COMPARING OF UTILIZATION SEROLOGICAL AND MOLECULAR TOOLS FOR DETECTION of BoHV-1 IN SPECIMENS FROM CLINICALLY SUSPECTED CATTLE AND BUFFALO.

El-Bagoury, G.F. [a]; El-Kholy, A.A. [b]; Sharawi, S.A. [a]; Saad, F.F. [a]

[a] *Department of Virology, Faculty of Veterinary Medicine, Benha University, Egypt.*

[b] *Veterinary Serum & Vaccine Research Institute, Abbasia, Cairo, Egypt.*

A B S T R A C T

A polymerase chain reaction (PCR), utilizing thymidine kinase (tk) primer set of bovine herpesvirus-1, virus isolation (VI) on MDBK cell culture, indirect immunofluorescence assay (IFA) were conducted for detection of BoHV-1 in suspected cattle and buffalo nasal swabs that obtained from four governorates in Egypt (Qalubeya, Menofya, Kafer El-sheikh and Beharia). A total of 9/93 (9.6%) of cattle and 4/65 (6.2%) of buffalo nasal swabs were BoHV-1 positive in this study. PCR was superior to immunodetection using IFA after VI procedure in cell culture. Besides, it was the most discriminative assay that detected BoHV-1 viral DNA extracted directly from (100%) of buffalo and (88.9) of cattle clinical specimens. These PCR negative viral isolates were regarded as antigenically related, yet genetically distinct, other ruminant herpesviruses that require further studies. Findings of this study emphasized the BoHV-1 tk based PCR as a sensitive, discriminative and rapid tool for detection of BoHV-1 infections, without confusion with other ruminant herpesviruses.

Keywords: BoHV-1, thymidine kinase, PCR, immunofluorescence.

(BVMJ-27(1):157-165, 2014)

1. INTRODUCTION

Bovine herpesvirus-1 (BoHV-1); an important contagious viral pathogen of domestic and wild bovine; is distributed worldwide exerting an economic impact on livestock industry. BoHV-1 is associated with a broad-spectrum of disease manifestations including; severe respiratory infection [infectious bovine rhinotracheitis (IBR)], conjunctivitis, pustular vulvovaginitis, balanopostitis, shipping fever and systemic infection in neonate calves (Wyler et al., 1989; Tikoo et al., 1995). In Egypt, since 1970s, BoHV-1 has been regarded as an enzootic source of substantial losses in feedlot and dairy farms; mainly due to pneumoenteritis, abortions and deaths (Madbouly and Hussein, 1997; Aly et al., 2003; El-Kholy, 2005). BoHV-1 is a member of the genus *Varicellovirus* of the sub family *Alphaherpesvirinae* within the family *Herpesviridae* (Studdert, 1990). All isolated BoHV-1 strains are belong to one single viral species, and are classified in three subtypes BoHV-1.1, BoHV-1.2 (1.2a and 1.2b) and BoHV-1.3. However, all subtypes are antigenically similar. BoHV-1.3, which is a neuropathogenic agent, has been re-classified as BoHV-5 (Magyar et al., 1993). BoHV-1 genome consists of a linear double-stranded DNA molecule of About 136 kilo bases (kb). A total of 73 open reading frames (ORF) have been clearly identified that codes for about 70 proteins, of which 33 are known to be structural and up to 15 are non-structural proteins (Muylkens et al., 2007; Biswasa et al., 2013). Bovine herpesvirus-1.1 Egyptian strain "Abu- Hammad" was characterized

on molecular basis by El-Kholy and Abdel Rahman (2006). Recently, development of a new molecular diagnostic assay for rapid and sensitive detection of BoHV-1 utilizing the loop-mediated isothermal amplification (LAMP) technique was adopted (El-Kholy et al., 2014).

2. MATERIALS AND METHODS

2.1. Virus and cells

A local Abu-Hammad Egyptian strain of BoHV-1 as tissue culture adapted virus was supplied by Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo. Madin Darby Bovine Kidney (MDBK) cell line was obtained from the National Animal Veterinary Service Laboratories, Ames, Iowa, U.S.A. and was maintained in minimum essential medium with Earle's salts (MEME, Sigma) supplemented with heat-inactivated 10% bovine calf serum (BCS, Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin.

