*Original Paper***Seroprevalence and molecular detection of isolated BoHV-1 among farm animals**Asmaa Mounier Elashmawy¹, Saad Sharawi Ali², Ayman Said El-Habbaa², Iman Mohamed Bastawey¹¹ Virology Department, Animal Health Research Institute, Dokki, Giza, Egypt² Department of Virology, Faculty of Veterinary Medicine, Benha University, Egypt**ARTICLE INFO****Keywords**

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ABSTRACT

Bovine herpes virus type 1 (BoHV-1) is an economically important, worldwide distributed pathogen of domestic and wild Bovidae. It causes respiratory tract manifestations and/or abortion. The present study was achieved to study seroprevalence and isolate BoHV-1 from suspected cattle, buffaloes, sheep and goats collected from three governorates in Egypt (Menofya, Kalubeya and Dakahlia) during the years 2017 and 2018. Serum and milk samples obtained from apparently healthy non-vaccinated animals were tested for detection of BoHV-1 specific antibodies by indirect ELISA method. A total of 78 (22.5%) serum samples and 46 (13.3%) milk samples out of 346 and 300 samples, respectively, were positive. Virus was isolated from nasal swabs and lung tissue samples on chorio-allantoic membrane (CAM) of 11-day-old specific pathogen free embryonated chicken eggs. It showed pin-point small foci, scattered on CAM membrane. Examination by electron microscope using positive staining methods showed the characteristic morphology of the herpes virion (an enveloped virus with 120 to 200 nm diameter). The viral isolate was confirmed to be BoHV-1 in a PCR assay using specific primers produced a fragment (575 bp) of gC encoding gene in the viral DNA genome.

1. INTRODUCTION

Bovine herpes virus-1 (BoHV-1) is one of the most important viral infections of cattle that cause severe respiratory symptoms, conjunctivitis, abortion, vulvovaginitis and balanoposthitis. BoHV-1 was classified as a member of family Herpesviridae and subfamily Alpha herpesvirinae (OIE 2008). The Outbreaks of respiratory illness in cattle due to BoHV-1.1 infections have occurred occasionally in Egypt since 1976 (Mahmoud et al., 2009; Elshemey and Hassan, 2010). The virus has also been isolated from other animals such as sheep (Mahmoud and Ahmed, 2009) and camel (Abou-Zaid et al., 2001).

In general, BoHV-1 is divided into 2 subtypes: respiratory isolates (BoHV-1.1) and genital isolates (BoHV-1.2) (Rijsewijk et al., 1999; Spilki et al., 2005). The BoHV-5, which was previously classified as BoHV-1.3, has historically been associated with acute meningoencephalitis in calves (Chowdhury et al., 2000; Traesel et al., 2013) and genital tract infections in cattle (Esteves et al., 2003). However, all subtypes are antigenically similar. Antigenically BoHV-1 is closely related to cervine herpesvirus-1 (CvHV-1), buffalo herpesvirus-1, and elk herpesvirus (Keuser et al., 2004).

Herpesviruses are large, enveloped, double-stranded DNA viruses (Harrison 2001). The BoHV1 genome encodes 73 recognized open reading frames (ORFs) within a 135301 bp double-stranded DNA genome. Ten genes code for glycoproteins and among them 6 are present in unique long (UL) region i.e. gK (UL53), gC (UL44), gB (UL27), gH(UL22),gM (UL10) and gI (UL1) and 4 are in unique short (US) region i.e. gG (US4), gD (US6),gI (Us7), gE

(US8) (Spilki et al., 2004). Glycoprotein C (gC) is one of the major glycoproteins present in the envelope of virion and plasma membrane of virus infected cell (Anonymous 2005), and it is the major protein involved in attachment to heparin like receptor on tissue culture cells (Traesel et al., 2013).

BoHV-1 can establish latency in the trigeminal ganglia and germinal centers of pharyngeal tonsils after infection. It should be considered that although such animals could clinically be normal after primary infection (Winkler et al., 2000); they may act as a potent source of infection to other healthy cattle. Stresses such as transportation, parturition and high ambient temperature can induce reactivation of the latent infection. Stress due to injection of steroids can also cause reactivation of the latent virus and leads to intermittent shedding of the virus into the environment (OIE 2008; Radostits et al., 2000).

