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DETECTION OF BOVINE HERPESVIRUS TYPE 1 IN SEMEN USING A RAPID AND SENSITIVE POLYMERASE CHAIN REACTION ASSAY

BY

AMIN, A. S. AND IBRAHIM, A. K.**

* Animal Reproduction Research Institute, Giza, Egypt.
** Fac. Vet. Med., Cairo Univ.

SUMMARY

A polymerase chain reaction (PCR) for the detection of bovine herpesvirus type 1 (BHV-1) was evaluated. Since bovine semen contains components that inhibit PCR amplification, a protocol was used to purify BHV-1 DNA from semen. The study to compare the PCR assay with the routinely used virus isolation method showed that the PCR assay is 100-fold more sensitive. In addition, the results of the PCR assay are available in 1 day, whereas the virus isolation method takes 10-17 days. It is concluded that the PCR assay is the most sensitive technique for laboratory detection of BHV-1.

INTRODUCTION

It is widely accepted that the conditions under which semen is frozen and preserved are conductive to maintaining viral infectivity (Chapman et al. 1979). As semen is widely distributed by the artificial insemination industry, the epidemiological risks of spreading bovine herpesvirus type 1 (BHV-1) infection are immediately apparent (Kahrs et al., 1976).

BHV-1,a herpesvirus infection of cattle, is known as a respiratory and genital tract disease. It has been associated with a wide variety of clinical syndromes e.g. pastular vulvovaginitis, abortion, metritis, balanoposthitis of bulls, infertility, tracheitis, conjunctivitis, kerato-conjunctivitis , encephalitis and fatal diseases of new born calves (Afsher, 1965; Gibbs and Rweyemamu, 1977 and Ludwing, 1983). In BHV-1 infections of the genital tract of bulls, BHV-1 replicates in the mucosa of the prepuce, penis and possibly in the distal part of the urethra causing decrease semen quality, orchitis and seminal vesiculitis (Sazegaard, 1970). Semen is most likely contaminated during ejaculation by virus that is shed from the infected mucosa (Elazhary et al., 1980). Insemination of cows with BHV-1 contaminated semen shortened estrous cycles, decreased conception rates and may cause histologically moderate to severe endometritis, infertility and abortion which occurs between the fourth and seventh month of gastation (Elazhary et al., 1980). Veneral spread of BHV-1 has also

been shown to occur under herd conditions (Deas and Johnston, 1973).

Reactivity of latent BHV-1 in carriers is periodic and has been shown to be associated with stress, such as occasioned by inter-current disease, transportation, cold, crowding ,vaccination or corticosteriods treatment (White and Snowdon, 1973; Darcel and Dorward, 1975 and Ackermann and Wyler, 1984). Under these circumstances, virus may be shed in the semen of seronegative as well as seropositive bulls (Straub, 1979). Therefore, serological status alone can not be used to screen bulls for use in artificial insemination centers.

To prevent transmission of BHV-1 by artificial insemination, only BHV-1 free should be used . The method that is routinely used to detect BHV-1 in bovine semen is virus isolation (Kahrs et al., 1980). Kupferschimed et al., (1986) and Bielamski et al., (1988) demonstrated that infectious dose of BHV-1 in semen for recipient cows is lower than the dose that be detected by the isolation method. Hence,, semen that is negative in virus isolation assays may still contain virus that can infect cows.

We anticipated that a polymerase chain reaction (PCR) assay would be more sensitive than the virus isolation method. In addition, a PCR assay could be more rapid than the virus isolation method. The objective of this study was to compare the sensitivity of the PCR assay with the sensitivity of the virus isolation methods.

MATERIAL AND METHODS

Semen samples:

Semen samples were collected and processed from BHV-1 free bulls at AI center in Animal Reroduction Research Institute. Semen was extended in sterilized egg yolk citrate, packed in straws, and frozen in liquid nitrogen. Each straw contained approximately 0.5ml of extended semen. Ten fold dilution series of BHV-1 was added to the semen (ten samples per each dilution). Following addition of the virus, the semen samples were stored at -20°C until assayed.

The prevalence of thirty semen samples were used for BHV-1 detection by using virus isolation method and PCR amplification assay.

Virus isolation:

The virus isolation method that used was described by Kahrs et al. (1980). Identification of the isolated virus was confirmed by staining with a fluorescein isothiocyanate conjugated BHV-1 antibodies [National Animal Center (NADC), Ames, Iowa, USA].

