Ovine Herpesvirus 2 Infection Causes Sudden Death and Abortion in Susceptible Animals

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Abstract: Malignant catarrhal fever (MCF) is a systemic disease of cattle belonging to genus Macavirus of the subfamily Gamma herpesvirinae. This genus includes alcelaphine herpesvirus 1 (AIHV-1) and ovine herpesvirus 2 (OvHV-2), the causative agent of sheep associated MCF which is worldwide because it is endemic in most sheep. Clinical signs vary depending on the species affected, the form of MCF contracted and the organ systems that are most affected by the lymphoproliferative disease. OvHV-2 was isolated from suddenly dead foals, calf-camels, cattle, buffalos and sheep. It was isolated also from aborted faetuses of mares, she-camels, cows and ewes. Isolation was achieved in cell cultures (MDBK, VERO and BHK21) and specific pathogen free-embryonated chicken eggs (SPF-ECE), yolk sac and chorioallantoic membrane (CAM) routes. Identification of the isolates was performed with negative staining electron microscopy (EM), agar gel precipitation test (AGPT) and virus neutralization test (VNT). Serological examination of the animals who abort was performed using VNT. They give positive results with observation that she-camels were having the highest titer of neutralizing antibodies may be due to part of the humoral immune response of camels is based on heavy chain antibodies (HC Abs). On conclusion, the current study showed an emergent infectious disease associated with OvHV-2 infection in horse and dromedary camels. Sequencing of the isolates is recommended to know if the virus underwent modifications that expanded to host range. Since MCF is worldwide, testing of the imported animals to Egypt is recommended.

Keywords: Ovine herpesvirus 2, sudden death, abortion, isolation, identification, serological examination.

1. Introduction:

Malignant catarrhal fever (MCF) is an acute, generalized and usually fatal disease previously thought to be restricted to mammals of the order Artiodactyla often members of the subfamilies Bovinae, cervidae, and suidae (Costa et al., 2009).

MCF is a systemic disease of cattle caused by multiple viruses belonging to new genus macavirus (previously known as Rhadinovirus) of the subfamily Gamma herpesvirinae (family Herpesviridae) (Li et al., 2005 and Davison et al., 2009). At least ten viruses are found in this genus (Crawford et al., 2002 and Li et al., 2005) including the alcelaphine herpesvirus 1 (AIHV-1) which is the causative agent of wildebeest (or African)- associated MCF (Plowright et al., 1960) and ovine herpesvirus 2 (OvHV-2) which is the causative agent of sheep-associated malignant catarrhal fever (SA-MCF) which is worldwide because it is endemic in most sheep (OIE, 2008).

Outbreaks of SA-MCF continue to cause production and economic losses in cattle and bison worldwide (Powers et al., 2005).

Clinical signs vary depending on the species affected, the form of MCF contracted (Powers et al., 2005) and the organ systems that are most affected by the lymphoproliferative disease. It can include fever, inappetence, ocular and nasal discharge, lymphoadenopathy, buccal and muzzle crustung, diarrhea, corneal opacity, signs of depression and neurologic signs. The disease can result in death peracutely (i.e., with 1 to 2 days of clinical onset or can result in clinical signs lasting from 1 to several weeks before death. However chronic form of the disease in cattle is possible as an apparent recovery from infection and subclinical infection. The disease in cattle has been reported in both sporadic and outbreak forms and European breeds are less susceptible compared with other ruminants (Moore et al., 2010).

The primary shedding route of OvHV-2 is through nasal secretions (Li et al., 2004). Nose–to–nose contact with sheep is the most efficient method of spread, but fomite transmission has also been reported (Moore et al., 2010). Cattle – to sheep – to cattle transmission has been effected on a number of occasions (Blood et al., 1983).

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

OvHV-2 infection has been demonstrated based on polymerase chain reaction (PCR) and blocking enzyme linked immunosorbent assay (ELISA). PCR which is sometimes not available in all laboratories, it
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 identify subviral components such as empty virions, which may be produced late in an infection (Hazelton and Gelderblom, 2003) and blocking ELISA (Dunowska et al., 2001) which 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 herpes viruses, 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 identify typical herpesviruses (Anthony and Werner, 1992).