Specific antibodies to BoHV-1 can be detected by serum-neutralization test or ELISA, two to four weeks after infection (Kaur and Chandra, 2016). Diagnostic samples for detection and isolation of BoHV-1 are lachrymal discharges, nasal and tracheal swabs and bronchial and lung tissue (Lojki et al., 2011).

BoHV-1 was successfully isolated on both embryonated chicken eggs via chorio-allantoic membrane route and MDBK cell line. The virus produced pocks on CAM of chicken embryos and characteristic CPE was observed in MDBK cell line (Samrath et al., 2016).

The use of electron microscopy to identify virus particles in clinical material is a rapid method for the diagnosis of BoHV-1 (Nandi et al. 2009).

Polymerase chain reaction (PCR) was applied to detect the viral DNA in nasal swabs and other tissues which has high

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sensitivity (Van Engelenburg et al., 1993; Moore et al., 2000; Belak and Hakhverdyan, 2006). Despite the high similarity, differentiation among BoHV-1.1, BoHV-1.2 and BoHV-5 can be achieved by molecular analysis of gC (Claus et al., 2005; Silva et al., 2007).

In the present study, we reported the prevalence of BoHV-1 among farm animals in serum and milk samples; Also, isolation and molecular detection of BoHV-1 in viral samples derived from clinically suspected animals from three governorates in Egypt (Menofya, Qalubeya and Dakahlia governorates).

2. MATERIAL AND METHODS

2.1. Field Samples:

2.1.1. Serum and milk samples:

Serum (n=346) and milk (n=300) samples were separately obtained from cattle, buffaloes, sheep and goats apparently healthy with no history of vaccination program against the disease. These animals were in different localities in Menofya, Kalubeya and Dakahlia governorates during years of 2017-2018. Serum and milk samples were prepared according to Wellenberget al. (1998) and stored at -20 °C until subjected to indirect ELISA to investigate the presence of BoHV-1 antibodies.

2.1.2. Viral samples:

Nasal swabs (n=253) and tissue samples (n=21) were collected from suspected animals showing signs of pneumo-enteritis (cough, nasal discharge with or without mild diarrhea and fever) and little showed lachrymal discharges and opacity of eyes beside the respiratory manifestation, were obtained from animals located at three different governorates in Egypt (Menofya, Kalubeya and Dakahlia), during the years 2017-2018. Nasal swabs collected under aseptic conditions and tissue samples collected as postmortem specimens of the lung from emergency slaughtered animals were prepared according to OIE (2008). These samples were preserved at -70 °C until used in trials for virus isolation in Embryonated chicken eggs then identification of positive samples with Electron microscope and PCR.

Indirect ELISA for antibody detection:

Indirect ELISA kit for detection of BoHV-1 antibodies were obtained from Bio-X Diagnostics, 5580 Jemelle, Belgium. It used anti-mammalian IgG peroxidase conjugates (portion G horseradish peroxidase-labelled). It was used for diagnosis of BoHV-1 antibodies in blood sera and milk. The test method and result interpretation were performed according to the manufacturer procedures.

2.2. Virus isolation:

Prepared viral samples were agitated to elute the virus and filtered through 0.45 Millipore filters and a volume of 0.2 ml was inoculated into chorio-allantoic membrane (CAM) route specific pathogen free (SPF) of embryonated Chicken Eggs (ECE) at 11 days old embryo. The inoculated eggs were incubated at 37°C, 2-3 serial blind passages were done and the harvested CAM with pock lesion was confirmed (OIE, 2016).

2.3. Electron microscopic examination of viral isolates:

An ultrathin section of infected CAM was prepared for examination using transmission electron microscopy (TEM). Sections of infected CAM were prepared according to Miller (1995) then cut by Reichert-Jung Ultra-cut 701701 Ultramicrotome. The selected ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate then examined with TEM (JEM2100-Joel-Japan).

2.4. Polymerase Chain Reaction (PCR) for isolates identification:

2.4.1. Extraction of viral DNA:

Viral DNA was extracted from infected fertile CAM homogenates according to QIAGEN amplification (QIAamp) DNA mini kit instructions. The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time is only 20 minutes.

