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DETECTION OF BOVINE HERPESVIRUS 1 (BHV 1) IN BOVINE SEMEN BY COMBINED POLYMERASE CHAIN REACTION AND *IN VITRO* PROTEIN SYNTHESIS

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirement

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Faculty of Veterinary Medicine

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Charlottetown, P.E.I.

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ABSTRACT

Bovine herpesvirus 1 (BHV 1) is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*. BHV 1 is an important cause of infectious bovine rhinotracheitis, infectious pustular vulvovaginitis and infectious pustular balanoposthitis. Infection with BHV 1 occurs worldwide and causes serious economic losses due to loss of animals, abortion, decreased milk production, and loss of weight. BHV 1 is frequently found in bovine semen and transmitted through artificial insemination. Semen is most likely contaminated during ejaculation by virus that is shed from the infected mucosa. Artificial insemination with BHV 1 contaminated semen can result in markedly reduced conception rates, shortened oestrous cycles, and endometritis. The linear-double stranded DNA genome of BHV 1 consists of approximately 140 kilobase pairs (kbp). Glycoprotein D (gD), a glycoprotein of 71kD, has been identified as a suitable candidate for the production of subunit vaccines, and as a major molecule on the surface of the virion and virus-infected cells. It induces a strong and consistent humoral and cellular immune response to BHV 1. The main objective of this study was to develop an improved method for detecting BHV 1 in bovine semen samples. The polymerase chain reaction (PCR) products amplified from the gD gene region of BHV 1 were transcribed and translated into gD peptides in an attempt to improve the sensitivity of direct virus detection in clinical samples.

The optimal amplification conditions for PCR were obtained when a reaction buffer containing 1.5 mM Mg^{2+} with pH 9.0, and TaqPlus Long PCR polymerase were used. GeneReleaser was used to release the BHV 1 DNA from the virus. When the PCR was used to detect BHV 1 in virus stock or BHV 1 in 1:50 diluted bovine semen, 10^{-5} (0.28 $TCID_{50}$) dilution of virus could be detected. The specificity of BHV 1 gD DNA was demonstrated by the fact that all the 13 strains of BHV 1 gave PCR products with the expected size of 1306 base pairs, and were confirmed by digestion with five restriction enzymes. However, BHV 1 DNA in high concentrations of bovine semen could not be detected by the GeneReleaser procedure because of inhibitory components in bovine semen. When Proteinase K in PCR buffer was used to purify and isolate BHV 1 DNA from bovine semen, virus diluted up to 10^{-2} (280 $TCID_{50}$) in 1:20 diluted bovine semen and 10^{-3} (28 $TCID_{50}$) in 1:50 diluted bovine semen could be detected. Even in 1:5, and 1:10 diluted semen samples, positive results were obtained for virus diluted 10^{-1}. A coupled transcription-translation system was used to express the gD protein using the PCR product that was produced by a 10-fold serial dilution of virus with 1:20 dilution of semen. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), virus diluted up to 10^{-3} could be detected by autoradiography. Since only 1/20th of volume of PCR product was used in the *in vitro* coupled transcription-translation, the expression of gD was 200-fold more sensitive than the PCR assay.

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DEDICATION

To my parents Jing Zhang and Haoting Zhou for their kindness, encouragement and support.

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ABBREVIATIONS

Abbreviation	Term
APS	ammonium persulfate
AVC	Atlantic Veterinary College
ATCC	American Type Culture Collection
BEHV	bovine encephalitis herpesvirus
BHV 1	bovine herpesvirus 1
bp	base pair
BRSV	bovine respiratory syncytial virus
CPE	cytopathic effect
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTPs	mixture of dATP, dCTP, dGTP, dTTP
dsDNA	double strained DNA
dTMP	deoxythymidine monophosphate
dUMP	deoxyurdine monophosphate
dUTP	deoxyurine triphosphate
dUTPase	deoxyuridine triphosphatase
EDTA	ethylenediaminetetraacetate

ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's Minimal Essential Medium
gD	glycoprotein D
HSV 1	herpes simplex virus 1
IBR	infectious bovine rhinotracheitis
IE	immediate early
IF	immunofluorescence
Ig	immunoglobulin
IPV	infectious pustular vulvovaginitis
Irs	internal inverted repeat
kbp	kilobase pair
kDa	kiloDalton
KV	killed virus
LR	latency related
MDBK	Madin Darby Bovine Kidney
Mg^{2+}	Magnesium ion
MLV	modified live virus
mRNA	messenger RNA
NP40	Nonidet P40
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
PPi	inorganic pyrophosphate
RNA	ribonucleic acid
SDS	Sodium dodecyl sulphate
$TCID_{50}$	Median Tissue Culture Infective Dose
TEMED	N,N,N',N',-tetra-methyl-ethylenediamine
tk	thymidine kinase
Trs	terminal inverted repeat
Ul	long unique region
Us	short unique segment
VN	virus neutralization
VP	viral protein

1 GENERAL INTRODUCTION

An outbreak of an apparently new upper respiratory disease of cattle in USA during the 1950s led to the recognition of a new disease "Infectious Bovine Rhinotracheitis" (IBR) (McKercher et al., 1955). Bovine Herpesvirus 1 (BHV 1) is an important cause of infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, and infectious pustular balanoposthitis. BHV 1 causes serious economic losses due to loss of animals, abortion, decreased milk production, and loss of weight (Wyler et al., 1989). BHV 1 is frequently found in bovine semen and can be transmitted through artificial insemination. The most common technique for detection of BHV 1 in the diagnostic laboratory is by virus isolation in cell culture (Wyler et al., 1989; Straub, 1990). The clinical samples are inoculated in susceptible cells, and the presence of BHV 1 can be confirmed by immunofluorescence (IF) or virus neutralization (VN) test (Leary and Splitter, 1992). However, the bovine semen is toxic to cell culture (Kahrs et al., 1977) and the virus isolation takes a long time. The polymerase chain reaction (PCR) has been applied for the direct detection of BHV 1 in clinical samples and it is considered to be more sensitive than virus isolation (Van Engelenburg, 1993; Masri et al.,1996). Our laboratory has established a PCR protocol utilizing primers in the thymidine kinase (tk) gene; the PCR product was used as a DNA probe in dot-blot and Southern blot hybridizations (Kibenge et al., 1994, Xia et al., 1995). In order to improve the sensitivity of detection of BHV 1 in bovine semen, we developed a protein synthesis

assay from the PCR product to detect BHV 1 in bovine semen.

1. 1 Characteristics of BHV 1

1.1.1 Virion structure

Bovine herpesvirus 1 (BHV 1) has an icosahedral nucleocapsid (95-110 nm in diameter) consisting of 162 capsomeres (12 nm long by 11.5 nm wide with an axial hole of 3.5 nm). The nucleocapsid is surrounded by an electron-dense zone, called the tegument, and by the bilayer of the envelope, forming rather pleomorphic virion of 150-200 nm diameter (Wyler et al., 1989).

1.1.2 Classification

Bovine herpesvirus 1 is a member of the family *Herpesviridae* based on the morphological features and physicochemical properties of the virus (Madin et al., 1956). The growth characteristics and genome structure have helped in classifying it as a member of the subfamily *Alphaherpesvirinae* (Roizman et al., 1992). BHV 1 used to be divided into three groups or subtypes based on the clinical manifestation in infected animals and restriction endonuclease analysis patterns of the BHV 1 DNA. Subtype 1 contained infectious bovine rhinotracheitis (IBR) strains; subtype 2 contained mostly infectious pustular vulvovaginitis (IPV) strains; and subtype 3 contained neurologic strains which are now called bovine encephalitis herpesvirus

(BEHV) (Brake and Studdert, 1985; Studdert, 1990), and is designated BHV 5 (Roizman et al., 1992). In cross-neuturalization tests, BHV 1 isolates exhibit one serotype regardless of their origin from IBR or IPV cases. According to the molecular properties of BHV 1, the BHV 1 strains now can be subdivided into three subtypes 1, 2a, and 2b, with restriction endonuclease analysis and monoclonal antibodies (Wyler et al., 1989). However, the subtyping does not show a strict correlation between the clinical origin of the isolate and its molecular subtype. In a recent report, 14 different strains of BHV 1 were compared by restriction fragment pattern analyses (Christensen et al., 1996). There was no correlation between genome type and clinical manifestation, and it was confirmed that there was no phylogenetic basis for a distinction between groups of strains associated with respiratory or genital disease.

1.1.3 The molecular structure of BHV 1

1.1.3.1 Genome of BHV 1

The bulk of the BHV 1 sequence, except for a small gap in UL36, has been determined (Schwyzer and Ackermann, 1996). The linear double stranded DNA genome of BHV 1 exhibits the typical arrangement of group D, because it consists of approximately 136-140 kilobase pairs (kbp) comprising a long unique region (Ul) of 104 kbp, and a short region which contains a unique segment (Us) of 10 kbp flanked by internal and terminal inverted repeats (Irs, Trs) of 11 kb each (Roizman et al., 1992). This allows

the Us sequence to invert relative to the Ul sequence, thus giving rise to two isomeric forms of the genome (Mayfield et al., 1983). The sequence comprises 67 unique genes and two genes, both duplicated, in the inverted repeats. Thus, BHV 1 encodes at least 69 proteins (Schwyzer and Ackermann, 1996). Most genes exhibit sequence homology and occur in the same order as with their counterparts in herpes simplex virus 1 (HSV 1). Therefore, and not to confuse the nomenclature further, the BHV 1 genes have been named after their HSV 1 homologues according to existing nomenclature (McGeoch et al., 1988).

1.1.3.2 Envelope glycoproteins

The BHV 1 genome contains at least 10 genes with the potential to encode glycoproteins gB, gC, gD, gE, gI, gH, gL, gG, gK, and gM. The predicted molecular weights of the corresponding gene products range from 17 to 101 kDa. However, their observed sizes are larger because they contain N- as well as O-linked oligosaccharides and may form structures similar to those specified by HSV 1 (Schwyzer and Ackermann, 1996). Because of their location in the virion envelope and on the surface of infected cells, these glycoproteins are important targets for the host immune response. Furthermore, they play important roles in pathogenicity by mediating entry of the virion into the host cell, cell fusion, and cell-to-cell spread of virus.

Glycoprotein B, a homologue of HSV 1 gB (Misra et al., 1988; Whitbeck et al., 1988),

is one of the major glycoproteins found in the virion envelope and the plasma membrane of virus-infected cells (van Drunen Littel-van Den Hurk et al.,1984; Marshall et al., 1986). Its gene maps in the Ul region of the genome and codes for a polypeptide of 932 amino acids with a fully mature product of 130 kDa. gB induces a strong neutralizing antibody response (Babiuk et al., 1987) and is recognized by CD4+ helper T lymphocytes (Hutchings et al., 1990).

Glycoprotein C is also a major glycoprotein presented in the envelope of the virion and in the plasma membrane of virus-infected cells (van Drunen Littel-van Den Hurk et al., 1984; Marshall et al., 1986). Glycoprotein C is synthesized as a 69 kDa partially glycosylated precursor which leads to the formation of a 91 kDa fully mature product containing both N-linked complex-type and O-linked oligosaccharides (van Drunen Littel-van Den Hurk and Babiuk, 1986). It induces a neutralizing antibody response and is recognized by CD4+ and CD8+ T lymphocytes (Denis et al., 1994). gC is considered to be involved in the attachment of the virus (Liang et al., 1991) through heparin-like receptors on tissue culture cells (Okazaki et al., 1991).

BHV 1 glycoprotein D gene maps in the Us region of the genome. Glycoprotein D consists of 417 amino acid residues and is a typical integral membrane protein containing a signal peptide of 18 amino acids at the amino terminus and a hydrophobic transmembrane domain between residues 361 and 389, which is followed by a 28-

amino acid cytoplasmic tail. The mature form of gD has a molecular weight of 71 kDa and contains both N-linked and O-linked oligosaccharides (van Drunen Littel-van Den Hurk et al., 1984) and exists as a monomer, or forms a dimer of 140 kDa. The N-linked glycosylation addition sites have been located at residues 41 and 102 (Tikoo et al., 1993). Glycoprotein D has been identified as a major molecule on the surface of the virion and virus-infected cells. It is essential for viral replication (Fehler et al., 1992) and is suggested to be involved in virus attachment (Liang et al., 1991) and penetration (Fehler et al., 1992; Chase and Letchworth, 1994), and induces a stronger and more consistent cellular immune response to BHV-1 than gB or gC (van Drunen Littel-van Den Hurk et al., 1994). It induces a strong neutralizing antibody response (Babiuk et al.,1987) and is recognized by both CD4+ and CD8+ T cells (Hutchings et al., 1990; Denis et al., 1994) which makes it a potential candidate for a subunit vaccine or a polynucleotide vaccine. PCR and nested PCR amplification targeting in a portion of the BHV 1 gD gene have been performed for detection of BHV 1 in clinical samples (Wagter et al., 1996; Wiedmann et al., 1993).

The glycoprotein E gene is also located in the Us region of the BHV 1 genome and encodes a 575 amino acid polypeptide with a predicted molecular mass of 94 kDa (Yoshitake et al., 1997). BHV 1 gE is involved in the cell-to-cell transmission of the virus (Yoshitake et al., 1997). Glycoprotein I, a 45 kDa glycoprotein is considered to form a complex with gE (Yoshitake et al., 1997).

Glycoprotein H, an essential component of the virion, is found in all herpesviruses and constitutes the second most highly conserved group of herpesvirus glycoproteins. Homologues of gH have been found to be required for virus penetration and cell-to-cell spread (Forrester et al., 1992). Recently, an accessory named glycoprotein L (Hutchinson et al., 1992) was identified as the viral glycoprotein that forms a complex with gH, which is necessary for attaining proper antigenic conformation and transportation of gH (Khattar et al., 1996). The remaining three glycoproteins gG, gK and gM have been expressed, but their functions are unknown.

