Serological and virological studies on Infectious Bovine Rhinotracheitis Virus infection (IBR) in cattle in some farms in Behera Governorate

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Abstract

Infectious bovine rhinotracheitis /infectious pustular vulvovaginitis (IBR/IPV) is a disease of domestic and wild cattle and associated with serious economic impact to the animal production. In this study Infectious bovine rhinotracheitis-1 (IBR-1) virus was isolated and identified from cattle in different areas in El-Behera Governorate. Some of these cattle were suffering from respiratory disorders during 2011-2012 and the others were apparently healthy. A total of 274 samples (52 nasal and 52 ocular swabs, 90 blood samples and 80 individual milk samples) were collected from opened farms and closed farms. By inoculation of swabs on MDBK cells, 10 suspected viral isolates showed clear cytopathic effect (CPE). The isolates were identified by Direct Fluorescent Antibody Technique (DFAT), five IBR viral isolates showed clear perinuclear inclusion bodies. Confirmation of the positive isolate by electron microscope revealed the presence of two herpes virions enclosed in a common envelop (Bar=50nm) .A total number of 90 serum samples and 80 skimmed milk samples were tested for IBR antibodies by using indirect ELISA. 43 serum and 35 skimmed milk positive samples were resulted. Antibody detection by ELISA is considered the most rapid, reliable, inexpensive and simple test and is particularly well suited for analysis of large number of samples.

Key words: *Infectious bovine rhinotracheitis , infectious pustular vulvovaginitis , MDBK cells.*

Introduction

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV) is a disease of domestic and wild cattle. It is caused by bovine herpesvirus 1(BoHV-1), which is a member of the genus Varicellovirus in the subfamily Alphaherpesvirinae, which belongs to the Herpesviridae family, order Herpesvirales. The viral genome consists of double-stranded DNA that encodes for about 70 proteins, of which 33 structural and more than 15 nonstructural proteins have been identified **(OIE, 2010)**. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity of the disease. BoHV-1 can be differentiated into subtypes 1.1, 1.2a and 1.2b **(Edwards *et al*., 1990)** by which BoHV-1 shares antigenic and genetic close relationships with other ruminant alphaherpesviruses **(Thiry *et al*., 2006).** Infection by BoHV-1 via the airborne route, lead to virus replication by high titres in mucous membranes of the upper respiratory tract and in the tonsils, subsequently, the virus disseminates to conjunctivae and reaches the trigeminal ganglia by neuronal axonal transport. After genital infection, BoHV-1 replicates in the mucous membranes of the vagina or prepuce, and becomes latent in the sacral ganglia. The viral DNA remains in the neurons of the ganglia, probably for the entire life of the host (status of latency or carrier). Stress, such as transport and parturition can induce reactivation of the latent infection. Consequently, the virus may switch between latent and lytic infection, it may be shed intermittently into the environment and spread to contact animals **(OIE, 2010)**. Vaccination is an effective means of control, but does not stop carrier animals from shedding virus so purchase of carrier animals is the main source of new infections **(Kilari *et al*, 2000** and **D'Arce *et al*, 2002)**.

The virus spread horizontally through sexual contact, artificial insemination, and aerosol transmission and it may also be transmitted vertically across the placenta. BoHV-1 can

cause both clinical and subclinical infections, depending on the virulence of the strain. The disease had been associated with a wide range of clinical symptoms including rhinotracheitis, abortion, infertility, conjunctivitis, encephalitis in calves and reduced milk yields in dairy cattle **(French, 1962).** Abortions may occur in the course of IBR during the third trimester of pregnancy **(Murphy *et al*., 1999).** Morbidity and mortality may reach 100% and 10%, respectively, particularly if complication occurs **(Radostits, *et al*., 2007).**

BoHV-1 infection can be identified by detection of the virus or viral antigen. Virus isolation occurs from blood on EDTA, nasal, pharyngeal, conjunctival, swabs, and in severe cases, from various organs collected at postmortem. In addition detection of a specific antibody response is an important way for the disease diagnosis. Various ELISAs (Enzyme-linked Immunosorbent Assay) are usually used for detecting antibodies against BHV1 in serum and milk, however, data from individual or pooled serum from non-milking groups will more precisely provide the herd's status **(Pritchard, 2001** and **OIE, 2002).**

In Egypt, detection of neutralizing antibodies against IBR virus for the first time in cattle suffering from respiratory syndrome was achieved by **(Hafez and Frey, 1973)**.The first isolation and characterization of IBR in Egypt was by **(Hafez *et al*, 1974** and **Mohamed , 1974)** who isolated IBR/IPV virus from Egyptian cattle with respiratory manifestation in Abou Hamad district, Sharkia Governorate, while **(Hafez *et al*., 1976)** isolated the virus from Friesian cattle suffering from genital form of the disease.