2.4.2. Polymerase Chain Reaction (PCR)

Oligonucleotide primers used in cPCR; They have specific sequence and amplify a specific product of gC gene of BoHV-1 (PF:CGGCCACGACGCTGACGA) and (PR:CGCCGCCGAGTACTACCC) (Esteves et al., 2008).

PCR Master Mix was prepared according to Emerald Amp GT PCR master mix (Takara) Code No. RR310Akit as follow:

Component	Volume/reaction
Emerald Amp GT PCR master mix (2x premix)	12.5 µl
PCR grade water	5.5 µl
Forward primer (20 pmol)	1 µl
Reverse primer (20 pmol)	1 µl
Template DNA	5 µl
Total	25 µl

Then the resulting mixture was subjected to optimized thermo cycling in a thermocycler as follow: pre-denaturing at 94°C for 3 min; denaturing at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, (35 cycles) followed by a final extension at 72°C for 5 min.

Agarose gel electrophoreses (Sambrook et al., 1989) with modification was done as 20 µl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel were transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

3. RESULTS

Prevalence of BoHV-1 specific antibodies in serum and milk samples among cattle, buffaloes, Sheep and Goats in 3 governorates in Egypt:

Using serum samples, 78 out of 346 (22.54%) were positive for BoHV-1 antibody detection using indirect ELISA. These positive results were distributed among the animal species as follow: 29 out of 144(20.14%) for cattle sera, 24 out of 105(22.86%) for buffaloes' sera, 11 out of 49 (22.45%) for sheep sera and 14 out of 48 (29.17%) for goat sera.

Detection of antibodies specific for BoHV-1 in sera of cattle were distributed as follow 11 out of 44 (25.00%) in Menofya governorate, 7 out of 35 (20.00%) in Kalubeya governorate and 11 out of 65 (16.92%) in Dakahlia governorate. Detection of antibodies specific for BoHV-1 in sera of buffaloes were distributed as follow 13 out of 40 (32.50%) in Menofya governorate, 5 out of 15 (33.33%) in Kalubeya governorate and 6 out of 50 (12.00%) in Dakahlia governorate. Detection of antibodies specific for BoHV-1 in sera of sheep were distributed as follow 3 out of 17 (17.65%) in Menofya governorate, 4 out of 19 (21.05%) in Kalubeya governorate and 4 out of 13 (30.76%) in Dakahlia governorate. Detection of antibodies specific for BoHV-1 in sera of goats were distributed as follow 4 out of 15 (26.67%) in Menofya governorate, 5 out of 17 (29.41%) in Kalubeya governorate and 5 out of 16 (31.25%) in Dakahlia governorate (table 1).

Using milk samples, 46 out of 300 (15.33%) were positive for BoHV-1 antibody detection using ELISA. These positive results were distributed among the animal species as follow: 13 out of 80 (16.25%) for cattle sera, 17 out of 115 (14.78%) for buffalo sera, 7 out of 51 (13.73%) for sheep sera and 9 out of 54 (16.67%) for goat sera. Detection of antibodies specific for BoHV-1 in milk of cattle were distributed as follow 7 out of 30 (23.33%) in Menofya governorate, 4 out of 20 (20.00%) in Kalubeya governorate and 2 out of 30 (6.67%) in Dakahlia governorate. Detection of antibodies specific for BoHV-1 in milk of buffaloes were distributed as

follow 10 out of 25 (40.00%) in Menofya governorate, 3 out of 50 (6.00%) in Kalubeya governorate and 4 out of 40 (10.00%) in Dakahlia governorate. Detection of antibodies specific for BoHV-1 in milk of sheep were distributed as follow 3 out of 15 (20.00%) in Menofya governorate, 2 out of 16 (12.50%) in Kalubeya governorate and 2 out of 20 (10.00%) in Dakahlia governorate. Detection of antibodies specific for BoHV-1 in milk of goats were distributed as follow 2 out of 14 (14.29%) in Menofya governorate, 4 out of 20 (20.00%) in Kalubeya governorate and 3 out of 20 (15.00%) in Dakahlia governorate. All the above-mentioned results were shown in table (2).