1.1.3.3 Viral-encoded enzymes

The thymidine kinases (tk) specified by many herpesviruses have attracted much attention because they are potential targets for chemotherapy or attenuation of vaccine strains and provide a useful selective marker for genetic studies. The herpesvirus tk differs from host cell enzyme activity in substrate specificity, thermostability, and in its ability to use different nucleotide triphosphates as phosphate donors (Weinmaster et al., 1982). In addition to confirming the virus-specific nature of this enzyme activity, it has been suggested that the tk activity is not essential for viral replication (Weinmaster et al., 1982). However, deletion of the tk gene reduces the virulence of the virus *in vitro* (Kit et al., 1986).

Another enzyme that is conserved among alphaherpesviruses is deoxyuridine

triphosphatase (dUTPase). This enzyme catalyses the degradation of dUTP to dUMP and inorganic pyrophosphate (PPi) and maintains the intracellular levels of dUTP. dUMP then can be converted into dTMP by thymidylate synthetase. Thus, dUTPase is a component of the *de novo* dTMP synthesis pathway. Isolation of a deletion mutant of dUTPase confirmed the gene assignment and suggested that this enzyme was not required for replication of the virus *in vitro* (Liang et al., 1993a).

Two other enzymes, ribonucleotide reductase and DNA polymerase, are involved in the replicating process of DNA viruses.

1.1.3.4 Other virus-encoded proteins

Analysis of proteins synthesized in BHV 1 infected cells treated with cyclohexamide and actinomycin D identified three proteins classified as immediate early (IE) proteins (Misra et al., 1981), BICP4 (Schwyzer et al., 1993), BICP0 (Wirth et al., 1992), and BICP22 (Schwyzer et al., 1994). VP8 is the most abundant viral protein found in the tegument of the virion and in virus infected cells (Misra et al., 1981 Marshall et al., 1986). Another protein gene sequenced is Btif, which has been shown to be involved in the activation of BHV 1 IE gene transcription (Carpenter, 1992).

1.1.4 Viral pathogenesis

Bovine herpesvirus 1 (BHV 1) is an important cause of infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, infectious pustular balanoposthitis, encephalitis, conjunctivitis, and abortion.

BHV 1 usually enters the body via the mucous membrane of the upper respiratory tract and the mucous membrane of the genital tract, and possibly by way of the conjunctival epithelium. It is not yet established if oral infections occur (Engels and Ackermann, 1996).

BHV 1 multiplies at the portal of entry in the mucosa of the respiratory tract, the genital tract or the eye. The disease symptoms may mainly be attributed to the destruction of infected cells due to viral replication. The highest virus titres are produced and excreted at this stage of the infection. However, the severity of clinical disease appears to be related to the viral strain, immunological status of the animal, environmental stressors and the natural condition of the animal (Tikoo et al., 1995).

The events involved in increasing susceptibility to secondary bacterial infections have been reviewed (Babiuk et al., 1988). Evidence is accumulating to support the contention that BHV 1 induces alteration of the host immune system and alterations

in the functional integrity of the respiratory tract environment, thereby aiding the colonization with bacteria, primarily Pasteurella.

BHV 1 can spread to a broader range of tissues and organs thereby causing a broader variety of diseases such as abortion and enteritis. A local infection may be followed by a viremia and subsequent generalized infection. BHV 1 can infect blood monocytes, where limited virus replication and release is possible. In addition, the virus is able to adsorb to lymphocytes which also may serve as vehicles (Nyaga and McKercher, 1979). BHV 1 may also enter the axons of local nerve cells, and then by neuronal spread through intercellular bridges (Pastoret et al., 1982) reach the neuronal bodies in the regional ganglions where latency can be established.

1.1.4.1 Virus replication

Initiation of BHV 1 infection of permissive cells takes place by the attachment of viral glycoproteins to cell surface receptors (Okazaki et al., 1987,1991; Liang et al., 1991, 1993b). Fusion of the viral envelope with the plasma membrane of host cells (Okazaki et al., 1986) releases the nucleocapsid and tegument into the cytoplasm of the cell, where they are transported to the nucleus. In the nucleus, the transcription of the viral genes starts in a temporally regulated manner. Tegument proteins, which may include Btif and VP8, along with cellular regulatory proteins, initiate transcription and translation of selected viral genes encoding immediate early proteins (Misra et al.,

1981) such as BICP0, BICP4 and BICP22. The function of these immediate early proteins is to induce transcription and translation of early proteins (Misra et al., 1981) such as thymidine kinase and DNA polymerase. Early proteins are predominantly involved in genome replication and the induction of the synthesis of late proteins that form the virion structural proteins, such as gC and VP8 protein. Replication of the genome and assembly of nucleocapsids occur in the infected cell nucleus (Hammerschmidt et al., 1988; Ludwig, 1983). These nucleocapsids become enveloped by budding through the nuclear membrane. The mature enveloped virus particles accumulate in the endoplasmic reticulum, the final processing of glycoproteins occurs in the Golgi complex and virions eventually reach the extracellular space by exocytosis (Roizman and Sears, 1995).

1.1.4.2 Latency

Herpesvirus latency is characterized by the fact that no infectious virus can be isolated and no viral antigen can be demonstrated in the latently infected cells.

After infection, BHV 1 can be spread from a local infection to the nervous system by entry of virus into peripheral neural cells. The virus reaches the sensory ganglion such as trigeminal and lumbosacral ganglions where latency can be established (Ackermann and Wyler, 1984). Lymph nodes and nasal mucosa also are considered to be sites of latency (Engels and Ackermann, 1996). Once infected, cattle must be regarded as

lifelong potential shedders of BHV 1. The latent virus represents a long-term reservoir in an immune host which becomes relevant upon reactivation. A wide variety of stimuli such as stress, transport, and treatment with corticosteroids may lead to reactivation from latency (Sheffy and Davies, 1972; Thiry et al., 1985, 1987). The reactivated virus is transported intra-axonally back to the periphery, to the original portal of entry, where it is available for transmission to other susceptible hosts. The reactivation of herpesvirus usually remains subclinical. The local immune response may be too weak to prevent virus shedding completely, depending on the period of time elapsed between the initial infection and reactivation. It may be necessary to vaccinate the host to interfere with the virus transmission at this stage.

It is unknown how the virus is maintained in the latent state, and how it becomes reactivated. Scientists working on the viral expression during virus latency believe that the latency related (LR) gene is the only transcriptionally active region of the viral genome in latently infected neurons (Rock et al., 1987, 1992; Kutish et al., 1990). Although LR RNA accumulates in the nucleus of latently infected sensory neurons, only a fraction of the RNA is polyadenylated (Hossain et al.,1995). Polyadenylated transcripts presumably account for the weak cytoplasmic signal observed by *in situ* hybridization (Kutish et al., 1990). The LR gene contains two open reading frames (ORF). LR ORF2 encodes a 40 kDa protein which is expressed during late stages of a productive infection, but ORF1 does not apparently express a protein (Hossain et al.,

1995). LR gene products inhibit cells in the G1 phase of the cell cycle (Schang et al., 1996). Since neurons which re-enter the cell cycle undergo apoptosis (Lee et al., 1994), it appears that the LR gene products enhance neuronal survival during virus latency.

1.1.4.3 Clinical symptoms

The incubation period after experimental BHV 1 infection is 2 to 4 days depending on the exposure dose and route. Infectious bovine rhinotracheitis (IBR) is the most commonly recognized disease caused by BHV 1. The disease is characterized by a sudden onset of high fever, increasing respiratory rate and persistent harsh cough, anorexia, depression, and in milking cows, by a severe drop in milk production and weight loss. In most infected cattle, a clear bilateral nasal discharge develops within a day or two and the mucosa of nares becomes hyperaemic (red nose). The profuse nasal discharge later becomes mucopurulent. Excessive salivation is noticed in some animals, but oral lesions are uncommon (Wyler et al., 1989).

BHV 1 also produces venereal infections in cattle. The genital disease is referred to as infectious pustular vulvovaginitis (IPV) in cows and infectious pustular balanoposthitis (IPB) in bulls. It develops 2-4 days after natural service, artificial insemination or contamination by the tail of a neighbouring infected animal. Genital manifestations of BHV 1 are characterized by mucosal inflammation of the vulva and

vagina in IPV and of the penis and prepuce in IPB. Frequent micturition and elevated tail are the first characteristic signs. Small pustules become visible in the vulvar and caudal vaginal region with an increased mucopurulent vaginal discharge (Wyler et al., 1989; Straub, 1979).

The most severe effect of BHV 1 as a reproductive pathogen is seen in pregnant animals. If the infection becomes systemic, the virus can pass through the uterine epithelium to the conceptus, leading to either embryonic death or abortion (Miller et al., 1991).

Encephalitis seems to be most prevalent in Australia and Argentina. The neurological signs are characterized by incoordination, muscular tremor, recumbency, aimless circling, ataxia, and blindness (Wyler et al., 1989). Conjunctivitis is usually associated with IBR but has been reported to occur separately or even in combination with genital tract infection (Straub, 1990). BHV 1 also causes other diseases such as enteritis, mastitis and endometritis (Wyler et al., 1989).

1.1.5 Epidemiology

1.1.5.1 Host range and geographic distribution

BHV 1 is widely distributed among cattle on all continents. Many wild species have

been found to be seropositive but distinct clinical signs have been observed only in cattle. Cattle are the principal reservoir of BHV 1 but serologic surveys in North America, Australia, and Europe have demonstrated BHV 1 antibody in numerous species of wild ruminants (Wyler et al., 1989). Seropositive animals represented the families *Bovidae, Cervidae, Giraffidae, Hippopotamidae* and *Suidae* (Wyler et al., 1989). Rabbits, neonatal skunks, and goats are susceptible and have been used as experimental models (Straub, 1990).

1.1.5.2 Transmission

BHV 1 infections are easily transmitted directly from one animal to another because large quantities of virus are shed essentially in the respiratory, ocular and reproductive secretions of infected cattle. The virus can be spread through nasal secretions or aerosol droplets containing the virus (Engels and Ackermann, 1996). Because of the mechanism by which the virus is spread, close contact among animals is responsible for the high rate of transmission. In feedlots, crowding and mixing of animals allow the efficient spread of the virus (Van Donkersgoed and Babiuk, 1991).

1.1.5.3 Virus shedding in semen

Bulls can start shedding BHV 1 from the prepuce between 2 and 7 days after primary intrapreputial infection. This first period of shedding can last several weeks. After the primary phase of infection, virus can often no longer be isolated (Huck et al., 1971).

Nevertheless, spontaneous intermittent virus shedding has been frequently observed. In addition, latently infected bulls can be triggered to re-excrete virus by stress, transport, or corticosteroid treatment. Such periods of spontaneous or artificially induced intermittent virus shedding often go clinically unnoticed and can be detected for prolonged periods, even for years after the primary infection.

Semen most probably becomes contaminated by virus that replicates in the mucosa of the prepuce and the penis, rather than by virus produced in the testis, epididymis, or accessory gland (Snowdon et al., 1965). In general, BHV 1 is excreted in much higher concentration in the primary phase of the infection than in later phases when shedding is often intermittent (Bitsch, 1973). Bitsch (1973) found virus titres in semen to be somewhat lower than in preputial washings. Titres ranging between 10^5 to $10^{8.5}$ Median Tissue Culture Infective Dose ($TCID_{50}$) per ml of preputial washing or semen have been found during the acute phase of natural or experimental infection. During periods of recurrent virus excretion, either spontaneous or induced by natural or artificial stimuli, virus titres can vary greatly; virus titres ranging between 10^1 and $10^{5.6}$ $TCID_{50}$ per ml of preputial washing or semen have been recorded (Bitsch, 1973; White and Shnowdon, 1973; Dennett et al., 1976). The dose required to infect cattle after intranasal or vaginal inoculation with a field strain of BHV 1 has been estimated to be 3.2 $TCID_{50}$ (van Oirschot, 1995).

The infectivity of BHV 1 remains stable during storage of semen in liquid nitrogen. No decrease in titre was observed during storage for one year (Chapman et al., 1979). BHV 1 in extended semen was well conserved at 4^0C for 7 days and at ambient temperature for 5 days. Drew et al. (1987) found no loss of infectivity after five freeze-thaw cycles.

1.1.7 BHV 1 vaccines

Presently a number of countries have embarked on a program to eradicate BHV 1. Previously the only tools that were available were the serological testing of animals and elimination of seropositive animals. Several countries in the European Union, like Denmark, Sweden, Finland, and Switzerland have eradicated BHV 1 by prohibiting vaccination and removing seropositive animals (Bosch et al., 1996). Unfortunately, this approach is not feasible in many countries with large cattle populations or where management practices result in the movement of cattle from one region to another. Antiviral drugs have been tested against BHV 1, mainly *in vitro*, and have been shown to have little effectiveness. One of the best approaches for eradication would be to develop an immunization program.

Bovine herpesvirus 1 vaccines can be divided into two categories: the conventional BHV 1 vaccines and the new generation BHV 1 vaccines. The new generation BHV

1 vaccines have been developed recently, and there are many papers focused on this field (Bosch et al., 1996; van Drunen Littel-van Den Hurk et al.,1994).

1.1.6.1 Conventional BHV 1 vaccines

The conventional vaccines are either a modified live (MLV) or a inactivated vaccine. Live vaccines contain an attenuated BHV 1 strain that is able to replicate in the host. The widely used live BHV 1 vaccine is from a BHV 1 strain that was modified by treatment with nitrous acid and subsequently selected on the basis of its temperature sensitivity (Zygraich et al., 1974), whereas the BHV 1 strain in inactivated vaccines has been rendered non-infectious and therefore does not replicate in the host. The virus strains in inactivated vaccines are grown in cell culture to high titres and subsequently inactivated by treatment with chemicals. It is necessary to include an adjuvant in a inactivated vaccine to induce an adequate immunity.