The virus neutralization test (VNT) has been the only test available for the presumptive diagnosis of MCF caused by AIHV-1 (Blood et al., 1983) and OvHV-2 (Bastawecy et al., 2013). It does not show any evidence of cross-reaction with antibodies to any other herpesviruses (Anthony and Werner, 1992). Paired serum samples, collected 3 to 4 weeks apart, should be taken for serology where 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).

Since OvHV-2 is endemic in most sheep and MCF is a problem in farmed bison, deer and susceptible cattle worldwide, a vaccine is eagerly sought (Russell et al., 2012). OvHV-2 was successfully isolated on Madden Derby bovine kidney (MDBK) cell culture from cattle, buffalos (Bastawecy and Abd El-Samee, 2012) and sheep (Bastawecy et al., 2013). Also it was isolated on choroiidantoic membrane (CAM) of embryonated chicken egg (ECE) (Bastawecy et al., 2013).

The purpose of the work reported here is the trial to isolate OvHV-2 from suspected animals that showing sudden death and from aborted fetuses with trial to use green monkey kidney (VERO) and baby hamster kidney (BHK21) cell cultures as an alternative to MDBK cell culture and we use yolk sac route along with CAM route for isolation. Identification of the isolates with conventional virological methods were attempted with detection of neutralizing antibodies in serum samples of the tested animals showing abortion.

2. Material and Methods:

Animals:

1. Sudden deaths of foals (6 months to 2 years, Cairo governorate), calf-camels (1 week up to 1 month, Sharkia governorate), Holstein cattle, buffalos and sheep (all ages and belonging to Giza, Sharkia and Gharbia governorates). All animals showing respiratory and or nervous signs with or without diarrhea.

2. Mares (Cairo governorate), she-camels (Sharkia governorate), cows and ewes (Giza, Gharbia and Sharkia governorates) showing abortion.

All tested animals were in direct or indirect contact with sheep.

Samples:

Internal organs (spleen, heart, lung, small intestine, kidney and liver) and lymph nodes of the suddenly dead animals and the aborted fetuses were transported to the laboratory on ice without delay.

Blood samples:

Five ml of blood was taken to separate serum samples from mothers (mares, she-camels, cows and ewes) of the aborted fetuses.

Serum is heated at 56°C for 30 minutes to inactivate complement, then stored at -20°C prior to antibody analysis.

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 (AHRI), Dokki, Giza, Egypt.

Cell cultures and Virus:

Madden Derby bovine kidney (MDBK), green monkey kidney (VERO) and baby hamster kidney (BHK21) cell cultures were provided by Virology Department, AHRI, Dokki, Giza, Egypt. These cell cultures used for isolation and VNT.

Ovine herpesvirus 2 isolated from Egypt by Bastawecy and Abd El-Samee (2012) was obtained from AHRI, Dokki, Giza, Egypt. The virus was titrated according to Reed and Muench (1938) and its titer was 10^7 TCID per ml.

Specific pathogen free – embryonated chicken eggs (SPF-ECE):

SPF-ECE were obtained from Poultry Department, AHRI, Dokki, Giza and subjected for inoculation with suspensions of the tested organs and lymph nodes.

Diagnostic Methods:

Isolation:

Isolation in MDBK, VERO and BHK21 cell cultures:

Suspensions of tested organs and lymph nodes were subjected for inoculation of the mentioned cell
culture according to Bastawecy and Abd El-Samee (2012).

The cell cultures 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 cell cultures were incubated at 37°C and examined for cytopathic effect (CPE) for 3 to 10 days. If no CPE is detected, cultures inoculated should be frozen and thawed 3 times and used for inoculation up to 3 blind passages.

