First Isolation and Identification of Ovine Herpesvirus 2 Causing Malignant Catarrhal Fever Outbreak in Egypt

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Abstract: Ovine herpesvirus 2 (Ov HV-2) was isolated for the first time from cattle and water buffalos during an outbreak of malignant catarrhal fever (MCF) in Egypt, 2012. The isolated virus was characterized as herpesvirus with negative staining electron microscopy (EM). Further identification using polymerase chain reaction (PCR) and nucleotide sequencing of the PCR product. GenBank confirmed it as ovine herpesvirus 2, complete genome with query coverage 100% and maximum identity 100% and ovine herpesvirus 2 strain BJ 1035, complete genome with query coverage 100% and maximum identity 99%. Separation of susceptible animals from sheep and goats specially during lambing is recommended and euthanasia of animals which were clinically infected with MCF is advised.
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1. Introduction

Malignant catarrhal fever (MCF) is an acute, highly fatal, infectious disease of domestic cattle, buffaloes, many wild ruminant species (Anthony and Werner, 1992) and pigs (Bremer, 2010).

Alcelaphine herpesvirus 1 (Al HV-1) and ovine herpesvirus 2 (OvHV-2) belongs to subfamily Gammaherpes virinae. They are the causative agents of MCF. These viruses were previously included in the genus Rhadinovirus, but have been reassigned recently to the genus Macavirus (Maclachlan and Dubovi, 2011) Latent infection of Gammaherpes viruses occurs in lymphoid tissue (Brooks *et al*., 1998)

In South Africa, approximately 90% of MCF polymerase chain reaction (PCR)-positive cases are associated with A1 HV-1 and only 10% with OvHV-2 (Bremer, 2010). In USA, the prevalence of OvHV-2 in adult sheep was 99% as determined by nested PCR (Li *et al*., 1995). OvHV-2 – associated disease occurs throughout the world (OIE, 2008).

The disease is characterized by the development of an erosive stomatitis, erosions in the upper respiratory tract, gastroenteritis, keratoconjunctivitis, encephalitis, cutaneous exanthema and lymph node enlargement. It may occur sporadically or in explosive outbreaks (Blood *et al*., 1983) where epizootics of acute high-morbidity syndrome may occur (Katz *et al*., 1991).

The epidemiology of the two major types of MCF viruses within their natural well adapted hosts differs significantly. Whereas intense virus shedding from the wildebeest occurs predominantly during the first 90 days of life, lambs do not shed virus until after 5 months of age. Wildebeest – associated MCF of cattle occurs most frequently in Africa during the wildebeest calving season whereas the sheep-associated form of MCF occurs year – round in cattle with a modestly increased incidence during the lambing season (Maclachlan and Dubovi, 2011).

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).

Clinically, a presumptive diagnosis of MCF can be made when nasal and ocular lesions are observed with a persistent high temperature, enlargement of the peripheral lymph nodes and terminal encephalitis particularly with a history of exposure to sheep, goats, antelope or wildebeest during parturition (Radostits *et al*., 2000).

Identification of MCF virus can be made by immuno-fluorescent (IF) staining of infected cells. Virus neutralization test (VNT) provides the most reliable method of specific virus identification. Electron microscopy may be used to morphologically identify herpesviral particles. (Anthony and Werner, 1992).

Alcelaphine herpesvirus-2 could be isolated in monolayer cultures of ruminant origin but OvHV-2 has never been identified formally (OIE, 2008) although it could be isolated by Schuler *et al* (1990). Also, OvHV-2 has been transmitted experimentally to rabbits and hamsters which develop lesions characteristic of MCF (OIE, 2008).

Viral DNA has been detected in clinical material from cases of MCF caused by both A1 HV-1 and OvHV-2 using the polymerase chain reaction (PCR), and this is becoming the method of choice for diagnosing the OvHV-2 form of the disease (OIE, 2008).