BHV-1 DNA extraction and purification from infected semen:

The BHV-1 DNA extraction and purification method that used was described by Loparaev et al., (1991). Briefly, a 100µl of semen was centrifuged at 12,000 xg for 30 s in a microcentrifuge. Two volumes of lysis buffer [0.15M NaCL,0.75% sodium-N-lauroylasarcosine and 1.5mg of proteinase K (Boehringer, Mannheim, Germany] per ml were then added to

the supernatant and the samples were incubated at 60°C for one hour. After centrifugation of the lysate at 12,000 xg for 30 s, an equal volume of 6 M NaCl was added to the supernatant and incubated for 5 minutes at room temperature. After incubation, 1.4 volumes of chloroform soamyl alcohol (24:1) was added, and the mixture was mixed for 1 minute and centrifuged at 12,000 xg for 5 minutes. Nucleic acids were precipitated from the supernatants that were obtained by adding 0.6 volume of isopropanol, and the mixture was centrifuged at 12,000 xg for 15 minutes at room temperature. The DNA pellet was further purified from organic contaminants by n-butanol extraction (Sawadego and Van Dyke, 1991). The pellet was resuspended in 100µl M Tris (pH 7.5)-1 mM EDTA (TE) buffer, and 10 volumes of n-butanol was added. Nucleic acids were precipitated by centrifugation at 12,000xg for 3 minutes. Finally, this pellet was dissolved in 50µl of TE.

Oligonucleotides:

The primer sequences are based on the sequence of the BHV-1 glycoprotein C (gpC) gene (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'-CTGCTGTTCGTAGCCCACAACG-3') and P2 (5'-TFTGACTTGGTGCCCATGTCGC-3).

DNA amplification and detection of PCR product:

PCR was performed according to the method described by Von Beroldingen et al. (1990) in 50µl of a reaction mixture containing a final concentration of 10 mM Tris (pH9.0), 50mM KC1, 0.01% gelatin, 1.9 mM $MgC1_2$, 5% (wt/vol) glycerol, 0.2mM deoxynucleoside triphosphates (Pharmacia, Piscataway, N.J.), 0.1µM primer 1, 0.1 uM primer 2, 10 U of Taq polymerase (Boehringer) per ml and µl of extracted BHV-1 DNA.. The PCR mixture were overlaid with 40µl of paraffin oil. The samples were subjected to 38 repreated cycles of amplification in a thermal cycler. The cycling conditions were as follows: Denaturation, 1 minute at 95°C; primer annealing 1 minute at 60°C; and extension, 1 minute at 72°C . Negative and positive control reactions were used. After amplification, a 10 µl was taken for electrophoresis on 1 2% agarose gel. The gels for relative sensitivity experiments were evaluated after being stained with ethidium bromide under UV light. A visible band of appropriate size was considered a positive reaction.

RESULTS

Detection of BHV-1 in infected semen: The relative sensitivities of virus isolation method and PCR amplification assay were determined by examined ten fold serial dilution of extended semen. The results are shown in the Figure and Table.(1).

Detection of BHV-1 in semen samples: Thirty semen samples were included in the study. The prevalence of BHV-1 as detected by virus isolation method and PCR amplification assay is summarized in Table 2.

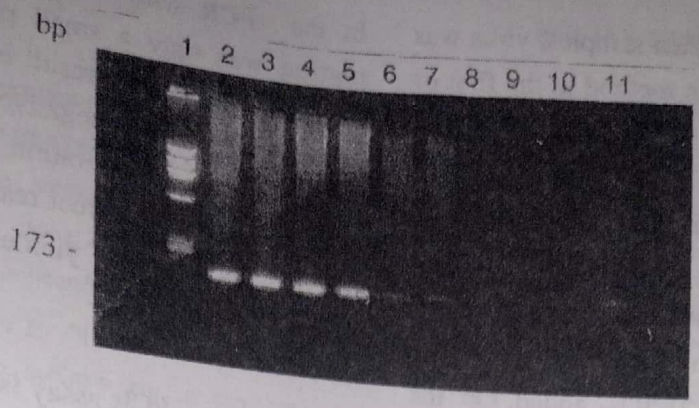
Table (1): Relative sensitivities of virus isolation method and PCR assay for detection of BHV-1 in experimentally infected semen

Virus added	No.of tested samples	Virus isolation				Virus detection by PCR	
		First passage	second passage	Third passage	%	Positive No.	Positive No.
10,000	10	10	ND[b]	ND	100	10	100
1000	10	10	ND	ND	100	10	100
100	10	8	2	ND	100	10	100
10	10	6	2	0	80	10	100
1	10	0	0	0	-	10	100
0.1	10	0	0	0	-	10	100
0.01	10	0	0	0	-	0	-
0.001	10	0	0	0	-	0	-

a) TCID50/0.5 ml of extended semen.
b) ND = Not Done.

