who mentioned that adolescent lambs between 6 and 9 months of age shed virus more frequently and intensively than adult. They also found that OvHV-2 replication may be controlled by a host – defence mechanism and stress reduction can help disease prevention in subclinically or mildly affected animals (OIE, 2008).

OvHV-2 was the cause of an outbreak in Egypt 2012 among cattle and buffalos and it continued to cause sporadically mortalities in all ages, abortions, skin lesions, hoof and mouth lesions, corneal opacity, respiratory signs, diarrhea and nervous manifestations. So many viral diseases were suspected: rift valley fever (RVF), lumpy skin disease (LSD), foot and mouth disease (FMD), infectious bovine rhinotracheitis (IBR), bovine viral diarrhea-mucosal disease (BVD-MD) and rabies but these cases were proven to be infected with OvHV-2 (unpublished data). This syndrome, is due to MCF ranged from mild to sever even sudden death especially in calves as the disease occurs in a number of forms, the " peracute form", the "alimentary tract form", the common, "head and eye form" and the" mild form", but these are all gradations.

Cases being classified on the prominent clinical signs where serial transmission with one strain of the virus, all of these forms may be produced as mentioned by Blood et al. (1983).

Animals clinically affected that die acutely may lack detectable serum antibodies and thus may have negative serologies (Anthony and Werner, 1992).

The association between OVHV-2 infection and SA-MCF has been demonstrated based on PCR and blocking ELISA assays. Since OvHV-2 has never been isolated in cell culture, and only very little genomic data are available, it is difficult to assess specificity and sensitivity of these assays (Dunowska et al., 2001). Therefore a definitive diagnosis for OvHV-2 is necessary. So we tried to establish a suitable virological techniques.

OvHV-2 was suspected to be the etiology of the unusual symptomatic infection of sheep. Isolation was achieved in MDBK cell culture, identification and confirmation was carried out with EM, AGPT which detected herpesviral infection and VNT which is the most reliable and specific test and gives no cross reaction with other herpesviruses although it is time consuming (Anthony and Werner, 1992).

Isolation was also tried in SPF-ECE and identification was attempted with AGPT which is a group specific test and less reliable than VNT but it gives result after 24 hours. So VNT was recommended for positive reactors in AGPT as stated by Anthony and Wener (1992).

Tissue samples and isolates were subjected for negative staining EM which identify herpesviral particles and positive staining EM for blood lymphocytes revealed cross section of typical herpesvirions as stated by Brooks et al. (1998). 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 present in the specimen (Hazelton and Gelderblom, 2003 and Bastawecy et al., 2007). AGPT could be used as group specific test to exclude viral infections suspected (other than herpesviral infection) on using their antisera when EM is not available.

Positive staining EM for blood lymphocytes supports this method of differential diagnosis which excludes infection with peste des petits ruminants (PPR), bluetongue (BT), border disease (BD) which were strongly suspected due to clinical signs. Because of this capability, EM must be a frontline method (Green et al, 2002) when EM is available.

Detection of herpesvirions among blood lymphocytes supports results described by Versteeg (1990) who stated that gamma herpesviruses replicates in lymphocytes and the cells are transformed and can replicate without limit. Also Baxter et al. (1993) found that OvHV-2 infection of

cattle and other ruminant results in T-lymphocyte proliferation and transformation. These data may help in giving detailed characterization of how the immune response controls OvHV-2 lytic replication in the sheep lung which will provide fundamental knowledge for the development of vaccine strategies which are needed by producers to protect clinically susceptible hosts from SA-MCF (Li et al., 2008).

Based on the clinical signs, epidemiological data, and laboratory diagnosis with virological methods(isolation, identification and confirmation) our results denoted that the tested sheep were infected with OvHV-2.

In conclusion, the current study illustrated that OvHV-2 could be isolated in vitro using SPF-ECE and MDBK cell culture which could be a building stone of accurate serology for surveillance in the natural host. Also when we have less experience with any infection for example; OvHV-2 infection of sheep especially those showing clinical signs which is unusual for reports obtained worldwide. EM is recommended as frontline to give " open view " along with trials for isolation and, identification and confirmation. Further studies as sequencing for the isolated OvHV-2 from symptomatically infected sheep in this study is recommended to know if this virus diverges over time or during circulation among susceptible hosts and comparing its sequence to that had been isolated before from cattle and buffalos in Egypt, 2012.

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