COMPARISON OF NUCLEIC ACID HYBRIDIZATION, POLYMERASE CHAIN REACTION, AND VIRUS ISOLATION FOR DETECTION OF BOVINE HERPESVIRUS 1 (BHV-1) IN BOVINE SEMEN

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Masters of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Jane Qin Xia

Charlottetown, P.E.I.

December, 1994

ISBN 0-315-99744-3

Canada

CONDITION OF USE

The author has agreed that the Library, University of Prince Edward Island, may make this thesis freely available for inspection. Moreover, the author has agreed that permission for extensive copying of this thesis for scholarly purposes may be granted by the professor or professors who supervised the thesis work recorded herein, or, in their absence, by the Chairman of the Department or the Dean of the Faculty in which the thesis work was done. It is understood that due recognition will be given to the author of the thesis and to the University of Prince Edward Island in any use of the material in this thesis. Copying or publication or any other use of the thesis for financial gain without the author's written permission and approval by the University of Prince Edward Island is prohibited.

Requests for permission to copy or to make any other use of material in this thesis in whole or in part should be addressed to:

Chairman of the Department of Pathology and Microbiology
Faculty of Veterinary Medicine
University of Prince Edward Island
Charlottetown, P.E.I.
Canada C1A 4P3.

SIGNATURE PAGES

iii-iv

REMOVED

ABSTRACT

Bovine Herpesvirus 1 (BHV-1) is an economically important viral agent causing infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious pustular balanoposthitis, encephalitis, conjunctivitis, enteritis, abortion and immunosuppression in cattle. The virus which is frequently found in bovine semen can be transmitted by artificial insemination internationally, and is correlated with reduced fertility and abnormal fetal development. Therefore, the detection of BHV-1 in artificial insemination centres and semen banks is of prime importance to the cattle industry. The objective of this study was to optimize virus isolation, dot blot hybridization and the polymerase chain reaction (PCR) for detection of BHV-1 in bovine semen and to compare the sensitivity of these three methods for detection of BHV-1 in bovine semen.

In bovine semen artificially infected with BHV-1, the samples diluted 1:20 in tissue culture medium showed the least cytotoxicity and inhibition of viral cytopathic effects, allowing detection of 1 $TCID_{50}$/100 µl of BHV-1 by virus isolation. The inhibition of PCR amplification of BHV-1 in bovine semen was eliminated by diluting the samples at 1:20 in tissue culture medium. The best PCR amplification was obtained when semen was diluted 1:20 and when reaction buffer of pH9.0 with 1.0 mM $MgCl_2$ was used. Under these conditions, the PCR followed by ethidium bromide staining could detect 1 $TCID_{50}$/100µl of BHV-1, whereas PCR followed by Southern blot hybridization could detect 0.01 $TCID_{50}$/100µl of BHV-1. The presence of foreign DNA, such as bovine sperm DNA or salmon sperm DNA increased the sensitivity of dot blot hybridization in detecting BHV-1, allowing detection of 20,000 $TCID_{50}$/100µl of BHV-1 in neat semen.

Two 18 month-old, BHV-1 seronegative bulls were experimentally infected with 2 ml of 10^5 $TCID_{50}$/50 µl of BHV-1 via their prepuces. The semen samples, nasal swabs, prepucial swabs and serum samples were collected 7 days before infection and on days 0, 4, 10, 20, 30, and 40 after infection. Only the semen sample of Bull 1 collected at day 4 was positive by dot blot hybridization, and semen samples from both bulls at day 4 were positive by either virus isolation or PCR with ethidium bromide staining. On the other hand, semen samples which were collected from both bulls on days 4, 10, 20, and 30, and from Bull 1 at day 40 after infection, were positive by PCR with Southern blot hybridization. PCR with Southern blot hybridization was the most sensitive method, detecting the virus for the longest period. Both bulls developed detectable antibody at day 10, which was later than the virus shedding at day 4 detected by virus isolation, dot blot hybridization and PCR. These observations confirmed the BHV-1 infection of bulls and emphasized the need for early and sensitive diagnostic methods.

This study showed that PCR with Southern blot hybridization is the most sensitive method for detection of BHV-1 in bovine semen, followed by virus isolation and PCR with ethidium bromide staining. The dot blot hybridization protocol used in this study was the least sensitive of all the methods studied.

DEDICATION

To my parents, my husband and our son, Benjamin Yihang Zhou.

ACKNOWLEDGMENTS

This research project was supported by Natural Science and Research Council of Canada (NSERC) / Agriculture Canada Research Partnerships Program, and Canadian Association of Animal Breeders. A graduate student stipend from the Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island is gratefully appreciated.

I extend my appreciation to members of my supervisory committee, Drs R.F.Markham, A.Lopez, and R.M.Lofstedt for their advice and critical reading of this manuscript. I particularly thank my co-supervisors, Drs. F.S.B.Kibenge and C.V.Yason for their guidance, encouragement and support throughout my programme.

I wish to thank the faculty members, staff and fellow students in Atlantic Veterinary College who helped me during this program. I especially thank Patricia McKenna and Dr. Biao Qian for their technical advice.

Finally, I gratefully acknowledge my husband, Chengfeng Zhou, for his love, understanding, support and acadamic discussions, also to our son, Benjamin Yihang Zhou for all the joy he brought to the family, and to all our family members for their love and support in many ways. The special gratitude goes to my mother, Hao Zhang, who gave me great help and inspiration, and made it possible to finish my program.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Abbreviation	Term
ATCC	American Type Culture Collection
AVC	Atlantic Veterinary College
BEHV	bovine encephalitis herpesvirus
BFT	bovine fetal testis
BHV-1	bovine herpesvirus-1
BHV-5	bovine herpesvirus-5
bp	base pair
CPE	cytopathic effect
cpm	counts per minute
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	mixture of dATP, dCTP, dGTP, and dTTP
dsDNA	double stranded DNA
dTTP	2'-deoxythymidine 5'-triphosphate
EB	ethidium bromide

ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EMEM	Eagle's minimal essential medium
FA	fluorescent antibody
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HEPES	N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid)
HSV-1	herpes simplex virus type 1
IBR	infectious bovine rhinotracheitis
ICP	infected cell protein
IF	*immunofluorescent*
IPV	infectious pustular vulvovaginitis
IR_S	internal repeat short segment
K	kilo
kbp	kilobase pair
LR	latency-related
MAb	monoclonal antibody
MLV	modified live virus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pst I	endonuclease from *Providencia stuartii*

RE	restriction endonuclease
SDS	sodium dodecyl sulfate
SSC	sodium chloride, sodium citrate solution
ssDNA	single stranded DNA
$TCID_{50}$	median tissue culture infective dose
TK	thymidine kinase
TR_S	terminal repeat short segment
U_L	unique long segment
U_S	unique short segment
VN	virus neutralization
VP	viral protein

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Bovine herpesvirus-1 (BHV-1) is the cause of infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious pustular balanoposthitis (IPB), and also has been incriminated in causing encephalitis, conjunctivitis, enteritis, and abortion in cattle (Wyler et al., 1989; Straub, 1990). BHV-1 also causes immunosuppression and infected animals become highly susceptible to secondary bacterial infections such as *Pneumonic pasteurellas*, the number one cause of mortality in feedlot cattle in North America (Yates, 1982; Donkersgoed and Babiuk, 1991). Like other herpesviruses, BHV-1 can establish latency in clinically normal animals, with subsequent intermittent episodes of re-excretion (Wyler et al., 1989). The virus cannot be eliminated from the host following infection, and vaccination can only prevent the clinical disease but not the establishment of latency (Wyler et al., 1989; Drunen Littel-van den Hurk et al., 1993). The virus is frequently found in bovine semen (Kahrs et al., 1980; Kupferschmied et al., 1986; Weiblen et al., 1992; Philpott, 1993) and can be transmitted by artificial insemination internationally (Philpott, 1993), when semen containing virus may be diluted and inseminated in many susceptible females (Drew et al., 1987). Infected semen is also associated with reduced fertility and abnormal fetal development (Wyler et al., 1989; Miller, 1991).

Therefore, the detection of BHV-1 in artificial insemination centres and semen banks is of prime importance to the cattle industry.