Trails for BoHV-1 isolation from clinical nasal swabs on CAM of SPF-ECE revealed that 8 out of 101 of cattle, 15 out of 76 of buffaloes, 3 out of 40 of sheep and 5 out of 36 of goat specimens were positive for viral isolation as shown in table (2). On the other hand, trails for BoHV-1 isolation from tissue samples (lung) demonstrated that 7 out of 15 from cattle and 2 out of 6 from buffaloes were positive for isolation on SPF-ECE. These findings were demonstrated in Table (3).

Virus infected CAMs showed pinpoint pock lesions scattered all over the membrane were observed at second passage. More severe changes were observed in CAM after serial passage which showed adaptation of BoHV-1 via CAM route. Pock lesions were more pronounced at third passage (Fig 1A).

Table 1 Prevalence of BoHV-1 specific antibodies in serum samples among cattle, buffaloes, Sheep and Goats in 3 governorates in Egypt by indirect ELISA

Governorate	Number of serum samples/ animal species examined using ELISA							
	Cattle		Buffaloes		Sheep		Goats	
	Total	Positive- %	Total	Positive- %	Total	Positive- %	Total	Positive- %
Menofya	44	11 (25.00%)	40	13 (32.5%)	17	3 (32.5%)	15	4 (26.67%)
Kalubeya	35	7 (20.00%)	15	5 (33.33%)	19	4 (21.05%)	17	5 (29.41%)
Dakahlia	65	11 (16.92%)	50	6 (12.00%)	13	4 (30.76%)	16	5 (31.25%)
Total	144	29	105	24	49	11	48	14

Table 2 Prevalence of BoHV-1 specific antibodies in milk samples among cattle, buffaloes, Sheep and Goats in 3 governorates in Egypt by indirect ELISA

Governorate	Number of milk samples/ animal species examined using ELISA							
	Cattle		Buffaloes		Sheep		Goats	
	Total	Positive- %	Total	Positive- %	Total	Positive- %	Total	Positive- %
Menofya	30	7 (23.33%)	25	10 (40.00%)	15	3 (20.00%)	14	2 (14.29%)
Kalubeya	20	4 (20.00%)	50	3 (6.00%)	16	2 (12.50%)	20	4 (20.00%)
Dakahlia	30	2 (6.67%)	40	4 (10.00%)	20	2 (10.00%)	20	3 (15.00%)
Total	80	13	115	17	51	7	54	9

Trials for isolation of suspected BoHV-1 on chorio-allantoic membrane (CAM) of specific pathogen free embryonated chicken eggs (SPF-ECE).

Table 3 Isolation of suspected BoHV-1 from viral samples on SPF-ECE.

Animal species	Type of Sample	Samples used for isolation on CAM of SPF-ECE	
		Total	*Positive
Cattle	Nasal swab	101	8
	Lung tissue	15	7
Buffalo	Nasal swab	76	15
	Lung tissue	6	2
Sheep	Nasal swab	40	3
Goat	Nasal swab	36	5

* Virus infected CAMs showed pin-point pock lesions scattered all over the membrane

Identification of BoHV-1 isolates using Electron microscope

BoHV-1 appeared spherical in shape enveloped virus with 120 to 200 nm diameter (Fig 1B).

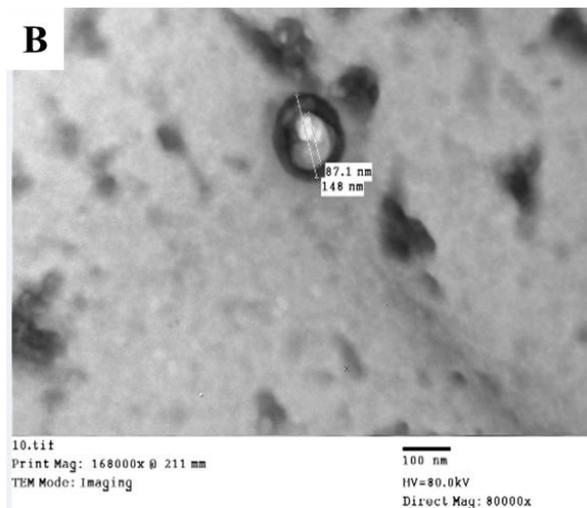
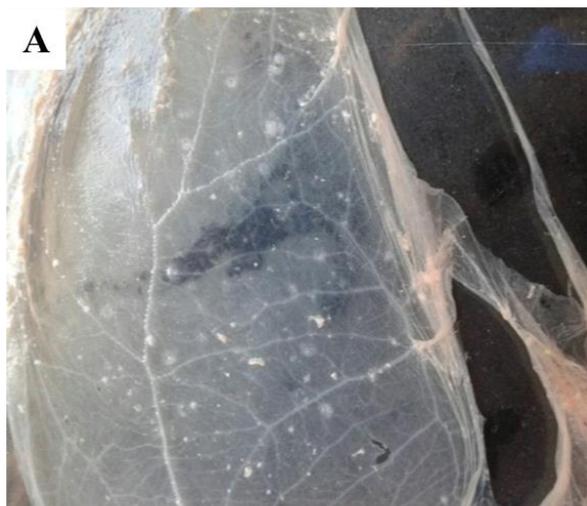