The widely used modified live vaccines may induce rapid protective immunity. The immunity after intramuscular or intranasal vaccination with MLV vaccine may develop in 2-3 days (Todd et al., 1972; Sutton, 1980). There is evidence that a certain degree of protection against challenge still exists at 6-9 months after vaccination with either MLV or inactivated vaccine (Sibbel et al., 1988). However, these vaccines do not always prevent infection or disease. MLV vaccines also cause some problems because of the potential of adverse post-vaccine reactions, such as mild clinical signs and virus

shedding with subsequent transmission to unvaccinated animals (Hyland et al., 1974). Furthermore, some MLV vaccines can cause abortion (Kelling et al., 1973), establish latency, and cause immunosuppression (Pastoret et al., 1980; Harland et al., 1992). On the other hand, inactivated vaccines do not provide early or complete protection and do not prevent latent infections by field strains (Babiuk et al., 1987).

1.1.6.2 New generation BHV 1 vaccines

1.1.6.2.1 Live and inactivated marker vaccines

A marker vaccine can be defined as a vaccine based on deletion mutants on one or more viral proteins, that allows the distinction between vaccinated and infected animals based on the respective antibody responses. Hence, a marker vaccine is used in conjunction with a test that detects antibodies against a glycoprotein that is lacking in the vaccine strain (van Oirschot et al., 1996).

Mutants of BHV 1 have been developed by deleting one or more of the non-essential glycoproteins, gC, gG, gI or gE. Mutants of BHV 1 from which the coding region for gG, gI, or gE were removed were strongly reduced in virulence for calves and may therefore be suitable to provide the basis for a marker vaccine (Rijsewijk et al., 1994). Based on the gE-negative mutant, both live and inactivated BHV 1 marker vaccines have been produced (Kaashoek et al., 1994, 1996; Strube et al., 1996). This kind of

vaccine can be used efficaciously in the early stages of a BHV 1 outbreak and demonstrates safety in all target animal categories, including pregnant animals (Bosch et al., 1996). The live gE-negative vaccine induced the best clinical protection as evidenced by the total absence of clinical signs and fever in cattle, and it reduced the shedding of challenge virus significantly more than did the inactivated vaccine (Bosch et al., 1996). With the conventional BHV 1 vaccines, it is impossible to monitor the prevalence of infection in a vaccinated population. However, marker vaccines offer the advantage of being able to evaluate the effect of vaccination on the circulation of field virus by measuring the antibodies against gE (Bosch et al., 1996)

1.1.6.2.2 Subunit BHV 1

A subunit vaccine only contains one or more proteins of a microorganism. The use of recombinant DNA technology offers possibilities to synthesize these proteins in large amounts (van Oirschot et al., 1996). Some glycoproteins of BHV 1 have been shown to be involved in stimulating protective immunity. A series of experiments with purified glycoproteins gB, gC and gD pointed to gD as the most promising candidate for a subunit vaccine, because it induced the highest level of immunity against disease and infection in cattle (van Drunen Littel-van Den Hurk et al.,1994).

Several vaccinations with an experimental gD subunit BHV 1 vaccine appeared to be highly efficacious, not only in prevention of clinical signs after challenge infection, but

also in preventing replication and subsequent excretion of challenge virus (van Orischot et al., 1996; van Drunen Littel-van Den Hurk, 1997). BHV 1 gD vaccine could engender solid mucosal immunity and induce an effective mucosal barrier to viral replication in cattle (Zhu and Letchworth, 1996).

1.1.6.2.3 Polynucleotide vaccine

In vivo gene transfer to living vertebrates has been described and successfully conducted in a number of different species using several target tissues. These systems were based on retroviral vectors to direct the integration of the desired gene into the chromosomes of the target tissue. More recently it has been demonstrated that functional gene transfer can be accomplished without the aid of retroviral vehicles by simply introducing plasmids initially designed for transfection of mammalian cells in culture into tissues of living animals (Wolff et al., 1990). This technology may be applied to immunize cattle with noninfectious plasmid DNA encoding BHV 1 protective proteins under appropriate conditions. A polynucleotide vaccine encoding BHV 1 gD was used to immunize the cattle and murine model (Cox et al., 1993; Lewis et al., 1997), which led to immune responses. A vector vaccine containing BHV 1 DNA fragment and a gene encoding the G protein of bovine respiratory syncytial virus (BRSV) induced protective immunity against a subsequent BRSV and BHV 1 infection (Schrijver et al.,1997). If these vaccines were available commercially, they could alter the economics of vaccination and vaccine production as well as allow

immunization under various management conditions.

1.2 Diagnosis

1.2.1 Serological tests for viral antibodies

BHV 1 is able to establish latent infections. The latent virus persists during the life of the animal and may be reactivated under certain stressful conditions. Shedding of the virus may or may not be accompanied with clinical signs (Bitsch et al., 1978; Pastoret et al., 1982). Clinical disease induced by BHV 1 can be controlled by vaccination. However, latent infection cannot be prevented by vaccination (Whetstone et al., 1986). Different strategies have been used to diagnose and control BHV 1 infection in European countries. In order to control and eradicate BHV 1, some countries only permit importation of cattle if the animals are seronegative for BHV 1. Currently, at some artificial insemination centres, only seronegative bulls may be used for collection of semen. Because of the necessity to detect latent virus carriers in control programs and in international trade activities, serological tests for BHV 1 specific antibodies must be sensitive as well as specific. Enzyme-linked immunosorbent assay (ELISA) is rapid, inexpensive and of relatively high specificity. It has become a prominent test for the serodiagnosis of viral infections, including BHV 1 (Riegel et al., 1987). ELISA have been used for detection of IgG and IgM antibodies of BHV 1. Blocking ELISA, which is highly sensitive, has also been developed (Kramps, 1993, 1994; Van

Wuijckhuise et al., 1998).

1.1.2 Viral antigen diagnosis

BHV 1 is frequently found in bovine semen and can be transmitted through artificial insemination. Semen is most likely to become contaminated during ejaculation by virus that is shed from the infected mucosa. Artificial insemination with contaminated bovine semen by BHV 1 can result in markedly reduced conception rates, shortened oestrous cycles, and induced endometritis (Kendrick et al., 1958; Miller et al., 1991). Therefore, the detection of BHV 1 in bovine semen is important to the cattle industry. The most common technique for BHV 1 detection in the diagnostic laboratory is virus isolation in cell culture (Wyler et al., 1989). The samples are inoculated into Madin Darby Bovine Kidney (MDBK) cells or other susceptible cells, and the presence of BHV 1 is detected by cytopathic effect (CPE) and can be confirmed by immunofluorescence or the virus neutralization test (Leary and Splitter, 1992). However, when semen is used as inoculum, difficulties arise. Seminal plasma is toxic to cell cultures (Kahrs et al., 1977; Pacciarini et al.,1988), and therefore semen is often prepared or treated to eliminate its toxicity when examined for the presence of virus. These treatments include dilution of semen, extensive washing after adsorption, centrifugation steps and trypsin or kaolin pretreatment of semen (Wyler et al., 1989).

The immunofluorescence technique has been applied for the detection of BHV 1 in

nasal smears, and in nasal, preputial, lung and tracheal mucosa (Silim and Elazhary, 1983; Terpstra, 1979). The main advantage of this technique is that it is rapid. Electron microscopy and immune electron microscopy are very reliable and rapid methods, but high amounts of virus are required for detection. ELISA with monoclonal antibodies has been used to detect BHV 1 (Collins et al., 1988). Brunner et al. (1988) compared several hybridization methods with immune electron microscopy and various cell cultures, and the result showed that virus isolation was the most sensitive of all these methods with which as little as 5 $TCID_{50}$ could be detected in 1:25 diluted semen.

1.2.3 Molecular diagnostic tests

Recent attention in diagnostic virology has been directed toward the development of nucleic acid techniques for the detection of virus in clinical specimens. Nucleic acid hybridization and polymerase chain reaction (PCR) were developed as ideal diagnostic tools for detection of BHV 1 in clinical specimens because they are fast, sensitive and specific.

Several hybridization formats such as dot-blot hybridization (Vilcek et al., 1993a; 1993b), *in situ* hybridization, and Southern blot hybridization (Kibenge et al., 1994; Xia et al., 1995) with radioisotope (Vilcek 1993a; Kibenge et al. 1994; Xia et al., 1995), biotin (Oreshkova et al., 1995) or digoxigenin (Vilcek, 1993a) labeled probes have been applied to the detection of BHV 1 in nasal swabs and semen. Our laboratory

has developed a diagnostic procedure using PCR with Southern blot hybridization (Kibenge et al., 1994; Xia et al., 1995) in which 0.01 $TCID_{50}$/100µl of BHV 1 could be detected in 1:20 diluted bovine semen.

1.2.3.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) process was discovered in April 1985 by Kary Mullis and was originally applied to the amplification of human β-globin DNA and to the diagnosis of sickle-cell anaemia (Saiki et al., 1985). The basic principle of PCR is amplification of the template (target) DNA. This is achieved by the use of synthetic oligonucleotides that correspond to sequences within the target and use of a thermostable DNA polymerase. The exponential increase in target is achieved by subsequent rounds of denaturation, primer annealing and extension by DNA polymerase (Latchman, 1995). After the amplification of DNA, the reaction is assayed for the specific product in agarose gel with ethidium bromide staining, by hybridization with a cloned probe or oligonucleotide probe, or by the digestion with a restriction enzyme. The widespread success of PCR as a technique comes from the fact that it is rapid, automated, efficient, sensitive, and specific.

Despite the apparent rapidity and simplicity of PCR, it has a relatively large number of associated variables that will affect the efficiency and specificity of the technique. These factors will affect the success of PCR technology. These parameters include the

concentration of magnesium and potassium chloride, dNTP, Taq polymerase, primer, pH, annealing temperature, and cycle number (Meena et al., 1995).

Because of the amplification power of PCR, it is critical to avoid even traces of contamination of the DNA containing the target sequence. Contamination of the PCR amplification reaction with products of previous PCR reactions (PCR carryover), cross-contamination between samples, and contamination with exogenous nucleic acids from the laboratory environment can create false-positive results.

Many recent papers have reported using PCR in basic science and clinical medicine. PCR applications in molecular genetics are numerous, with major applications being molecular cloning, mutagenesis and sequencing. PCR has also been applied in clinical analysis of genetic disease, infectious disease and cancer (Xu and Larzul, 1991). In medical microbiology and infectious disease diagnosis, PCR has now been used to detect numerous bacteria, viruses, fungi, and parasites in clinical specimens. Especially in virology, this method is now being widely used to detect the presence of viral DNA or RNA genomic material in tissue biopsies or secretions, or to characterize different isolates of a virus. It has also been used in viral typing, monitoring drug and treatment efficacy, characterization of genetic variants, identification of new viruses, and simultaneous detection of multiple viral agents (Williams and Kwok, 1992).

The PCR method has also been applied for the direct detection of BHV 1 in clinical samples. The detection of BHV 1 gB, gC, gD and thymidine kinase (tk) DNA by PCR has been described (Vilcek et al, 1993c; Wiedmann et al., 1993; Kibenge et al., 1994). Vilcek developed the first PCR assay for detection of BHV 1 (1993c) with primers from the BHV 1 gB gene. The first nested PCR assay targeting a portion of the gD gene was reported for the detection of BHV 1 in artificially inoculated samples of extended and raw semen (Wiedmann et al., 1993). Another nested PCR was developed for the detection of BHV 1 in bovine semen (Masri et al., 1996). This assay could detect BHV 1 DNA in semen at 0.25-2.5 $TCID_{50}$. A highly sensitive detection of BHV1 in semen was facilitated by purifying BHV 1 DNA from semen (van Engelenburg, 1993). Three to five molecules of BHV 1 DNA in 50 µl of bovine semen could be detected by PCR. Other PCR assays for detection of BHV 1 using different primers and different treatments of bovine semen have been reported (Vilcek et al., 1994; Wagter et al., 1996; Santurde et al., 1996).

Our laboratory has established a PCR protocol utilizing primers in the tk gene; the PCR product was used as a DNA probe in dot-blot and Southern blot hybridizations (Kibenge et al., 1994, Xia et al., 1995). 0.01 $TCID_{50}$/100µl of BHV 1 could be detected in 1:20 diluted bovine semen by using this method.

1.3 Research objectives

The main objective of this study was to develop an improved method for detecting bovine herpesvirus 1 (BHV 1) directly in clinical samples. This entailed polymerase chain reaction (PCR) amplification of DNA of the BHV 1 glycoprotein D (gD) gene, and transcription-translation of the PCR products. The specific objectives were as follows:

1 To optimize conditions for PCR amplification of DNA of the gD gene of BHV 1 grown in cell culture.

2 To establish conditions for PCR amplification of DNA of the gD gene of BHV 1 in bovine semen.

3 To transcribe and translate the PCR product and compare the sensitivity with PCR.

2 MATERIALS AND METHODS

2.1 Virus sample and cell culture

2.1.1 Virus strains

The thirteen BHV 1 strains used included LA, Colorado 1, Kit, and NG dltk which were obtained from American Type Culture Collection (ATCC, Rockville Maryland, USA); B 347-10804 was isolated from cattle in Diagnostic Virology, AVC; DV 196, DV 197, DV 199, DV 200, DV 202, DV 203, DV 206, and DV 207 which were isolated from cattle in Sackville Animal Pathology Laboratory, Sackville, New Brunswick.

2.1.2 Cell culture

The thirteen BHV 1 strains were propagated in Madin Darby Bovine Kidney (MDBK) cells. Briefly, the cells were grown in Eagle's Minimal Essential Medium (EMEM) (Sigma, St. Louis, Mo., USA) containing 100 unit/ml of penicillin, 100 μg/ml of streptomycin, 25 μg/ml of fungizone (GIBCO BRL Canada, Burlington, Ontario), and 10% fetal bovine calf serum, and maintained in EMEM with the same concentration of antibiotics with 2% fetal calf serum in 75 cm^2 flasks. The flasks were incubated at 37°C with 5% CO_2 atmosphere. A monolayer had formed in 24 hours, and a 1:10 diluted stock virus in EMEM was used to infect the cells. The virus was harvested

after 3 to 4 days post-infection when a viral cytopathic effect (CPE) was observed. BHV 1-induced CPE occurs rapidly and is characterized by cell-rounding, syncytia formation and cell detachment from the vessel surface. When 95% CPE was obtained, the flasks were then frozen and thawed 3 times. Virus was clarified by centrifugation at 1100 xg for 20 min, and the supernatant was stored in 1.5 ml aliquots at -80^{0}C.