The current methods used to detect the presence of BHV-1 in bovine semen include the "Cornell Semen Test" (Schultz et al., 1982) and virus isolation (Wyler et al., 1989). The "Cornell Semen Test" is expensive, time consuming, and does not identify the specific samples (ie., the bull) which contain the virus because usually more than 100 semen samples are pooled prior to testing. Virus isolation in cell culture is impeded by the cytotoxicity of semen and its inhibition of viral cytopathic effects (CPE) (Wyler et al., 1989; Ackermann et al., 1990; Weiblen et al., 1992). Clearly, there is a need for rapid and inexpensive diagnostic tests for the detection of BHV-1 in bovine semen.

In recent years, molecular based techniques, such as dot-blot hybridization and the polymerase chain reaction (PCR) have stimulated strong interest in the area of diagnostic virology (Paul, 1990; Wolcott, 1992; Williams and Kwok, 1992). Several laboratories have described the detection of BHV-1 genome by various hybridization techniques, using different cloned BHV-1 DNA fragments as probes (Dorman et al., 1985; Dunn et al., 1985; Pacciarini et al., 1988; Brunner et al., 1988). Pacciarini et al. (1988) found hybridization of DNA extracted from semen samples with a radioactively labeled specific BHV-1 DNA fragment to be a very sensitive method. However, Brunner et al. (1988) compared several hybridization methods with immuno

electron microscopy and various cell culture techniques, and concluded that the most sensitive method was a special cell culture technique. The PCR assay for BHV-1 detection was described by Vilček (1993) who used 22 bp oligomers from the BHV-1 gI gene as primers. A nested PCR assay targeting a portion of BHV gIV gene was reported by Wiedmann et al. (1993) and was found to be comparable to the Cornell Semen Test for detection of BHV-1 in bovine semen. Engelenburg et al. (1993) reported a PCR assay using a primer pair which targets BHV-1 gIII gene, and found the PCR was more rapid and sensitive than a virus isolation method. Recently, PCR to the thymidine kinase (TK) gene region was developed by Kibenge et al. (1994) and Yason et al. (1995). Three BHV-1 strains from American Type Culture Collection (ATCC) and 9 BHV-1 field isolates from Atlantic Canada were detected by this PCR protocol (Kibenge et al., 1994). The specificity of the primers for the BHV-1 TK gene was confirmed by failure to amplify DNA in a BHV-1 TK deletion mutant strain, 2 strains of BHV-4, a feline herpesvirus, an equine herpesvirus, a bovine adenovirus, and noninoculated bovine cell cultures (Kibenge et al., 1994). In the present study, this newly developed BHV-1 TK PCR was optimized and tested in bovine semen. The PCR product was labeled and used as a probe for detection of BHV-1 in bovine semen by dot blot hybridization.

1.2 Aims of this study

The main objective of this study is to compare dot blot hybridization, PCR and virus isolation in detecting BHV-1 in bovine semen. The specific objectives are as follows:

1. To optimize dot blot hybridization, PCR and virus isolation for detection of BHV-1 in artificially infected bovine semen.

    a) Optimization of virus isolation to eliminate the cytotoxicity and viral inhibition factors in bovine semen.

    b) Optimization of dot blot hybridization to increase the sensitivity of BHV-1 detection in semen.

    c) Optimization of the sample preparation and reaction conditions for PCR to eliminate the PCR inhibition factor present in bovine semen and increase the sensitivity.

2. To compare the sensitivity of the optimized dot blot hybridization, PCR and virus isolation for detection of BHV-1 in bovine semen artificially infected with BHV-1.

3. To compare the sensitivity of dot blot hybridization, PCR and virus isolation for detection of BHV-1 in the semen of bulls experimentally infected with BHV-1.

1.3 Historical perspective and current knowledge on bovine herpesvirus 1 (BHV-1)

1.3.1 Historical background and classification

An outbreak of an apparently new upper respiratory disease of cattle was described in USA in the 1950's (Schroeder and Moys, 1954; Miller, 1955). The disease was named infectious bovine rhinotracheitis (IBR) (McKercher et al., 1955), when a viral agent was isolated (Madin et al., 1956). This virus was considered to be the same agent that caused infectious pustular vulvovaginitis (IPV), which was also known in Europe as exanthema coital vesiculosum ("Bläschenausschlag") (Gillespie et al., 1959). Besides IBR and IPV, the virus was also found to be involved with other conditions such as balanoposthitis, conjunctivitis, abortion and enteritis (Wyler et al., 1989). The virus could produce a wide range of illnesses similar to that caused by human herpesviruses (Mohanty, 1978), and met the morphological criteria of the herpesvirus group (Armstrong et al., 1961). It was designated "bovine herpesvirus 1" (BHV-1) a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae* (Roizman et al., 1981).

Bovine herpesvirus 1 used to be divided into three groups or subtypes based on the clinical manifestations and restriction endonuclease analysis patterns: Subtype 1 causes primarily respiratory infections; subtype 2 causes primarily genital infections (Engels et al., 1981; Metzler et al., 1985; Misra et al., 1983). Subtype 3 which is primarily associated with neurologic infections (Metzler et al., 1986), is now called

bovine encephalitis herpesvirus (BEHV) (Brake and Studdert, 1985) and classified as bovine herpersvirus 5 (BHV-5) (Roizman et al., 1992). However, it cannot be assumed that a specific clinical syndrome is definitely caused by a specific virus subtype (Donkersgoed and Babiuk, 1991).

1.3.2 Clinical infection of BHV-1

1.3.2.1 Infectious bovine rhinotracheitis

Infectious bovine rhinotracheitis is the most prominent and best-known disease caused by BHV-1. The incubation period described by different authors varies from 2-3 days (McKercher, 1973) to 20-60 days (Chow et al., 1956) depending on exposure dose, route and other factors (Kahrs, 1977). The disease is characterized by fever, increased respiratory rate and persisting harsh cough, anorexia, depression, and in milking cows, by a severe drop in milk production and emaciation (Gibbs and Rweyemamu, 1977). A clear discharge from nares develops within a day or two and the mucosa of the nares becomes hyperaemic hence the name "red nose" (Miller, 1955). In the early stages the profuse nasal discharge is clear but later becomes mucopurulent. Excessive salivation is noticed in some animals, but oral lesions are uncommon (Gibbs and Rweyemamu, 1977). In newborn calves, BHV-1 can also cause ulcerative stomatitis, esophagitis, and rumenitis, with or without focal hepatic necrosis (Dungworth, 1993).

The tracheitis could be detected by auscultation, but lung sounds are normal. The acute stage of the disease usually lasts from 5 to 10 days after which most animals recover rapidly. In approximately 10% of affected animals the respiratory form of IBR may be complicated with secondary bacterial pneunomia or superimposed viral infections. The animals that develop secondary bacterial pneunomia often die. These conditions are more likely to occur in the stressful environment of feedlots (Wyler et al., 1989). Conjunctivitis can be uni- or bilateral and is associated with large amount of purulent discharge in cattle with typical IBR. The conjunctivae are hyperaemic and oedematous, and in severe cases eversion of the eyelids occurs (Gibbs and Rweyemamu, 1977).

1.3.2.2 Infectious pustular vulvovaginitis/balanoposthitis

Although BHV-1 is primarily associated with respiratory disease, it also affects the reproductive system. Infectious pustular vulvovaginitis is another classic syndrome of BHV-1 infection. It was also known as "Bläschenausschlag" in German-speaking countries as early as 19th century (McKercher, 1963). Acute IPV usually develops 1-3 days after mating and is obviously painful, for frequent micturition and tail swishing are usually tne first characteristic signs noticed. On closer examination the vulva is seen to be oedematous and hyperaemic with small pustules disseminated over the mucosal surface. In some cases, the pustules coalesce to form a yellowish-white fibrinous membrane. The membrane soon becomes detached resulting in the formation of ulcers. During this time, the vulva may be swollen. A small amount

of exudate is discharged from the vulva throughout the course of the disease. The acute stage of the disease lasts from 2 to 4 days, during which time the animal may have a fever and reduced milk production (Kendrick, 1958). The virus can also cause severe necrotizing lesions in the uterus and ovary, and can produce temporary infertility (Miller, 1991).