Serological tests as VNT, IF, complement fixation test, agar gel precipitation test (Anthony and Werner, 1992) and competitive inhibition enzyme linked immunosorbent assay (OIE, 2008) could be used to identify animals infected with MCF virus but some of which may be asymptomatic virus carriers such as sheep and goats .Those animals clinically affected with MCF that die actually may lack detectable serum antibodies. The aim of the present study is the accurate diagnosis of suspected cases to be infected with MCF and with history of exposure to sheep during lambing. They are assumed to be infected with OvHV-2. Trials for isolation of the causative virus were attempted with identification using negative staining EM, PCR and nucleotide sequencing of the PCR product.

2. Material and Methods:

Animals:

Foreign breed cows and water buffalos of all ages and both sex were subjected for this study. They belonged to Gharbia, El-kalyobia and Alexandria governorates, Egypt. These animals were suffering from fever (41-41.5 C), rapid pulse rate (100-120/min), and clinical signs as nasal and ocular discharges, erosions in nasal and oral mucosae, excessive salivation of ropey and bubbly saliva, hyperemia and edema of conjunctiva, injection of scleral vessels, bilateral corneal opacity, arthritis severe inflammation of the hoof, lameness, trembling, in-coordinated gait and sometimes nystagmus.

Samples:

Tongue epithelia were collected from the affected animals for isolation and identification. Samples were submitted to the laboratory on ice without delay.

Cell Culture:

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

Diagnostic Methods:

Isolation:

Samples were subjected for inoculation onto MDBK cell culture according to Anthony and Werner (1992). The cells were maintained in Eagle's essential medium containing 2% fetal calf serum, 100 mg of streptomycin per ml and 100 IU of penicillin 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.

Negative staining electron microscopy:

Negative staining EM was conducted according to Payment and Trudel (1993). Supernatants of the tongue epithelia and suspensions of the inoculated MDBK cell cultures showing CPE were mixed with a droplet of 3% phosphotungestic acid (PTA). A copper grid coated with carbon formvar was dipped into the mixture. After drying, the grid was examined by EM.

Polymerase Chain Reaction and Sequencing of PCR Product:

The PCR products were constructed and sequenced by Macrogen, Southern Koria and PCR study was performed according to Dunowska *et al*., (2001). Viral DNA was extracted from the tongue epithelia of affected animals and infected MDBK cells. A set of primers for OvHV-2 (glycoprotein B) were ORF8 F 5'-GGGCCTTTATCTAACGTATGAGA-3'

ORF8 R 5'-TCACAATGCAAACACTTATGAGTAA-3'

Reaction conditions for PCR were 94 C/2min. (1X). 10 cycles were performed (94 C for 10 seconds, 60 C for 30 seconds, 72 C for 2 minutes), followed by 20 cycles with the same denaturation and annealing conditions, but with 5 sec. added to each successive elongation cycle and a final elongation step (72 C for 7 min). The PCR products were electrophoresed and subjected for nucleotide sequencing.

3. Results:

Animals:

The tested animals showed erosions on the tongue (which were local or diffuse and the epithelium was fragile leaving eroded and hyperemic surface), pressed head, corneal opacity (Fig. 1), teats with dry tenacious scabs (Fig. 2) and ulcers on the gum below the incisors (Fig. 3).

Isolation:

The inoculated MDBK cell cultures revealed CPE characterized by formation of multinucleated syncytial giant cells which degenerate rapidly by contraction and rounding followed by detachment from the surface (Fig. 4).

Negative Staining Electron Microscopy:

Herpesviral particles were detected in supernatants of tongue epithelia and suspensions of inoculated MDBK cell cultures revealing CPE and subjected for EM (Fig. 5).