Fig 1A CAM showing pin-point pocks.

Fig 1B Detection of BoHV-1 under electron microscope on ultrathin section on CAM of SPF-ECE.

Molecular identification of BoHV-1 using polymerase chain reaction (PCR):

Both BoHV-1 reference strain and suspected isolates from inoculated SPF-ECE were subjected for PCR for amplification using polymerase enzyme with the upstream and down-stream primers specific for gC gene. Electrophoresis of the amplified products revealed the presence of specific PCR product at the correct expected size (575bp). BoHV-1 reference strain and the cattle and buffalo isolates had the same size of specific fragment without significant differences between the strains as represented in Fig. (2).

4. DISCUSSION

BoHV-1 virus infected host is persistently infected and established life-long latency upon recovery, so it represents the virus reservoir that may be reactivated at intervals and spread the virus infection (Ackermann and Engels 2006).

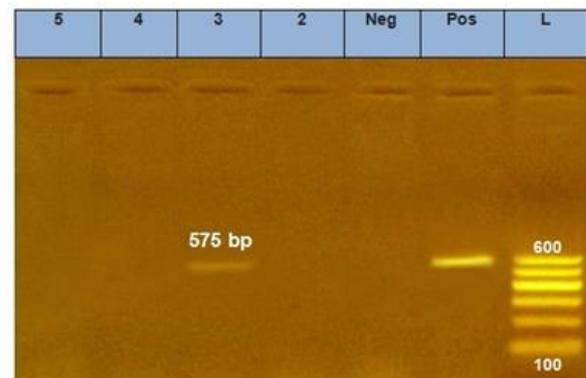


Fig 2 Agarose gel electrophoresis of PCR amplicons for gC gene of BoHV-1 separated on 1.5% agarose gel and stained with ethidium bromide (Positive PCR amplicons are at the size of approximately 575 bp). Lane L denotes for 100 bp Marker. Lane Pos: Positive control. Lane Neg: negative control. Lane 3: Positive sample.

The major problem of herpesviral infections is the carrier status they induce in the animals they prevail in, consequent to which the presence of antibodies in an animal may not indicate an active infection. Considering this major drawback in serum based tests, detection of virus or its antigen becomes mandatory to designate any animal as positive for BoHV-1 (Anonym, 2008; Chandranaik et al 2010). The present study was carried out for detecting Bovine herpesvirus-1 (BoHV-1) antibodies in serum and milk samples of different animal species (cattle, buffaloes, sheep and goats) reared in 3 governorates at Egypt by using indirect ELISA technique. The greater sensitivity of ELISA is due to the fact that it detects both neutralizing and non-neutralizing antibodies and showed high specificity and sensitivity acting on antibodies against whole antigen of BoHV-1 giving positive results with low titers of antibodies that may not be detected by SNT that is agreed with (Pritchard, 2001 and Solis-Calderon et al., 2003; Madbouly and Abd El-Raof, 2004; Lamy, 2008; Taha, 2011). ELISA and particularly Indirect ELISA is considered to be technically superior as a routine diagnostic and sensitive test for the examination of large number of serum samples for the presence of antibodies to BoHV-1 (Van Wuijckhuise et al., 1998; Cho et al., 2002; Nandi and Kumar, 2011) and used as a way of sero-epidemiology in many countries including Egypt (Aly et al., 2003). These results agreed with that of Madbouly and Abd El-Raof (2004), Bastawecy et al. (2005), Boelaert et al. (2005), Abd El-moniem et al. (2006), El-Elie (2008) and Jacevičius et al. (2010) who used a commercial indirect ELISA kits and found that ELISA is more specific for seroprevalence than virus-neutralization (VN) test for detection of antibodies against BoHV-1.