In Holland, BHV-1 was isolated from bulls with infectious pustular balanoposthitis (IPB) and orchitis, and it was found that the isolate produced IPV in heifers (Bouters et al., 1960). Outbreaks of IPB have also been described in bulls at artificial insemination centres (Huck et al., 1961; Loretu et al., 1974). The virus has been isolated from apparently clinically normal bulls (Saxegaard, 1966, 1970; Orischot et al., 1993) and stored samples of frozen semen collected from clinically normal bulls (Spradbrow, 1968). IPB also develops after an incubation period of 1-3 days. Lesions similar to those seen in the vagina and vulva develop on the mucosa of the penis and prepuce. Secondary bacterial infection of these lesions is usual. In uncomplicated cases, healing occurs within 10-14 days, but some animals may lose their libido or find erection and ejaculation painful and take several weeks before resuming regular mating (Gibbs and Rweyemamu, 1977).

1.3.2.3 Abortion

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 and kill the conceptus, leading to either embryonic death and resorption or abortion (Miller, 1991). The abortion usually occurs after an incubation period of 3-6 weeks, mainly between the 5th and 8th month of pregnancy. Under field conditions about 25% of pregnant cattle may abort during a disease outbreak (Wyler et al., 1989). Focal hepatic necrosis is the most characteristic lesion in bovine fetuses aborted during BHV-1 infection (Dungworth, 1993).

1.3.2.4 Encephalitis

Encephalitis was originally identified as a clinical syndrome of BHV-1 infection on the basis of CPE in cell culture (Eugster et al., 1974), positive fluorescent antibody staining using conjugated BHV-1 antisera, and serum neutralization tests (Friedli and Metzler, 1987). More recently the encephalitic isolates have been categorized as bovine herpesvirus 5 (BHV-5) (Roizman et al., 1992) according to the restriction enzyme patterns and DNA dot blot hybridization analysis (Engels et al., 1986).

Diarrhoea, enteritis, dermatitis and mastitis have also been observed occasionally during BHV-1 infection (Wyler et al., 1989). Mild or subclinical infection of BHV-1 is also common (Gibbs and Rweyemamu, 1977). The virus also causes immunosuppression of the host which results in high mortality due to severe secondary bacterial infections (Yates, 1982). The clinical aspects of BHV-1 had been reviewed in detail by Gibbs and Rweyemamu (1977), Yates (1982), Wyler et al (1989), and Straub (1990).

1.3.3 Pathogenesis

Usually, the virus enters the body via the mucous membranes of the upper respiratory tract causing IBR, while IPV/IPB results from infection via the mucous membranes of the genital tract. Another possibility is the entry of the virus by way of the conjunctival epithelium. Oral infection has been suspected but not confirmed (Straub, 1990). The virus replicates at the primary sites of infection causing lesions (Gibbs and Rweyemamu, 1977). *In vitro* experiments have shown that BHV-1 can replicate not only in epithelial cells, but also in cells of the submucosa and connective tissue (Yates, 1982). It was suggested that IPV/IPB was caused by introduction of the virus to the mucosa of the genital tract by coitus or other external agents, rather than from a viraemic phase associated with a BHV-1 lesion elsewhere in the body. This is due to the fact that no virus could be detected in the genital tracts of the calves inoculated intranasally. Furthermore, the genital and respiratory infections due to BHV-1 rarely occur together in the same animal or even in the same herd (Straub, 1990).

After primary infection, BHV-1 is transported subsequently by monocytes and probably other white blood cells via the blood stream to the target organs (Nyaga and McKercher, 1979). Viraemia is weak and transient, a phenomenon which might be due to the small number of infected leucocytes in the circulation (Peter et al., 1966). Another route of spread may occur along peripheral nerves as with herpes simplex virus, especially in establishing latency (Wyler et al., 1989). Ganglioneuritis was

observed following BHV-1 inoculation (Narita et al., 1981, 1982) and BHV-1 DNA was found in neurons of trigeminal and sacral ganglia (Ackermann et al., 1982). The significance of viral spread through intercellular bridges is not yet clear. It was presumed that this spreading mechanism may be important for viral propagation after reactivation because during this stage, cell to cell transmission may be shielded from neutralizing antibodies (Roizman et al., 1992). It may be hypothesized that a spread through intercellular bridges only plays a role in local infections and not in systemic infections.

Events leading to abortion are maternal infection, viraemia, placental infection, peracute generalized fetal infection and finally fetal death (Kendrick, 1973; Miller, 1991).

1.3.3.1 Latency

Like other herpesviruses, BHV-1 can establish a latent or persistent infection in sensory ganglionic neurons of the infected host (Ackermann et al., 1982; Rock et al., 1986). The virus can persist in a latent state for the lifetime of an infected host without virus shedding, or it can be periodically reactivated, and cause extensive clinical disease to the host with concomitant virus shedding (Homan and Easterday, 1983; Rock et al., 1982).

After entry into the host, BHV-1 replicates initially in epithelial cells at the site of infection and then spreads by retrograde axonal transport to the sensory ganglia where it can either replicate or establish latency. Virus can be reactivated by natural or artificial stimuli which stress the animal., e.g. transport (Thiry et al., 1987), parturition (Thiry et al., 1985), immunosuppressive treatment with corticosteroids (Sheffy and Davies, 1972), superinfection with another microorganism (Msolla et al., 1983) or treatment with dexamethasone (Narita et al., 1981). Reactivation may lead to replication in the peripheral nervous system, following which the virus may be transported either back to the periphery or to the central nervous system (Fraser and Valyi-Nagy, 1993).

A latency-related (LR) transcriptional activity has been found in trigeminal ganglia of rabbits which have been experimentally infected with BHV-1 (Kutish et al., 1990). This LR gene maps to a region of the viral genome which overlaps immediate-early transcription unit 1 and inhibits its activity (Bratanich et al., 1992). The promoter which regulates expression of the LR gene is contained within a 980-bp *Pst*I fragment (Jones et al., 1990). Viral infection also positively regulates LR promoter activity. The cis-acting sequence within the LR promoter is responsible for a neuronal cell type expression, nerve growth factor and the trans-activation by viral or virus-induced factors (Bratanich and Jones, 1992). Besides the viral genes and the neuronal cellular factors, the immune system of the host is assumed to be involved in establishment

and reactivation of latency of herpes simplex virus (Fraser and Valyi-Nagy, 1993). On the other hand, the role of the immune system in latency of BHV-1 is not clear.

1.3.3.2 Immunosuppression

Some viral infections may temporarily suppress humoral and/or cell-mediated immune responses. Herpesvirus infections, including BHV-1 infection, are immunosuppressive. The mechanisms involved in such generalized immunosuppression are not fully understood, but may result from the replication of the virus in lymphocytes and/or macrophages (Fenner et al., 1993). It was found that BHV-1 can cause a drop in T cells and null cells and depression in responses to T-cell mitogens. The virus also interferes with alveolar macrophage function by depressing macrophage-mediated cytotoxicity and interleukin-1 synthesis, and thus easily allows secondary bacterial infections in stressed calves (Tizard, 1992; Forman et al., 1982; Bielefeldt et al., 1986; Jericho and Carter, 1985).

1.3.4 Molecular biology of the virus

1.3.4.1 Genome structure

The BHV-1 genome is a linear double-stranded DNA molecule of approximately 140 kilobase pairs (kbp) which exhibits the typical arrangement of group D herpesvirus genomic DNA (Roizman et al., 1992). It is composed of a unique long segment U_L (104 kbp) and a unique short segment U_S (11kbp) flanked by the inverted repeat

sequence IR_S and TR_S (Mayfield et al., 1983). The short region can invert its orientation relative to U_L, resulting in the existence of two isomeric forms (Wirth et al., 1989). Nothing is known about possible functions of this flip-flop mechanism, and the biological implications of this inversion are not known either.