PCR and Sequence Analysis of PCR Products:

Supernatants of tongue epithelia and suspensions of inoculated MDBK cell cultures which showing CPE and revealed herpesvirus particles in negative staining EM, revealed PCR positive for OvHV-2 which when sequenced, they revealed OvHV-2, complete genome with query coverage 100% and maximum identity 100% and OvHV-2 strain BJ1035, complete genome with query coverage 100% and maximum identity 99% (Fig. 6) using NCBI software when they were submitted to GenBank.

Fig. (1): Cow showing pressed head, excessive salivation of ropey and bubbly saliva, corneal opacity, and fragiled tongue epithelium leaving hyperemic and eroded surface.

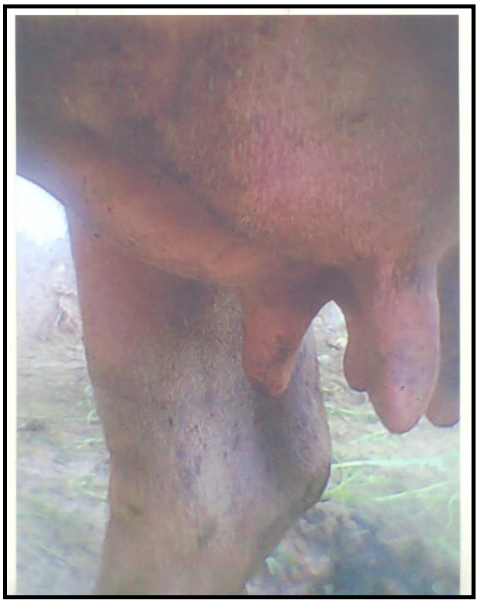

Fig. (2): Teats with dry tenacious scabs.

Fig. (3): Cow showing ulcers on the gum below the incisors.

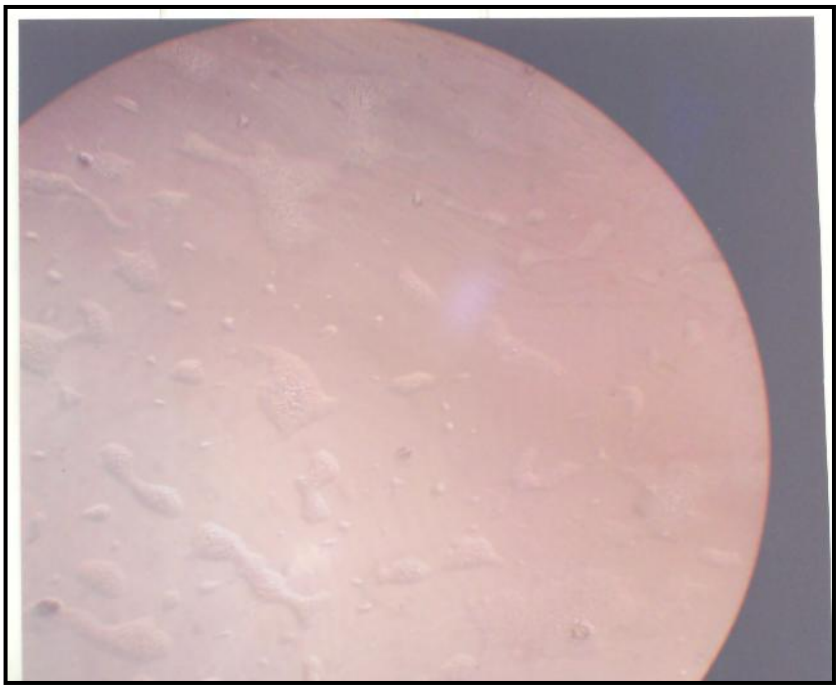

Fig. (4): **Inoculated MDBK cell culture revealed CPE characterized by formation of multinucleated syncytial giant cells.**