2.2. Clinical specimens

Nasal swabs were collected from cattle (n=93) and buffalo (n=65) represented suspected animals showing signs of respiratory manifestation (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 farms located at four governorates in Egypt (Qalubeya, Menofya, Kafer El-sheikh and Beharia governorates) to be screened for BoHV-1; Samples were collected during the winter months of the year 2013. All nasal swabs were collected, aseptically as possible, in 3 ml cold viral transport medium (VTM, cell culture medium containing antibiotic-antimycotic mix as 1% and 2% bovine serum to protect virus from inactivation). Then, swabs were squeezed against wall of container then removed from soaked fluid. Then, all samples were centrifuged in cooling centrifuge 4˚C at 2000 rpm for 20 minutes. The supernatant fluid was collected in sterile screw-capped vials and then kept at -20˚C until used.

2.3. Trails for BoHV-1 isolation

According to method described by (Singh et al., 1983), a half volume fraction from each specimen was filtered through 0.45 µm filters before being inoculated in tissue culture plates. Each nasal swab specimen was inoculated into duplicate wells MBDK cell cultures, the inoculated plates were incubated at 37˚C for one hour to allow virus adsorption. Then the maintenance media with 2% new born calf serum was added to each well of the plate then the plates were re-incubated at 37˚C and microscopically examined daily to detect development of the (CPE).

2.4. Indirect Immunofluorescence assay (IFA)

The IFA technique was carried out in tissue culture microtitration plates as a duplicate per sample according to (Van Donkersgoed and Babiuk, 1991) with minor modifications. The MBDK cells, inoculated with positive culture supernatants, washed with PBS (pH 7.6); fixed with fixation buffer for 30 minutes, re-washed with PBS and reacted for an hour with the reference rabbit anti-BoHV-1 polyclonal antiserum (diluted 1:100 in PBS, pH 7.6). Then, MBDK cells were washed with PBS (pH 7.6) and probed with Fluorescence Isothiocyanate labelled anti-Rabbit IgG (diluted to 1:50 in PBS). Microtitration plates were washed in three changes of PBS, mounted with 50% buffered glycerol, protected from light and examined using fluorescence microscope for greenish yellow fluorescence.

2.5. Extraction of viral DNA

Extraction of BoHV-1 DNA from nasal swabs by using QIAamp® DNA Blood Mini kit according to the manufacturer's protocol with minor modifications; DNA for the PCR assay was extracted directly from the other fractions of the same crude specimen VTMs without the filtration step

and culture supernatants of the local BoHV-1 infected MDBK cells (as positive control virus).

2.6. Polymerase Chain Reaction (PCR)

Amplification of BoHV-1 tk gene using Polymerase Chain Reaction (PCR); One set of primers (synthesized by Operon Biotechnologies group) was used for PCR amplification; Tow oligonucleotide primers used for PCR of BoHV-1,1 tk gene (Moore et al, 2000).

tk1 (Forward); 5' TGGTACGGACGCCTTAAGTGG 3'

tk2 (Reverse); 5' GTTGATCTCGCGGAGGCAGTA 3'

PCR reaction mixture was prepared by mixing the following components; (Coral buffer, Q buffer (QIAGEN, Germany, Cat. No. 201223), dNTPS, Forward (PH) primer, Reverse (PH) primer, Taq polymerase enzyme (5 U/ 1 µl) (ACT Gene, Catalog No. E2100, Extracted DNA, Nuclease free water was added to obtain (50µl total volumes). Then the resulting mixture was subjected to optimized thermocycling in a thermocycler (Biometra, Germany) as shown in table (2).

2.7. Detection of amplified PCR product using agarose gel electrophoresis

15µl of amplified product was analyzed by electrophoresis using a 1.5 % agarose gel (100 volt for 50 minutes) with 1X TAE running buffer (Sambrook et al., 2001). Gel was stained with ethidium bromide (0.5µg/ml), visualized on ultraviolet transilluminator and the size of DNA bands were determined compared to 100 bp DNA ladder.

