APPLICATION OF MOLECULAR DIAGNOSTIC TECHNIQUES FOR THE DETECTION OF BOVINE HERPESVIRUS-1 (INFECTIOUS RHINOTRACHEITIS VIRUS)

BY

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SUMMARY

Infections with bovine herpesvirus type 1 (BHV-1) occur worldwide and cause serious economic losses due to loss of animals, infertility, abortions, decreased milk production and loss of body weight. In the present study, a polymerase chain reaction (PCR)- probe hybridization assay was applied in order to improve detection methods for the laboratory diagnosis of animal infections with BHV-1. The method is based on the PCR amplification of a highly conserved DNA fragment (430 base pairs) within the glycoprotein gD of BHV-1. The practical applicability of the assay was evaluated using field isolates from infected sheep as well as experimentally infected bovine semen that were tested. The specificity (the viral origin of the PCR product) of the assay was assessed by dot blot hybridization with an internal probe. The PCR amplification allowed specific detection of BHV-1 genome in all isolates tested. The assay detected BHV-1 DNA in cell culture supernatants and infected semen with titres of 1 $TCID_{50}$/ml. The results suggest that PCR- probe hybridization assay could be used as a rapid, sensitive and specific laboratory testing procedure for the detection and diagnosis of animal infections with BHV-1.

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1), a member of the Alfaherpesvirinae, is an important pathogen of ruminants and is classified into three subtypes according to the clinical manifestation produced (Wyler et al., 1989). BHV-1.1 (subtype 1) causes primarily respiratory infections (infectious bovine rhinotracheitis [IBR], BHV-1.2 induces respiratory and genital infections (infectious pustular vulvovaginitis [IPV] in females and infectious balanoposthitis [IBP] in males), and BHV-1.3 causes primarily neurological infections. The encephalitic BHV-1.3 subtype was reclassified as BHV-5 (Roizman, 1991). All of these subtypes are capable of inducing a latent state in clinically normal animals (Pastoret et al., 1986). Upon reactivation of latent

infection, the virus is re-excreted in respiratory, ocular or genital secretions, including semen (Van Oirschot et al., 1993).

Currently, virus isolation in cell culture, serum neutralization test (SNT) and fluorescent antibody technique (FA) are used to diagnose BHV-1 in both acutely and latently infected animals (Straub, 1990). These techniques are labor intensive, time consuming, insensitive and do not provide rapid diagnostic results (Drew et al., 1987, Zhou et al., 1999, Cândido et al., 2000). Detection of BHV-1 in semen cell culture technique or "the Cornell Semen Test" (Schulz et al., 1982) has proven to be not absolutely reliable due to the natural cytotoxicity of seminal plasma, overall cost and its inability for wide scale application such as semen monitoring (Kahrs et al., 1977, Xia et al., 1995).

Recent research has focused on development of a reliable diagnostic tests for the detection of BHV-1 in biological samples. The PCR assay represents an excellent tool for the fast and sensitive detection of BHV-1 genome in biological and clinical specimens (Yason et al., 1995, Smits et al., 2000). The present study was initiated to study the application of a PCR amplification-probe hybridization assay as a highly sensitive and rapid detection method for the diagnosis of animal infections with BHV-1.

MATERIALS AND METHODS

Animals and sampling

30 nasal and vaginal swabs were collected aseptically as possible from sheep of local breeds kept at different localities. The animals were presented with mild respiratory (nasal and ocular discharges, rhinitis, and cough) and reproductive (mucoid vaginal discharges and vulvovaginitis). Swabs were transported to the laboratory in minimum essential medium (MEM) with 3% horse serum and antibiotics. Afterwards, samples were centrifuged at 3, 000 rpm for 15 minutes. The supernatants were frozen at –70°C until used for further testing.