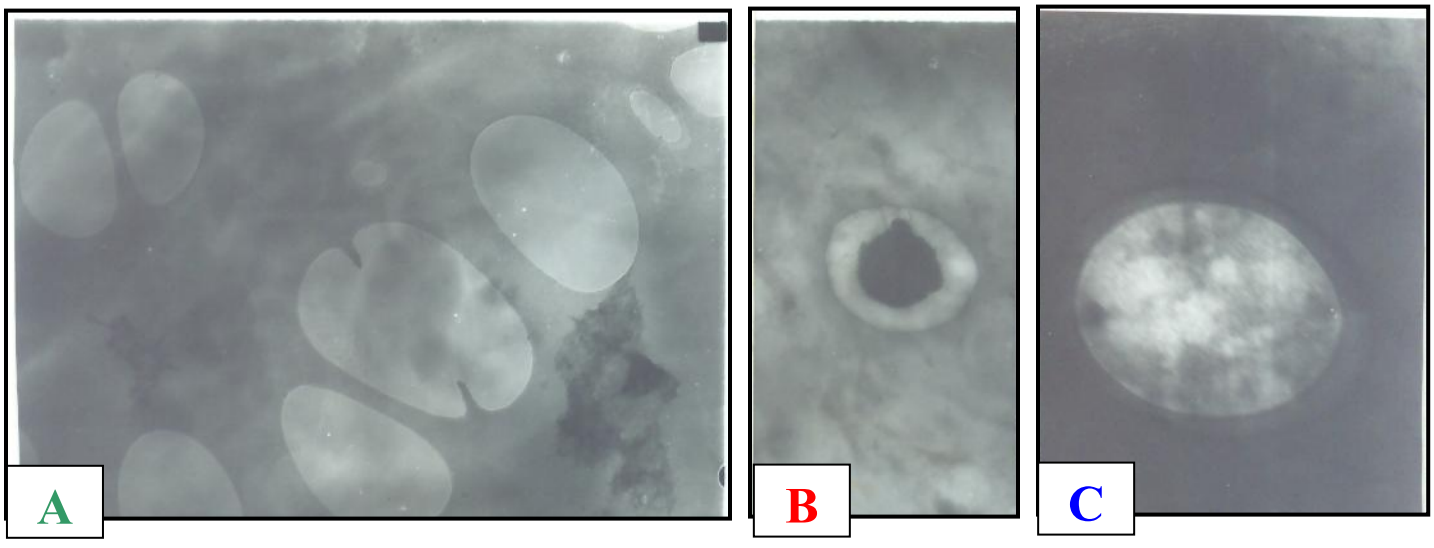

Fig. (5): **Electron micrographs showing:**

(A): **An intact, negatively stained herpesviral particles, the intact envelop is not permeable to negative stain. (35.000 X)**

(B): **Herpesviral particle showing envelop surrounding cubic capsid. (21.000 X)**

(C): **Capsid containing DNA permeated with negative stain and appears as thread-like structures on the surface of the core. (56.000 X)**

Descriptions

Legend for links to other resources: UniGene EGEO GGene SStructure MMap Viewer PubChem BioAssay

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
DQ198083.1	Ovine herpesvirus 2, complete genome	3.285e+04	3.285e+04	100%	0.0	100%	
AY839756.1	Ovine herpesvirus 2 strain BJ1035, complete genome	3.212e+04	3.220e+04	100%	0.0	99%	
JN133502.1	Bovine herpesvirus 4 strain V.test long unique region, complete sequence	58.4	58.4	0%	0.002	100%	
AF318573.1	Bovine herpesvirus 4 long unique region, complete sequence	58.4	58.4	0%	0.002	100%	

Fig. (6): Result of neocleotide sequencing submitted to Gen Bank illustrated OvHV-2, complete genome with query coverage 100% and maximum identity 100% and OvHV-2 strain BJ1035, complete genome with query coverage 100% and maximum identity 99%.

4. Discussion:

A tentative diagnosis of MCF can be made based on clinical signs and a history of contact with sheep, goats or alcelaphine especially during the period of parturitions in these species (Anthony and Werner, 1992).