3. RESULTS

3.1. Viral isolation:

The trails for BoHV-1 isolation (VI) from clinically suspected cattle (n=93) and buffaloes (n=65) clinical nasal swab specimens on MDBK cells revealed that (85/93) of cattle and (62/65) of buffaloes specimens were negative for viral isolation in this study. Only 8 of cattle and 3 from buffaloes nasal swab specimens, suspected viral isolates were cytopathogenic on MDBK cells (Table 2). Microscopic detection of suspected BoHV-1 isolates was based on their ability to induce variable degree of cytopathic effect (CPE) after three successive blind passages in cultured MDBK cells (cell rounding, cells aggregation and grape-like clusters of rounded cells with cell detachment and death). These findings were demonstrated in Table (2), Figure (1) and Figure (2).

3.2. Serological identification of suspected BoHV-1 isolates on MDBK cells by indirect florescent antibody assay (IFA):

Similar to VI; IFA assay equally identified all of the 3 buffalo and only 6 from 8 cattle suspected viral isolates as BoHV-1. While, IFA failed to identify 2 cattle nasal swab suspected viral isolates which were cytopathogenic on MDBK cells during the procedure of VI (Table 3). The samples were also clearly positive by IFA indicating that intact viral antigen was present (Positive result indicated as apple greenish yellow fluorescence against a dark background) Figure (3).

3.3. Molecular identification of BoHV-1using PCR assay:

Using PCR assay could detect all viral isolates that were positive for VI and IF assays. Moreover, it detected other additional viral isolates that were negative by VI and IFA manifested as one extra cattle isolate and another buffalo isolate from nasal swabs as well as the two cattle isolates where cytopathogenic during viral isolation procedure but failed to be immunodetected with IFAT. However PCR assay fail to detect one suspected viral isolate that was cytopathogenic on MDBK cells and positive with IFAT (Figure 4) and (Table 3).

Table (1): The PCR amplification was conducted in the thermocycler using the following cycling protocol.

Step	Temperature	Time	Cycles
Initial Denaturation	96oC	3 minutes	1
Denaturation	95oC	45seconds	35
Annealing	63oC	45seconds	
Extension	72oC	45seconds	
Final Extension	72oC	10minutes	1

Table (2): Isolation and propagation of BoHV-1 from nasal swab specimens of suspected clinically diseased animals with three successive blind passages on MDBK cell culture.

Governorate	Total number of specimens for viral isolation	Number of positive specimens	Number of positive samples propagated in MDBK cell culture					
			1st passage		2nd passage		3rd passage	
			No.	%	No.	%	No	%
Kalubeya	45	0	0	0%	0	0%	0	0%
Menofya	33	2	2	6.0%	2	6.0%	2	6.0%
Kafer El-sheikh	32	4	4	12.5%	4	12.5%	4	12.5%
Beharia	48	5	7	14.6%	5	10.4%	5	10.4%
Total	158	11	13	8.2%	11	6.9%	11	6.9%

Table (3): Comparative assays polymerase chain reaction (PCR), indirect immune-fluorescence assay (IFA) and viral isolation (VI) for detection of bovine herpsvirus 1(BoHV-1) in clinical nasal swab specimens obtained from four governorates in Egypt.

Governorates	Total number of specimens	Cattle					Buffalo				
		Total number	Positive samples	VI	IFAT	PCR	Total number	Positive samples	VI	FAT	PCR
Kalubeya	45	25	0	0	0	0	20	0	0	0	0
Menofya	33	18	2	2/2	2/2	1/2	15	0	0	0	0
Kafer El-sheikh	32	21	3	3/3	2/3	3/3	11	2	1/2	1/2	2/2
Beharia	48	29	4	3/4	2/4	4/4	19	2	2/2	2/2	2/2
Total	158	93	9	8/9	6/9	8/9	65	4	3/4	3/4	4/4

Figure (1): Normal (non- inoculated) MDBK cell culture (Magnification power 100X). Stained with H&E.