Virus isolation (VI) and identification

Madin-Darby bovine kidney (MDBK) cells were grown on MEM supplemented with 10% bovine serum. Viral isolation was done according to the procedures described by kahrs (1977). Samples were considered BHV-1-negative by virus isolation if no cytopathic effect (CPE) was obtained after 3 passages. The ATCC-VR 864 Colorado strain of BHV-1 (Kindly provided by Dr. B. Liess, Institute of Virology, Hannover Veterinary School, Hannover, Germany) was used as a reference.
Virus in supernatants of putatively BHV-1 positive field samples was identified by:
-Virus neutralization test (VNT): using reference specific BHV-1 antiserum (obtained from the National Animal Disease Laboratory, Ames, IA, USA) according to methods previously described by Carbrey and Lee (1966).
-Direct fluorescent antibody (FA) test: using a fluorescein isothiocynate-conjugated BHV-1 antibodies (obtained from the Central Veterinary laboratory, Weybridge, UK) according to methods previously described by Majewska et al. (1984).

In Vitro seeding of semen with BHV-1

To assess the capability of detection of BHV-1 in semen and the sensitivity of

PCR amplification, the reference Colorado strain of BHV-1 (10^6 $TCID_{50}$/ml) was 10 fold serially diluted in BHV-1 free bovine raw semen. After centrifugation for 30 seconds at 10, 000 rpm, the seminal plasma were used for DNA extraction.

PCR-assay

The PCR-method was essentially done as described by Wagter et al. (1996). The procedure is briefly as follows:

DNA isolation

A100 µl aliquot of supernatants from cell cultures infected with BHV-1 reference, field isolates or seminal infected plasma was thawed and incubated with 100µl of proteinase-K reagent (1.5 mg/ml proteinase-K, 0.75% sodium N-laurylsarcosine in 0.15 mol/L NaCl) for 60 min 55°C.. The supernatant (approximately 150µl) was mixed with an equal volume of a mixture of phenol, chloroform and isoamyl alcohol (25.0:24.0:1.0 v/v) and centrifuged (10 min, 10, 000 rpm).

The supernatant aqueous fraction was transferred to a 1.5 ml microcentrifuge tube and mixed with equal volume of chloroform and isoamyl alcohol (24.0:1.0 v/v). After further centrifugation (10 min, 10, 000 rpm), the aqueous layer was aspirated and the DNA was precipitated with 0.2 volumes of sodium acetate (3.0 mol, pH 5.2) and 2 volumes of absolute chilled ethanol. After an overnight incubation at -20°C, the samples were centrifuged (15 min, 10, 000 rpm). The DNA pellet was washed with 200µl of 70% ethanol, dried and resuspended in 50µl of ultrapure water. The concentration of extracted DNA was determined by the measurement of the absorbance at 260 nm.

Primers

The primers were based on the sequence of the conserved gD region of BHV as described by Tikoo et al. (1990): 5'-GTC GAG GTG CGC TAC GCG ACG-3'; 5'-CCG CGA GCC GCG CCG AGT TTG CA-3'. The primers were synthesized and supplied by New England Biolabs (Beverly, MA, USA).

Amplification

The PCR was performed in 100µl volumes using a thermocycler (T gradient thermoblock, Biometra, Goettingen, Germany). Final concentrations of 1µmol/L of each primer and 0.1mmol/L of deoxynucleoside-triphosphates (dNTPs), 1X PCR buffer (1.5 mmol/L $MgCl_2$, 50 mmol/L KC1, 10 mmol/L Tris-HCl, pH 8.3, and 0.01% w/v gelatin), 2.5 units of Taq polymerase (Promega, Madison, WI, USA) and10µl of sample DNA were added to the each PCR tube. Amplification was started with 30 seconds at 94°C, to obtain full denaturation of DNA, Followed by 35 cycles of 15 seconds at 94°C, 15 seconds at 67°C and 60 seconds at 72°C and completed with final extension of 5 minutes at 72°C.

To test the specificity of the selected PCR primers, negative control reactions with DNA extracts of uninfected MDBK cell cultures, uninfected bovine semen, BHV-4, as well as BVDV cDNA were tested. To check for carry-over contamination (false-positive PCR results), a sample with no DNA template (H_2O instead) was tested in parallel with every test series.