2.1.3 Quantification of viral infectivity

Ten-fold serial dilutions of the stock virus preparation ranging from 10^{-1} to 10^{-8} were made in EMEM with antibiotics. Fifty microlitres of each dilution was added to each well of a 96-well microtitre plate, and 50 µl of MDBK cells in growth medium was added to each well on the plate. Each dilution was performed in quadruplicate. These plates were incubated at 37^{0}C in a CO_2 incubator and monitored by microscopic observation of the wells daily for 4 days. After 4 days, the plates were stained for 10 minutes in a solution of 1% crystal violet in 70% ethanol. The plates were rinsed with water and dried before reading results. The virus titre was calculated using the Karber method of estimating the Median Tissue Culture Infective Dose ($TCID_{50}$) (Section 3.1).

2.1.4 Semen and *in vitro* seeding with BHV 1

2.1.4.1 Semen

Fresh BHV 1 free semen was collected by electroejaculation from a bull at the Atlantic Veterinary College. The semen samples were stored in 1 ml aliquots at -80^{0}C until use. The serum of the bull had been assayed by immunofluorescence technique method, and the result had demonstrated that it was seronegative for BHV 1 antibody.

2.1.4.2 Semen in EMEM

Semen volumes of 20, 10, 5, and 2 µl were diluted to a total volume of 90 µl with EMEM, and a 90 µl aliquot of neat semen was used without dilution. Ten-fold serial dilutions of BHV 1 LA strain stock culture containing $10^{6.05}$ $TCID_{50}$/100 µl were made in EMEM. Ten microlitres of each 10-fold serial dilution from 10^0 to 10^{-7} was then added to each of the diluted semen samples (Table 1). Ten-fold serial dilutions of BHV 1 LA strain stock culture without bovine semen were also made in EMEM. The sample was vortexed to mix the contents.

2.1.4.3 Semen in PCR buffer

Semen volumes of 20, 10, 5, and 2 µl were diluted to a total volume of 90 µl with 2 different PCR buffers. PCR buffer one consisted of 0.75% sodium N-laurylsarcosine, 0.15M NaCl (Wagter et al., 1996); PCR buffer two consisted of 50 mM KCl, 10 mM

Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP40, and 0.45% tween 20 (Higuchi, 1989). A 90 µl aliquot of neat semen was used without dilution. Ten-fold serial dilutions of BHV 1 LA stock culture containing $10^{6.05}$ $TCID_{50}$/100 µl were made in PCR buffer. Ten microlitres of 10-fold serial dilutions from 10^0 to 10^{-7} was added to each of the diluted semen samples (Table 1). The sample was vortexed to mix the contents.

2.1.5 Calculation of the $TCID_{50}$ in different dilutions

Since 10 µl of 10-fold serial dilutions from 10^0 to 10^{-7} of BHV 1 LA strain stock culture of $10^{6.05}$ $TCID_{50}$/100 µl was diluted 10 times in a total volume of 100 µl, the virus dilutions from 10^{-1} to 10^{-8} contained the following virus amounts, respectively, $10^{5.05}$, $10^{4.05}$, $10^{3.05}$, $10^{2.05}$, $10^{1.05}$, $10^{0.05}$, $10^{-.95}$, and $10^{-1.95}$ $TCID_{50}$/100 µl.

Table 1. Preparation of artificially infected bovine semen samples with different dilutions of BHV1.

Dilution of virus stock[1]	Dilution of semen (in EMEM or PCR buffer)[2]					
	undiluted	1:5	1:10	1:20	1:50	EMEM
10^{-1}	A1	B1	C1	D1	F1	E1
10^{-2}	A2	B2	C2	D2	F2	E2
10^{-3}	A3	B3	C3	D3	F3	E3
10^{-4}	A4	B4	C4	D4	F4	E4
10^{-5}	A5	B5	C5	D5	F5	E5
10^{-6}	A6	B6	C6	D6	F6	E6
10^{-7}	A7	B7	C6	D7	F7	E7
10^{-8}	A8	B8	C8	D8	F8	E8

[1] BHV 1 LA strain stock culture containing $10^{6.05}$ $TCID_{50}$/100 µl.

[2] All the semen samples were diluted in EMEM or PCR buffer; undiluted semen denotes 90 µl undiluted bovine semen with 10 µl dilutions of BHV 1.

2.2 Preparation of samples for PCR

2.2.1 GeneReleaser

A proprietary nucleic acid releasing cocktail, GeneReleaser (Bioventures Inc., Murfreesboro, Tennessee), that releases DNA in a form suitable for amplification by PCR was used with a PCR thermocycle program protocol as previously described (Kibenge et al., 1994). GeneReleaser greatly simplifies the amplification of genomic DNA by avoiding the requirement to purify DNA. Two point five microlitres of virus stock culture diluted in EMEM (Section 2.1.4.2) or virus seeded in the semen (2.1.4.3) was lysed in a total volume of 12.5 µl in which 10 µl of GeneReleaser was added. The thermocycle program was run according to the thermocycler manufacturer's procedure. After the thermocycle program, the specimens were stored at either 4^0C or -20^0C until they could be amplified by PCR.

2.2.2 Proteinase K

The semen sample (Section 2.1.4.3) was digested with a final concentration of 100 µg/ml of Proteinase K for 60 min at 56^0C, and then processed by one of the following two methods:

Method 1: The sample was heated for 10 min in boiling water to denature the Proteinase K, and was then centrifuged at 13,000 xg for 30 min. The supernatant was

harvested for use in PCR.

Method 2: Following Method 1, but prior to PCR, the supernatant was extracted with an equal volume of phenol-chloroform-isoamyl (25:24:1). The aqueous phase was precipitated with 1/10 volume of 3M Na-Acetate (pH 5.2) and 2 times volumes of 100% ethanol and kept at -80^{0}C for at least 2 hours. The DNA pellet was washed in 70% ethanol and vacuum dried. The DNA was resuspended in 100 µl of sterile deionized distilled (dd) water before use in PCR.

2.3 Polymerase chain reaction (PCR) assay

2.3.1 Oligonucleotide primers

The pair of primers, FC and RB, used in the PCR assay was based on the published nucleotide sequence of the gD gene of the P8-2 strain of BHV 1 from nucleotides 77 to 1341 (Tikoo et al., 1990). The forward primer, FC, is located from nucleotides 77 to 100 and also contained the T7 RNA polymerase promoter sequence (5'-GTA AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AG-GG GCG AAC ATG CAA GGG CCG ACA T); the reverse primer, RB, which contained only nucleotides of gD gene of BHV 1 is located from nucleotides 1315 to 1341 (5'-CGC TGA CCC GGG CAG CGC GCT GTA GTT). These primers give a PCR product 1306 bps long. The primers were diluted in sterile deionized distilled water to a

concentration of 20 mM and stored at -20^{0}C until used in PCR.

2.3.2 PCR reagents

Either the Taq polymerase (5 U/µl) (Bio-Can Scientific, Mississauga, Canada), or the TaqPlus Long polymerase mixture (5 U/µl) consisting of an optimized blend of Taq 2000™ DNA polymerase and cloned Pfu DNA polymerase (Stratagene, Jolla, California) was used in conjunction with 5X Taq polymerase buffer E (300 mM Tris-HCl, 75 mM $(NH_4)_2SO_4$, 7.5 mM $MgCl_2$, pH 9.0), sterile deionized distilled water, the dNTP mixture (dATP, dCTP, dGTP, and dTTP 2.5 mM each) (Invitrogen Corporation, Carlsbad, California), and glycerol (Sigma, Oakville, Ontario) diluted to 50 % in sterile deionized distilled water before use, and AmpliWax™ PCR gem 100 (Perkin Elmer Cetus, Montreal, Canada).

The PCR was performed in the DNA thermocycler 480 (Perkin-Elmer Canada, Dartmouth, Nova Scotia) in GeneAmp™ reaction tubes (Perkin Elmer Canada).

2.3.3 DNA amplification

After 10 µl GeneReleaser was used to release viral DNA in 2.5 µl of each previously prepared sample (Section 2.2.1), 37.5 µl of PCR mastermix was added and the PCR amplification was carried out in a final volume of 50 µl. The PCR mastermix consisted of 11 µl sterile deionized distilled water, 10 µl 5× PCR buffer (7.5 mM

$MgCl_2$, pH 9.0), 10 µl 50% glycerol, 1 µl 20 mM primer 1 (FC), 1 µl 20 mM primer 2 (RB), 4 µl dNTP mixture (2.5 mM each), 0.5 µl Taq polymerase or TaqPlus Long DNA polymerase. When bovine semen sample was used, virus DNA was released with GeneReleaser or treated with Proteinase K (Section 2.2.2). When Proteinase K in PCR buffer was used to treat the semen instead of GeneReleaser, 10 µl deionized distilled water was added instead of GeneReleaser. One drop of Ampliwax was added on top of the reagents to seal the reaction mixtures. The thermocycler was run using the following inter-linked thermocycle programs: Delay temperature at 94°C for 2 min, a first cycle of 94°C for 3 min, 61°C for 1 min and 72°C for 3 min, followed by 34 cycles of denaturation at 95°C for 1 min, primer annealing at 61°C for 1 min and extension at 72°C for 3 min, and a last cycle of 95°C for 1 min, 61°C for 1 min and 72°C for 10 min, then the soak temperature at 4°C. The PCR mixtures were stored at -20°C.

2.3.4 Agarose gel electrophoresis analysis

Fifteen microlitres of PCR product was analysed using 1.2% agarose gel electrophoresis (125 V for 2 hours) in Tris-borate buffer (0.045 M Tris, 0.045 M boric acid, 0.001 M EDTA, pH 8.0). DNA in the gel was visualized under ultraviolet (UV) light after ethidium bromide staining.

2.3.5 Optimization of amplification conditions

2.3.5.1 Reaction buffer

The PCR optimized buffer kit (PCR optimizer™, Invitrogen Corporation, San Diego, California, USA) was used to determine the optimal $MgCl_2$ and pH in the reaction buffer. Sixteen reaction buffers from A to P with different concentrations of $MgCl_2$ and pH were used (Table 2); the buffers of pH 8.5, 9.0, 9.5 and 10.0 with $MgCl_2$ concentration of 1.5 mM, 2.0 mM, 2.5 mM and 3.5 mM were tested with a concentration of BHV 1 of 10^{-2}.

2.3.5.2 Selection of the optimal polymerase enzyme

Two types of Taq DNA polymerase were used for PCR: Taq polymerase and TaqPlus Long polymerase. BHV 1 LA strain was diluted from 10^{-1} to 10^{-8} in EMEM. Buffer E, determined to contain optimal pH and $MgCl_2$ (Section 3.2.1) was used. Other conditions were the same as used before for optimal PCR amplification (Table 3).

Table 2. The pH and $MgCl_2$ concentration of the sixteen reaction buffers tested using PCR OptimizerTM Kit (Invitrogen).

pH	Concentration of Mg^{2+} (mM)			
	1.5	2.0	2.5	3.0
8.5	A	B	C	D
9.0	E	F	G	H
9.5	I	J	K	L
10.0	M	N	O	P

Table 3. Comparison of Taq polymerase and TaqPlus Long polymerase in PCR.

	Dilution of BHV 1 in EMEM							
Taq DNA polymerase	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
TaqPlus Long polymerase	A1	A2	A3	A4	A5	A6	A7	A8
Taq polymerase	B1	B2	B3	B4	B5	B6	B7	B8

2.4 Restriction enzyme analysis of gD DNA

2.4.1 Extraction of gD DNA from the PCR product

The PCR product was put into a microcentrifuge tube, and extracted with equal volume of phenol-chloroform-isoamyl (25:24:1). The DNA in the supernatant was precipitated with 1/10th volume of 3M Na-Acetate (pH 5.2), and 2 volumes of 100% ethanol and kept at -80^{0}C for at least 2 hours. The DNA was washed in 70% ethanol and vacuum dried. The DNA was resuspended to 10 µl of TE buffer, pH 8.0, and kept at -80^{0}C until used in restriction enzyme analysis (Section 2.4.2).

2.4.2 Digestion of gD DNA by restriction enzyme

Ten microlitres of DNA, 2 µl restriction buffer, 2 µl restriction enzyme SalI, ApaI, DraIII, SmaI or SacII (New England Biolabs Inc. Mississauga ON Canada), and 6 µl TE buffer were added to a microcentrifuge tube, and incubated for 2 hours at 37^{0}C. The reaction was stopped by heating to 65-75^{0}C for 10-15 minutes, and 10 µl of the reaction product was analysed by 2.5% agarose gel electrophoresis.

2.5 *In vitro* expression of gD protein

2.5.1 Reticulocyte lysate system

In vitro protein synthesis was performed using a T7 RNA polymerase-coupled reticulocyte lysate system (TNT; Promega, Madison, WI USA). The TNT lysate system includes TNT rabbit reticulocyte lysate, TNT reaction buffer, TNT T7 RNA polymerase, amino acid mix minus methionine, and 1mM luciferase control DNA, 0.5 mg/ml. 1000 Ci/mmol ^{35}S-methionine (Amersham Canada Limited, Oakville, Ont.) was used to label the protein.

In order to reduce the chance of RNase contamination, gloves were worn during the experiments, and microcentrifuge tubes and pipette tips were treated with diethyl pyrocarbonate (DEPC) water. The reagents were stored at -70^{0}C, and the TNT lysate was not frozen/thawed more than twice.