Isolation in SPF-ECE:

Suspensions of the tested organs and lymph nodes were subjected for inoculation of SPF-ECE (6-8) days old via the yolk sac route according to Blood et al (1983) and 10-12 days old via the CAM route. According to Versteeg (1990) and eggs were examined daily for stunted, oedematous, haemorrhagic or lacerated embryos (yolk sac route) and for bok lesions and thickened (oedematous) CAM from 3 to 7 days.

Identification:

Negative staining electron microscopy (EM):

Negative staining EM was conducted according to Payment and Trudel (1993). The inoculated cell cultures (MDBK, VERO and BHK(K)) showing CPE 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.

Agar gel precipitation test (AGPT):

It was carried out according to Payment and Trudel (1993) using the suspensions of the tested organs and lymph nodes, the inoculated cell cultures (MDBK, VERO and BHK(K)) showing CPE and the SPF-ECE isolates suspensions (Bock lesion, in case of CAM route inoculation and embryos, in case of yolk sac route inoculation). All of them considered as tested antigens against positive OvHV-2 antiserum. Preimmune serum of the rabbits used for the preparation of OvHV-2 antiserum was used as a negative control.

Virus neutralization test (VNT) for identification of cell culture isolates:

The test was carried out with the MDBK, VERO and BHK(K) cell culture isolates only according to Payment and Trudel (1993) using OvHV-2 antiserum prepared in rabbits and preimmune serum are used as control positive and negative sera respectively.

3. Results:

Results of gross pathology:

Prominent white foci were seen in kidney, heart and liver of suddenly dead calf-camels (Fig1 a, b, c).

Results of isolation in cell cultures:

The inoculated MDBK, VERO and BHK(K) cell cultures revealed CPE characterized by formation of multinucleated syncytial giant cells (MDBK). The CPE began after 3 to 5 days (Fig. 2a) and it became more clear after 5 to 10 days where syncytia degenerate by contraction and rounding followed by detachment leaving large bare cell – free areas. CPE could be detected from as early as four days after inoculation of VERO cell cultures (Fig. 2b). CPE consisted of small foci of refractile cytomegalic cells. These then appeared to coalesce to form discrete syncytia of varying size containing up to 100 or more nuclei. The syncytia of VERO cells were usually surrounded by refractile cytomegalic cells and did not tend to detach as readily from the monolayer as those in MDBK cells.

After inoculation of BHK(K) cell culture, CPE could be detected as early as 2 or 3 days and cells tend to globose or detach (Fig.2C) and giant cells could be detected specially in subsequent passages.

Results of isolation in SPF-ECE:

CAM route:

Typical appearance of bock lesions in the CAM (Fig. 3a ) on the third day of inoculation. The numerous lesions were small, white and circumscribed and on prolonged incubation for 7days, the lesions enlarged considerably and the CAM became oedematous.

Yolk sac route:

Embryos were stunted, edematous, lacerated, deformed and or haemorrhagic (Fig.3b).

Results of negative staining EM:

Herpesviral particles were detected in the inoculated cell cultures (MDBK, VERO and BHK(K)) after having CPE where several intact negatively stained herpesvirions occurred (Fig.4).

Results of agar gel precipitation test (AGPT):

A clear precipitation lines (positive results) appear between the supernatants of organ and lymph node samples, cell culture (MDBK, VERO or BHK(K)) or SPF-ECE (Bock lesion or embryos supernatant) isolates and OvHV-2 antiserum (Control positive) and no precipitation lines appear on using the negative control serum (preimmune serum of the rabbit).

Results of virus neutralization test (VNT):

MDBK, VERO and BHK(K) 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 neutralized by OvHV-2 antiserum where complete neutralization of all isolates were occurred.
(no CPE) after the inoculation of the virus – positive serum mixture on MDBK, VERO or BHK_{21} cell cultures.

**Results of virus neutralization test (VNT) for detection of neutralizing antibodies:**

All serum samples tested were positive. She-camels serum samples showed notably the highest titers of neutralizing antibodies.

![Fig. (1): Prominent white foci were seen in kidney (a), liver (b) and heart (c).](image1)

![Fig. (2):](image2)

(a) : Inoculated MDBK cell cultures revealed CPE characterized by contraction and rounding of syncytia before detachment.