Analysis of amplification product

Electrophoresis

The PCR products were analyzed by electrophoresis in 1.5% (w/v) agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer and ethidium bromide staining (Sambrook et al., 1989). The 1Kb DNA ladder (Gibco BRL Life technologies, Rockville, MD, USA) was used as size marker to check the size of the amplicon. The gels were read by eye over a UV light and photographs were taken to record the results. The expected PCR product length was 430 base pairs

Dot blot hybridization

In some experiments, dot blot hybridization analysis of the amplified product was done to confirm the specificity and sensitivity of the PCR reaction. Dot blots of PCR product DNA were made using nylon, positively charged membranes (Sigma Co, St. Louis, MO, USA). BHV-1 Colorado strain purified DNA was used as a template in a PCR amplification reaction as described above to generate a PCR-derived specific probe. DNA dotting on membrane, probe labelling, hybridization and the detection procedures were performed using non-radioactive DIG-labelling and detection kit (Boehringer Mannheim Corp. Indianapolis, IN, USA) according to the supplier's instructions. Briefly, blots (membranes) were air dried and baked for hour at 80°C.

The membranes were prehybridized for 4 hours at 68°C in hybridization solution (5%SSC, 0.1% (w/v) N-laurylsarcosine, 0.02% (w/v) SDS and 1% (w/v) blocking reagent). After overnight hybridization at 68°C with DIG-labelled probe, membranes were washed twice with 2X SSC; 0.1% (w/v) for 5 minutes at room temperature and then twice with 0.1X SSC;0. 1% (w/v) for 15 minutes at 68°C. After washing, membranes were air dried and subsequently subjected for detection of the bound probe.

RESULTS

Out of the 30 nasal and vaginal swab field samples from sheep presented with mild respiratory and reproductive manifestation, 8 were found to be positive by virus isolation on MDBK cell culture. The virus titers ranged from $10^{2.7}$ to $10^{5.4}$ $TCID_{50}$/ml (Table1). All of the 8 isolates were further identified as BHV-1 by VNT and FA assays.

In the present study, the coding region of BHV-1 gD gene was selected as target for PCR amplification-based detection for BHV-1. After PCR amplification conditions were optimized, it was possible to amplify the expected 430 bp fragment. BHV-1 genome was detected by the PCR assay in all field isolates from sheep as demonstrated by the presence of a single band of 430 bp (Table1 and Fig.1a). To test the specificity of the selected primers, BHV-4 DNA and BVDV cDNA were also subjected to the PCR assay but did not render any specific products. The identity of the PCR product was additionally confirmed by dot blot hybridization with a non-radioactive internal probe (Fig.1b).

To determine the PCR sensitivity, a 10 fold dilution series of purified DNA was subjected to PCR. As little as 10 fg of the BHV-1 specific DNA was detected by PCR on ethidium bromide-stained gel (Fig.2).

Fig.3.shows the results of PCR amplification to BHV-1specific detection in experimentally infected bovine raw semen. No amplification product was detected by agarose gel electrophoresis on DNA samples from BHV-4 infected semen.

PCR sensitivity was also studied by using DNA extracted from a 10 fold dilutions of supernatant from BHV-1 infected cells. As shown in Fig.4, the 430 bp fragments were visualized from 10^5 to 1 $TCID_{50}$. The sensitivity of PCR detection of BHV-1 in infected raw semen was equivalent to that of PCR assay in cell culture medium.

DISCUSSION

Bovine herpes virus1(BHV-1) is a major pathogen of cattle that is distributed worldwide and that causes various syndromes like infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, infectious balanoposthitis, and neurological infections. Although BHV-1 mainly infects cattle, several studies have reported on various BHV-1infections in sheep and goats (Lehmkuhl et al., 1985; Wafula et al., 1985; Whetstone and Evermann, 1988; Engels et al., 1992).

The existence of BHV-1 infections in various ruminant species other than bovine species is a major threat for eradication schemes (Ros and Belak, 1999). Therefore, There is a need for sensitive and specific diagnostic tests for detection of the presence of the virus in order to achieve effective control of BHV 1-induced diseases.

Virus isolation (VI) in tissue culture is the used method for detection of BHV-1 in clinical samples or semen but the principal drawbacks of the method is the length of time required to obtain results.