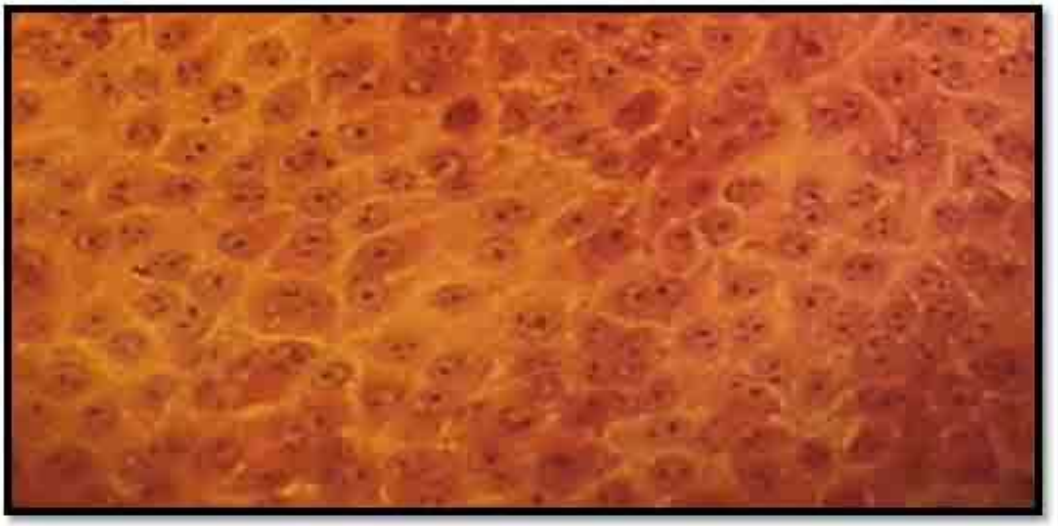


Figure (2): MDBK cell culture inoculated with suspected BoHV-1 virus specimens showing cytopathic effect (CPE; cell rounding, cells aggregations and formation grape- like clusters of rounded cells) stained with Hematoxyline and Eosin, (Magnification power 100X).

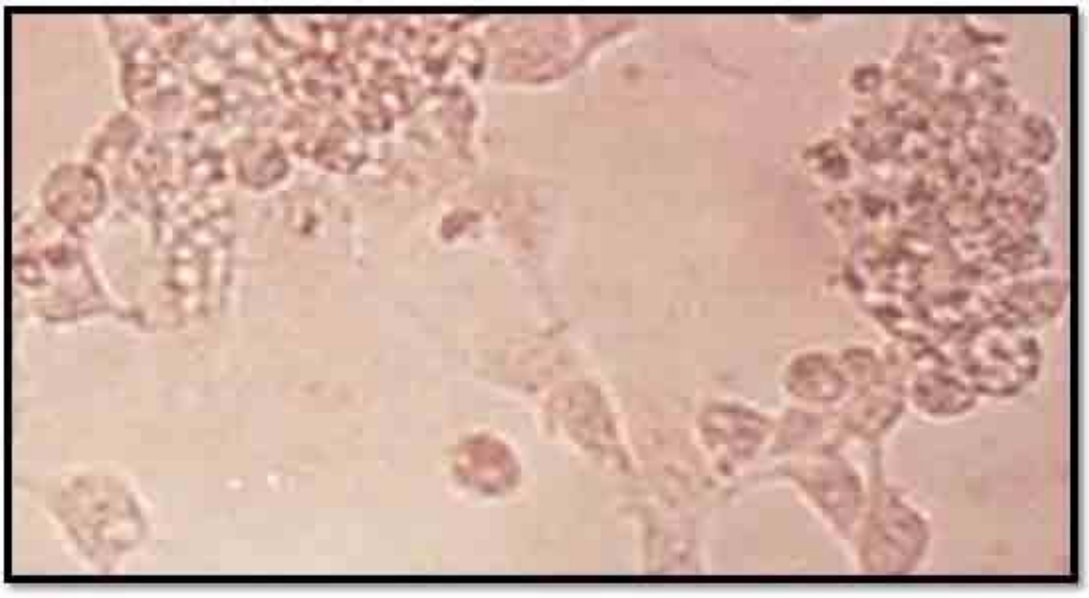


Figure (3): Indirect immunofluorescence (IF) of MDBK cells infected with suspected BoHV-1 viral isolates from bovine clinical nasal swabs specimens, reacted with reference anti-BoHV-1 antiserum and probed with FITC-labeled anti-rabbit IgG. (Positive result indicated as apple greenish yellow fluorescence against a dark background). Magnification power was 400X.

