

Isolation and identification of bovine herpes virus -1 (BHV-1) from semen of foreign breeds bulls

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Bovine herpes virus-1 (BHV-1) was isolated from bull semen by inoculation onto chorioallantoic membrane of specific pathogen free eggs. The isolated virus was identified by agar gel precipitation test, Dot ELISA, pock reduction and neutralization test, as well as by histopathology. The isolated virus was propagated on Madin Darby Bovine Kidney cells and identified by polymerase chain reaction. In sero-survey for BHV-1 antibodies on 1091 collected serum samples 188 sera showed clear precipitation lines by AGPT.

The extensive use of artificial insemination (AI) in cattle since the 1930 has facilitated exchange of desired genetic characteristics (e.g. milk production, body conformation) both nationally and internationally. However possible contamination of semen and dissemination by semen of bovine pathogens are of primary concern to cattle breeders and regulatory authorities in countries where AI is practiced. The microbial pathogens associated with bovine semen, including bacteria, fungi, protozoa and virus may be present in bull semen and transmitted to susceptible animals at insemination. The preservation of semen increased the national and international distribution of semen and possibility of spreading diseases among cattle populations. Measures taken by the industry and by regulatory authorities to prevent or control microbial contamination of semen have included maintaining groups of bulls free from specific diseases and adding antimicrobial agents to extended semen. Viruses may be excreted into any of the fluids or secretions of the body including semen which consists of sperm cells suspended in fluids originating from various sites in the genital tract of the male. Also viruses are readily preserved under the conditions of freezing and storing bovine semen thus permitting the spread of infectious viruses to the inseminated cattle. As it is not always practical to exclude genetically superior bulls from AI centers based on their infection with or exposure to specific viruses, the need often arises to

examine individual batches of semen for the presence of virus using reliable laboratory tests (Afshar and Eaglesome, 1990). Bovine herpes virus -1(BHV-1) has been the herpes virus most often found in bull semen and considered one of the most dangerous viruses within family *Herpesviridae* and able to establish latency in sensory neurons of sacral ganglia. This work was conducted on semen samples. BHV-1 was isolated and identified. Sero-prevalence of BHV-1 was also investigated.

Material and methods

Samples. Total of 19 fresh semen samples were collected from foreign breed bulls (Friesian and South brown) located at Beni-Suef Governorate, and subjected for freezing and thawing several times(3-5 cycles) then centrifuged at 3000 rpm for 10 minutes, the seminal plasmas were collected and stored at -20 °C until used for virus isolation.

Total of 20 preputial swabs were collected from bulls located at Beni-Suef Governorate. Collected preputial swabs in PBS were centrifuged and the obtained supernatant were stored at -20°C until used for virus isolation. These samples were used for virus isolation according to (Van Oirschot *et al.*, 1995) by inoculation into Specific Pathogen Free (SPF) embryonated chicken eggs obtained from (Nile SPF farm, Koam Oshiem, Fayoum, Egypt).

Total of 1091 serum samples were collected from 7 centers in Beni-Suef Governorate, these samples were used for detection of bovine herpesvirus-1 antibodies by agar gel

precipitation test using 1% agarose in saline solution according to (Beard, 1982).

Dot ELISA. It was done according to (Hawkes, *et al.*, 1982) by dotting 3-5 μ L of prepared antigen suspension on nitrocellulose membrane (NCM) followed by blocking of the free protein binding site using 1% Tween80. The blocked membrane was incubated in 10% antiBHV-1 hyperimmune serum then washed three times before secondary incubation in anti species conjugated with peroxidase. Then the NCM was immersed in working substrate solution, and the reaction was stopped by washing under tap water.

Histopathology. Both trypsinized membrane test (Madbouly *et al.*, 1997), and thin section of infected CAMs were stained and examined under light microscope to detect intranuclear inclusion bodies.

Pock reduction test. The infectivity titers of isolated viruses were done according to (Hitchner and White, 1958), 1000EID₅₀ of the titrated viruses were mixed with 4 neutralizing unit of BHV-1 hyperimmune serum before inoculation into CAM of ECE.

Virus propagation in MDBK. The isolated viruses were propagated on MDBK cell line and the produced cytopathic effects (CPE) were recorded.