Depending on the number of passages in tissue culture, the procedure may take one to three weeks to be completed. Moreover, VI-based tests are subject to the usual difficulties in cell culture such as cytotoxicity and/bacterial contamination. In this study, a PCR-probe hybridization assay was applied as an alternative detection method for the diagnosis of animal infection with BHV-1.

The choice of the DNA template target in PCR-based detection of BHV-1 could reduce specificity, as a consequence of the differences among BHV-1 strains, including mutations or deletions demonstrated by the different restriction enzyme pattern of BHV-1 (Wyler et al., 1989). The PCR primer set used in this study was designed to target a highly conserved coding region of BHV-1 genome (gD). The gD is a glycoprotein of herpesvirus that show a high degree of homology at the DNA among BHV-1 strains (Tikoo et al., 1990). Therefore, no genomic variation is expected in the unique long regions of BHV-1 where the gD is located. Since DNA was amplified from the tested BHV-1 references strain and all 8 field isolates from sheep with various clinical manifestations, it is clear that the target sequences lies within a relatively conserved area of the BHV-1 genome of all isolates.

Therefore, from a practical point of view the designed PCR primers could be used to detect BHV-1 as the method is not affected by strain differences. The

specificity of PCR amplification primers for BHV-1 DNA was demonstrated by the fact that all tested BHV-1 references strain and all 8 field isolates from sheep gave PCR products with the expected size of 430 bp, whereas DNA from a closely related herpesvirus (BHV-4) and a non related virus (BVDV) were not amplified. The possibility of cross contamination during PCR procedures can not be excluded. Negative control samples that were used (water, uninfected MDBK cell supernatants) did not show a positive reaction. Therefore, it is unlikely that false-positive results were produced because of a lack of specificity.

The specific verification of the PCR amplification products was confirmed when amplified products of the expected size reacted positively with the corresponding BHV-1-specific digoxigenin-labelled DNA probe in the dot blot hybridization assay (Fig1b).

The diagnostic applicability of the PCR assay was also evaluated with bovine raw semen experimentally infected with BHV-1. In this study, the PCR assay is based on the purification of BHV-1 DNA from semen fraction free from spermatozoa DNA. Van Engelenburg et al. (1993) have shown that in BHV-1 contaminated semen, most of the BHV-1 can be found in the seminal fluid and large amount of background DNA may inhibit PCR. Therefore, spermatozoal DNA was excluded from the reaction by centrifugation. The fast DNA isolation in conjuction with the PCR makes the assay suitable for processing large number of semen samples for monitoring semen for BHV-1 infection.

The specificity of the PCR assay was demonstrated by the fact that BHV-1 free semen samples were negative. The sensitivity for virus detection in semen by PCR assay was equivalent in cell cultures where a unique DNA band corresponding to 1TCID50 was visualized by ethidium bromide- stained agarose gels. The results presented in this study confirmed that an optimized PCR procedures for BHV-1 detection in semen allowing rapid, sensitive and specific viral detection in bovine semen samples. Comparison with virus isolation showed that PCR detected BHV-1 virus in more samples and during longer periods of time (Van Engelenburg et al., 1995). Furthermore, the PCR-probe hybridization results were obtained in 2 working days, while virus isolation usually took up to 2 weeks to obtain a diagnosis.

Advantages of using PCR-probe hybridization assay that is based on highly specific primers for BHV-1 are its sensitivity, speed and cost effectiveness which makes this method a useful alternative to conventional diagnostic tests for detection of BHV-1 in cell cultures and clinical samples form infected animals.

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Table1: Results of viral isolation, identification and PCR-probe hybridization assays performed on virus reference strains and local field isolates.

