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Serological and Molecular Identification of Infectious Bovine Rhinotracheitis Virus Isolation and Adaptation in Embryonated Chicken Eggs

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Abstract

Infectious bovine rhinotracheitis virus (IBRV) is a major pathogen in livestock animals and had led to significant economic losses to the industrial production worldwide. However, IBR symptoms are not life-threatening. The present study was achieved to make seroprevalence and isolation of bovine herpes virus type (BHV-1) from nasal and ocular swabs samples of suspected cows and buffaloes, identification by serological and polymerase chain reaction (PCR). Total of 380 blood samples were collected from suspected 287 cattle and 93 buffaloes in different districts at Beni-suef and El-Fayoum governorates in winter 2017. Nasal and ocular discharge swabs were collected from cattle and buffaloes with clinical respiratory signs (Nasal discharge, cough, lachrymal discharge with or without mild diarrhea and higher body temperature). A total of 106 (27.89%) samples were positive by indirect ELISA and the positivity of 80 (27.87%) samples from cattle and 26 (27.96%) samples from buffaloes located at different centers at Beni-Suif and El-Fayoum governorates. Virus was isolated from nasal and ocular discharges swabs samples and it was adapted in chorioallantoic membrane (CAM) of 11-day-old embryonated chickens eggs and in MDBK cells. Virus-infected CAM showed congestion, edematous vacuole, thickening small foci ranged from 2 to 3 mm in diameter, scattered on CAM membrane and MDBK cell line inoculated blind serial passages at 3rd passage showed characteristic of cytopathic effect (CPE). Identification of virus isolated on CAM and infected cell culture fluid gave precipitation against positive specific anti-BHV-1 immune serum by AGPT and clear blue zone by Dot ELISA, absent of pock lesion in pock reduction test (PRT), and confirmed by PCR with product size of 175 bp. Finally BHV-1 virus was isolated from nasal and ocular discharges of cattle and buffaloes but the further extensive study still need for clear final classification by phylogenic analysis.

Keywords: Bovine herpes virus-1; Infectious Bovine Rhinotracheitis virus; ELISA; AGPT; PCR Dot ELISA

Introduction

Infectious bovine rhinotracheitis (IBR), caused by bovine herpesvirus 1(BHV-1 [1]. BHV-1 is one of the most important viral infections of cows and buffaloes all over the world [1]. It is a member of the Varicellovirus genus of the subfamily Alphaherpesvirinae which is belong to Herpesviridae family [2]. BHV-1 is a member of the Varicellovirus genus of the subfamily Alphaherpesvirinae which is belong to Herpesviridae family, the viral genome consists of double stranded (ds DNA) that code for about 70 proteins, of 33 structural and 15 nonstructural proteins, an envelope glycoprotein, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity. BHV-1 can be differentiated into three subtypes belong to one single viral species [3-5].

The BHV-1 is excreted through nasal, conjunctiva secretion, semen and aborted placenta an acute infection, it get latent in the sensory ganglia of the animal [6]. In general, cattle above 6-8 months of age are infected with BHV-1 after decreasing of their maternal immunity [6,7]. However, all subtypes are antigenically similar BHV-3, which is a neuropathogenic agent, has been re-classified as BHV-5 [7,8]. Despite the development of antiBHV-1 antibodies, the virus can establish latency in the trigeminal or sacral ganglia and germinal centers of pharyngeal tonsils after infection, animals considered clinically normal after primary infection [9]. But they remain a source of infection to other healthy cattle. Reactivation of BHV-1 from latent infection due to stresses factor as transportation, parturition, high ambient temperature and due to injection of anti-inflammatory corticosteroids leads to shedding of the BHV-1virus into environment causes infections by direct or in direct contact [4,10]. Immune suppression as results of BHV-1 infections leads to secondary bacterial infections; as a result BHV-1 is an important co-factor in the bovine respiratory disease complex [10,11].