(b) : Inoculated VERO cell cultures revealed CPE characterized by small foci of refractile cytomegalic cells which then colaeose to form discrete syncytia of varying size containing nuclei.

(c) Inoculated BHK_{21} cell cultures revealed CPE characterized by cells that tend to globose or detach.

![Fig. (3):](image3)

(a) : Inoculated SPF–ECE, CAM route revealed bock lesions which are numerous white and circumscribed and enlarged on prolonged incubation and the CAM became edematous.

(b) : Inoculated SPF–ECE, yolk sac route revealed stunted and haemorrhagic embryo.
Discussion:

Malignant catarrhal fever (MCF) is found worldwide wherever susceptible hosts mix with reservoir species (Russell et al., 2009).

OVHV-2 was isolated from acutely infected cattle and buffalos (Bastawecy and Abd El-Samee, 2012) and sheep showing clinical signs of MCF (Bastawecy et al., 2013) 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 and also most of the previous studies stated that sheep were carrier without showing clinical symptoms. So a molecular approach was the method of choice to verify histopathological findings (Taus et al., 2006 and Taus et al., 2007).

OVHV-2 continued to cause sporadically mortalities in all ages, abortions, skin lesions, hoof and mouth lesions, lameness, arthritis, diarrhea, enlarged lymph nodes, nervous manifestations and respiratory signs among cattle, buffalos and sheep where isolation of OVHV-2 was achieved from the mentioned cases (unpublished data) where some viral diseases were suspected as rift valley fever (RVF), lumpy skin disease (LSD), foot and mouth disease (FMD), infectious bovine rhinotracheitis (IBR), bovine viral diarrhea - mucosal disease (BVD-MD), bovine leukosis (BL), bovine ephemerall fever (BEF), rabies, bluetongue (BT), rinderpest, peste des petits ruminants (PPR), border disease (BD), contagious pustular dermatitis (CPD), sheep pox (SP) and rota or corona viral infections but these viral disease were excluded by laboratory diagnosis. These viral diseases were suspected due to MCF ranged from mild to sever even sudden death of all ages 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 and serial transmission with one strain of the virus all of these forms may be produced as mentioned by (Blood et al., 1983).

Natural transmission of OVHV-2 from latently infected sheep to susceptible animal is predominately through the shedding of the virus in nasal secretions (Li et al., 2004) specially during lambing (Maclachlan and Dubovi, 2011) and also adolescent lambs appear to shed high numbers of virions and virus shedding seems to peak when most of the lamb crop is 6-9 months old (Moore et al., 2010). These data explain our observations that most cases in Egypt occurs in late winter and spring (lambing time). Also, late summer and fall months (lamb crop is 6 to 9 months old). However cases continue all the year round due to the long incubation period in cattle (Anthony and Werner, 1992).

Although Tylopoda have previously been considered not susceptiable to OVHV-2, we show that dromedary camels may be infected by this virus. Also, Costa et al. (2009) found that horses may be infected by this virus although animals of the order Periossodactyla family equidae have previously been considered not susceptible to OVHV-2.

MCF could be the cause of abortions and sudden deaths as reported in pigs (OIE, 2008). Therefore we suspected that OVHV-2 may be the cause of sudden deaths and abortions in the present study among the tested cattle, buffalos, sheep, horses and dromedary camels.

The current study represented that horses and dromedary camels could be infected with OVHV-2 due to this virus may underwent recent modifications that expanded to host range as stated by Costa et al. (2009).

Our results presented that suddenly dead calf-camels showed white foci in kidney, liver and heart due to lymphoproliferative infiltration of the non lymphoid tissues as renal cortex and peripheral areas of liver as stated by OIE (2008).

This study, presented that VERO and BHK21 cell cultures could be used for isolation of suspected cases
of OvHV-2 infection as an alternative for MDBK cell culture which may have defects as being sometimes not free of contaminating agents as the non cytopathic BVD virus (Mc Vey et al., 2013) or its contamination with viruses causing MCF which is worldwide. So VERO and BHK21 cell cultures could facilitate isolation of OvHV-2 because these cell culture were already used in isolation for most of the suspected viral diseases.