Reference strains and BHV-1 field isolates tested	Source	Test			
		VI	VNT*	FA**	PCR
BHV-1Colorado strain	ATTC-VR 864 Reference strain	10^6	+	+	+
Field isolate #1	N.S	$10^{2.7}$	+	+	+
Field isolate #2	N.S	$10^{4.2}$	+	+	+
Field isolate #3	N.S	$10^{5.4}$	+	+	+
Field isolate #4	N.S	$10^{4.7}$	+	+	+
Field isolate #5	N.S	$10^{3.4}$	+	+	+
Field isolate #6	N.S	$10^{5.1}$	+	+	+
Field isolate #7	V.S	$10^{3.5}$	+	+	+
Field isolate #8	V.S	$10^{3.2}$	+	+	+
BHV-4	Reference strain	10^5	nd	nd	-
BVDV	Reference strain	10^6	nd	nd	-

N.S = Nasal swab
* Using reference specific BHV-1 antiserum
V.S = vaginal swab
** Using fluorescein isothiocynate-conjugated BHV-1 antibodies
VI (viral isolation) titers in $TCID_{50}$/ml nd = not done

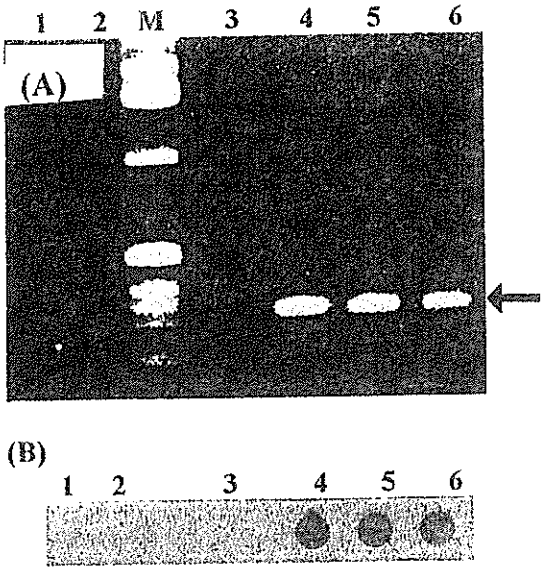

Fig.1. Specificity of PCR amplification for detection of BHV-1. A) A 10µl sample of each PCR product was subjected to gel electrophoresis. No specific amplification from BHV-4 DNA (Lane 1) and BVDV cDNA (lane 2) by PCR. Lane M, 1 Kb DNA ladder as a molecular weight marker. Lane 3, BHV-1 uninfected cell supernatant as negative control. Lane 4, BHV-1 Colorado strain. Lane5, represents BHV-1 isolate from nasal swab. Lane6, represents BHV-1 isolate from vaginal swab. The 430-bp amplified fragment is indicted by an arrow on the right of the panel. B) Dot blot hybridization analysis of the PCR products with the BHV-1 internal DIG probe. Lanes 1,2,3-6 as in panel A.