There are different methods for the diagnosis and identification of BHV-1, serological and polymerase chain reaction (PCR) is more sensitive, specific and relatively rapid in comparison to virus isolation [8,12]. In Egypt, since 1970, BHV-1 has been enzootic source of substantial losses in dairy farms and feedlot animals mainly due to pneumonia, abortions and deaths [13,14]. The present study was achieved to make seroprevalence and isolation of bovine herpes virus type (BHV-1) from nasal and ocular swabs samples of suspected cows and buffaloes, identification of the isolated virus by serological and polymerase chain reaction (PCR).

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Material and Methods

Sample Preparation

A total of 380 blood samples without anticoagulant were collected from 287 cattle and 93 buffaloes with or without IBR respiratory signs and are not vaccinated with BHV-1, in different location at Beni-Suef and El-Fayoum governorates during winter months 2017. IBR respiratory signs were includes (Nasal discharge, cough, and lachrymal discharge with or without mild diarrhea). 5 ml blood samples were taken from the jugular vein by vacationer tubes without anticoagulant, and held at room temperature for approximately one hour at slant to allow the separation of blood serum. Samples kept at -20°C until use for the detection of antibodies in indirect ELISA [15,16]. Nasal and ocular swabs samples were collected from 147 (cattle and buffaloes) showed IBR symptoms in 3 ml Eagle's Minimum Essential Medium (EMEM) for each sample containing 1% new fetal bovine serum (FBS) and 100 IU/ml mycostatin, 100 IU/ml penicillin and 100 µg/ml streptomycin, 20 IU/ml kanamycin then centrifuged in cooling centrifuge 4°C at 3000 rpm for 15 m the supernatant fluid was kept at -20°C for virus isolation.

Reference viruses and Reference antiserum

Local Egyptian strain of BHV-1 virus was kindly obtained from Veterinary Serum and Vaccine Research Institute, Abbassia, Egypt. Positive anti BHV-1 antiserum was kindly obtained from Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Egypt. Rabbit anti-species (cattle and buffaloes) immunoglobulin peroxidase conjugate was kindly obtained from ELISA unit, Animal Health Research Institute, Dokki, Giza, Egypt. (Sigma Chemical Co. USA).

Indirect ELISA

It was used for the detection of antibody against BHV1 in cattle and buffaloes serum and was used according to [8,17]. Diluted to1/100 then each sample was added to the micro-plate wells. The contents of the wells were homogenized by gentle shaking of the plate, and then the plate was covered with a lid of aluminium foil and incubated for 1 h. Washing solution was diluted (1:100) with distilled water. The plate was washed by washed buffer 3 successive time. The conjugate was diluted 1/1000 with dilution buffer. 100 µl of diluted conjugate was dispensed per well, and incubated for 30 m. Washed three successive times.100 µl of revelation solution was placed in each well, and the plate was incubated for 20 m. 100 µl of stop solution was dispensed per well. The optical density was read at 450 nm in a multi-well plate reader (EXL 800 Biotic, USA).

Agar gel perceptions test (AGPT)

The AGPT was performed according to [18-20]. One and half gram of agarose (DIFICO) and one and half gram glycine were added to 100 ml distilled water, the mixture was boiled in water bath until dissolving of the agarose, the agarose medium was left at room temperature until reaching 45°C then poured in Petri dishes 5 cm in diameter. After solidification, of the agarose in the petridishes 7 wells of 6 mm in diameter had been cut. The central well was filled with reference BHV-1 homogenized harvested pock lesion and the 4 peripheral wells were received serum, positive and negative control sera in upper and lower wells. The central well was filled with anti-BHV-1 positive serum and the 4 peripheral wells were received from homogenized CAM with pock lesion also, positive and negative control antigens in upper and lower wells then incubated at 37°C in a humid chamber and examined from12 to 72 h.