Identification and confirmation was carried out with AGPT, EM 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 performed in SPF-ECE (CAM and yolk sac routes) and identification was achieved 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 Werner, (1992).

Cell culture (MDBK, VERO and BHK21) isolates were subjected for negative staining EM which identify herpesviral particles. 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 diagnosis of different agent present in the isolate supernatant (Hazelton and Gelderblom, 2003; Bastawecy et al., 2007; Bastawecy and Abd El-Samee, 2012). This method of differential diagnosis to exclude other suspected diseases. Because of this capability, EM must be a frontline method (Green et al., 2002) when EM is available but when it is not available, AGPT could be used as group specific test to exclude viral infections suspected (other than herpesviral infection) on using their antisera.

Serological diagnosis were attempted to know if MCF induced by OvHV-2 is the cause of abortion in mares, she-camels, cows and ewes. Ideally, paired serum samples, one early during acute phase of infection and a second obtained during convalescence but in case of abortion, acute serum samples are generally not available because the initial infection may be asymptomatic and may occur before abortion. Indeed, when abortion occurs, the serological titer of the animal has often reached its maximum and it is not possible to show a seroconversion (James and Potgieter, 1985). Serum samples were tested by VNT to detect neutralizing antibodies to OvHV-2 where VNT is serotype specific and gives no cross reactions with members of Gamma herpesvirinae (Blood et al., 1983).

All examined serum samples were positive with an observation that she-camels were having the highest titer of neutralizing antibodies. This result may be due to part of the humoral immune response of camels and llamas is based largely on heavy chain antibodies (HC Abs) where the light chain is totally absent. These unique antibody isotypes interact with the antigen by virtue of only one single variable domain, referred to as VH. Despite the absence of the VH-VL combinatorial diversity, these HC Abs exhibit, broad antigen binding repertoire by enlarging their variable regions (Muyldermans, 2001). Dromedary camels have three IgG subtypes where Ig G1 consists of two light plus two heavy chains but Ig G2 and Ig G3 (HC Abs) lack light chains and the CH1 domain (Hamers – Casterman et al., 1993). HC Abs constitute about 75% of camel immunoglobulins (Rahborizandeh et al., 2005). HC Abs are able to grasp their targets just as firmly as normal antibodies do, with affinities for their targets, virtually equal to a full antibody 10 times their size so dromedary camels could be used for production of hyperimmune serum as a prophylactic and therapeutic for viral diseases (Bastawecy and Abd El-Samee, 2007) where these antibodies are smaller than conventional antibodies prepared in different animals even monoclonal antibodies hence the name nanobodies. So they can easily permeat blood brain barrier and attack the virus within the cells (Muyldermans et al., 2001; Bastawecy and Abd El-Samee, 2007).

The possibility for isolation of OvHV-2 gives a chance for vaccine preparation. Also isolation helps for preparation of diagnostic serological tests to detect animals which survived with MCF for an extended period of time or even recovered from all clinical signs and are persistently infected with OvHV-2 and can later become clinically ill again (Gollnick et al., 2007). Moreover, isolation helps to assess specificity and sensitivities of PCR and blocking ELISA assays (Dunowska et al., 2001).

On conclusion, the current study illustrated that OvHV-2 could be isolated in vetro using MDBK, VERO and BHK21 cell cultures and SPF-ECE (CAM and yolk sac routes) and the isolates are used for isolation in SPF-ECE (CAM and yolk sac routes) and the isolates are used for surveillance in natural host after its identification, confirmation and titration. EM is recommended as a frontline to give (open view) if it is available. Our results showed an emergent infectious disease associated with OvHV-2 infection in horse and dromedary camels. Sequencing of the isolates is recommended to know if the virus underwent modifications that expanded to host range. Since MCF is worldwide, testing of imported animals to Egypt is recommend.

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