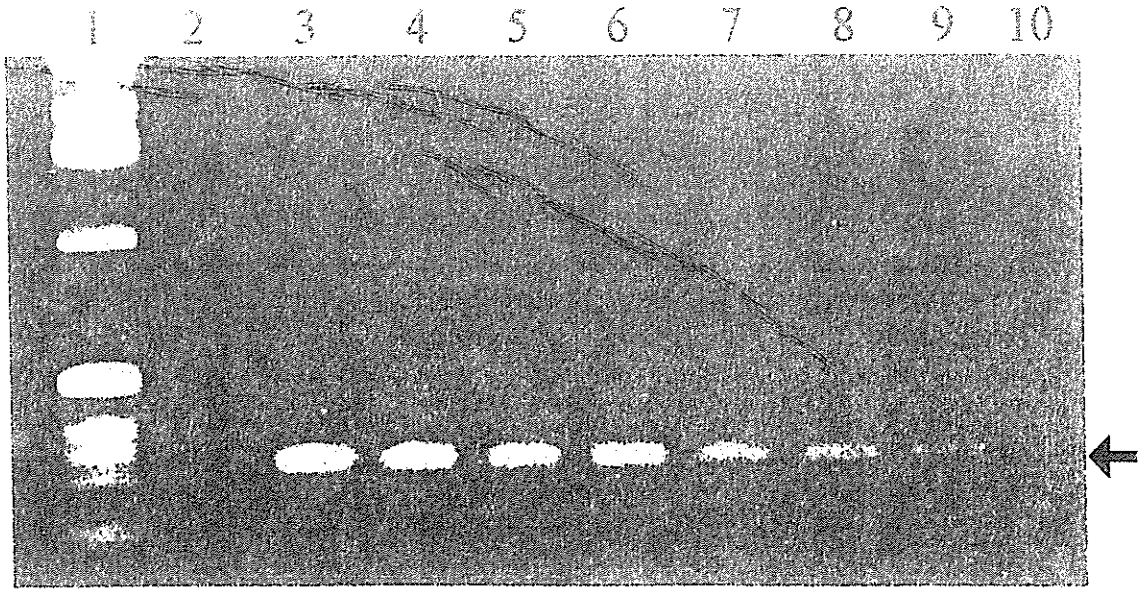

Fig.2. Specificity and sensitivity of PCR amplification for detection of BHV-1 purified DNA. Ten fold serial dilutions (from 10 ng to 1fg) of the purified BHV-1 DNA were assayed by PCR. A 10µl sample of each PCR product was subjected to gel electrophoresis. Lane1, Kb DNA ladder as a molecular weight marker. Lane2, BHV-1 uninfected cell s supernatant as negative control. Lanes 3 to 10, serial DNA dilutions. The 430-bp amplified fragment is indicted by an arrow on the right of the panel.

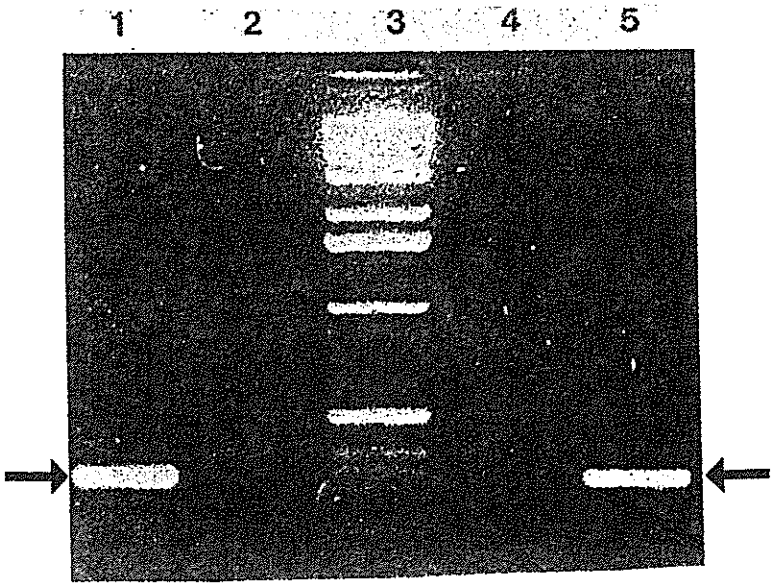

Fig.3. Specificity of PCR amplification to BHV-1detection in experimentally infected bovine raw semen. A 10µl sample of each PCR product was subjected to gel electrophoresis. Lane1, BHV-1 infected cell supernatant as positive control. Lane 2, BHV-4 infected raw semen. Lane3, molecular weight marker. Lane 4, uninfected raw semen as negative control. Lane5, BHV-1 infected raw semen. The 430-bp amplified fragment is indicted by an arrow on the right of the panel.