Virus Isolation

Isolation of the virus: *In vitro*, nasal and ocular swabs were agitated in the transport medium to elute virus and left at room temperature for 30 minutes and filtered through 0.45 Millipore filters and a 200 µL volume of supernatant from the processed swabs was inoculated into chorio-allantoic membrane route (CAM) specific pathogen free (SPF) of embryonate Chicken Eggs (ECE) which obtained from (Nile SPF, Koam oshiem, Fayoum, Egypt). The inoculated eggs were incubated at 37°C, 2-3 serial blind passages and the harvested CAM with pock lesion was confirmed [19,20]. 100 µL volume of supernatant from the processed swabs was inoculated into monolayer of MDBK cell culture and incubated at 37°C for 1 h, the cells were rinsed, maintenance medium was added and the cultures were subsequently incubated at 37°C in an incubator with 5% CO_2. The cell cultures were observed daily for CPE daily under inverted microscope.

Infectivity Titers of Isolated Viruses

Virus titration was conducted using infectivity method according to [21]. The titer of the virus was expressed as log 10 TCID50/ml and tenfold serial dilution was prepared from the supernatant fluid of the infected MDBK cell by adding 0.1 ml of the isolated virus to 0.9 ml of maintenance media to obtain 1:10 dilution then 0.1 of the first dilution was transferred to the next tube with thoroughly mixing and 0.1 of this dilution was transferred to the next tubes and this step was repeated till 10-10 dilution 0.2 ml from each dilution was inoculated to 5 well containing confluent MDBK cell. The negative control wells were contained cells and media only. The plate was incubated at 37°C after the adsorption of the inocula and was examined daily for CPE. Also, about 0.5 ml of each dilution was inoculated on 4 ECE (0.1 ml for each egg). The inoculated eggs were incubated at 37.5°C with 80% humidity in egg incubator with daily candling during the incubation period (5days). Egg infective dose 50 (EID50) were calculated. The virus titre was calculated according to Reed and Munch formula.

Serum Neutralization Test

This test was done according to [21]. Infectivity titer of the isolated virus was calculated and then 0.4 ml from this dilution was incubated with 0.4 ml anti-BHV-1 hyper immune serum, left at room temperature for 30 m. Virus-serum mixture was inoculated on to 4 CAM (0.2 ml per egg) of SPF of ECE day 11-12 old embryos. ECE were incubated at 37.5°C for 4-5 days with checking daily with Candler. At the end of incubation CAMs had been harvested and examined for the pock reduction lesion. Control positive (inoculated with reference strain of BHV-1) and control negative (inculcated with PBS) were done.

Dot Enzyme-linked Immunosorbent Assay

It was done according to the method described by [22,23]. Nitrocellulose membrane (NCM) was prepared by placing it on the top of a 96 wells ELISA plate. The NCM was pressed by the tip of the finger to form the required number of impression circle, then, washed by distilled water for 3 successive times at 3 m and then dried in the room temperature. After drying NCM, 5 µL of prepared homogenate antigen suspension was dropped into each impression, then dried for 15-20 m

at the room temperature, then washed for 3 m with Tris Buffer Saline (TBS), dotted NCM was blocked by using blocking solution for 60 m at room temperature, blocked NCM was placed in clean sterile vehicle and then covered by anti BHV-1 hyper immune serum diluted 1:10, then incubated at room temperature for one hour with slight shaking. After the end of incubation the unbounded antibodies removed by washing NCM three successive times with TBS-Tween 80 for 10 m of each, NCM was impressed in the anti-species immunoglobulin conjugated with peroxidase diluted 1:1000 then incubated for one hour at room temperature with slight shaking. Excess amount of the secondary antibodies were removed by washing the membrane three times with TBS-Tween 80 for 10 minutes for each. The positive colored reaction was developed by immersing the NCM in 4-chloro-1-Naphthol working substrate solution. Blue dots were appeared within 5-15 m after incubating the NCM in working substrate solution in a dark place. The reaction stopped by washing the membrane under running tap water.

Extraction of Viral DNA

DNA was extracted using the commercially available DNA extraction kit (Qiagen, USA), as indicated in the manufacturer's protocol. DNA extraction from the samples was extracted from harvested homogenate CAM with clear pock lesion and from supernatant culture of MDBK cell DNA was isolated using QIAamp DNA Mini Kit (Qiagen Ltd USA). The DNA concentration and purification was measured using spectrophotometer and final elution of DNA was done in 50 µL of elution buffer and stored at -20 for long term use.