Aim of the study:

- Investigation on IBR disease situation in El-Behera Governorate through the isolation of the virus on tissue culture then its identification by direct fluorescent antibody technique (DFAT) and the electron microscope.
- Serological detection of antibodies against IBRV by using indirect ELISA Kit on serum and milk samples collected from infected and apparently healthy cattle.

Materials and Methods

Samples: A total of 274 samples were collected from, Behera Governorate during years (**2011-2012) (Table 1).**

a-Swabs: a total of 74 nasal and ocular swabs from open farm (the animals were reared in open yard) and 30 samples from closed farm (the animals were housed in partitions and not allowed to get out) were collected from apparently healthy and diseased cattle (Calves & dairy) with symptoms of nasal, lacrimal discharges. The samples were taken in viral transport medium (Eagle's Minimum Essential Medium) with foetal calf serum (5%) and antibiotics (Penicillin 100,000 I.U. & Streptomycin, 100 mg per liter of medium). Samples are clarified by centrifugation at 1500 x g for 10 minutes **(Mahmoud and Ahmed, 2009 & OIE, 2010) (Table 1).**

b- Blood samples: 90 Blood samples were collected from healthy and diseased cattle for separation of serum which used for detection of antibodies against IBRV by using Indirect-ELISA kits **(Table 1).** Blood samples were centrifuged in the laboratory at 1500 x g for 10 min. Sera were separated and stored at -20°C until tested. **(OIE, 2010).**

c- Milk samples: A total of 80 individual milk samples were collected from lactating cattle. Skimmed milk was obtained by centrifugation of whole milk at 1500 x g at 4°C for 20 min and subsequent collection of the milk below the fat layer. Skimmed milk samples were stored at -20°C until examined by Indirect-ELISA **(SVANOVIR IBR- Ab ELISA kit) (Ghazy, *et al*, 2007) (Table 1).**

Tissue Culture: Madin Derby Bovine Kidney (MDBK) cell line were obtained kindly from Virology Department, Animal Health Research Institute, Giza, Egypt and used for IBRV isolation.

Fluorescine isothiocynate conjugate: Specific anti-IBR hyperimmune serum conjugated with fluorescine isothiocynate **(Eappel organon , Tecknika Crop West Cheste)** was supplied by the ELISA unit and viral strain bank, Animal Health Research Institute, Giza, Egypt , used for IBRV identification by direct

fluorescent antibody technique (DFAT).

ELISA Kit: A commercially available Screening **SVANOVIR IBR- Ab** ELISA kit which is designed to detect IBR specific antibodies (IgG) in bovine serum and milk samples.

Table (1). Samples collected from cattle suffered from respiratory signs and apparently health from El-Behera Governorate (2011-2012).

Farms	Animal case	Species	Age	Swabs		serum	Millk
				Nasal	Ocular		
Open farm	Apparently healthy	calves	3-12 M	9	9	31	-
	Apparently healthy	Dairy	3-5Y	15	15	22	33
	diseased	Dairy	3-5Y	13	13	15	20
	Total			37	37	68	53
Closed farm	Apparently healthy	Dairy	3-5Y	10	10	12	18
	diseased	Dairy	3-5Y	5	5	10	9
	Total			15	15	22	27
Over all Total				52	52	90	80

M =Month Y= Year

Isolation &identification of IBRV:

Isolation of the Virus: Collected and prepared nasal and ocular swabs from cattle, were subjected to virus isolation via inoculation on Madin–Darby bovine kidney cell line (MDBK) as described by **(Brunner *et al*. 1988),** inoculated cells were incubated at 37°C and examined daily under inverted microscope to observe the cytopathic effect (CPE) .

Identification of the virus:

1- Direct Fluorescent antibody technique (DFAT). Inoculation of suspected samples (nasal and ocular swabs) on MDBK cell line and examined daily till the appearance of CPE. The harvested cell virus attachment smeared on glass slide and dried on air then fixed by cold acetone, DFAT was applied according to (Moore ***et al*. 2000)**. Fixed cells were mounted by anti-BHV-1 fluorescine isothiocynate conjugated serum and incubated for one hour at 37°C with humidity. Cells were washed with phosphate buffer saline, mounted with 50% glycerol buffer saline and examined under fluorescent microscope.