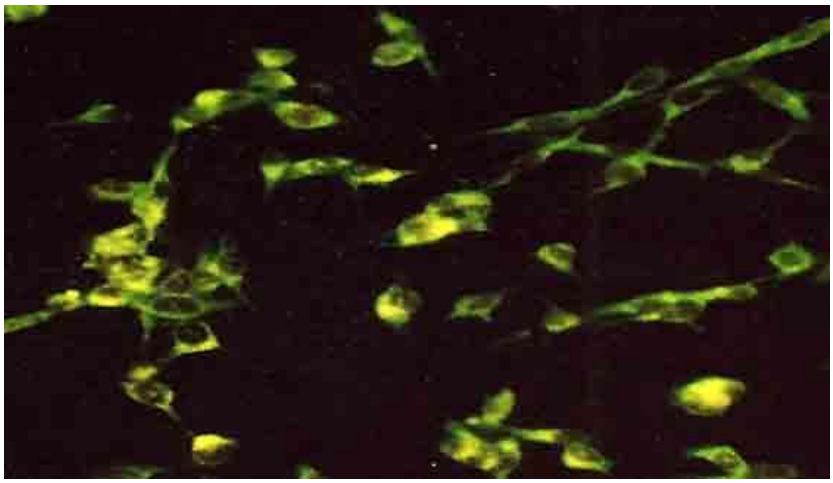
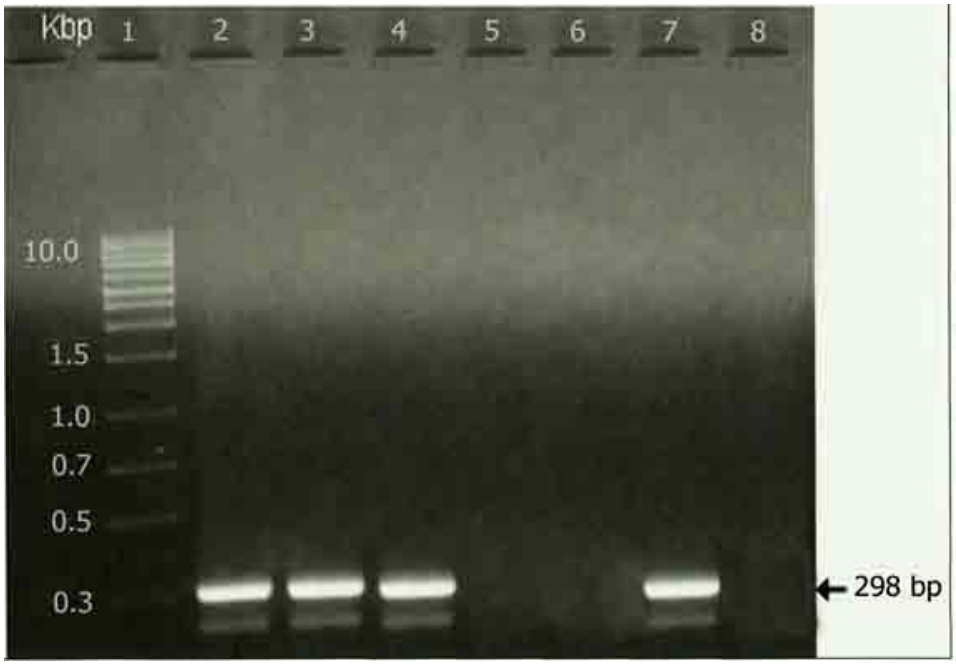


Figure (4): Agarose gel electrophoresis of PCR amplicons from tk gene coding sequence of BoHV-1 genome separated on 1.5% Agarose gel and stained with ethidium bromide; lanes, (1) Gene Ruler 100pb DNA ladder (consists of repeats of 100 bp fragment size); (2), (3) and (4) BoHV-1positive nasal swab specimens; (5) And (6) BoHV-1 negative nasal swab specimen; (7) Genomic DNA from local BoHV-1 Abu Hammad strain (positive control); (8) Negative control genomic DNA from non-infected MDBK cells. (Positive PCR amplicons are at the size of approximately 298 bp).