Within the cells, BHV-1 genomes serving as templates for DNA replication are circular molecules. Progeny DNA is formed as head-to-tail concatemers which are subsequently cleaved into unit-length BHV-1 DNA (Hammerschmidt et al., 1988). In replicative intermediates, therefore, template DNA as well as progeny DNA exhibit covalently joined left and right genomic termini. The mechanisms involved in circularization and concatemer cleavage are still poorly understood (Hammerschmidt et al., 1988). An immediate-early transcription over joined genome ends was observed during the lytic infection, which suggested that circles are present at the initial stage of infection (Fraefel et al., 1993).

The present size estimates of the genome and unique long and short segments are based on restriction endonuclease (RE) mapping; their range may in part reflect the properties of individual BHV-1 strains, and in part minor differences in methodology and interpretation. Restriction endonuclease mapping was also used to tentatively classify BHV-1 into subtypes (Bratanich et al., 1991; Engels et al 1981): BHV-1.1 (IBR-like strains) and BHV-1.2 (IPV-like strains). It was found that both subtypes have abortifacient property (Miller et al., 1991a) and the RE patterns changed during

host animal passage by acute infection or reactivation of latency (Whetstone et al., 1989). These observations suggest that application of RE analysis in studies of BHV-1 has limitations.

The BHV-1 genome encodes more than 40 polypeptides (Metzler et al., 1985; Misra et al., 1981), but only a few of them have been linked to specific genes. So far, the nucleotide sequence of the whole genome is not available, but the complete DNA sequence of the U_S (Leung-tack et al., 1994), and the genes coding for the gI (Lawrence et al., 1986), gII (Simard et al., 1990), gIII (Fitzpatrick et al., 1989), gIV (Tikoo et al., 1990) and gH glycoproteins (Meyer et al., 1991), thymidine kinase (Bello et al., 1992), DNA polymerase (Owen and Field, 1988), four immediate-early proteins (Wirth et al., 1991), and the VP8 major protein (LaBoissiere et al., 1992) have been described. The functions of these genes and their products are discussed below.

1.3.4.2 Glycoproteins

Eleven glycoproteins were identified in the virion envelope of the BHV-1 and in the plasma membrane of infected cells (Drunen et al., 1986). These glycoproteins have important biological and immunological functions, for example, they affect attachment and penetration of the virus into the cell (Manservigi et al., 1977; Sarmiento and Spear, 1979) and mediate the immune responses of the host to the viral infection (Norrild et al., 1980).

Glycoprotein gI of BHV-1, with apparent molecular weight of 130K, belongs to a group of homologous glycoproteins that have been detected in all herpesviruses analyzed to date and is essential for viral replication (Drunen Littel-Van Den Hurk et al., 1 '92). A number of functions have been ascribed to this glycoprotein, such as attachment and penetration into susceptible cells (Liang et al., 1991a), induction of cell to cell fusion (Fitzpatrick et al., 1988, 1990) and the induction of neutralizing antibodies (Drunen Littel-van den Hurk and Babiuk, 1985). It is also a major target for the immune response of the host during BHV-1 infection (Collins et al., 1985; Van Drunen Littel-van den Hurk and Babiuk, 1986) and it confers protection from BHV-1 challenge in cattle (Babiuk et al., 1987; Drunen Littel-van den Hurk et al., 1990), which makes it a suitable subunit vaccine candidate.

Little is known about gII for which only two non-neutralizing monoclonal antibodies (MAbs) have been obtained so far (Drunen Littel-van den Hurk et al., 1984; Friedli and Metzler, 1987).

Glycoprotein gIII, a homologous glycoprotein to gC of herpes simplex virus, is a major viral glycoprotein of approximately 90K and assembles into homodimers. It is located on the virion envelope and on the surface of infected cells (Drunen Littel-van den Hurk et al., 1984; Marshall et al., 1986; Okazaki et al., 1986). It has been proposed to be a member of an immunoglobulin superfamily. Therefore, it is presumed to be implicated in the complex host-virus relationships governing the

immunology of the virus infection (Fitzpatrick et al., 1989). Although gIII is not an essential protein for virus propagation, it plays a dominant role in virus attachment to a heparin-containing cellular receptor in cell culture (Liang et al., 1991b), and appears to be responsible for maintaining virus replication efficiency in cattle (Liang et al., 1992). The glycoprotein is also a major target of immune responses at both the humoral and cellular levels (Babiuk et al., 1987; Fitzpatrick et al., 1989).

Glycoprotein gIV is an integral component of the virion envelope and appears to be essential for virus replication (Fehler et al., 1992). It is the major glycoprotein of approximately 71K which elicits high titers of complement-independent neutralizing antibodies (Babiuk et al., 1987; Hughes et al., 1988), followed by gIII and gI in that order. It has also been implicated in virus adsorption (Hughes et al., 1988; Liang et al., 1991a), penetration (Fehler et al., 1992; Hughes et al., 1988), and cell fusion (Tikoo et al., 1990).

Three glycoproteins of BHV-1 other than glycoproteins gI, gII, gIII and gIV, with relative molecular weights of 108, 93, and 42 K, were identified by MAb analyses (Baranowski et al., 1993). These glycoproteins were detected in infected cell lysates. Some of the anti-108K glycoprotein MAbs neutralized BHV-1 infectivity and some of the non-neutralizing MAbs were able to reduce plaque development. It was presumed that these glycoproteins are involved in viral entry into the cell and in cell-

to-cell spread of the virus (Baranowski et al., 1993). There is no published information regarding the other glycoproteins.

1.3.4.3 Thymidine Kinase

Like other herpesviruses, the BHV-1 genome encodes a viral thymidine kinase (TK). Although it is not essential for viral replication *in vitro* or *in vivo* (Weinmaster et al., 1982; Kit, 1985; Mittal and Field, 1989), it appears to play an important role in pathogenicity and has attracted much attention because it is a potential target for chemotherapy or attenuation of vaccine strains, and provides a useful selective marker for genetic studies and diagnosis of BHV-1 infection. The BHV-1 induced TK can be distinguished from host cell TK by its ability to use CTP in place of ATP as the phosphate donor (Wyler et al., 1989). This virus-specific enzyme is required for the activation of the antiviral agent Acyclovir. In order for Acyclovir to inhibit viral DNA synthesis, it must first be phosphorylated to Acyclovir monophosphate by the virus-encoded thymidine kinase and this activation confers a high degree of selectivity on the drug (Elion, 1982). Furthermore the enzyme appears to be important in the establishment and maintenance of neural latency (Field, 1985) and TK negative mutants generally have markedly reduced neuropathogenicity (Field and Owen, 1988). The ability to attenuate otherwise virulent viruses by chemically or genetically altering the functioning of their viral TK genes has been an important avenue of vaccine development (Kit and Qavi, 1985; Kit et al., 1985; Kit and Kit, 1986). The locations and nucleotide sequences of the TK gene of different BHV-1

strains have been reported (Kit and Kit, 1986, Bello et al., 1987; Mittal and Field, 1989; Smith et al., 1990; Bello et al., 1992). Comparison of these sequences revealed major differences which have been attributed to sequencing errors (Mittal and Field, 1989; Smith et al., 1990). A primer pair was designed from the TK gene region for detection of BHV-1 by PCR (Kibenge et al., 1994; Yason et al., 1995). This primer pair was specific for BHV-1 strains, and did not amplify BHV-4 strains, and a feline and an equine herpesvirus (Kibenge et al., 1994).

1.3.4.4 Regulation of BHV-1 gene expression

Like other herpesviruses, the expression of BHV-1 genes during infection in cell culture is temporally regulated (Seal et al., 1991). The viral proteins, can be categorized as immediate early (α), early (β) or late (γ) depending upon the order of their synthesis in the infected cell (Carpenter and Misra, 1991). The α genes are transcribed first in the absence of virus protein synthesis, and functional α gene products are required for the expression of β genes, while β gene expression occurs at the start of virus protein synthesis and prior to virus DNA synthesis. The products of β genes shut off α gene expression and turn on the expression of γ genes. The γ genes require the onset of virus DNA synthesis. The γ gene products, mostly structural components of the virion, turn off the expression of β genes. At least one γ gene product designated as an α trans-inducing factor has been introduced into cells during infection to induce α gene transcription (Roizman and Sears, 1990; Seal et al.,

1991). The late gene products can be divided further into 2 subclasses. The γ1 genes are expressed earlier than γ2, and are affected minimally if viral DNA replication is interrupted. The γ2 genes are expressed later in infection and are not expressed if viral DNA synthesis is blocked (Carpenter and Misra, 1991).