2- Electron microscope: Tissue culture suspension of positive IBRV isolate was identified by negative staining electron microscope according to (**Caroline *et al*, 1992),** at **VACCSERA Institute.**

Detection of IBR Virus antibodies by indirect ELISA in serum and milk samples:

The serum and milk samples were tested for presence of antibodies against BHV-1 with a SVANOVIR IBR- Ab ELISA kit according to its instructions. The principal of the test depends on that the plates are coated with IBRV antigen, the samples under examination are added. If the suspected sample contains antibodies against IBRV, the antibodies will bind to the antigen; the conjugate will bind to this antigen antibody complex, and then give blue colour with the substrate solution. The reading occurs at 450nm. Calculation of corrected Optical Density (COD) of controls and samples by**:**

COD = OD of +ve, -ve controls or samples (coated with IBRV antigen) - OD of wells coated with the control antigen

The calculation of percent of positivity(PP) of the samples according to the equation **PP =COD of sample / COD of +ve control x 100**

For the serum samples, the sample consider negative if the PP is <10, doubtful, (10-19) and positive ≥ 20. While for the milk samples, the sample consider negative if the PP is < 3, doubtful, (3-5) and positive >5.

Results

Isolation of IBR Virus

Inoculation of the nasal and ocular swabs in Madin–Darby bovine kidney cell line (MDBK), revealed a total of 10 positive isolates, showed characteristic CPE within 2-5 days, characterized by grape like clusters of rounded cells gathered around a hole in the monolayer; sometimes giant cells with several nuclei can be found **(Table 2)**. **Figure (1-b).**

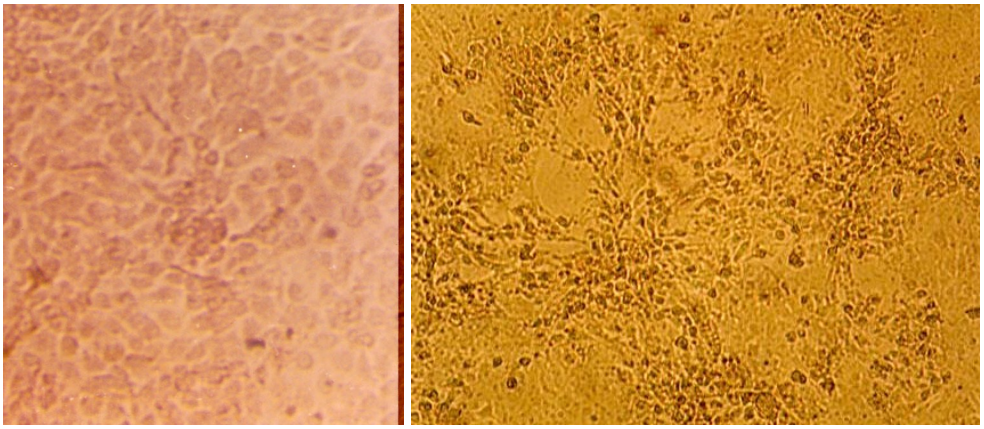
Virus Identification by Direct Fluorescent antibody technique (DFAT).

Five positive isolates out of 10 give clear perinuclear yellowish green fluorescent granules by using the direct fluorescent antibody technique (DFAT) **.(Table 2) (Figure 2).**

Virus Identification by electron microscope

Confirmation of the positive isolate by electron microscope revealed the presence of two herpesvirions enclosed in a common envelop (Bar=50nm) (Magnification, 60,000 x) **(Figure 3).**

(a)



(b)

Figure (1) (a). None infected MDBK cell. **(b).** MDBK cells infected with IBRV showing grape like clusters of rounded cells gathered around a hole in the monolayer (400x)

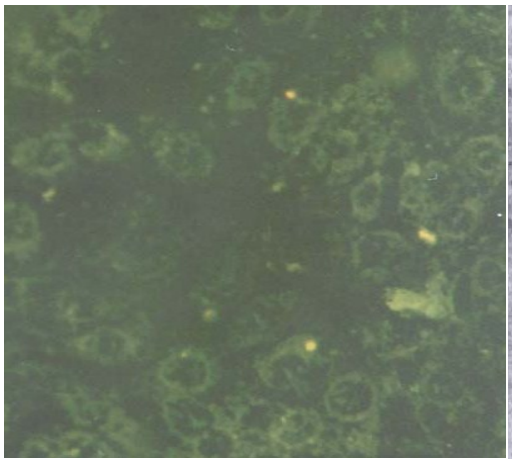


Figure (2). Perinuclear yellowish green fluorescent granules (400x)