4. DISCUSSION

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 (Van Oirchot, 1996; Mettenleiter, 2006). In the present study, the trails for BoHV-1 isolation on MDBK cells successed and revealed that (8) of cattle and (3) from buffaloes nasal swab specimens (Table 2) suspected viral isolates were cytopathogenic on MDBK (Figure 2).The failure to detect infectious virus in two of cattle samples and one of buffalo samples was most likely due to the fact that they were all submitted as swabs. Samples in this form occasionally do not arrive at the laboratory until 2-3 days after they are taken and may dry out resulting in the loss of viable virus (Moore et al., 2000; Taha, 2011). This, also, could be attributed to poor growth of viruses in cell cultures or presence of cytotoxic elements in the processed samples (Jiang and Lee, 1993).Virus isolation on cell cultures remains the gold standard for detection of many viruses in clinical specimens. However, it is laborious, costly, time-consuming (1–3 weeks) and depends on the presence of infectious virus, less sensitive and impractical for large scale screening of animals (Belák and Ballagi-Pordány, 1993). Moreover, it requires a confirmatory immunoassay for detection of the viral antigen (El-Kholy et al., 2014). Immunodetection of BoHV-1 antigen, using IFA was comparatively inferior to the molecular detection of viral DNA and viral isolation procedure because IFAT needs viral antigen to be present in intact cells. Problems associated with poor quality microplates preparations such as lack of sufficient cell numbers and poor drying or fixing procedures may hamper the efficient detection of BoHV-1 antigen by IFAT (Moore et al., 2000; El-Kholy, 2005). Findings of this study showed that the PCR assay was the most discriminative and relevant assay that identified all BoHV-1 viral isolates from (89.9%) cattle (8/9) and (100%) buffalo (4/4) specimens (Table 3); PCR has become an important diagnostic tool for veterinary virologists; In addition to speed, specificity and sensitivity, PCR offers an additional advantage in the case of BoHV-1 in that the present absolute requirement for good quality diagnostic samples is eliminated given the fact that only short sequences of target nucleic acid are necessary for detection. As well as, it is inexpensive alternative that is applicable to large numbers of specimens (Moore et al., 2000; El-Kholy, 2005; Oliveira et al., 2011; Nandi and Kumar, 2011). In view of the one sample was repeatedly negative by PCR although it was cytopathogenic on MDBK cells and positive with IFA; assuming correct interpretation of the test results in these cases, these may represent false negative PCR results. Alternatively, the samples may have contained only extensively degraded or insufficient quantities of viral DNA for detection; as well as, this sample might be shown to contain inhibitory factors that might otherwise prevent PCR (Moore et al., 2000).

Results obtained herein coincide with recent reports regarding the presence and circulation of BoHV-1among cattle and buffalo herds in Egypt as well as molecular importance of the tk gene based PCR assays for pathogenesis and epidemiological studies of BoHV-1 infections (Vilcek et al, 1994; Yason et al., 1995; Rocha et al., 1998; Moore et al., 2000; El-Kholy, 2005).

5. REFERENCES

Aly, N.M., Shehab, G.G., Abd El-Rahim, I.H.A. 2003. Bovine viral diarrhoea, bovine herpesvirus and parainfluenza-3 virus infection in three cattle herds in Egypt in 2000 Rev. sci. tech. Off. int. Epiz., 22: 879-892.

Belak, S., Ballagi-Pordany, A. 1993. Application of the polymerase chain reaction (PCR) in veterinary diagnostic virology. Vet. Res. Commun. 17: 55-72.