Polymerase Chain Reaction (PCR)

PCR specific primers of BHV-1 were used glycoprotein B (gB) gene as target gene as protocol using by [24,25]. To amplify the target sequence primers for BHV-1 glycoprotein B (gB) primers were (F, 5'-TGT GGA CCT AAA CCT CAC GGT-3'; R 5'-GTA GTC GACAG ACC CGT GTC-3'), 1 µl (25 pmol) of each primers, 5 µl of the extracted DNA was added to 45 µl of PCR mix containing 2.5 U of Taq DNA polymerase, 5 µL of 10X PCR buffer, 1.5 µl of 50 mM MgCl2, 1 µl of 10 mM of dNTPs mix, The reaction was run under the following thermal cycling program: Pre-denaturing at 94°C/3 min; denaturing at 94°C/1 min, annealing at 60°C/1min, extension at 72°C/1min repeated (35 cycles) followed by a final extension at 72°C for 5 m. Negative and positive control reactions were used. Agarose gel was performed according to Maniatis et al. (1982). The DNA products were shown under ultraviolet (UV) light of the PCR products in a 2% agarose with ethidium bromide (0.3 mg/ml) and used ladder 100 bp molecular weight marker.

Results

The IBR suspected cattle and buffaloes were suffering from one or more clinical signs; abortion, respiratory signs, ocular symptoms, fever, mastitis and diarrhea. 80 serum samples from cattle were positive, the highest positive samples were (n-45, 32.14%) by indirect ELISA and (n-42, 30%) by AGPT while buffaloes had 26 positive serum samples the highest positive cases (n-14, 26.4%) by indirect ELISA and (n-11, 20.1%) by AGPT. The overall presences of BHV-1 antibodies were 27.87% and 27.96%, in cattle and buffaloes respectively as in Table 1. IBR respiratory disorders, including, high body temperature (39.5 to 41°C), nasal, ocular discharges, severe rhinitis and conjunctivitis were positive to BHV-1 virus or apparently healthy.

Species	Location	Sera from cattle and buffaloes	Indirect ELISA	AGPT
			Number of positive % sera	Number of positive % sera
Cattle	Beni-Suef	147	35 (23.9)	34 (29.9)
	El-Fayoum	140	45 (32.14)	42 (30.0)
Buffaloes	Beni-Suef	40	12 (30.0)	9 (22.5)
	El-Fayoum	53	14 (26.4)	11 (20.7)
Total		380	106 (27.89)	96 (25.26)

Table 1: Comparing between IELISA and AGPT for detection of BoHV-1 antibody in serum samples of cattle and buffaloes.

Data presented in Table 1 showed that the positive percentage of serum samples for antiBHV-1 antibodies were (27.89) by indirect ELISA while (25.26) were positive by AGPT from cattle buffaloes. The highest percent of ELISA in detection of antibody prove the superiorly test of ELISA than AGPT.

Isolation of bovine herpes virus 1 (BHV-1) on CAM of SPF and MDBK cell line

BHV-1 isolate on CAM of ECE-SPF and MDBK cell. After three to fifth blind passages CPE granulation of cells and rounding, at 72 h post inoculation more cells were found to be rounded and there was development of syncitia. As the incubation period progresses to 96 and 120 h respectively, more than 90% of cells revealed intense CPE like rounding, syncytia and lysis of the cells. Percent of virus isolation were 16 (10.88%) and 18 (12.24%) from ocular and nasal swabs samples of cattle and buffaloes respectively. These results were demonstrated in Table 2.

Discussion

Bovine herpes virus-1(BHV-1) is a member of family herpesviridae which causes severe economic losses in livestock worldwide due to weight losses, insufficient feed conversion, and abortion, temporary

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reduction in body condition, milk yield as well as secondary bacterial bronchopneumonia and deaths [25]. Although IBR symptoms can be prevent by vaccination but the latent infection is not prevented, once animal lifelong potential shedder of BHV-1 [26,27].