Ovine herpesvirus 2 was suspected to be the etiology of MCF outbreak in Egypt since there is no evidence of contact for cattle and buffalos with wildebeest but all the infected animals were in contact with sheep and goats during lambing. Cattle and buffalos developed clinical signs characteristic for MCF, ranged from mild to sever even sudden death especially in calves due to the disease occurs in a number of forms, the peracute, 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).

Previous investigations among dairy cattle supports our observation in the disease severity of the susceptible animals showed that although OvHV-2 infection was positively associated with the development of MCF, not all OvHV-2 positive cattle developed disease (Dunowska *et al*., 2001). Another observation was the continuous of cases to occur for months because of the long incubation period (OIE, 2008) and the greatest incidence of the disease is during late winter, spring and summer months (Blood *et al*., 1983).

We found that buffalos are more susceptible and these results agreed with OIE (2008) because OHV-2 appears to fit an emerging pattern in which a mild or subclinical infection with herpesvirus that is lethal for other animal species provides an advantage not to the virus, but also to its host (Brown, 1997) where morbidity and mortality are according to the species (Bratanich *et al*., 2012).

Our trial for isolation of the etiology of MCF was succeeded although most references mentioned its difficulty for isolation but some stated that lymphoblastoid cell lines propagated from affected animals contain OvHV-2 specific DNA and virus particles have been observed in these cells (OIE, 2008). Due to most monolayer cultures of ruminant origin are probably susceptible and develop CPE (OIE, 2008), we use MDBK cell cultures for isolation. The causative agent was identified as herpesvirus using negative staining electron microscopy as mentioned by Anthony and Werner (1992). EM has the advantages of ease for sample preparation and rapid analysis (same day result) and the undirected "open view" of electron microscopy allows rapid morphologic identification and differential diagnosis of different agent present in the specimen (Hazelton and Gelderblom, 2003; Bastawecy *et al*., 2007). Because of this capability, EM must be a frontline method (Green *et al*., 2002).

This step of identification excludes infection with foot and mouth disease (FMD) which is suspected due to presence of oral and hoof lesions.

Further identification was performed with PCR and sequencing. Primers used in ORF 75 (coded for tugment protein) PCR do not react with A1 HV-1 (Li *et al*., 1994). However, they may not react with all isolates of OvHV-2 and furthermore, they may be able to cross react with viruses similar to OvHV-2 (Dunowska *et al*., 2001).

Glycoprotein B (encoded by ORF 8) is one of the most conserved herpesvirus glycoproteins (Pereisa, 1994). It plays a role in virus entry and spread between cells. The gB sequence has been used for estimating, phylogeny between herpesviruses and it is predictive of the more accurate phylogenetic relationships based on the analysis of several conserved genes (Mc Geoch *et al*., 1995). These primers were designed on the partial OvHV-2 sequence from ORF 6 to ORF 9 determined (Dunowska *et al*., 2001).

Neocleotide sequence obtained in the present study when it was submitted to the GenBank, it confirmed it as ovine herpesvirus 2, complete genome with query coverage 100% and maximum identity 100% and ovine herpesvirus 2 strain BJ 1035, complete genome with query coverage 100% and maximum identity 99%. Our results are in agreement with Mc Geoch *et al*. (2000) who mentioned that herpesviruses appear to co-evolve with their host and thus diverge over time for alphaherpesviruses and betaherpesviruses but less clear for members of Gamma herpesvirinae.

On conclusion, the current study illustrated that OvHV-2 was responsible for MCF outbreak in Egypt, 2012 as sheep associated form. When we have less experience with disease as MCF which may be misdiagnosed with other diseases, negative staining electron microscopy is recommended as frontline to give "open view" along with trials for isolation and confirmation with PCR and sequencing. Because of the field observation that sheep and goats are important (potential reservoir species) in the spread of the disease especially during lambing, separation of susceptible animals from them is recommended. Since animals clinically infected with MCF may remain virus carriers, euthanasia of animals which were clinically infected with MCF is advised.

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