2.5.2 Coupled transcription-translation

The reaction components consisted of 25 µl TNT rabbit reticulocyte lysate, 2 µl TNT reaction buffer, 1 µl TNT reaction T7 RNA polymerase, 1 µl amino acid mixture minus methionine, 4 µl ^{35}S-methionine, 1 µl of 10 u/µl RNasin ribonuclease inhibitor, 2.5 µl DNA template from PCR product (Section 2.3.3), and 13.5 µl nuclease-free H_2O to a total volume of 50 µl. The reaction mixture was incubated at 37^{0}C for 90 minutes

and then kept at -20 until used.

2.5.3 Analysis of gD protein

The labeled translated proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels which comprised of 3% stacking gel containing 30:0.8 acrylamide and bis methylene acrylamide, 10% SDS, H_2O, N,N,N',N'-tetra-methyl-ethylenediamine (TEMED) (Bio-Rad Laboratories Mississauga, ON Canada), and 10% ammonium persulfate (APS) (Bio-Rad laboratories Mississauga, ON Canada) and 12.5% resolving gel containing 30:0.8 arcylamide and bis methylene acrylamide, 3.0 M Tris pH 8.8, 10% SDS, H_2O, TEMED and 10% APS in running buffer (1X Tris-glycine pH 8.3, 10% SDS, H_2O). Ten microlitres of the translation reaction was added to 10 µl of SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue 4% 2-mercaptoethanol just before use). The samples were heated at 100^{0}C for 5 minutes to denature proteins. Fifteen microlitres of the denatured sample was loaded on to the SDS-polyacrylamide gel, as was 5 µl of prestained SDS-PAGE standards (Bio-Rad laboratories, Richmond, CA USA) containing myosin 202 kDa, β-galactosidase 116 kDa, bovine serum albumin 67 kDa, ovalbumin 50.1 kDa, carbonic anhydrase 35.7 kDa, soybean trypsin inhibitor 29.3 kDa, lysozyme 21.2 kDa, and aprotinin 7.5 kDa. Electrophoresis was carried out at a constant current of 15 mA until the sample passed through the stacking gel. The current was then raised to 30 mA and electrophoresis

was continued until the bromophenol blue dye had run off the bottom of the gel. The gel was dried under vacuum for 3 hours at 80^{0}C and then exposed on X-Omat AR film (Kodak film) at room temperature for 48 hours.

3. RESULTS

3.1 Quantification of viral infectivity

The thirteen BHV 1 strains including strains LA, Colorado 1, Kit, NG dltk, B 347-10804, DV 196, DV 197, DV 199, DV 200, DV 202, DV 203, DV 206, and DV 207 were propagated in MDBK cells. All virus strains were titrated in microtitre plates to obtain the Median Tissue Culture Infectious Dose ($TCID_{50}$). Virus titres ranged between $10^{5.75}$ and $10^{7.50}$ $TCID_{50}$ /50 µl (Table 4). The titre of LA strain was $10^{5.75}$ $TCID_{50}$/50 µl ($10^{6.05}$ $TCID_{50}$ /100 µl).

Here is an example (Table 5) using the Karber method of estimating $TCID_{50}$. The LA strain stock culture was determined using the Karber method:

Negative logarithm of $TCID_{50}$ of the LA strain stock culture:

=Negative logarithm of highest virus concentration - (sum of % mortality at each dilution/100 - 0.5) x (logarithm of dilution)

Table 4. Determination of viral infectivity ($TCID_{50}$) of 13 BHV 1 strains.

Virus	Strain designation	Source	$log_{10}TCID_{50}$/ 50 µl
BHV 1	LA	ATCC (VR-188)	5.75
BHV 1	Colorado-1	ATCC (VR-864)	6.50
BHV 1	Kit	ATCC (VR-2066)	6.75
BHV 1	NG dltk	ATCC (VR-2112)	6.75
BHV 1	B 347-10804	New Brunswick	6.25
BHV 1	DV 196	Nova Scotia	7.00
BHV 1	DV 197	Nova Scotia	6.50
BHV 1	DV 199	New Brunswick	6.25
BHV 1	DV 200	New Brunswick	7.50
BHV 1	DV 202	Nova Scotia	6.50
BHV 1	DV 203	Nova Scotia	5.75
BHV 1	DV 206	Nova Scotia	6.00
BHV 1	DV 207	New Brunswick	7.00

Table 5. The result of cytopathic effect by BHV 1 LA strain

Virus dilution	CPE (No.wells with CPE/No.well inoculated)	
10^{-1}	4/4	(100%)
10^{-2}	4/4	(100%)
10^{-3}	4/4	(100%)
10^{-4}	4/4	(100%)
10^{-5}	4/4	(100%)
10^{-6}	1/4	(25%)
10^{-7}	0/4	(0%)
10^{-8}	0/4	(0%)

$=-1-(1+1+1+1+1+0.25-0.5)x \log_{10}10$

$=-1-(5.25-0.5)x1$

$=-5.75$

$TCID_{50}$ titre$=10^{5.75}/50$ µl

3.2 Optimization of PCR amplification conditions

The entire open reading frame (ORF) of the gD gene (1265 bp) was targeted for PCR amplification of DNA of BHV 1 strains in cell culture. The PCR primers for use were selected from the published nucleotide sequence of the gD gene of the P8-2 strain of BHV 1 (Tikoo et al., 1990). The PCR sense primers typically contained an additional T7 bacteriophage promoter sequence at the 5' end. A modification of the PCR procedure routinely used in our laboratory to obtain specific amplification of BHV 1 DNA in the range of 1 pg to 10 fg of the total DNA (Yason et al., 1995) was used. Conditions were optimized to allow amplification of the whole coding region of the gD gene. Optimization of amplification conditions was necessary because of the different primers used.

3.2.1 Optimization of PCR buffer

In order to improve the specificity and the sensitivity of the PCR with the new primers (FC/RB), we determined the optimum composition of the PCR buffer, by testing various Mg^{2+} concentrations and pH. Sixteen reaction buffers with different pH and $MgCl_2$ concentrations were tested. Three reaction buffers gave a positive result, buffer C (2.5 mM of $MgCl_2$ at pH 8.5), buffer D (3.0 mM of $MgCl_2$ at pH 8.5) and buffer E (1.5 mM of $MgCl_2$ at pH 9). The best amplification result with the primer pair FC/RB was obtained when the reaction buffer contained 1.5 mM of $MgCl_2$ at pH 9.0 (Table 6).

Table 6. Optimization of pH and $MgCl_2$ concentration of the PCR buffer for detection of BHV 1 in EMEM by PCR.

pH	Concentration of $MgCl_2$ (mM)			
	1.5	2.0	2.5	3.0
8.5	–	–	+	+
9.0	++[1]	–	–	–
9.5	–	–	–	–
10.0	–	–	–	–

[1] ++ denotes strong positive; + denotes positive; - denotes negative for PCR amplification.

3.2.2 Selection of Taq DNA polymerase

Two types of Taq DNA polymerases, TaqPlus Long polymerase and Taq polymerase were tested using 10-fold serial dilutions of LA strain. TaqPlus Long polymerase allowed detection of the BHV1 LA strain diluted up to 10^{-5} (Table 7). In comparison, Taq polymerase allowed detection of BHV 1 LA strain diluted only up to 10^{-3}. Thus, the sensitivity of PCR was increased 100 times when the TaqPlus Long polymerase was used (Table 7).

Table 7. Comparison of Taq DNA polymerases.

Taq DNA polymerase	Dilution of BHV 1 LA strain in EMEM [1]							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
TaqPlus Long polymerase	+[2]	+	+	+	+	–	–	–
Taq polymerase	+	+	+	–	–	–	–	–

[1] Ten-fold serial dilutions of LA strain ($10^{6.05}$ $TCID_{50}$/100 µl) from 10^{-1} to 10^{-8} were used.

[2] + denotes positive; - denotes negative for PCR amplification.

3.3 Specificity and sensitivity of the PCR amplification

3.3.1 Determination of sensitivity of PCR for detection of BHV 1 in stock culture

In order to determine whether the coding region of the BHV 1 gD gene was a suitable target for PCR-based detection of BHV 1 and the sensitivity of PCR amplification, 10-fold serial dilutions (from 10^{-1} to 10^{-8}) of a BHV 1 LA strain ($10^{6.05}$ $TCID_{50}$ /100 µl) diluted in EMEM were used as a source of DNA template (Section 2.1.4.2). The PCR with the gD primer pair could detect BHV 1 LA strain at a 10^{-5} dilution ($10^{0.05}$ $TCID_{50}$/100 µl). Since only 2.5 µl (1/40th) of diluted virus was used for PCR amplification (Section 2.3.3), as little as 0.28 $TCID_{50}$ (10^{-5} dilution) of virus could be detected by 1.2% agarose gel electrophoresis with ethidium bromide straining (Section 2.3.4) (Fig 1).

3.3.2. Determination of the specificity of PCR for gD gene

In order to test if the PCR product of gD gene is conserved among BHV 1 strains, 13 BHV 1 strains diluted to 10^{-1} in EMEM were used as DNA templates in PCR. The specific PCR product of 1306 bps was obtained for all 13 BHV 1 strains (Fig 2). For all 13 BHV 1 strains, digestion of the 1306 bp-fragment with restriction enzymes SalI, ApaI, DraIII, SmaI and SacII, yielded fragments with sizes as predicted by the published gD sequence of strain P8-2 (Tikoo et al., 1990). This demonstrated that there were no apparent differences among these 13 BHV 1 strains in the PCR products of the gD gene (Fig 3).

Fig 1. Determination of sensitivity of PCR for detection of BHV 1 in stock culture. Lane M denotes molecular weight marker; lane 1-8 denote 10-fold serial dilutions of BHV 1 LA strain from 10^{-1} to 10^{-8}.

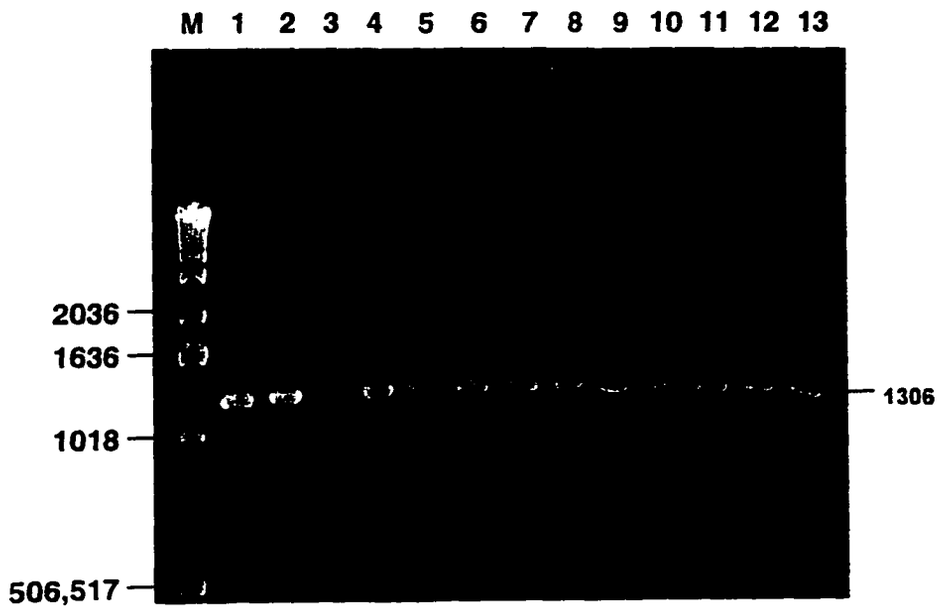

Fig 2. Determination of specificity of the PCR for gD gene. 10^{-1} dilution of 13 BHV 1 strains diluted in EMEM was used in PCR. Lane M denotes molecular weight marker; lanes 1-13 denote 13 BHV 1 strains: LA, Colorado 1, Kit, NG dltk, B 347-10804, DV 196, DV 197, DV 199, DV 200, DV 202, DV 203, DV 206, and DV 207.

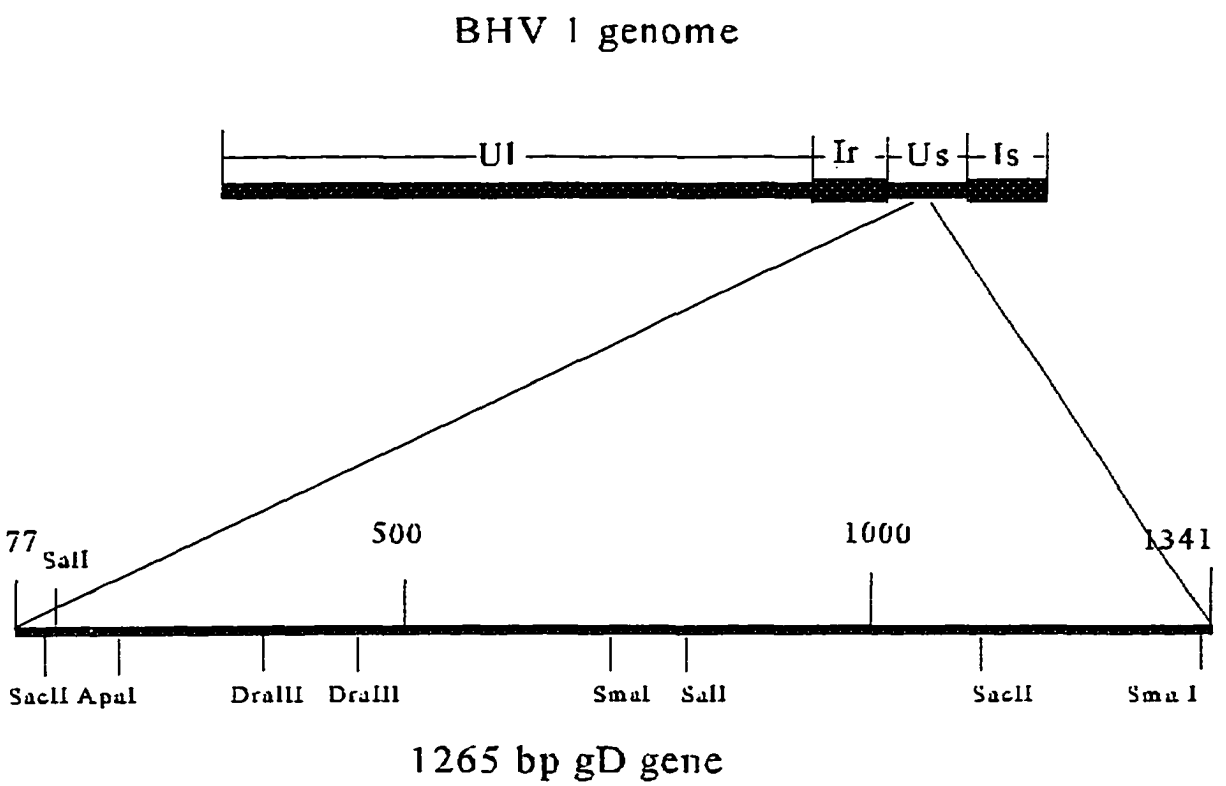

Fig 3. BHV 1 genome and a gross restriction map of the gD gene of BHV 1 strain P8-2. Nine restriction enzyme sites are shown in gD gene that may be digested by five restriction enzymes, SalI, ApaI, DraIII, SmaI and SacII.