Species	Location	Nasal and ocular swabs		Virus isolation				Identification by		
				CAM positive %		MDBK positive %		Dot ELISA	AGPT	PCR
		NS	OS	NS	OS	NS	OS			
Cattle	Beni-suef	49	49	5 (10.23)	6 (12.24)	6 (12.24)	7 (14.28)	+	+	+
	El-fayoum	38	38	2 (5.11)	3 (7.81)	2 (5.26)	3 (7.90)	+	+	+
Buffaloes	Beni-suef	29	29	6 (20.60)	5 (17.24)	6 (20.62)	6 (20.60)	+	+	+
	El-fayoum	31	31	3 (9.60)	4 (12.90)	4 (12.90)	5 (16.12)	+	+	+
Total		147	147	16 (10.88)	18 (12.24)	18 (12.24)	21 (14.28)	+	+	+

Table 2: Isolation and Identification of BHV-1 from nasal ocular discharge samples swabs on CAM of ECE and adaptation in MDBK cells.

In Egypt BHV-1 was isolated from cattle suffering from a respiratory syndrome, the majority of animals population is not subjected to vaccination for BHV-1 but few number of private farms used cattle master vaccine for controlling viral infection [12,28]. Examined cattle and buffaloes by indirect ELISA were 27.87% and 27.96%, positive for BHV-1 in cattle and buffaloes. Seropositive samples for BHV-1 virus antibodies gave highlighted the circulation of virus among the livestock population as in Table 1, these results were in agreement with [28,29]. Found that serosurvey of BHV-1 antibodies in sera of domestic animals during months of 2001 and they found 21.9% of cattle sera were positive results by ELISA [30,31]. they found that seropositive against BHV-1 was 19% by using SNT (19%) and by ELISA test (21.9%) was attributed to high sensitivity of both these two tests (SNT and ELISA),with considerable variation in the percentages of positive clinical signs in the present study may relate to natural infection, type of viral strain, age susceptibility and environmental factors. Also, it may be due to the secondary bacterial infection, which leads to rise of severity of the disease and appearance of different clinical signs in the same case.

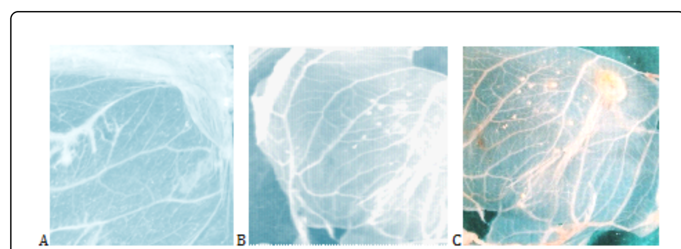

Figure 1: A, B and C pock lesions, area of opacity at the site of inoculation and generalized edema in the CAM of commercial ECE. Well-developed characteristic pock lesions of large and small size appeared rounded in shape with opaque raised edge and depressed gray central area of necrotic large necrotic foci, there are small foci ranged from in diameter scattered all over the membrane.

Virus isolated on CAM from nasal swabs samples 16 (10.88%) and 18 (12.24%) out from 147 samples after 3rd blind passages produced pock lesions as in Figures 1A-1C. CAM showed pock lesion become thick, edematous and congested at the 1st passage level but typical pocks lesion was observed onto CAM on later passages. More severe changes were observed in CAM after serial passages, indicating adaptation of BHV-1 on to CAM and this result is in agreement with [32].