Variation in the rate of prevalence of BoHV-1 antibodies among animals in the tested governorates are depending on environmental condition, animal movement and the chance of exposure of these animals to BoHV-1 infection taking in consideration the breeding of cattle, sheep and goats together under Egyptian field conditions which are the main factors that help the transmission of BoHV-1 infection from cattle to sheep and goats and vice versa.

Trials for virus isolation of BoHV-1 were also done, nasal and lung tissue samples were collected from animals distributed throughout different districts of three governorates at Menofya, Kalubeya and Dakahlia in years 2017-2018. A total of 253 Nasal swabs samples and 21 lung tissues samples were collected from animals with different

ages, breeds, sex and different season for virus isolation. Serial passage of these samples were done using SPF-ECE by CAM route BoHV-1 suspected samples developed changes after inoculation in embryonated chicken egg. Virus infected CAM showed Small foci ranged from 1 to 2 mm in diameter, scattered all over the membrane were observed. Out of 253 Nasal swabs used for virus isolation in egg only 31 samples from different animal species (8 from cattle, 15 from buffaloes, 3 from sheep and 5 from goat) gave characteristic changes in CAM after inoculation. and from 21 total number of tissue samples (lung tissue sample) used for virus isolation in egg only 9 samples (7 from cattle, 2 from buffaloes), this result was in agreement with (Zeedan et al., 2017) as they isolated the virus from nasal and ocular discharges swabs samples and it was adapted in chorio-allantoic membrane (CAM) of 11-day-old embryonated chickens eggs.

Further identification of virus infected chorio-allantoic membranes by electron microscope using positive staining showed characteristic morphology spherical shaped enveloped virus with 120 to 200 nm diameter. This result agreed with the study showed that BoHV-1 is an enveloped virus with 120 to 300 nm diameter (Roziman and Pellett, 2001).

In a range of routine diagnostic submissions of the BoHV-1, classical virological assays are facing several problems because of the genetic and antigenic relatedness of ruminant herpesviruses together with the presence of latent infected carriers; PCR has the primary advantages of being more sensitive, more rapid; discriminative and relevant assay as it could be performed in 1-2 days and could detect DNA in latently infected animals (Studdert, 1999, Van Oirschot, 1996 and Mettenleiter, 2006).

Sensitive PCR assays specific for gC were performed The sensitivity of the designed gC specific primers was 75 per cent and compared with the published primers targeting gB gene whose sensitivity was 66 percent (Ranganatha et al., 2011).

Isolates from nasal swabs and lung tissue samples from cattle and buffaloes were positive with gC specific primer set of BoHV-1. The other isolates from sheep and goat nasal swab samples gave negative result by PCR examination using specific primers for gC gene, this mean the virus isolates may be another virus within Herpesviridae family rather than BoHV-1. These results agreed with the studies showed that glycoprotein C gene of BoHV-1 is highly conserved in all the isolates and it can be used as a target for designing of primers for diagnosis of BoHV-1 infection (Sobhy et al., 2014).

The genomic DNA products of the BoHV-1 reference strain and obtained isolates from PCR for amplification using Taq polymerase enzyme with specific primers revealed the presence of specific PCR product at the correct expected size (575 bp) upon electrophoresis of the amplified products.

These results denoted that BoHV-1 are circulating among animals in examined governorates. The disease was reported in cattle and buffaloes in Egypt (Moussa et al., 1990; Youssef, 1997; Nawal et al., 2003; Madbouly and Abd El-Raof, 2004; El-Kholy and Abdelrahman, 2006; Lamya, 2008; Mahmoud and Ahmed, 2009 and Mahmoud et al., 2009).

So, it is concluded that the virus is spread due to active infection rather than vaccination as the samples were taken from non-vaccinated animals and ELISA technique could be used for serosurvey as it is accurate, sensitive and specific

for detection of BoHV-1 antibodies. Also, PCR is the technique of choice for diagnosis of BoHV-1 as it is rapid, sensitive and more specific than conventional techniques.

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