3.4 Direct detection of BHV 1 in semen by PCR

3.4.1 Standard method for detection of BHV 1 in semen

The sensitivity of the PCR assay for detecting BHV 1 in bovine semen was determined by mixing undiluted and 1:5, 1:10, 1:20 and 1:50 dilutions of bovine semen in EMEM with 10^{-1} dilution of BHV 1 LA strain (Section 2.1.4.2), using the GeneReleaser procedure to release the BHV 1 DNA (Section 2.2.1). Only the 1:50 dilution of semen showed a positive result (Fig 4), indicating that semen has an inhibitory effect on the PCR assay. Following PCR on 10-fold serial dilutions of BHV 1 LA strain containing 1:50 semen, a band was visualized up to 10^{-5} dilution (equivalent to 0.28 $TCID_{50}$) (Fig5).

3.4.2 PCR using semen sample treated with Proteinase K

Because there are some inhibitory components in semen, different methods were investigated to reduce the inhibitory effect by extraction of BHV 1 DNA from the bovine semen. Proteinase K in PCR buffer two(Sections 2.2.2) was used to treat 1:50 bovine semen with 10-fold serial dilutions of BHV1 LA strain (Section 2.1.4.3), followed either directly by PCR or by extraction with phenol-chloroform-isoamyl and precipitation with ethanol in high salt prior to use in PCR (Section 2.2.2). Use of Proteinase K-treated semen, either directly in PCR or after extraction of the DNA gave similar results; the sensitivity of detection was 10^{-3} of virus dilution (Figs 6 and 7).

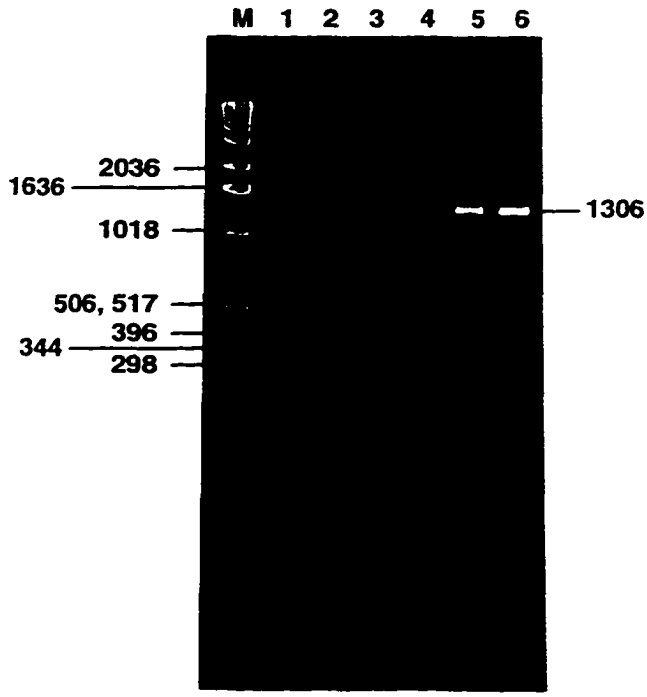

Fig 4. Determination of sensitivity of PCR for detection of BHV 1 in bovine semen using the GeneReleaser procedure. Lane M denotes molecular weight marker; lanes 1-6 denote raw semen, 1:5, 1:10, 1:20, 1:50 diluted semen, and no semen. BHV 1 LA strain of 10^{-1} dilution was used in every sample.

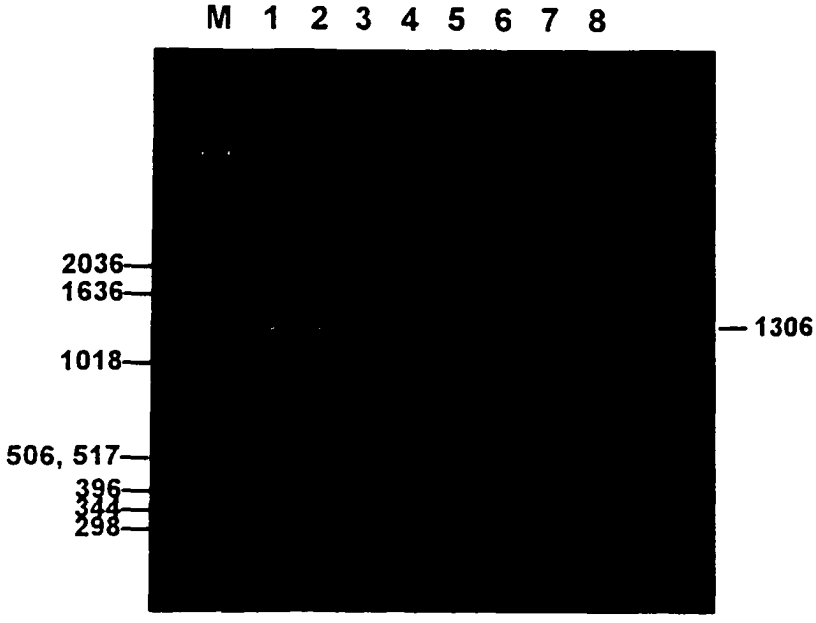

Fig 5. Determination of the sensitivity of PCR for detection of BHV 1 in 1:50 diluted bovine semen using the GeneReleaser procedure for releasing viral DNA. Lane M denotes molecular weight marker; lanes 1-8 denote 10-fold serial dilutions of BHV 1 LA strain from 10^{-1} to 10^{-8} in 1:50 diluted bovine semen.

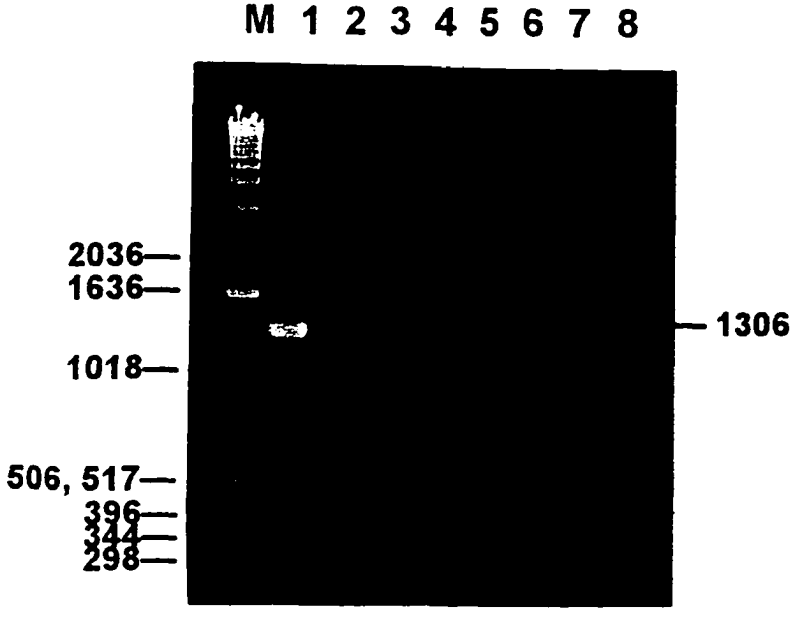

Fig 6. Determination of the sensitivity of PCR for detection of BHV 1 in 1:50 diluted bovine semen using the Proteinase K procedure (with phenol-chloroform extraction for releasing viral DNA). Lane M denotes molecular weight marker; lanes 1-8 denote 10-fold serial dilutions of BHV 1 LA strain from 10^{-1} to 10^{-8} in 1:50 diluted bovine semen.

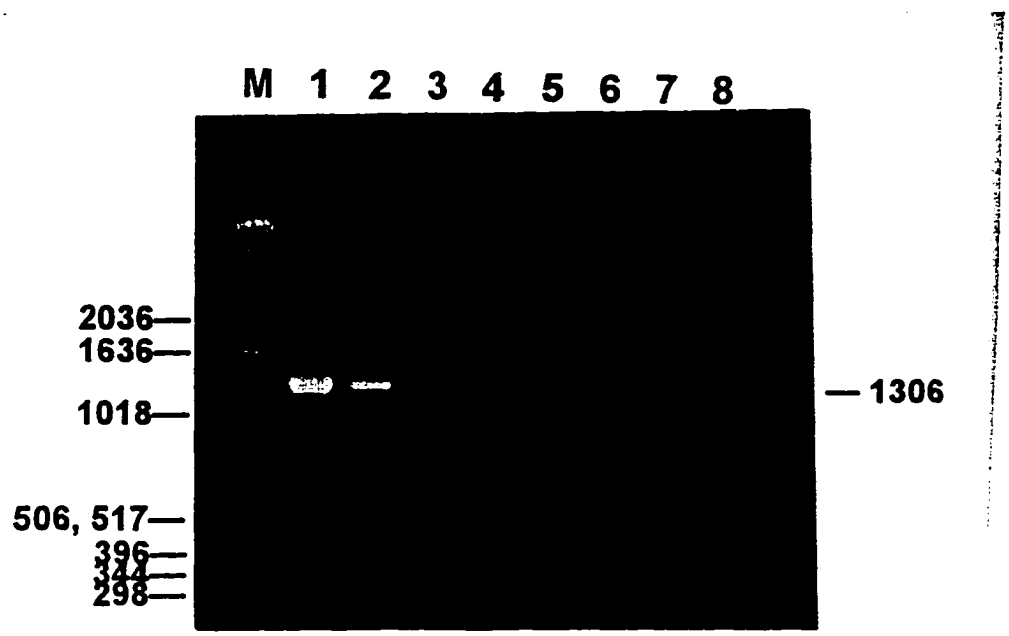

Fig 7. Determination of the sensitivity of PCR for detection of BHV 1 in 1:50 diluted bovine semen using Proteinase K procedure (without phenol-chloroform extraction for releasing viral DNA). Lane M denotes molecular weight marker; lanes 1-8 denote 10-fold serial dilutions of BHV 1 LA strain from 10^{-1} to 10^{-8} in 1:50 diluted bovine semen.

All semen samples containing 10^{-1} dilution of virus and respectively including neat semen, 1:5, 1:10, 1:20, or 1:50 diluted semen in only PCR buffer with nonionic detergents (PCR buffer two) yielded a PCR product (Fig 8). Ten-fold serial dilutions of the virus were then prepared in semen diluted 1:5, 1:10, 1:20 and 1:50 in PCR buffer (Section 2.1.4.3). In 1:5 and 1:10 diluted semen sample, only virus diluted 10^{-1} could be detected (Table 8); in 1:20 diluted semen sample, virus diluted up to 10^{-2} (280 $TCID_{50}$) could be detected (Fig 9); in 1:50 diluted semen samples, virus diluted up to 10^{-3} (28 $TCID_{50}$) could be detected (Fig 8, Table 8).

3.4.3 Comparison of methods of preparation of semen sample for PCR

When GeneReleaser was used to prepare the virus culture sample (Section 2.2.1), up to a 10^{-5} dilution (0.28 $TCID_{50}$) of virus in EMEM could be detected. The same 10^{-5} dilution (0.28 $TCID_{50}$) of virus could be detected in 1:50 diluted bovine semen. However, BHV 1 DNA in undiluted or in a high-concentration bovine semen could not be detected by PCR (Table 9). When Proteinase K in PCR buffer was used to prepare the sample (Section 2.2.2), 10^{-2} (280 $TCID_{50}$) dilution of virus in 1:20 diluted semen and 10^{-3} (28 $TCID_{50}$) dilution of virus in 1: 50 diluted semen could be detected. However, in undiluted bovine semen, and in high concentration of 1:5 and 1:10 diluted bovine semen, only virus diluted 10^{-1} (2800 $TCID_{50}$) gave a positive result by PCR (Table 8).