Serum neutralization test. It was done according to (Hitchner *et al.*, 1958) by mixing 4 neutralizing units of BHV-1 hyperimmune serum with each dilution (10^{-1} - 10^{-8}) of the isolated viruses.

Polymerase chain reaction (PCR). It was done according to (Ibrahim *et al.*, 2001). The primer sequences was designed based on the sequence of the BHV-1 glycoprotein C (gpC) gene according to (Fitzpatrick, *et al.*, 1989). The specific primers were synthesized using DNA synthesizer (Institute for Molecular Biology and Genetic Engineering, ARC, Egypt). The sequences of the oligonucleotides are P1 (5 – CTG CTG TTC GTA GCC CAC ACG-3) and P2 (5 –TGTGACTTGGT GCCCA TGT CGC-3). BHV-1 DNA extraction and purification method were done according to (Galik *et al.*, 1990), DNA amplification and detection of PCR product were done according to the method described by (Von-Beroldingen *et al.*, 1990) bands were visualized after electrophoresis of 10

μ l of the PCR product in a 2% agarose stained with ethidium bromide (0.3mg/ml) DNA fragments were examined under UV light.

Detection of bovine herpesvirus-1 antibodies in serum samples. It was done by agar gel precipitation test according to (Beard, 1982).

Results

BHV-1 was isolated from semen samples (2 out of 19) showed characteristic pock lesion on inoculated CAM. The titers were 7.8, and 6.2 for the large (L) and small (S) pock lesions respectively. Preputial swabs failed to produce any characteristic pock lesions on inoculated CAM. Well developed characteristic pock lesions of large size appeared rounded in shape with opaque raised edge and depressed gray central area of necrosis. These foci ranged from 3-5mm in diameter, beside small foci ranged from 1-2mm in diameter scattered all over the membrane (Fig.1). Pock lesions (large and small) were picked up and propagated separately for 3 successive passages to produce clones of the same size.

The isolated viruses were propagated on MDBK cell line and produced CPE in the form of rounding and aggregation of the infected cell endings with dispatched areas of infected cells in the cell sheath, and characteristic intranuclear inclusion bodies which appeared acidophilic in color with margined chromatin condensation on the peripheral margin near the nuclear membrane (Fig.2).

The isolated viruses were identified by AGPT, Dot ELISA, pock reduction and neutralization tests (using specific anti BHV-1 hyper-immune serum), and polymerase Chain reaction (PCR) tests. The infectivity titer of L and S isolates was decreased by 4 and 2.8 (\log_{10}) respectively when incubated with specific antiBHV-1 hyper-immune serum.

The electrophoretic pattern of BHV-I PCR product where M (100-1000 bp molecular weight marker). Lane 1:(S) isolate, Lane 2:(L) isolate, Lane 3: negative control (non infected MDBK cells) and Lane 4: positive control BHV-I strain, (Fig. 3).

Sero-survey for BHV-1 antibodies. A 1091 serum samples were collected from cattle located at 7 centers at Beni-Suef Governorate and subjected for AGPT for detection of BHV-1 antibodies (Table 3).

Table (1): Isolation and infectivity titer of BHV-1 from seminal fluids

Type of Samples	No of inoculated samples	No of +ve	% of +ve	Type of pock lesion		EID50/ml (Log 10)	
				large	Small	Large (L)	Small (S)
Semen samples	19	2	10.5%	+	+	7.8	6.2
Preputal swabs	20	0	0%	-	-	0	0

Table (2): Identification of the isolated BHV-1.

Test type	Result
AGPT	Clear line of precipitation in both large and small pock lesions.
Dot ELISA	Blue dots with both L and S pock lesions
Pock reduction	+
Neutralization index	4 and 2.8L pock lesions (4),and S pock lesions (2.8)
PCR	+

Table (3): Results of prevalence of BHV-1 antibodies in cattle sera by AGPT.

Locality	No of tested samples	No of +ve sample	% of +ve samples
Naser center	427	72	16.86%
El-Wasta center	138	27	19.56%
El-Fashn center	84	8	9.52%
Beba center	55	17	30.9%
Ehnsia center	64	14	21.87%
Somosta center	256	40	15.62%
Beni-Suef center	67	10	15%
Total	1091	188	17.2 %



Fig. (1): Small and large pock lesions in infected CAM.