Of the genes of BHV-1 which have been mapped and sequenced, 4 belong to the immediate-early kinetic class. The α genes are grouped in two divergent transcription units with start sites located in the inverted repeats (Wirth et al., 1991; Wirth et al., 1992; Fraefel et al., 1993). Transcription unit 1 specifies three alternatively spliced BHV-1 transcripts, with a common noncoding leader sequence under the control of a single promoter (Wirth et al., 1991; Wirth et al., 1992; Fraefel et al., 1993). Transcription unit 2 specifies an α RNA which encodes BHV-1 infected cell protein 22 (BICP 22), the homolog of ICP 22 of herpes simplex virus type 1 (HSV-1) and related proteins of other herpesviruses (Fraefel et al., 1994).

The BHV-1 virion contains an abundance of a protein that has been designated VP8, which is also the most abundant viral protein in infected cells, and has been shown to be located in the tegument (Marshall et al., 1986). The structure of VP8 showed considerable homology with the product of the UL47 reading frame of herpes simplex virus 1, which encodes major tegument proteins VP13/VP14 (Carpenter and Misra, 1991; LaBoissiere et al., 1992)

1.3.5 Epidemiology and economic importance

1.3.5.1 Geographic distribution

Descriptions of clinical signs and antibody prevalence studies indicate a worldwide distribution of BHV-1 infections (Straub, 1990). Low sporadic to enzootic disease occurrence is reported from many countries in America, Europe, Asia, Australia and New Zealand (Ludwig, 1983).

BHV-1 infection causes serious economic losses all over the world due to animal death, abortions, decreased milk production and loss of weight. IBR outbreaks cost American farmers $ 25,000,000 annually, whereby the greatest detriment is a drop in milk production and secondary loss due to abortion. In the United Kingdom, the direct losses to the beef industry due to respiratory diseases, including IBR, could be as much as £ 5.7 million annually. In Switzerland, abortions were the main causes of loss of income and an eradication program costing 110 million SFr.is in its final stages (Wyler et al., 1989).

1.3.5.2 Transmission and BHV-1 in semen

The virus can be easily transmitted directly from one animal to another through respiratory, ocular and reproductive secretions of infected cattle, where large

quantities of virus are shed. Because of this mechanism, close contact among animals is responsible for the high rate of transmission (Donkersgoed and Babiuk, 1990). In feedlots, crowding and mixing of animals allow the efficient spread of the virus (Donkersgoed and Babiuk, 1990).

A further source of infection is semen. BHV-1 is the most commonly reported viral contamination in semen (Philpott, 1993). It can persist in frozen semen for over a year (Chapman et al., 1979). Both clinical and subclinical infections are followed by persistent latent infections that can be reactivated with virus shedding in semen (Kahrs et al., 1976; Orischot et al., 1993) . The risks of transmission are increased by the techniques of artificial insemination, where a single ejaculate may be diluted and inseminated into many susceptible cows (Kupferschmied et al., 1986). When susceptible cattle are inseminated with BHV-1 contaminated semen, the infection can be introduced into previously uninfected herds or areas (Miller, 1991). Although insemination with contaminated semen does not assure full blown infection (Kahrs et al., 1977), it might result in reduced fertility and abnormal fetal development (Parsonson and Snowdon, 1975; Miller, 1991).

Some countries require imported semen to be collected from BHV-1 seronegative bulls. However, there is no assurance that seronegative bulls are not shedding virus (Kohler and Kubin, 1972; Kahrs et al 1976, 1977). Several countries also require

imported semen to be certified free of BHV-1. But the current methods for detection of BHV-1 in semen lack sensitivity, and are time consuming and expensive.

1.3.6 Control

The control of BHV-1 infection is usually a combination of management procedures and a vaccination program (Donkersgoed and Babiuk, 1991). The aim of every control program is either to control the clinical disease or to eliminate the viral infection (Wyler et al., 1989). The prevalence and severity of BHV-1 infections in different countries has a direct impact on control and eradication programs (Wyler et al., 1989). Control programs depend also on conditions of animal breeding and management, i.e. different programs may be useful and necessary for dairy cattle farms, calf-breeding farms, fattening farms, artificial insemination and embryo transfer centres (Wyler et al., 1989). Some of the management procedures for BHV-1 respiratory infection have been reviewed by Donkersgoed and Babiuk (1991).

1.3.6.1 Vaccination

Vaccination is commonly used worldwide for control of BHV-1 infection. Only Switzerland and Denmark, and one district in France control BHV-1 infection by eradication without vaccination (Wyler et al., 1989). Other countries are running a combined vaccination / eradication program, and emphasize seronegativity of animals kept in artificial insemination centres and bull breeding herds (Kahrs, 1977; Straub, 1990; Donkersgoed and Babiuk, 1991).

Several BHV-1 modified live virus (MLV) vaccines are available for intranasal or intramuscular administration as single or combination products. The MLV vaccines produce a rapid immune response, relatively long duration of immunity and can induce local mucosal immunity (Todd et al., 1971; Jericho et al., 1982; Bordt et al., 1976; Sutton, 1980). However, several problems have been reported with MLV vaccines. In some cases, MLV vaccines have failed to prevent BHV-1 induced respiratory tract disease and conjunctivitis in cattle (Curtis and Angulo, 1974; Hyland et al., 1975; Jensen et al., 1976). This may be due to the improper storage causing inactivation of the MLV vaccines. Besides being easily inactivated by physical or chemical agents, changes in the genomes of BHV-1 vaccines have been reported after one passage in the host and these changes varied according to the tissue from which the virus was isolated (Whetstone et al., 1989). Vaccination of cattle with MLV vaccine can reduce the clinical signs, but cannot prevent either the vaccine strain or the field strain of BHV-1 from establishing latency nor to be reactivated (McKercher and Crenshaw, 1971; Kucera et al., 1978; Pastoret et al., 1980). Some MLV vaccines can cause abortion and should not be administered to pregnant animals (Mitchell, 1974; Miller et al., 1991b). There is also evidence that MLV vaccines of BHV-1 induce immunosuppression in cattle, resulting in increased susceptibility to other infections (Yates, 1982).

Another group of BHV-1 vaccines is inactivated vaccines. This group of vaccines does not cause abortion, immunosuppression or latency, although it does not prevent

the establishment of a latent infection by field strains (Donkersgoed and Babiuk, 1991). The major concern of the inactivated BHV-1 vaccines is their lack of efficacy. These vaccines are generally not used in feedlot cattle because natural exposure to the virus and disease may occur before adequate immunity develops 7 - 10 days following administration of the second dose (Hjerpe, 1990).

Recently, molecular biology and protein purification techniques have been employed in the development of genetically engineered attenuated BHV-1 vaccines as well as nucleic acid-free subunit vaccines. The crude BHV-1 envelope proteins, the purified glycoproteins, and glycoprotein mixtures were tested as subunit vaccines (Morein and Simons, 1985; Israel et al., 1988; Trudel et al., 1988; Drunen Littel-van den Hurk et al., 1990; Drunen Littel-van den Hurk et al., 1992b). With the genetic engineering approaches, it is possible to identify important virulence factors of potential vaccine agents, and then delete the gene(s) encoding these factors, and propagate the recombinant virus in culture (Liang et al., 1991b,c). These approaches include nucleic acid metabolism mutants (Kit and Qavi, 1985; Kit et al., 1986; Whetstone et al 1992; Liang et al., 1993) , glycoprotein gene mutants (Liang et al., 1991b; Rijsewijk et al., 1992; Engelenburg et al., 1992), viral tegument protein gene mutants (Goding and O'Hare, 1989; Carpenter and Misra, 1991;Zhang et al., 1991), and cytokine-expression mutants (Ramshaw et al., 1987). In general, the virulence factors to be deleted should also be non-essential for virus replication, so that the deletion does not constitute a lethal effect on mutant viruses (Drunen Littel-van den

Hurk et al., 1990). Also, in order to facilitate the vaccination strategy for BHV-1, the vaccine strain should have a marker so that vaccinated animals can be readily differentiated from naturally infected animals (Wyler et al., 1989).