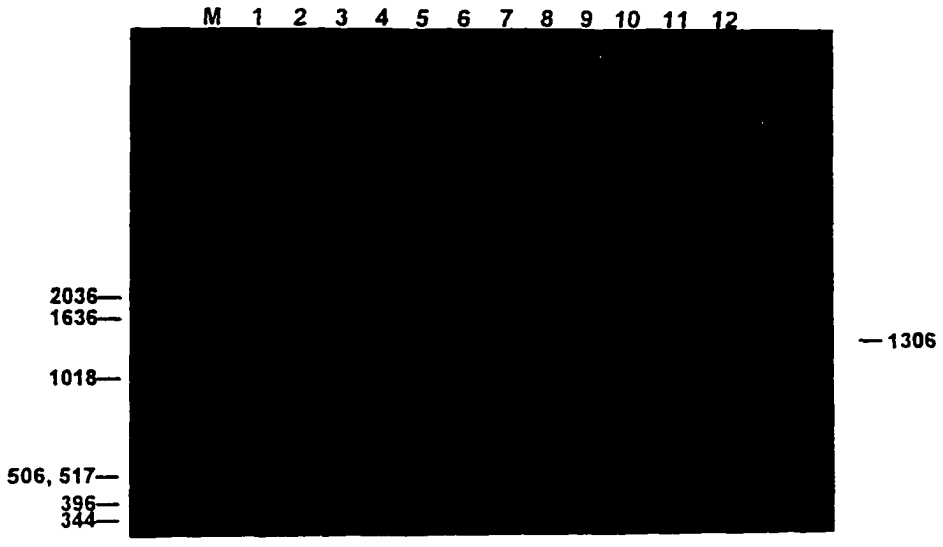

Fig 8. Determination of the sensitivity of PCR for detection of BHV 1 in bovine semen using Proteinase K procedure. Lane M denotes molecular weight marker; lanes 1-6 denote neat semen, 1:5, 1:10, 1:20, 1:50 diluted semen and no semen in PCR buffer one; lanes 7-12 denote neat semen, 1:5, 1:10, 1:20, 1:50 diluted semen and no semen in PCR buffer two. BHV 1 LA strain 10^{-1} dilution was used in every sample.

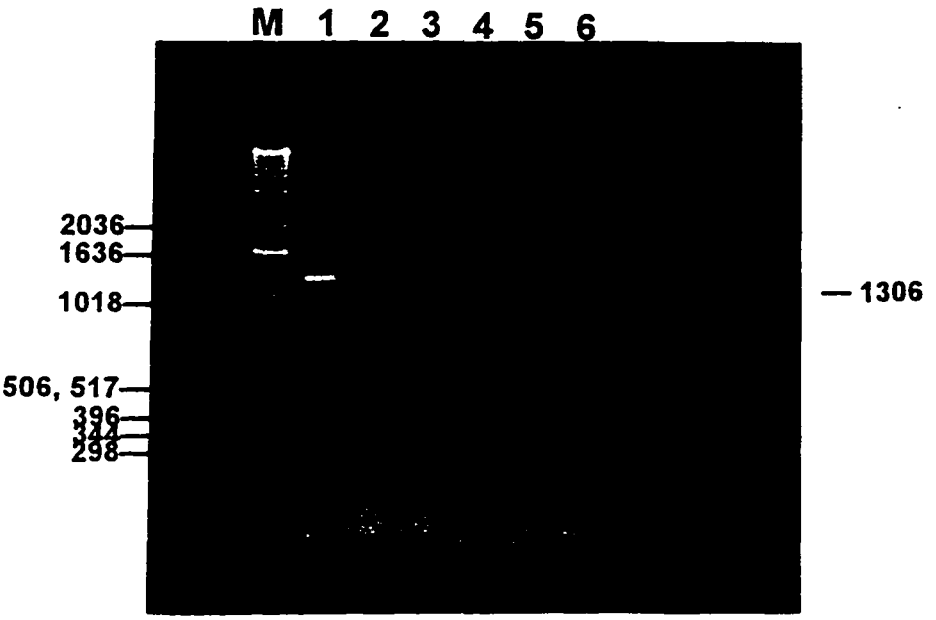

Fig 9. Determination of the sensitivity of PCR for detection of BHV 1 in 1:20 bovine semen using Proteinase K procedure. Lane M denotes molecular weight marker; lanes 1-6 denote 10-fold serial dilutions of BHV 1 LA strain from 10^{-1} to 10^{-6} in 1:20 diluted bovine semen.

Table 8. PCR amplification of gD DNA in artificially infected bovine semen samples with different dilutions of BHV1 using Proteinase K procedure

Dilution of virus stock	Dilution of semen (in PCR buffer)					
	undiluted	1:5	1:10	1:20	1:50	EMEM
10^{-1}	+[1]	+	+	+	+	nd
10^{-2}	nd	_	_	+	+	nd
10^{-3}	nd	_	_	_	+	nd
10^{-4}	nd	_	_	_	_	nd
10^{-5}	nd	_	_	_	_	nd
10^{-6}	nd	_	_	_	_	nd
10^{-7}	nd	nd	nd	nd	_	nd
10^{-8}	nd	nd	nd	nd	_	nd

[1] + denotes positive; - denotes negative; nd denotes not done.

Table 9. PCR amplification of gD DNA in artificially infected bovine semen samples with different dilutions of BHV 1 using GeneReleaser procedure.

Dilution of virus stock	Dilution of semen (in EMEM)					
	undiluted	1:5	1:10	1:20	1:50	EMEM
10^{-1}	–	–	–	–	+[1]	+
10^{-2}	nd	nd	nd	nd	+	+
10^{-3}	nd	nd	nd	nd	+	+
10^{-4}	nd	nd	nd	nd	+	+
10^{-5}	nd	nd	nd	nd	+	+
10^{-6}	nd	nd	nd	nd	–	–
10^{-7}	nd	nd	nd	nd	–	–
10^{-8}	nd	nd	nd	nd	–	–

[1] + denotes positive; - denotes negative; nd denotes not done.

3.5 Detection of BHV 1 by *in vitro* coupled transcription-translation of gD PCR product

In order to improve the sensitivity of detection of BHV 1 in bovine semen, the coupled transcription-translation system was used to express the gD DNA into protein. The translation products were analysed by SDS-PAGE. A 42 kDa polypeptide was obtained by *in vitro* coupled transcription-translation. Using this method, virus diluted up to 10^{-3} in 1:20 diluted semen could be detected by autoradiography (Fig 10). When compared to PCR that gave a result when virus was diluted up to 10^{-2} (280 $TCID_{50}$) in 1:20 diluted semen, this method was 10 times more sensitive than PCR. However, since only 1/20th of the volume of PCR reaction was used in transcription-translation reaction (Sections 2.3.3 and 2.5.2), it implies that this method was 200 times more sensitive than PCR, ie, this method could detect 1.4 $TCID_{50}$ of virus in 1:20 diluted semen.

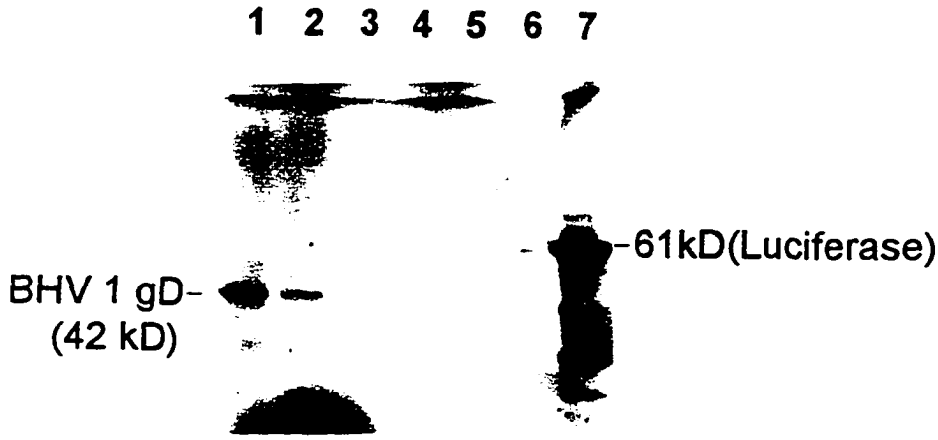

Fig 10. The sensitivity of detection of BHV 1 in 1:20 diluted bovine semen by protein amplification. Lanes 1-6 denote 10-fold serial dilutions of BHV 1 LA strain from 10^{-1} to 10^{-6} in 1:20 diluted bovine semen; lane 7 denotes luciferase control.

4 DISCUSSION

Bovine herpesvirus 1 (BHV 1) is an important cause of respiratory infection and genital infection. Infection with BHV 1 occurs worldwide and causes serious economic losses due to mortality, abortions, decreased milk production and loss of weight (Wyler et al., 1989). BHV 1 is frequently found in bovine semen and is transmitted through natural service and artificial insemination. The current technique for BHV 1 detection in semen in the diagnostic laboratory lacks sensitivity and is both time-consuming and costly. The PCR method has been applied for the direct detection of BHV 1 in clinical samples and has been shown to be more sensitive than the virus isolation (Van Engelenburg et al., 1993; Masri et al., 1996). In order to improve the sensitivity of detection of bovine herpesvirus (BHV 1) in bovine semen, a protein amplification assay from the polymerase chain reaction (PCR) product was developed.

4.1 The preparation of sample

With BHV 1 infection 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. Semen is most likely contaminated during ejaculation by virus particles shed from the infected mucosa rather than by virus particles produced in the testis, epididymis or accessory gland (Snowdon et al., 1965). Using the PCR to detect BHV 1 in infected semen, Van Engelenburg et al. (1993) confirmed that the BHV 1 appeared in the semen fluid

fraction. However, a few BHV 1 particles were found to be part of the nonsperm cell and sperm head fractions. There are no significant differences in infectivity between artificially contaminated bovine semen and naturally infected bovine semen. Using the artificially infected bovine semen, it is possible to accurately determine the sensitivity of detection of BHV 1 in bovine semen, since the amount of the virus ($TCID_{50}$) to be added is predetermined.

In this study, BHV 1 LA strain was propagated in MDBK cells, and the viral infectivity was determined to be $10^{6.05}$ $TCID_{50}$ /100 µl. Different dilutions of virus from 10^{-1} to 10^{-8} in raw semen, as well as in semen diluted to 1:5, 1:10, 1:20 and 1:50 in EMEM or PCR buffer were examined.

4.2 Polymerase chain reaction

We developed a method of amplification of a specific glycoprotein of the BHV 1 from the PCR product in bovine semen. The method was based on the PCR amplification of a highly conserved gD sequence of the virus. The gD gene targeted in the PCR assay was selected for the following reasons: (a) gD has been identified as a major molecule on the surface of the virion and virus-infected cells. It is essential for viral replication (Fehler et al., 1992), is thought to be involved in attachment (Liang et al., 1991) and penetration (Fehler et al. 1992; Chase and Letchworth, 1994), and induces a strong and consistent cellular immune response to BHV 1 (van Drunen Littel van

Den Hurk, et al., 1994). (b) The gD of BHV 1, a glycoprotein of 71 kDa, is a very stable antigen whose epitopes do not change under selective pressure. Moreover, several laboratories have made monoclonal antibodies to gD of BHV 1 which can facilitate detection by ELISA, of the gD polypeptide following coupled *in vitro* transcription-translation of PCR product. (c) gD can be used as a subunit vaccine and gD DNA can be used to immunize cattle.

Despite the apparent simplicity of PCR, it has a relatively large number of associated variables that will affect the efficiency and specificity of the test. These factors will affect the success of PCR technology, and since the two factors are interlinked, parameters affecting both need to be optimized. These factors or parameters include the concentration of magnesium and potassium chloride, dNTP, Taq polymerase, and primer, pH, annealing temperature, and cycle number (Meena et al., 1995). The standard protocol was developed by Kibenge et al. (1994) for detection of BHV 1 using the primer pair selected in the coding region of BHV 1 tk gene (Kibenge et al., 1994; Yason et al., 1995). Although a similar bovine semen sample was used in the present study, the PCR condition was optimized by using the new primer pair in the coding region of the BHV 1 gD gene and two kinds of Taq polymerase. The optimal magnesium ion concentration for each primer set should be determined by PCR with varied magnesium concentrations (Meena et al., 1995), since Mg^{2+} is one of the most important ingredients in a PCR reaction which is required by Taq polymerase enzyme.

Magnesium is also bound by template DNA, primers and dNTPs. The concentration of magnesium ions in the PCR reaction affects primer annealing, DNA melting temperature, and Taq polymerase enzyme activity. Based on this reasoning, a commercial PCR Optimizer Kit (Invitrogen) was used to optimize the pH and Mg^{2+} concentration of PCR with GeneReleaser for BHV 1 in cell culture. The reaction buffer which contained 1.5 mM of $MgCl_2$ at pH 9.0 gave the best amplification result.

Although these optimal conditions were used in PCR, it could only detect virus diluted up to 10^{-3}, possibly because the long DNA sequence is difficult to amplify. The Stratagene's TaqPlus Long™ PCR polymerase improves the efficiency of conventional PCR amplifications by increasing the overall reliability and yield of PCR products. The TaqPlus Long PCR system also allows for the successful amplification of difficult templates and for the synthesis of long targets up to 35 kbp in length from a variety of DNA templates. Considering the length of the template, a longer extension time of 3 min instead of 1 min was used. A more sensitive result of detection of virus diluted up to 10^{-5} was obtained with TaqPlus Long DNA polymerase.

Because of the amplification power of PCR, it is critical to avoid even traces of contamination of the DNA containing the target sequence. Contamination of the PCR amplification reaction with products of previous PCR reaction (PCR carryover), cross-contamination between samples, and contamination with exogenous nucleic acids from

the laboratory environment, can create a false-positive result. Precaution is most important, and the general preventative measures and reduction of such PCR carryover contamination approaches include: pre-aliquoting reagents, using pipettes exclusively for specific steps of the reaction, using positive-displacement pipettes, and using tips with barriers to prevent contamination of the pipette barrel. The physical separation of the amplification reaction preparation from the area of amplified product analysis can minimize the possibility of such contamination. Addition of multiple negative control reactions without any target DNA added is essential as quality control.

A proprietary nucleic acid release cocktail, GeneReleaser, is a polymeric material that can release DNA suitable for PCR from cells and other environments in ten minutes. The use of GeneReleaser in PCR can eliminate cross-contamination and reduce the sample preparation time. GeneReleaser also sequesters inhibitors released during cell lysis, as well as preservation agents which interfere with the amplification process. The lysis is accomplished directly in the amplification tube on a thermocycler. When PCR was used on virus from tissue culture, the results showed that PCR could detect 10^{-5} dilution (0.28 $TCID_{50}$) of virus in virus culture, or in 1:50 diluted bovine semen. However, BHV 1 in undiluted and high concentrations of bovine semen could not be detected by the GeneReleaser procedure, because of inhibitory components in bovine semen. The virus in bovine semen diluted 1:50 was detected by PCR possibly because

the inhibitory components may have been reduced by diluting the semen and allowing GeneReleaser to sequester the remaining inhibitory components.