Table (2): Prevalence of BHV-1 in 30 semen samples as determined by virus isolation method and PCR amplification assay.

Test method	No. (%) positive	No. (%) confirmed positive by PCR
Culture	2 ((6.7)	2 (6.7)
PCR	4(13.4)	----



Sensitivity of detection PHV-1 in semen by PCR Lane 1, mol, wt marker; 2-9, amplification of the 173-bp fragment from 10,000 -0.001 $TCID_{50}$/0.5ml; 10, negative control; 11 Positive control.

DISCUSSION

To prevent the spread of BHV-1, all possible hygienic precautions should be taken including the monitoring of semen of bulls in artificial insemination units for the presence of this virus samples to prevent transmission of virus to the recipient cow (Elazhary et al., 1980). Antibiotics added to semen do not affect the viability of the virus and deep-freezing of semen provides optimum conditions for preservation of virus (Elazhary et al., 1980).

Monitoring semen for the presence of any virus would not be beneficial unless the procedure was shown to be valid . The method presently used on a routine basis to identify BHV-1 contaminated semen samples is the virus isolation method, which takes 7-21 days to complete (Kahrs et al., 1980). The virus isolation method requires three tissue culture passages which increases the probability of contamination betwen passage and precludes the isolation of slow growing strains of BHV-1 The PCR assay can identify BHV-1 contaminated semen in one day and that could be more sensitive than the virus isolation method.

BHV-1 strains that have mutated or deleted primer binding sites might exist. To minimize the risk that the primer-binding sites are not identical for every BHV-1 strain, we chose our primers in a conserved region of the gpC gene (Fitzpatrick et al., 1989).

BHV-1 contaminated semen samples were used for a comparison of the sensitivities of the virus isolation method and the PCR amplification assay. Ten fold dilution series of the sample were made, and one-half of each dilution was tested by the virus isolation method and the other half was tested by the PCR amplification assay. The result (Table 1) indicated that, when 100 $TCID_{50}$ or

more was added to the semen samples, virus was recovered by virus isolation method in the first or second passage.

While, when the concentration of virus added was reduced to 10 $TCID_{50}$ virus was recovered from 80% only of the samples. However, virus was not recovered from any infected semen samples when less than to $TCID_{50}$ added. On the other hand, the results of the PCR-based assay indicated that, the BHV-1 could be detected when a virus concentration were 0.1 $TCID_{50}$ or more in the infected semen samples Fig. and Table (1). All these results indicated that, sensitivity of the pCR amplification assay was 100 fold greater than the virus isolation method for detection of BVH-1 in semen. The results of other studies have shown the exquisite sensitivity and specificity of the PCR in detection of BHV-1 DNA too (Vileck, 1994; Santurde, 1996 and Sreenivasa, 1996).

The results in Table (2) shows that, the PCR detected BHV-1 DNA in 4 out of 30 semen samples, while, 2 samples only were positive by the virus isolation method. The same table shows that, the prevalence of infection with BHV-1 as determined by PCR and virus isolation method were 13.4% and 6.77%, respectively. Compared with the PCR, the culture method had sensitivity of 50% (Table 2). There was no evidence of a systemic error introduced by the order in which the samples were taken . A wide variety of culture methods are used in different laboratories, resulting in considerable differences in performance (Robinson and Thomas, 1991).

In the PCR with culture samples, the usable portion was only a small part of the samples. Therefore, a positive result can be considered as confirmatory, but a negative result can not. In view of these consideration, we concluded that the PCR gave the correct results. This means that the culture method yielded 2 false negative results.

In conclusion , PCR assay is a sensitive and rapid method for the detection of BHV-1 in semen. In our study, this assay is more sensitive than the routinely used virus isolation method. Moreover, the presently used virus isolation method takes 7-21 days, while the PCR assay can be performed in one day. Although a more extensive comparison is needed, it can be concluded that the PCR assay may be a good alternative to the virus isolation method for the detection of BHV-1 in semen.

REFERENCES

Ackermann, M. and Wyler, R. (1984): The DNA of an IPV strain of bovine herpesvirus-1 in sacral ganglia during latency after intracaginal infection. Vet. Microbiol. 9:53-63.

Afshar, A. (1965): Virus diseases associated with bovine abortion and infertility. Vet. Bull. 35: 735-752.