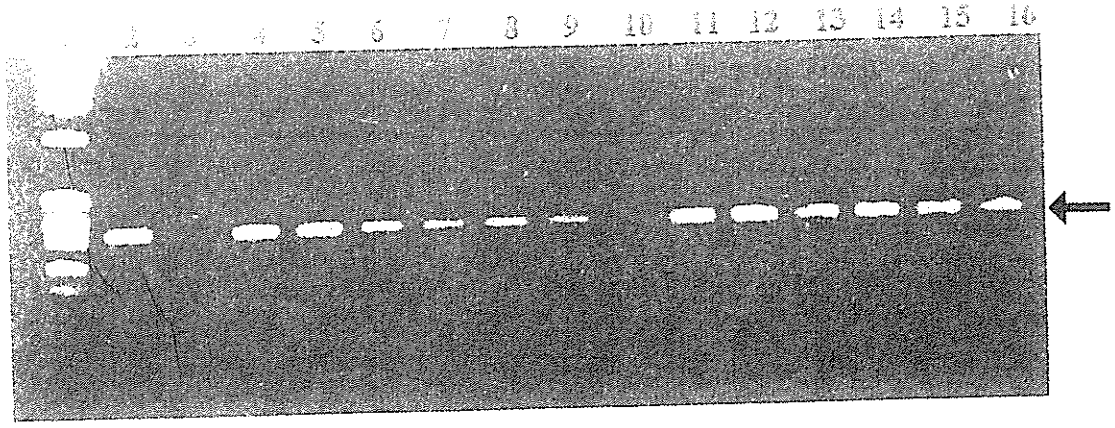

Fig 4. Sensitivity of PCR amplification to BHV-I detection in the supernatant of infected cell cultures and experimentally infected bovine raw semen. Ten fold serial dilutions (from 10^5 to $1TCID_{50}$) of each sample type were assayed by PCR. A 10µl sample of each PCR product was subjected to gel electrophoresis. Lane1, 1 Kb DNA ladder as a molecular weight marker. Lane2, BHV-1 DNA (10 ng) was used as a positive control. Serial dilutions from cell culture supernatant (Lanes 4 to 9), serial dilutions from raw infected semen (Lanes 11 to16). DNA from MDBK non-infected cell (Lanes3 and10) was also subjected to PCR rendering no product. The 430-bp amplified fragment is indicted by an arrow on the right of the panel.

الملخص العربي

تطبيق طرق تشخيص جزيئيه للكشف عن فيروس مرض التهاب القصبة الهوائية المعدي

سيد احمد حسن سالم* و جمال سليمان رضوان**

تتسبب الإصابة بالفيروس المسبب لمرض التهاب القصبة الهوائية المعدي في المجترات في حدوث خسائر اقتصادية كبيره نتيجة لنفوق الحيوانات و انخفاض الخصوبة و الإجهاض و انخفاض إنتاج اللبن. استهدفت هذه الدراسة إمكانية تطبيق استخدام اختبار تفاعل إنزيم البلمرة ومجس تهجين الحمض النووي الديوكسى ريبوزى كاختبارات حساسة في محاولة لإيجاد طرق لتشخيص بديله للكشف عن الإصابة بالفيروس المسبب لمرض التهاب القصبة الهوائية المعدي وبالتالي يمكن اتخاذ إجراءات تحكم فعالة للحد من انتشار الإصابة بهذا المرض.

وقد اعتمد اختبار تفاعل إنزيم البلمرة المستخدم في هذه الدراسة علي تضاعف الحمض النووي بمنطقة الجين المترجم للجليكوبروتين (gD) والتي تتميز بثبات التسلسل النيكلوتيدي بجينوم الفيروس. و تم تقييم القدرة التشخيصية للاختبار في الكشف عن جينوم فيروس مرض التهاب القصبة الهوائية المعدي باستخدام عترات مرجعية قياسية للفيروس و أخرى حقلية معزولة من أغنام مصابة بأعراض مرضية تنفسية و تناسلية و كذلك سائل منوي بقري تم حقنه بالفيروس بعدوى تجريبية معمليا. وقد أمكن باستخدام اختبار تفاعل إنزيم البلمرة الكشف بتخصص عن جينوم فيروس مرض التهاب القصبة الهوائية المعدي المستخلص من جميع العترات المرجعية و الحقلية المعزولة في ك من مزارع الخلايا و السائل المنوي. ولقد تم التأكد من تخصص الكشف عن الجينوم المميز لفيروس مرض التهاب القصبة الهوائية المعدي باستخدام اختبار تفاعل إنزيم البلمرة بإجراء اختبار التهجين النقطي مع مجس الحمض النووي المحضر من عترة مرجعية قياسية للفيروس.

دلت نتائج هذه الدراسة علي الدراسة إمكانية استخدام تقنيات تفاعل إنزيم البلمرة ومجسات تهجين الحمض النووي كطرق تشخيص جزيئيه سريعه و ذات حساسية فائقة و متخصصة في الكشف عن إصابة الحيوانات المجترة بالفيروس المسبب لمرض التهاب القصبة الهوائية المعدي.