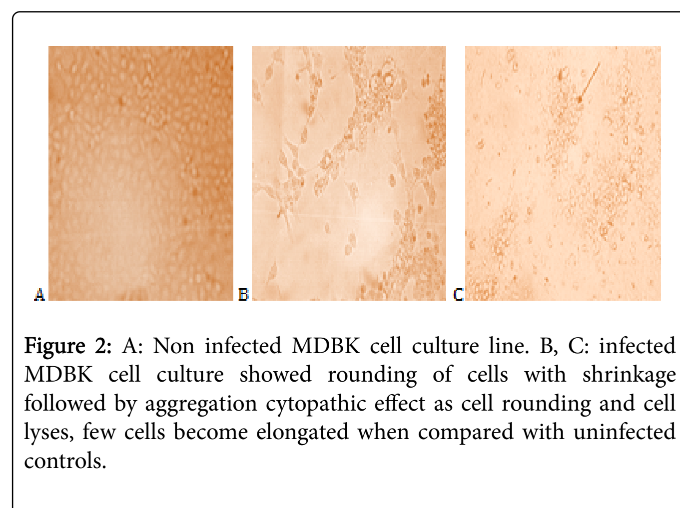

Figure 2: A: Non infected MDBK cell culture line. B, C: infected MDBK cell culture showed rounding of cells with shrinkage followed by aggregation cytopathic effect as cell rounding and cell lyses, few cells become elongated when compared with uninfected controls.

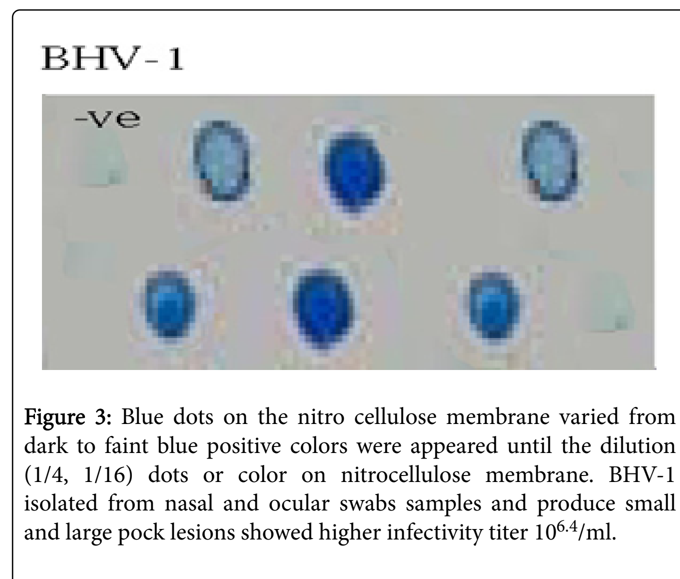

Figure 3: Blue dots on the nitro cellulose membrane varied from dark to faint blue positive colors were appeared until the dilution (1/4, 1/16) dots or color on nitrocellulose membrane. BHV-1 isolated from nasal and ocular swabs samples and produce small and large pock lesions showed higher infectivity titer $10^{6.4}$/ml.

Isolation BHV-1 from samples collected from dairy herds showed genital form of infection by inoculation on CAM of ECE, from nasal ocular discharge from cattle and buffaloes as in Tables 1 and 2 may be explain that infection of these animals by high doses of virulent BHV-1 and this explanation is agree with [33]. Who said that although sensory neurons in the trigeminal ganglia are the primary site of BHV-1, latency, viral genomes are detected in the tonsils of the latently infected calves and stress factors resulted in activation from site of latency [34]. Found lower percentages than the present study with results of 53.84% and the same percentage with the present study for nasal swabs (44%) were recorded [35].