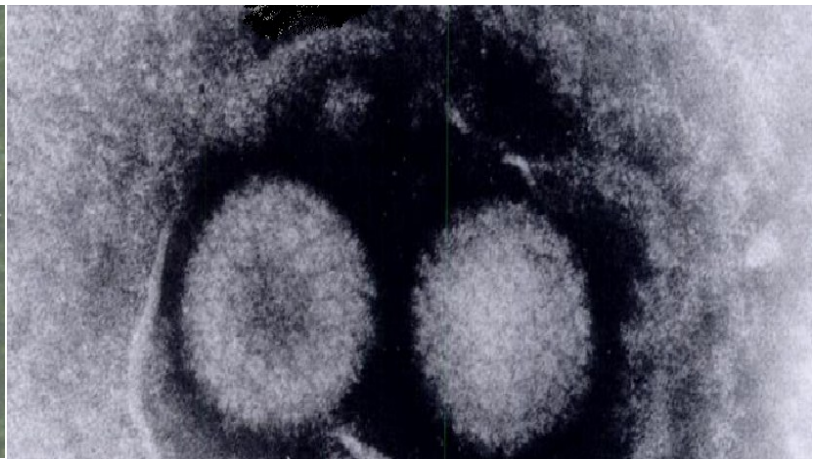


Figure (3). Two herpesvirions enclosed in a common envelop (Bar=50nm) (Magnification 60, 000x)

Table (2). Results of isolation and identification of BHV-1 on Tissue culture and identification by DFAT

Type Of Farm	Animal case	Species	No. of samples	Positive	
				CPE in T.C.	DFAT
Open farm .	Apparently healthy	calves	18	-	-
	Apparently healthy	Dairy	30	1	1
	diseased	Dairy	26	3	1
	Total		74	4	2
Closed farm	Apparently healthy	Dairy	20	1	-
	diseased	Dairy	10	5	3
	Total		30	6	3
Over all Total			104	10	5

Detection of IBR Virus antibodies by indirect ELISA in serum and milk:

Detection of the antibodies against BHV-1 in the serum and milk samples by using **SVANOVIR IBR- Ab** ELISA kit., revealed a number of positive samples (43 and 35) with a percent of positivity (47.8 & 43.7) in the serum and milk samples respectively **(Table3).**

Table (3). Detection of antibodies to BHV-1 IN Serum and milk samples by using ELISA

Type of samples	No. of samples	Positive ELISA	Negative ELISA	% of positive
Serum	90	43	47	47.8%
Milk	80	35	45	43.7%
Total	170	78	92	45.8%

Discussion

Although the symptoms of IBRV are mainly non-life threatening but it is an economically important disease as infection may cause a drop in production and affect trade restrictions **(Winkler *et al*., 2000).**

A definitive diagnostic procedures in the laboratory are aimed at detecting the causative virus (or viral components) and the specific antibodies they induce. Nevertheless, because of latent infection induced by BoHV-1, detection of antibodies could be sufficient for the determination of the BoHV-1 status of individual animals. For virus isolation, various cell cultures of bovine origin are used, for example, secondary lung or kidney cells or the Madin–Darby bovine kidney cell line (MDBK). The virus produces a cytopathic effect in 2–4 days **(OIE, 2010).**

In our study, 10 samples showed characteristic CPE by propagation on monolayer of MDBK cells, the CPE within 2-5 days, in the form of grape like clusters of rounded cells gathered around a hole in the monolayer; sometimes giant cells with several nuclei can be found (**Table 2) (Figure 1-b).** These results agreed with the results of (**Clyde, 1992, Amal *et al*, 2008, Xingnian & Kirkland, 2008 and Mahmoud** and **Ahmed, 2009).**

An alternative method of virus identification is the direct detection of BoHV-1 antigen in cells by an immunofluorescence or immunoperoxidase test with conjugated specific antiserum **(Kaashoek *et al*., 1994 & OIE, 2010)**. In our study virus identification by Direct Fluorescent antibody technique (DFAT) revealed that 5 isolates out of 10 give clear perinuclear yellowish green fluorescent granules **(Table 2) (Figure 2).** This agreed with the study which stated that DFAT showed that virus replication takes place entirely within the cytoplasm in association with structure formed modified en-

doplasmic reticulum **(Gray and Nettleton, 1987, Amal, *et al*, 2008** and **Mahmoud *et al*, 2009)**

Although the isolated IBRV strain can be identified by specific immunofluorescent assay, electronic microscope examination can also be used (**China, 2011)**. In This study IBRV was confirmed by negative staining electron microscope, revealing presence of two herpesvirions enclosed in a common envelop (Bar=50nm) (Magnification, 60,000x). These results agreed with **Caroline *et al*, (1992).**

Several types of BHV-1 ELISA tests are commercially available and most of them are also suitable for detecting antibodies in serum and milk samples but have some limitations. However, indirect ELISAs optimized for use with bulk milk samples in cows and can indicate reliably the BoHV-1 status of these animals.