Bielanski, A.; Loewen, K.G. and Hare, W.C.D. (1988): Inactivation of bovine herpesvirus-from in vitro infected bovine semen. Theriogenology. 30:649-657.

Chapman, M.S.; Lucas, M.H.; Hebert, C.N. and Goody, R.G. (1979): Survival of infectious bovine rhinotrachitis virus in stored bovine semen. Vet. Sci. Comm. 3: 137-139.

Darcel, C. and Dorward, W.J. (1975): Recovery of infectious rhinotracheitis virus following corticosteroid treatment of vaccinated animals. Can. Vet. J. 16: 87-88.

Deas, D.W. and Johnstone, W.S. (1973): The isolation and transmission of the virus of infectious bovine rhiniotrachitis/ infectious pasteular vulvovaginitis. Vet. Rec. 92: 636-639.

Elazhary, M.A.S.Y.; Lamothe, P.; Silim, A. and Roy, R.S. (1980): Bovine herpesvirus type 1 in the sperm of a bull from a herd with fertilityy problems. Can. Vet. J. 21: 336-339.

Fitzpatrick, D.R.; Babiuk, L.A. and Zamb, T.J. (1989): Nucleotide sequence of bovine herpervirus type 1 glycoprotein gIII, a structural model for gIII as a new member of the immunoglobulin superfamily, and implication for the homolgous glycoproteins of other herpesviruses. Virology. 137: 46-57.

Gibbs, E.P.J. and Rweyemamu, M.M. (1977): Bovine herpesviruses. Part I: Bovine herpesvirus 1. Vet. Bull 47: 317-343.Kahrs, R.F.; Schultz, R.D. and Bean, B.H. (1976): Epidemiologic considerations regarding the detection of infectious bovine rhinotrachitis virus in bovine semen. Proc. 19th Am. Meet.Am. Vet. Lab. Diag. 385-394.

Kahrs, R.F.; Gibbs, E.P.J. and Larsen, R.E. (1980): The search for virus in bovine semen. Theriogenology 14: 151-165.

Kupferschmied, H.U.; Kihm, U.; Bachman, aP.; Muller, K.H. and Ackermann, M. (1986): Transmission of IBR/IPV virus in bovine semen: a case report . Theriogenology. 25: 439-443.

Loparaev, V.N.; Carter, M.A.; Monken, C.E.; Velpandi, A> and Srinivasan, A. (1991): An efficient and simple method of DNA extraction from blood and cell lines to identify infectious agents. J. Virol. Methods. 34: 105-112.

Ludwing, H. (1983): Bovine herpesviruses. In 'The Herpesviruses', edited by Roizman, Planum Press, N.Y./London, Vol,2,pp. 135-214.

Robinson, D. and Thomas, J. (1991): Laboratory techniques for the diagnosis of BHV-1. Genitourin. Med. 67: 256-266.

Santurde, G. (1966): Rapid and high sensitivity test for direct detection of bovine herpesvirus-1 genome in clinical samples. Vet. Rec. 49: 81-92.

Sawadego, M. and Van Dyke, W. (1991): A rapid method for the purification of deprotected oligodeoxynucleotides. Nucleic Acid Res. 19: 674.

Saxegaard, F. (1970): Infectious bovine rhinotrachitis/ infectious pastular vulvovaginitis (IBR/IPV) virus infection of cattle with particular reference to genital infections. Vet. Bull. 40: 605-611.

Sreenivasa, B.R. (1996): Direct detection of bovine herpesvirus-1 DNA from cell culture fluids using the polymerase chain rweaction. Indian. J. Exp. Biol. 34: 1169-1171.

Straub, O.C. (1979): Persistence of Infectious bovine rhinotrachitis-infectious pastular valvovaginitis virus in the respiratory and genital tract of cattle. Comp. Immun. Microbiol. Infect. Dis. 2: 285-294.

Vileck, S. (1994): Rapid detection of bovine herpesvirus-1 (BHV-1) using the polymerase chain reaction. Vet. Microbiol. 42: 53-64.

Von Beroldingen, C.H.; Blake, E.T.; Higuchi, R.; Sensabaugh, G.F. and Erlich, H.A. (1990): Application of PCR to the analysis of biological evidence, pp. 209-223. In Erlich, H.A. (ed), PCR technology: principles and application for DNA amplification. Stockton Press, New York.

White, M.B. and Snowdon, W.A. (1973): The breeding record of cows with a batch of semen contaminated with infectious bovine rhinotrachitis virus. Aus. . Vet. J. 49: 501-506.