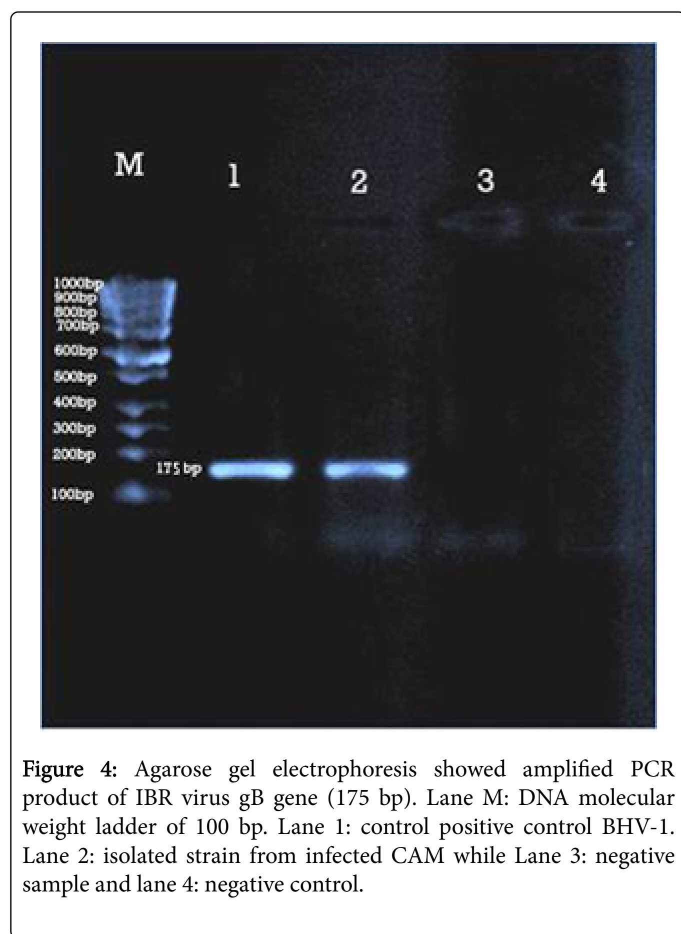

Figure 4: Agarose gel electrophoresis showed amplified PCR product of IBR virus gB gene (175 bp). Lane M: DNA molecular weight ladder of 100 bp. Lane 1: control positive control BHV-1. Lane 2: isolated strain from infected CAM while Lane 3: negative sample and lane 4: negative control.

However, BHV-1 infected monolayer was had CPE which includes rounding, aggregation, syncytia formation as in Figures 2A-2C with infectivity titers was $10^{6.4}$ and result is in agreement with [32,33]. Isolation of BHV-1 from serologically positive apparently healthy cattle and buffaloes are important where they provide useful and reliable methods for indication of prevalence of infection [34]. Animal positive BHV-1 antibodies are considered as a carrier for virus and potential shedding virus in their milk or from nasal and ocular discharge or from vaginal secretion [36]. Explained that the difficulty of isolation BHV-1 from nasal swabs was probably because of the decline of concentration of virus excreted from respiratory routs and this concentration increases if the nasal swabs have been taken from suspected animals during stress period [37]. Besides that, the shedding of virus occurs between the third and sixth days (during the early acute stage of the disease) so that the nasal swabs should be taken early when the discharge is more serious rather than mucopurulent [38]. The positive result obtained from isolated virus and Egyptian BHV-1 DNA amplification PCR products indicate that the gB gene is specific, sensitive and accurate for detection. BHV-1 in suspected samples from infected animals. BHV-1 isolates were identified by SNT (Pock reduction test) using control BHV-1 positive serum and control negative serum. Serum neutralization test (Pock reduction test) is one of the conventional methods which are widely used for identification of BHV-1 infection. Blue dot was appeared by Dot-ELISA on the homogenate of infected CAM and supernatant MDBK cell as in Figure 3. Further confirmation of identification of the isolated strains of BHV-1 was done using PCR which is lies on its sensitivity particularly during the later phases of infection, using glycoprotein B gene based primer gave PCR product results at 175 bp and this result was in agreement with (Figure 4) [39,40]. Finally, BHV-1 was isolated from nasal and ocular discharge samples collected from infected cattle and buffaloes at El-Fayoum and Beni-Suef governorates.

Conclusion

IBR virus strain was isolated from nasal and ocular discharges swabs from cattle and buffaloes with respiratory signs in Egypt. The isolated IBR virus strain was produced pock lesions on CAM and characteristic CPE in MDBK cell line. Isolated virus similar to Egyptian BHV-1strain, they gave blue dots by Dot ELISA, reduction or absent pock lesions by serum neutralization test, clear precipitation line in Agar gel precipitation test (AGPT), and followed by identification by PCR using set of designing primers targeting glycoprotein (gB) specific for BHV-1 and gave 175 bp in agarose electrophoresis pattern which is similar to Egyptian strain of BHV-1.

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