Biswasa, S., Bandyopadhyayb, S., Dimria U., Patrac, P.H. 2013. Bovine herpesvirus-1 (BHV-1) a re-emerging concern in livestock: a revisit to its biology, epidemiology, diagnosis, and prophylaxis. Veterinary Quarterly, 33: 68–81.

El-Kholy, A.A. 2005. Molecular and Immunological Detection of Bovine Herprsvirus-1 in clinical specimens. The Egyptian Journal of Immunology, 12:125-136.

El-Kholy, A.A., Abdel Rahman, K., Soliman, H. 2014. Rapid detection of BoHV-1 genomic DNA by loop-mediated isothermal amplification assay. Journal of Virological Methods 204: 81–85.

El-Kholy, A.A. Abdel Rahman, K.A. 2006. Genetic characterisation of the Egyptian Vaccinal strain Abu-Hammad of Bovine herpesvirus-1. Rev. sci. tech. Off. int. Epiz., 25: 1081-1095.

El-Kholy, A.A., Rady, D.I., Abdou, E.R., Elseafy, M.M., Abdel Rahman, K.A.,Soliman, S. 2013. Construction, Characterization and immune-genicity of a glycoprotein E negative Bovine herpesvirus-1.1 Egyptian strain "Abu- Hammad". Journal of Virological Methods, 194: 74– 81.

Jiang, B., Lee, C. 1993. Rapid and effective processing of tissue specimens for PCR using glass fiber filter paper. Biotechniques, 15: 38 - 39.

Madbouly, H.M., Hussien, M.M. 1997. Isolation of BHV-1, from Dairy herd Genital form of infection. Alex J. Vet. Scien., 13: 439-446

Magyar, G., Tanyi, J., Hornyak, A., Batha, A. 1993. Restriction endonuclease analysis of Hungarian bovine herpesvirus isolates from different clinical forms of IBR, IPV and encephalitis. Acta.Vet. Hung., 41:159–170.

Moore, S., Gunn, M., Walls, D. 2000. A rapid and sensitive PCR based diagnostic assay to detect BHV-1 in routine diagnostic submissions. Vet. Microbiol., 75:145–153.

Muylkens, B., Thiry, J., Kirten, P., Schynts, F., Thiry, E. 2007. Bovine herpesvirus 1 infection andinfectious bovine rhinotracheitis. Vet. Res., 38: 181-209.

Mettenleiter, T.C. 2006. Intriguing interplay between viral proteins during herpesvirus assembly or: The herpesvirus assembly puzzle. Vet. Microbiol., 113:163–169.

Nandi, S., Kumar, M. 2011. Comparison of ELISA and virus neutralization test in assaying serum antibodies to bovine herpesvirus 1. Acta Virol, 55: 175-177.

Oliveira, M.T., Camposa, F.S., Dias, M.M., Velho, F.A., Freneau, G.E., Brito, W.M.E.D., Rijsewijk, F.A.M., Franco, A.C., Roehe, P.M. 2011. Detection of bovine herpesvirus 1 and 5 in semen from Brazilian bulls. Theriogenology, 75: 1139–1145.

Rocha, M.A., Barbosa, E.F., Guimaraes, S.E.F., Neto, E.D., Gouveia, A.M.G. 1998. A high sensitivity nested PCR assay for BHV1 detection in semen of naturally infected bulls. Vet. Microbiol., 63: 1-11.

Sambrook, J., Russel, D.W. 2001. Molecular Cloning: A Laboratory Manual, Third Edition by Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schwyzer, M., Ackermann, M. 1996. Molecular virology of ruminant herpesviruses, Vet. Microbial. 53:17–29.

Singh, B.K., Kant, R., Tongaonk, S.S 1983. Adaptation of IBR virus in madine darby bovine kidney cell line and testing buffalo sera for neutralizing antibodies.

Studdert W. 1999. Bovine encephalitis herpes virus. Vet. Res. 126: 21-22.

Taha, A. S. 2011. Some studies on Bovine Herpesvirus -1. Ph.D thesis, faculty of Veterinary Medicine, Benha University.