1.3.6.2 Antiviral drugs

A wide variety of drugs have been tested for their antiviral effects on herpesviruses, particularly herpes simplex virus, and have been shown to be effective or partially effective *in vitro* or *in vivo* (Clercq et al., 1980, 1981; Myers et al., 1982; Richards et al., 1982). However, when these drugs were tested against BHV-1, mainly *in vitro*, most of them were shown to be ineffective in nontoxic doses (Babiuk et al., 1983). Interferon is one of the few agents that had antiviral effects against BHV-1 (Wyler et al., 1989). Treatment of calves with bovine recombinant α1 interferon prior to challenge with BHV-1 reduced dramatically the clinical disease by preventing secondary bacterial infections (Babiuk et al., 1985). Even though interferon-treated animals shed slightly less virus from their nasal passages than did untreated animals, this reduction was not significant (Babiuk et al., 1985). It was suggested that interferon had a greater immuno-modulatory effect than a direct antiviral effect (Babiuk et al., 1985).

1.4 Laboratory diagnosis

1.4.1 Viral antigen detection

The most common technique for BHV-1 detection in the diagnostic laboratory is virus isolation in cell culture (Wyler et al., 1989; Straub, 1990). When the samples, usually nasal, eye and genital swabs or fresh tissues from cases of abortion, are inoculated in primary bovine cell cultures, BHV-1 can cause characteristic herpesviral cytopathic effect (CPE). However, the identity of the virus needs to be confirmed by immunofluorescent (IF) or virus neutralization (VN) test (Leary and Splitter, 1992). Virus isolation used to be considered as the most sensitive method for BHV-1 detection (Edwards et al., 1983; Brunner et al., 1988; Donkersgoed and Babiuk, 1991). The disadvantage of this technique is the dependence on cell culture which needs a long "turn-around" time (Wyler et al., 1989). Moreover, latent infection in the nervous system cannot be detected (Kahrs et al., 1980; Bratanich and Jones, 1992).

More effort has been directed toward detection of BHV-1 in bovine semen by virus isolation, since seminal plasma has been shown to be toxic for cell cultures and contains CPE inhibitors (Kahrs et al., 1977; Kupferschmied et al., 1986; Pacciarini et al., 1988; Brunner et al., 1988). Several variations of cell culture techniques, with modifications of semen preparation and treatment have been tested. These include dilution of semen (Brunner et al., 1988; Weiblen et al., 1991), extensive washings

after adsorption (Darcell et al., 1977; Drew et al., 1987), centrifugation steps (Brunner et al., 1988) and trypsin pretreatment of semen to eliminate toxicity (Bielanski et al., 1988). Several methods have been shown to be useful, but the sensitivity was still unsatisfactory.

Some other techniques, such as electron microscopy (EM), various IF techniques and immunoperoxidase staining of fixed tissues or of impression smears were tested as alternatives to virus isolation (Wyler et al., 1989). These methods are rapid, but not as sensitive as virus isolation (Wyler et al., 1989). In addition, IF is often masked by autofluorescence of tissues, such as lung (Donkersgoed and Babiuk, 1991).

1.4.2 Specific viral antibody detection

In the diagnosis of BHV-1 infections, serological tests are used to detect the antibodies to the virus. For the exportation of animals and semen, cattle may be required to be antibody negative, which means that they have never been infected and thus not latently infected; or alternatively if vaccinated, show no rise in antibody titre, which means not acutely infected or showing reactivation of latent virus (Detegt et al., 1993).

For a long time the neutralization test has been the most commonly used test for the detection of BHV-1 specific antibodies, and still is the reference test in eradication programs (Wyler et al., 1989). Deregt et al. (1993) stated that the sensitivity of the

neutralization tests could be improved by increasing the incubation time and using complement. The disadvantage of the neutralization test is its dependence on cell culture. Alternative tests have been evaluated, such as IF techniques, agarose gel diffusion tests, and various enzyme-linked immunosorbent assay (ELISA) techniques (Wyler et al., 1989). Meanwhile the ELISA has been used to replace other serological tests, since it does evade use of cell culture and is rapid and economical (Wyler et al., 1989; Ackermann et al., 1990). But false positive results of ELISA were reported at an artificial insemination centre (Darcel, 1992), and therefore the reliability of ELISA needs to be further evaluated.

The biggest disadvantage of serological tests for BHV-1 detection is that maximal virus replication and shedding occur between three and six days after infection, which is before development of detectable antibody (Donkersgoed and Babiuk, 1991). Also some latently infected animals have very low antibody titers, or may even test seronegative, if they are not stressed and virus reactivation does not occur over a long period of time (Deregt et al., 1993). Virus isolation from the semen of seronegative bulls has been reported (Kupferschmied et al., 1986; Guerin, 1989).

1.4.3 Cornell Semen Test

In the Cornell Semen Test, pooled samples of semen are inoculated into susceptible calves or sheep (Schultz et al., 1982). Subsequent serological testing of the inoculated

animals can reveal the types of pathogens that were present in the semen sample. This method has several disadvantages. First it is not possible to recognize which specific samples are contaminated, since usually more than 100 semen samples are pooled together (Schultz et al., 1982). Moreover, semen is usually extended to high dilutions before it is stored in straws, and because only one or two semen straws per ejaculate are examined, small amounts of BHV-1 would probably not be detected (Drew et al., 1987). In bulls with subclinical BHV-1 infection, virus titers in nine straws varied between 10 and 1,000,000 median tissue culture infective dose ($TCID_{50}$) indicating that not all straws from one ejaculate contain virus, particularly when the virus titre is low (Orischot et al., 1993). Second, animal isolation facilities are required and subsequently the overall cost of this test is fairly high. Thirdly, seroconversion of the animals inoculated with semen takes up to 3 weeks, resulting in long turnaround time (Schultz et al., 1982).

1.4.4 Molecular bio-techniques in diagnostic virology

In the last decade, an intensive effort has been made to exploit the detection of viral nucleic acid as a diagnostic tool. Molecular biotechniques, such as nucleic acid hybridization and polymerase chain reaction (PCR), were extensively employed (Paul, 1990; Wolcott, 1992; Williams and Kwok, 1992). These methods are considered to be rapid, sensitive, specific and relatively less labour intensive for detection of virus in many situations, such as when the virus grows poorly in tissue culture or present in low titer, or the viral nucleic acid is not sufficiently expressed

for detection by immunochemical methods, or the virus is incorporated into the host genome, or the virus establishes a latent infection (Williams and Kwok, 1992).

1.4.4.1 Nucleic acid hybridization

Nucleic acid hybridization is one of the nucleic acid based detection methods that is becoming increasingly popular. The principle is that single stranded DNA (ssDNA) will hybridize by hydrogen bonded base pairing to another strand of DNA (or RNA) of complementary sequence. The specific viral genome can be detected by a radio-isotope, biotin or digoxigenin labeled, chemically synthesised probe or a probe cloned in a bacterial plasmid or bacteriophage (Paul, 1990; Wolcott, 1992). There are several different formats for nucleic acid hybridization, for example, dot-blot hybridization, in situ hybridization and Southern blot hybridization (Williams and Kwok, 1992). Several laboratories have described the detection of BHV-1 genome by various hybridization techniques, using different cloned BHV-1 DNA fragments as probe (Dorman et al., 1985; Dunn et al., 1986; Rock et al., 1986; Andino et al., 1987 ; Pacciarini et al., 1988; Belák et al., 1988). The BHV-1 DNA was detected in epithelial cells of nasal swabs by dot-blot hybridization (Dorman et al., 1985) or in situ hybridization (Dunn et al., 1986). Belák et al (1988) also used samples of nasal or preputial swabs from infected cattle for detection of BHV-1 DNA by direct filter hybridization. Pacciarini et al (1988) found hybridization of DNA extracted from semen samples with a radioactively labeled specific BHV-1 DNA fragment to be a very sensitive method. However, Brunner et al. (1988) compared several

hybridization methods with immunoelectron microscopy and various cell culture techniques, and concluded that the most sensitive method was a special cell culture technique. Vilček et al (1993) also pointed out that detection of BHV-1 in nasal swabs and nasal washings by virus isolation in cell cultures was more sensitive and reliable than hybridization, but it was more time consuming and laborious.