As mentioned previously, BHV 1 is mostly present in the seminal fluid fraction, but is also present in nonsperm cells and adsorbed to spermatozoa. When whole semen is used to detect BHV 1 by PCR, there are inhibitory components in the semen that inhibit the PCR amplification. It is not known what the inhibitory components are. Some protocols have been developed to purify and isolate BHV 1 DNA from bovine semen to overcome the inhibitory effect of bovine semen, such as using Chelex 100 (Walsh et al., 1991), NP-40 with Proteinase K (Masri et al., 1996), Proteinase K with NaI (van Engelenburg et al., 1993), or centrifugation to remove spermatozoa and nonsperm cells (Xia et al., 1995).

Proteinase K in PCR buffer was used to purify and isolate BHV 1 DNA from bovine semen, using phenol-chloroform extraction and ethanol precipitation or centrifugation. After PCR, the same result was obtained using both methods when the virus was diluted up to 10^{-3} in 1:50 diluted bovine semen. Although the standard purification procedure would provide a more purified DNA for optimal PCR, there is a loss of variable amounts of DNA during the extraction and ethanol precipitation steps. Thus it was decided to use simple centrifugation and use the supernatant in PCR.

Several publications have reported that the detection of BHV 1 in bovine semen by PCR is more sensitive than virus isolation, and that the assay could provide a result within 24 hours. Masri et al. (1996) used the nested PCR assay to detect BHV 1 DNA in extended semen inoculated at 0.25 $TCID_{50}$ per 0.5 ml. Van Engelenburg et al. (1993) developed maximum sensitivity of a PCR assay on the separated seminal plasma from an extended semen sample. By this method, three to five molecules of BHV 1 DNA in 50 µl of bovine semen could be detected. All these methods used virus diluted in a fixed dilution of bovine semen. In the present study, we used varying dilutions of semen added to 10-fold serial dilutions of BHV 1. The results showed that the semen dilutions of 1:20 and 1: 50 were ideal for detection of BHV 1 using PCR. Virus diluted 10^{-2} (280 $TCID_{50}$) and 10^{-3} (28 $TCID_{50}$) could be detected. However, in undiluted bovine semen or at 1:5 and 1:10 dilutions of bovine semen, only virus diluted 10^{-1} gave a positive result by PCR. Compared to GeneReleaser, Proteinase K in PCR buffer could possibly improve the sensitivity of PCR for undiluted and high-concentration bovine semen, but it was not efficient for low-concentration bovine semen.

An interesting phenomenon was observed when BHV 1 was diluted in EMEM or in low concentration of 1:50 diluted bovine semen. The PCR was more sensitive when using GeneReleaser than the Proteinase K procedure. This suggests that GeneReleaser was better for releasing viral DNA from virus particles than Proteinase K. In contrast,

when BHV 1 was diluted in high-concentration bovine semen, the PCR was more sensitive when using the Proteinase K procedure than the GeneReleaser procedure. This demonstrated that Proteinase K was better for reducing the inhibitory effect of bovine semen than GeneReleaser. Although this result demonstrated that PCR amplification was better by using Proteinase K than GeneReleaser for preparation of samples in high-concentration bovine semen, it does not mean that Proteinase K is better than GeneReleaser for preparation of the semen sample for PCR. In this study, when Proteinase K in PCR buffer was used to treat BHV 1 in bovine semen, the phenol-chloroform extraction and ethanol precipitation or centrifugation was used to purify the BHV 1 DNA. However, lysis by GeneReleaser was accomplished directly in the amplification tube. If the standard purification procedure or purification by centrifugation had been used here, the result would have been significantly different. Even if BHV 1 were in high-concentration bovine semen, the PCR amplification would be less sensitive with Proteinase K than with GeneReleaser.

4.3 Analysis of PCR product

The PCR primers were designed to target the complete ORF of gD gene and the specificity for BHV 1 gD DNA was demonstrated by the fact that all the 13 strains gave PCR products with the expected size of 1306 base pairs. In order to further confirm if these PCR products were of the gD gene sequence and if there were some differences among the 13 strains, five restriction enzymes were selected according to

the restriction enzyme sites in the coding region of gD (Tikoo et al., 1990). The restriction enzyme digestion result demonstrated that all 13 PCR products were gD gene products and there were no apparent differences among these 13 strains.

4.4 BHV 1 glycoprotein D

In order to improve the sensitivity of direct virus detection, a new method, using expression of gD protein from the PCR product with a coupled transcription-translation system was applied. The TNT lysate system greatly simplifies the process and reduces the time to 1-2 hours to obtain an *in vitro* translation product.

In the present study, a T7 bacteriophage promoter sequence was added to the 5' end of the gD gene forward primer. The T7 promoter was amplified with the gD gene using PCR which allowed the synthesis of RNA copies from the PCR product using T7 RNA polymerase. The endogenous mRNAs in rabbit reticulocyte lysate were destroyed, so that the translational activity of the reticulocyte lysate becomes completely dependent on exogenously added RNA. The linear DNA templates from the PCR amplification were used to express gD. The protein product of approximately 42 kDa was obtained from the PCR product of BHV 1 gene which was in accordance with the molecular weight of unglycosylated protein predicted from the nucleotide sequence (Schwyzer and Ackermann 1996).

BHV-1 gD consists of 417 amino acid residues and is a typical integral membrane protein containing a signal peptide of 18 amino acids at the amino terminus and a hydrophobic transmembrane domain between residues 361 and 389, which is followed by a 28-amino acid cytoplasmic tail. The mature form of gD has a molecular weight of 71 kDa and contains both N-linked and O-linked oligosaccharides (van Drunen Littel-van Den Hurk et al., 1984). When BHV 1 is expressed in cell culture, gD is synthesized as a 63 kDa partially glycosylated precursor that leads to the formation of a 71 kDa mature protein (van Drunen Littel-van den Hurk and Babiuk, 1986).

Expression of BHV 1 gD in *Escherichia coli* and eukaryotic cells either transfected with recombinant plasmids or infected with recombinant vaccinia, adenovirus, or baculovirus has been reported (van Drunen Littel-van Den Hurk et al., 1993; Tikoo, et al., 1990). However, the production of the full-length gD did not achieve the desired levels, usually the full-length gD and truncated gD were produced together (van Drunen Littel-van Den Hurk et al., 1997).

In this study, only a single unglycosylated gD peptide was obtained, because the coupled transcription-translation system lacks the functions of modification and glycosylation. In order to have obtained as a full-length glycoprotein, it would have been necessary to add canine microsomal membranes to the TNT system to allow for cotranslational and post-translational modifications. Although the microsomal

membranes can yield the mature structural and functional protein (Datta and Dasgupta, 1994), the more complex post-translational modifications (including glycosylation) are not achieved by the microsomal membrane (Prentice et al., 1997).

The results in the present study showed that a single band was obtained when semen diluted 1:20 with the virus dilution up to 10^{-3} was used. In comparison, PCR product yielded a positive result in the same semen dilution with the virus diluted up to 10^{-2}. Since only 1/20th of volume of PCR product was used in the *in vitro* coupled transcription-translation, the expression of gD was 200-fold more sensitive than the PCR assay. If the transcription-translation of PCR product had been used to detect BHV 1 in 1:50 diluted bovine semen treated by GeneReleaser, the sensitivity would be up to 10^{-6} of virus dilution (corresponding to 0.0014 $TCID_{50}$). To further improve the sensitivity, because there are only seven sites for methionine in BHV gD amino acid sequence, we may use other amino acids such as leucine or a combination of methionine and leucine as the radioactive-labeled amino acids. In particular, with the current level of ELISA technology, it would be possible to considerably enhance the sensitivity of the transcription-translation of PCR product. Current efforts are directed to establishing an ELISA for detection of the gD polypeptide using a pair of specific monoclonal antibodies.

4.5 Summary of results and conclusions

Combined PCR and *in vitro* protein synthesis assay was used to detect BHV 1 in bovine semen. The method was based on the PCR amplification of a highly conserved glycoprotein gD sequence of the virus. The forward primer contained the T7 promoter sequence to allow for transcription of the PCR product. The PCR amplification conditions were optimized for Mg^{2+} concentration, pH, and TaqPlus Long PCR polymerase. GeneReleaser, a proprietary nucleic acid releasing cocktail that releases DNA from virus particles in PCR, can eliminate cross-contamination and reduce the sample preparation time. GeneReleaser was used to release the BHV1 DNA from the virus particles. When the PCR was used to detect BHV 1 in virus culture, 0.28 $TCID_{50}$ in virus culture could be detected. The specificity of BHV 1 gD DNA was demonstrated by the fact that all the 13 strains of BHV 1 gave PCR products with the expected size of 1306 base pairs, and it was further confirmed by five restriction enzyme analyses. When PCR was used to directly detect BHV 1 in bovine semen, a 1:50 dilution of bovine semen with virus diluted up to 10^{-5} gave a positive result. However BHV 1 DNA in high-concentration bovine semen could not be detected by the GeneReleaser procedure because of inhibitory components in bovine semen.

When Proteinase K in PCR buffer was used to purify and isolate BHV 1 DNA from bovine semen and then the supernatant was used in PCR, the results showed that semen diluted 1:20 or 1: 50 was ideal for detection of BHV 1 using PCR. Virus

diluted up to 10^{-2} (280 $TCID_{50}$) and 10^{-3} (28 $TCID_{50}$) could be detected. Even in 1:5 and 1:10 dilution of semen, positive results were obtained for the virus dilution 10^{-1}.

A coupled *in vitro* transcription-translation system was used to express the gD using the PCR product that was produced by a 10-fold serial dilution of virus in 1:20 dilution of bovine semen. After the SDS-PAGE gel, a 42 kDa gD peptide could be visualized by autoradiography when virus was diluted up to 10^{-3}. These results demonstrated that a combined PCR and *in vitro* protein synthesis was more sensitive than traditional PCR for direct detection of BHV 1 in bovine semen.

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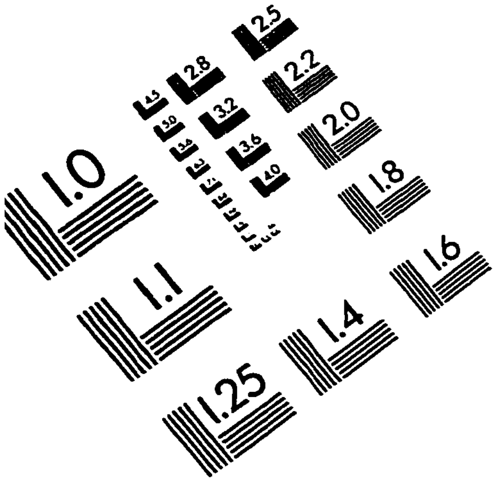
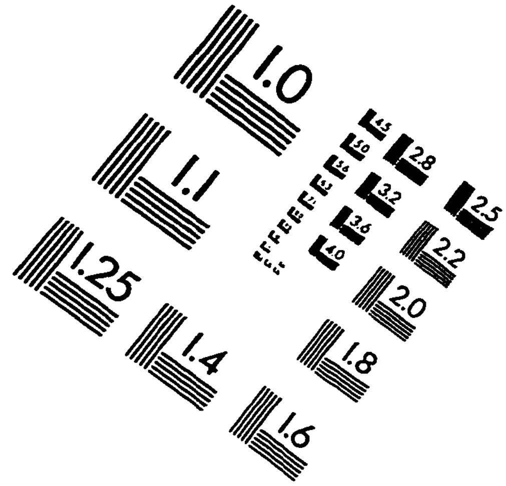
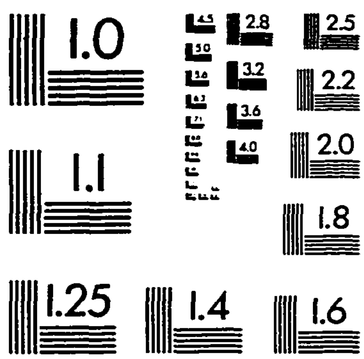
IMAGE EVALUATION TEST TARGET (QA-3)







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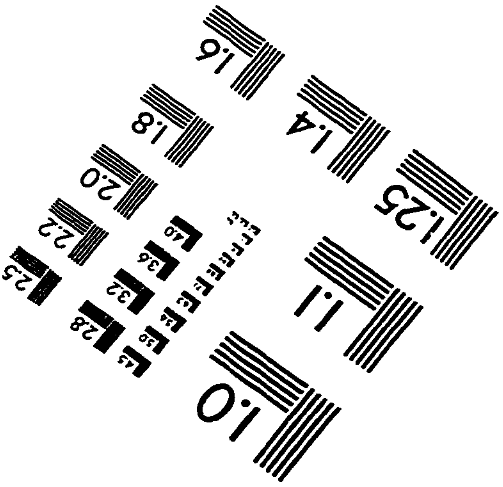
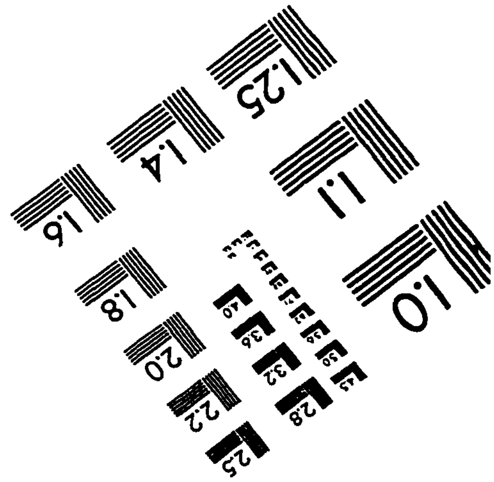
6"





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