Tikoo, S.K., Campos, M., Babiuk, L.A. 1995. Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control. Advances in Virus Research 45: 191-223.

Van Donkersgoed, J., Babiuk, L. 1991. Diagnosis and managing the respiratory form of IBR. Vet. Med., 1: 86 -94.

Van Oirschot, J.T., Kaashoek, M.J., Rijsewijk, F.A., Stegeman, J.A. 1996. The use of marker vaccines in eradication of herpesviruses. J. Biotechnol. 44: 75–81.

Vilcek, S., Nettleton, J.A., Herring, J.A., Herring, A.J. 1994. Rapid detection of bovine herpesvirus 1 (BHV-1) using the polymerase chain reaction. Vet. Microbiol., 42: 53– 64.

Wyler, R., Engels, M., Schwyzer, M. 1989. Infectious bovine rhino-tracheitis/ vulvovaginitis. In: Wittmann, G. (Ed.), Herpesvirus Diseases of Cattle, Horses and Pigs. Kluwer Academic Publishers, Boston, Dordrecht, London, pp: 1–72.

Yason, C.V., Harris, L.M., Mckenna, P.K., Wadowska, D., Kibenge, S.B. 1995. Establishment of conditions for the detection of bovine herpesvirus1 by the polymerase chain reaction using primers from the thymidine kinase region. Can.J.Vet.Res., 59: 94-101.

الكشف المقارن باستخدام وسائل سيرولوجية وجزيئية عن فيروس الهربس البقري نوع-1 في عينات من ابقار وجاموس مشتبه الإصابة

جبر فكري الباجوري[1]، علاء عبدالمنعم الخولي[2]، سعد شعراوي علي[1] ، فؤاد فؤاد سعد[1]

[1] كليه الطب البيطري-جامعه بنها، [2] معهد بحوث المصل واللقاح بالعباسية-القاهرة

الملخص العربي

تم اجراء اختبارات: تفاعل البلمرة المتسلسل الموجه للتتابع النيوكليوتيدي المشفر لبادئ الثيامدين كاينيز (TK) من جينوم فيروس الهيربس البقري-1 (BoHV-1)، والعزل الفيروسي، والفلورسنت المناعي الغير مباشر (IFA)، وذلك للكشف المقارن عن فيروس الهيربس البقري-1 في المسحات الأنفية من أبقار وجاموس محتمل اصابتها بالفيروس، من محافظات مختلفة في مصر وهي (القليوبية، المنوفية، كفر الشيخ والبحيرة). وقد أظهرت النتائج أن 9 من 93(9.6 %) من عينات الأبقار، و4 من 65 (6.2%) من عينات الجاموس كانت موجبة للعدوى بالفيروس في هذه الدراسة. وكذلك وجد أن تفاعل البلمرة المتسلسل كان متفوقاً على اختباري الكشف المناعي في خلايا الزرع النسيجي، وكان كذلك الأكثر تمييزاً واعتماداً في الكشف عن جميع فيروسات الهيربس البقري-1 (100%) المعزولة من العينات الإكلينيكية للجاموس و(88.9) للأبقاروقد ثبت أن جميع هذه المعزولات الفيروسية السالبة لإختبار البلمرة المتسلسل أنها ليست فيروس الهيربس البقرى -1 وإنما قد تكون من مجموعة فيروسات الهيربس الخاصة بالمجترات التى تتشابه أنتيجينياً ولكنها تختلف وراثياً مع فيروس الهيربس البقرى -1 ، إن نتائج هذه الدراسة قد فخمت فائدة إختبار تفاعل البلمرة المتسلسل الخاص بجين الثيامدين كاينيز (tk) لفيروس الهيربس البقري -1 كأداة حساسة وتمييزية وسريعة للاستخدام في الدراسات الوبائية وبرامج السيطرة الخاصة بعدوى فيروس الهيربس البقري -1 ، بدون الخلط المحير مع فيروسات الهيربس للمجترات الأخرى.

(مجلة بنها للعلوم الطبية البيطرية: عدد 27(1):157- 165, سبتمبر 2014)