1.4.4.2 Polymerase chain reaction (PCR)

Since the rapid, nonisotopic nucleic acid based diagnostic systems could only be developed for target sequences which were highly abundant, the application of nucleic acid hybridization in clinical diagnosis for viral diseases had long been a problem, until the *in vitro* amplification system, PCR was developed (Wolcott, 1992; Williams and Kwok, 1992). PCR is a method for *in vitro* amplification of specific DNA sequences. Synthesis of the target DNA sequence is directed by two oligonucleotides which bracket the target sequence on opposite strands of the DNA. By using a thermostable DNA polymerase, the reaction can be repeatedly cycled through alternating thermal DNA denaturation, and primer extension reaction steps in a single tube until sufficient amplification of the target sequence has been achieved. Typically, a 10-fold amplification of the target can be achieved in 2-4 h through 30 thermal cycles (Wright and Wynford-Thomas, 1990; Wolcott, 1992; Williams and Kwok, 1992).

Reports on the application of PCR to the diagnosis of virus infections are also accumulating at an exponential rate (Kocher and Wilson, 1991). PCR has the potential and demonstrated usefulness for detection of both DNA and RNA viruses of various virus families (Williams and Kwok, 1992). The method has great advantage in detection of uncultivable viruses, neonatal viral infection, and latent viral infections. 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 to the detection of BHV-1. The first PCR assay was described by Vilček (1993) who used 22 bp oligomers from the BHV-1 gI gene as primers. A nested PCR assay targeting a portion of BHV-1 gIV gene was reported by Wiedmann et al (1993) and was found to be comparable to the Cornell Semen Test for detection of BHV-1 in bovine semen. Another PCR assay for detection of BHV-1 in bovine semen was described by Engelenburg et al. (1993). By using a primer pair which targets BHV-1 gIII gene, these authors were able to detect 5 molecules of BHV-1 DNA in 50 µl of bovine semen, and found PCR to be more rapid and sensitive than a virus isolation method (Engelenburg et al., 1993). In order to develop a sensitive, specific and rapid diagnostic method, and differentiate the BHV-1 field strains and TK-ve strains which have potential to be used as vaccine strains, a PCR protocol utilizing TK gene based primers was developed and the PCR product was used as a DNA probe in dot blot and Southern

blot hybridizations (Kibenge et al., 1994; Yason et al., 1995). This method was found to be sensitive, specific and rapid for detection of BHV-1 in cell culture supernate (Yason et al., 1995), and it could detect several strains of BHV-1 and differentiate BHV-1 from other bovine herpesviruses (Kibenge et al., 1994). The application of this method for detection of BHV-1 in bovine semen and how it compares to virus isolation and dot blot hybridization is the subject of this thesis.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cells, virus and semen

2.1.1 Cells

Bovine fetal testis (BFT) cells, passage 2-4 were used in this study. The primary cell culture of BFT were obtained by processing fresh bovine fetal testes, using standard tissue culture procedures (Yason et al., 1995). Briefly, the bovine fetal testes from a local abattoir (Hub Meat Packers, Moncton, New Brunswick) were transported to the laboratory in Eagle's minimal essential medium (EMEM) (Sigma, St. Louis, Mo., USA) containing 50 μg/ml of Penicillin. 50μg/ml of Streptomycin, and 4 μg/ml of Fungizone (GIBCO BRL Canada, Burlington, Ontario). The testes were washed three times in the same media and rinsed three times in phosphate buffered saline (PBS; 0.52 M NaCl, 0.053 M Na_2HPO_4, 0.015 M KH_2PO_4, pH 7.0). They were then digested at 37^0C for 1 h in 100 ml using Dispase (Boehringer Mannheim, Laval, Quebec). The mixture was filtered through three layers of sterile gauze and centrifuged for 10 min at 700 X g. The pellet was resuspended with 60 ml of growth medium consisting of L-15 (Leibovite, ICN Biomedicals Inc., Costa Mesa, California, USA) with 10% fetal bovine serum (FBS Bockneck, Toronto, Ontario), and was then seeded into three 75ml tissue culture flasks (Fisher Scientific, Montreal, Quebec). The primary BFT cells were incubated at 37^0C for 5-7 days. Before harvesting, the BFT cells were suspended in freezing medium consisting of L-15 with 20% FBS and

10% dimethylsulfoxide (DMSO; Fisher Scientific), aliquoted and stored in liquid nitrogen.

2.1.2 Virus

The virus strain used in this study, BHV-1 LA ATCC/1, was obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA) and was passaged once in BFT cells.

2.1.3 Bovine semen

The bovine semen was obtained from Eastern Breeders (Fredericton, New Brunswick) and several straws were pooled before aliquoting. The semen was aliquoted into 2 ml in each tube and stored at -76^{0}C. For experiments using semen artificially infected with BHV-1, the samples were prepared as shown in Table 1. Ten-fold serial dilutions from 10^{-1} to 10^{-8} of the virus infected tissue culture supernate were prepared in neat semen, semen diluted 1:5, 1:10, 1:20, and 1:50, and in EMEM (Table 1). One milliliter aliquotes of these samples were stored at -76^{0}C until use.

Table 1. Bovine semen samples artificially infected with BHV-1 for use in this study.

Dilution of virus stock[a]	Neat[b]	1:5[b]	1:10[b]	1:20[b]	1:50[b]	EMEM[b]
10^{-1}	A1	B1	C1	D1	E1	F1
10^{-2}	A2	B2	C2	D2	E2	F2
10^{-3}	A3	B3	C3	D3	E3	F3
10^{-4}	A4	B4	C4	D4	E4	F4
10^{-5}	A5	B5	C5	D5	E5	F5
10^{-6}	A6	B6	C6	D6	E6	F6
10^{-7}	A7	B7	C7	D7	E7	F7
10^{-8}	A8	B8	C8	D8	E8	F8
Control[c]	A9	B9	C9	D9	E9	F9

[a] The concentration of the BHV-1 LA stock was 10^6 $TCID_{50}$/100µl.

[b] All the semen samples were diluted in EMEM; Neat denotes undiluted bovine semen.

[c] Samples without virus were used as negative controls.

2.2 Virus isolation

2.2.1 Optimization of virus isolation procedure

Two methods, a standard virus isolation procedure and a modified virus isolation procedure were compared to determine the optimal condition for isolation of BHV-1 from bovine semen. As shown in Table 1, the sensitivity of virus isolation was evaluated by diluting virus from 10^{-1} to 10^{-8} dilution of the virus in neat, 1:5, 1:10, 1:20, and 1:50 semen dilutions, and in EMEM.

2.2.2 Standard virus isolation procedure

For use in virus isolation, the frozen BFT cells were thawed and propagated in growth medium for 1-2 days in 24-well plates with round coverslips. The cell monolayers were washed three times with PBS, and then inoculated with 100 µl of each sample preparation. After 1 h incubation at 37^0C, maintenance medium which consisted of EMEM with 2% FBS was added, followed by further incubation at 37^0C for 5 days with daily microscopic examination for CPE.

2.2.3 Modified virus isolation procedure

This procedure was modified by Darcell et al. (1977) from the standard procedure above, in order to eliminate the cytotoxicity and viral inhibitor in bovine semen. In the modified procedure, the cell monolayers were washed three times with PBS after

1 h incubation with the samples, and before the maintenance medium was added. CPE was also examined daily for 5 days.

2.2.4 Fluorescent antibody (FA) test

The BHV-1 CPE on BFT cells was confirmed using the fluorescent antibody (FA) test as follows: Coverslips were harvested at 5 days post inoculation, rinsed with PBS, and fixed in acetone for 10 min at room temperature. Five microlitres of diluted 1:10 fluorescein isothiocyanate (FITC) conjugated antibody against BHV-1 (VMRD Inc, Pullman, Washington, USA) was placed on the coverslips, and incubated at 37^0C for 30 min in a humidified chamber. They were then washed in commercially available wash buffer (VMRD) for 10 min, briefly rinsed in distilled water and were mounted on glass slides with mounting medium (VMRD). They were then examined using fluorescence microscopy (Axioplan 451888, Carl Zeiss Inc., Don Mills, Ontario).