With respect to our study, serological identification of IBRV antibodies in the serum and milk samples revealed a number of positive samples, 43 and 35 samples with a percent of positivity 47.8 & 43.7% in the serum and milk samples respectively **(Table3).** These results agreed with **(OIE, 2010),** which stated that various indirect ELISAs are most widely used for antibody detection in serum or plasma, and milk samples. Different studies stated that, ELISA can detect antibodies to IBRV in bulk tank milk and serum samples to monitor dairy herd's status. (**Nakashly, 1981& OIE, 2002)**. **Obando *et al.* (1999)** used a commercial indirect ELISA kit and found that ELISA is more specific for seroprevalence than virus-neutralization (VN) test. In addition**, Bastawecy *et al.* (2005)** developed indirect ELISA for detection of antibodies against BHV-1.

Finally Immunoenzymatic assays have many advantages because they are cheep, reliable and quick to perform, especially when applied to milk. Thus, ELISA may be a useful tool in large scale screening and eradication programs giving insight to the local IBR and BVD infection status. **Ghazy, *et al*, 2007).**

As all Herpesvirdae, BHV-1 remains latent in infected animals and may recur under certain stress condition. Shedding of the virus may or may not be accompanied with clinical signs, which explain the positive results of CPE and FAT for the samples from apparently healthy animals. Latency allows the virus to persist and the introduction of a latently infected carrier into a non infected herd is the best way to spread the disease .In our study the number of positive results in closed farms is more than that in the open farm. This agreed with the study stated that the stock density and mixing of the animals in closed farms allow the virus to spread **(Solis-Calderon, *et al*, 2003) .**

In addition, due to the virus latency that is normal criteria of BHV-1, the identification of serologically positive apparently healthy animals, provide a useful indicator of infection status. **(Muylkens *et al*., 2007, Eiras *et al*. 2009 and Badiei, *et al*, 2010).**

The results of this study clearly indicate that BHV-1 is subclinical prevalent virus in cattle in Egypt. ELISA is quick and reliable test for diagnosis of BHV-1, highly sensitive and could be used in large scale for screening and eradication programs

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دراسات سيرولوجية وفيروسية على فيروس التهاب القصبة الهوائية المعدى فى الابقار فى بعض المزارع فى محافظة البحيرة

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الملخص العربى

يعتبر فيروس التهاب القصبة الهوائية المعدى من الفيروسات التى تصيب الابقار المستأنسة والمتوحشة ولها تأثير اقتصادى على الانتاج الحيوانى.فى هذه الدراسة تم عزل الفيروس والتعرف عليه بتجميع عينات من الأبقار من مناطق مختلفة فى محافظة البحيرة ،بعض هذه الابقار كانت تعانى من اعراض تنفسية وبعضها كانت سليمة وذلك خلال عام 2011-2012. تم تجميع عدد 274 عينة (52 مسحات أنفية، 52 مسحات من العين، 90 عينة دم لفصل السيرم و80 عينة لبن) من مزارع مفتوحة ومغلقة. بتمرير المسحات على خلايا الزرع النسيجى اعطت 10 عينات تغيرات باثولوجية على الخلايا. باستخدام اختبار الفلورسنت المشع المباشر للتعرف على وجود فيروس التهاب القصبة الهوائية المعدى كانت خمسة عينات ايجابية. تم التعرف على شكل الفيريون VIRIONباستخدام الميكروسكوب الالكترونى. بفحص 90 عينة سيرم و 80 عينة لبن منزوع الدسم للاجسام المناعية للفيروس باستخدام اختبار الاليزا الغير مباشر كانت 43 عينة سيرم و 35 عينة لبن منزوع الدسم ايجابية للاجسام المناعية ضد فيروس التهاب القصبة الهوائية المعدى. يعتبر اختبار الاليزا الاسرع،الابسط ،الاوفر ومناسب لفحص عدد كبير من العينات.