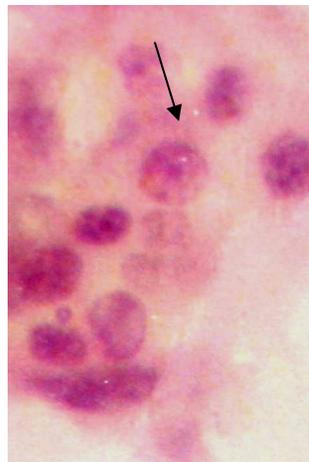


Fig.(2): Intranuclear inclusions in MDBK infected cells.

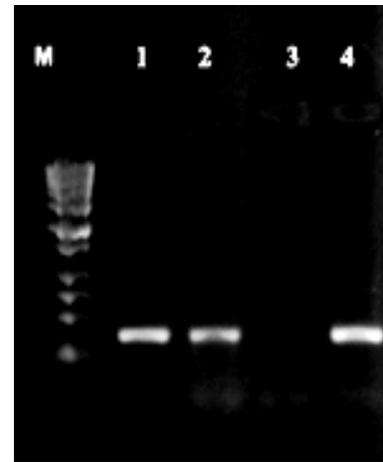


Fig. (3): PCR results showed 200bp product in Lane 1:(S) isolate, Lane 2:(L) isolate, Lane 3: negative control (non infected MDBK cells) .

BHV-1 antibodies could be detected in 188 out of 1091 tested serum samples (17.2 %) collected from cattle by AGPT where positive samples produced clear precipitation lines.

Discussion

Bovine herpes virus 1 is a member of family *Herpesviridae* which induces severe economic losses, these losses are not only due to death, but also due to weight losses, insufficient feed conversion, abortion, loss of newborn, temporary reduction in body condition and milk yield as well as secondary bacterial bronchopneumonia (Madbouly and Abd-El Raof, 2004). In Egypt the majority of domestic animal population is not subjected to vaccination for BHV-1. Previously and since few years ago only small number of private farms used cattle master vaccine for controlling BHV-1 and other viral infection (Madbouly and Abd-El Raof, 2004) but now this vaccine is prohibited to enter Egypt by the authorities. Pneumo 3&4 vaccines which include BHV-1 are now currently manufactured by Serum and Vaccine Production Institute Abbassia and used in small scales for controlling the disease in private farms. After primary infection of bulls, the virus was disseminated in semen (Thibier, 1988) therefore in the artificial insemination (AI) industry, the virus is of major concern, because there is a potential risk of transmission to cows during insemination (Van Engelenburg *et al.*, 1995).

In this work nineteen semen samples and twenty preputial swabs were collected, from apparently healthy bulls located at Beni-Suef Governorate, for virus isolation, in addition to 1091 serum samples collected from cattle located at Beni-Suef Governorate were used for the detection of BHV-1 antibodies. Data presented in Table (1) showed clearly large and small pock lesions were appeared on the CAM of the inoculated SPF ECE from semen samples (2/19). The large pock lesions are rounded in shape with opaque raised edge and depressed gray central area of necrosis ranged from 3-5mm in diameter. Beside these foci small foci ranged from 1-2mm in diameter scattered all over the membrane are seen (Fig.1). The characters of the observed pock lesions in this study was agree with those described by (Madbouly and Hussein, 1997) who successfully isolated BHV-1 from samples collected from dairy herds showed genital form of

infection by inoculation on CAM of ECE. The size of pock lesions that appeared on the CAM may be denote to only one virus isolate or to two virus isolates. The infectivity titers of these isolated viruses were determined on ECE by CAM route, it was $10^{7.8}$ and $10^{6.2}$ for L and S isolates respectively (table 1). Identification of isolated virus was done by AGPT, Dot-ELISA, pock reduction and neutralization tests by using specific anti BHV-1 hyper immune serum (Table 2). The isolated viruses that produced large pock lesions or small pock lesions reacted well with the used specific anti BHV-1 hyperimmune serum in the serological tests used for virus identification, and this may be explained by the presence of two strains of BHV-1. The isolated viruses produced CPE in MDBK in the form of rounding and aggregation of the infected cell endings with dispatched areas of infected cells in the cell sheath, and characteristic intra nuclear inclusion bodies which appeared acidophilic in color with margined chromatin condensation on the peripheral margin near the nuclear membrane. These observed CPE are in agreement with those observed by (Miller, *et al.*, 1965).

On the other hand PCR was used for identification of the isolated viruses (L and S) which gave positive results (Fig. 3), these results agreed with those observed by (Ibrahim, *et al.*, 2001). The importance of PCR lies on its sensitivity particularly during the later phases of infection (Smits, *et al.*, 2000), rapid detection of BHV-1 in bull semen (Van Engelenburg, *et al.*, 1993), easier to perform than the standard virus isolation in bovine semen (Rocha *et al.*, 1998) and can be cost-effective method to minimize the risk of transmission of the virus by semen in endemic countries with BHV-1 (De Gee *et al.*, 1996). Also PCR may be a good alternative to virus isolation especially when bovine semen has to be screened for BHV-1 prior to artificial insemination (Grom *et al.*, 2006). The isolation of BHV-1 from semen of apparently normal bulls may be explained by the previous infection of these bulls either by ascending infection from infected cows showed vulvo-vaginitis or descending infection (by latency) after primary acute respiratory infection of these bulls.

On applying sero-survey for BHV-1 antibodies in serum samples collected from different localities at Beni-Suef Governorate using

AGPT, .188 out of 1091 tested serum samples (17.2%) showed clear precipitation lines (Table3).

These results were in agreement with (Madbouly and Amal-Abd El- Raof, 2004) who made sero-survey of BHV-1 antibodies in sera of domestic animals during months of 2001 and they found 21.9% of cattle sera were reacted positive by ELISA test. Mousa , *et al.*, (1990) made serological survey on the prevalence of BHV-1 in domestic animals in Egypt during the period Oct.1985-Sept.1988 and they found that 19% of screened serum samples from cattle were reacted positive by using serum neutralization test (SNT) the high percentage of infection reported by (Mousa , *et al.*, 1990) using SNT (19 %) and by (Madbouly and Amal Abd El-Raof, 2004) by using ELISA test (21.9 %) was attributed to high sensitivity of both these two tests (SNT and ELISA) in a comparison with our results (17.2 % positive) by AGPT. The risk of using contaminated semen for insemination of Egyptian cows leads to increase the incidence of BHV-1 in our Egyptian cattle population and difficult control with consequently increasing the economical losses .The Egyptian authorities (the Ministry Of Agriculture, and the General Organization of Veterinary Services(GOVS) must take their decisions and implementation steps to prevent this risk through: periodical virological examination with replacement of infected bulls other uninfected and treatment of semen with antiBHV-1 drugs. From all these previous discussed data ,we could concluded that the disease (BHV-1) is widely spread in our country ,and the economic losses by this virus triggered us to say that BHV-1 is inducing high risk in cattle population in Egypt, and control of the disease is not through vaccination with live attenuated vaccine due to latency of the virus in vaccinated animals but we suggest the use of inactivated viral vaccine prepared from locally isolated strain and supplemented with good adjuvant in additional to better diagnoses of latency cases .

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عزل وتصنيف فيروس الهربس البقري من السائل المنوي لطلانق السلالات الأجنبية

تم عزل فيروس الهربس البقري من السائل المنوي لطلانق السلالات الأجنبية على الغشاء اللقائقي لأجنة البيض المخصب الخالي من الميكروبات . وتم التعرف على الفيروس المعزول باختبارات الترسيب الهلامي في الاجار والايضا النقطية واختزال البثرات والتعادل المصلي وكذلك بالفحص الهستوباثولوجي. ثم تم تمرير الفيروس المعزول على خلايا كلى الابقار وتم التعرف عليه باختبار تسلسل البلمرة . تم عمل مسح سيرولوجي للكشف عن الاجسام المناعية لفيروس الهربس البقري في 1091 عينة سيرم باستخدام اختبار الترسيب الهلامي في الاجار واطهرت 188 عينة خطوط ترسيب واضحة في الاجار .