2.3 Dot blot hybridization

2.3.1 Optimization of dot blot hybridization procedure

Bovine semen samples A1-A4, D1-D4, F1-F4 and A9, D9, F9 (Table 1) prepared in section 2.1.3 were used to determine the optimal dilution of bovine semen for use in dot blot hybridization for detection of BHV-1. Two different sample volumes, 0.1 ml and 2 ml, were used.

2.3.2 Viral DNA extraction and preparation of dot blots

The viral DNA was extracted using the procedure described by Kibenge (1992) with modifications as follows: the samples were centrifuged at 735 X g for 30 min at 4^0C to remove the cells. The virus was pelleted from the supernate by ultracentifugation at 134,000 X g for 4 h at 4^0C in SW28 rotor (Beckman, Mississauga, Ontario) and the pellet was resuspended in 500 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0). The virus was digested by adding 50 µl of 20 mg/ml Proteinase K (GIBCO BRL), 25 µl of 10% sodium dodecyl sulfate (SDS; Boehringer Mannheim, Laval, Quebec) with incubation at 37^0C for 18 h (Kibenge et al., 1994). Viral DNA was extracted by phenol-chloroform and was precipitated in ethanol. Briefly, an equal volume of Tris-buffered phenol/chloroform/isoamyl (25:24:1) was added to the sample, and the mixture was vortexed for 30 s, and centrifuged at 8000 X g. The top aqueous layer was re-extracted once in phenol/chloroform/isoamyl then in chloroform/isoamyl (24:1), and the DNA was precipitated from the aqueous phase by adding 0.1 vol. of 3M sodium acetate, pH 5.2 and 2 vols. of cold absolute ethanol (Commercial Alcohol Ltd. Quebec) and leaving at -70^0C for at least 2 h. Before use, the DNA was washed once with 70% ethanol, dried for 5 min in a Speedvac System (Savant, Farmingdale, New York, USA), and resuspended in 200 µl of TE buffer. One microlitre of RNase A (Pharmacia Biotech. Inc., Baie d'Urfé, Québec) was added to each sample and incubated at 37^0C for 1 h, followed by phenol/chloroform extraction and ethanol precipitation as before. The DNA free of RNA was resuspended in 200 µl TE buffer and stored at -20^0C prior to use.

The purified viral DNA was denatured by boiling for 3 min and quickly chilled on ice for 10 min. It was then applied on nylon membranes prewetted with 20X SSC solution (3M NaCl, 0.3M sodium citrate, pH 7.0), using a 96-well Bio-Dot microfiltration unit (Bio-Rad). The nylon membranes were baked at 80^0C in a vacuum oven (Precision Scientific Inc., Chicago, Illinois, USA) for 2 h to bind nucleic acid to the membranes.

2.3.3 Preparation of the BHV-1 DNA probe

A PCR product 183 bp long from the BHV-1 TK gene (Kibenge et al., 1994) was radiolabeled to make the probe. Fifteen microlitres of the PCR product were run on 1% low-melting temperature agarose gel. The band was visualized by staining with ethidium bromide (EB) and the DNA was recovered as described by Falson (1992). Briefly, bands were carefully cut from the agarose gel in the smallest volume possible. The volume was estimated by weight and an equal amount (w/v) of TE buffer, pH 8.0 was added. The agarose was melted at 65^0C for 5 min. Then an equal volume of Tris-buffered phenol was added and the mixture was centrifuged at 8000 X g in a microfuge for 3 min. The aqueous layer was re-extracted with phenol/chloroform and the DNA was recovered from the aqueous layer by precipitation with 0.1 vol. of LiCl (Fisher Scientific) and 2.5 vol. of cold absolute ethanol at -70^0C overnight. The DNA was then radiolabeled using the Random Primer Labeling Kit (GIBCO BRL Canada) following the manufacturer's protocol. Five microlitres of the PCR product in TE buffer were heated in a boiling water bath for 5 min and quickly

cooled on ice for 10 min. To the solution, 2 µl dCTP, 2 µl TTP, 2 µl dGTP, 15 µl of random primer buffer, 5 µl [α-^{32}P] dATP (Amersham, Oakville, Ontario) and 13 µl distilled water were added. One microlitre of Klenow fragment was added and the mixture was incubated for 1 h at room temperature. The reaction was stopped by adding 5 µl stop buffer, and the volume was brought to 102 µl by adding 47 µl distilled water. The mixture was passed through a spun column (glass wool, Biogel; Bio-Rad, Richmond, California, USA) by centrifugation at 200 X g for 5 min to remove the unincorporated radioisotope label. Two microlitres of the solution were taken into 4 ml of scintillation counting fluid (Beckman) to determine the radioactivity in counts per minute (cpm).

2.3.4 Prehybridization and hybridization:

Before hybridization with the probe, the filter was treated with prehybridization buffer (5X SSC, 5X Denhardt's [0.1% wt/vol Ficoll, 0.1% wt/vol polyvinylpyrrolidone, 0.1% wt/vol bovine serum albumin], 50mM sodium phosphate (pH 6.8), 0.1% wt/vol SDS, 250 µg/ml salmon sperm DNA, 50% vol/vol formamide, 100 µg/ml tRNA) overnight at 42^{0}C. The buffer was drained and fresh hybridization buffer, which is similar to the prehybridization buffer but contained 1 X Denhardt's solution, 20 mM sodium phosphate, pH 6.8, and 0.2% SDS, was added at a rate of 1 ml/10 cm^2 of filter. Equal volumes of probe solution and hybridization buffer were mixed, heated for 2 min in a boiling water bath, and snap frozen on ice for 10 min. The probe was then added to the hybridization bag containing the filters, to a final concentration of

3 X 10^6 cpm per filter. The filters were incubated overnight at 54^0C in a shaking water bath.

Excess and non-specifically bound probe were removed by high stringency washing. This consisted of washing in 2X SSC/0.1% SDS twice for 15 min each at room temperature, 0.1X SSC/0.1% SDS twice for 15 min each at room temperature and 0.1X SSC/0.1% SDS twice for 30 min at 55^0C. The filter was blotted on Whatman paper (Whatman Int. Ltd., Maidstone, England) to remove excess fluid. Filters were subjected to direct autoradiography using Kodak X-Omat diagnostic film (Picker International, Dartmouth, Nova Scotia) at -70^0C for 72 h.

2.3.5 Dot blot hybridization of salmon sperm DNA artificially infected with BHV-1

Bovine sperm DNA was extracted from 2 ml of neat bovine semen following the methods described in section 2.3.2 for viral DNA extraction. The DNA pellet was resuspended in 200 µl TE buffer and quantitated in the GeneQuant RNA/DNA Calculator (Pharmacia) . An equivalent amount of salmon sperm DNA (Sigma) was diluted in TE buffer. Ten-fold serial dilutions of BHV-1 stock were then prepared in the neat and the 1:20 diluted salmon sperm DNA solutions. These samples and the samples with equivalent amounts of virus in EMEM were processed for dot blot hybridization as described in sections 2.3.2 and 2.3.4.

2.4 Polymerase chain reaction (PCR)

2.4.1 PCR reagents

The PCR was performed in a DNA Thermocycler 480 (Perkin-Elmer Canada, Dartmouth, Nova Scotia) in GeneAmp™ reaction tubes (Perkin Elmer Canada). The reagentsy *Taq* DNA polymerase (5U/μl), 10X *Taq* polymerase buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1% triton X-100), and 25 mM $MgCl_2$ were purchased from one source (Bio-Can Scientific, Mississauga, Ontario). The dNTP mixture, dATP, dCTP, dGTP, and TTP (20mM each) were prepared from 100 mM stocks (Pharmacia). Ampliwax gems were from Perkin Elmer Cetus, glycerol was from Sigma, and Primers IBR TK1 and IBR TK2 (Kibenge et al., 1994) were purchased from the Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta. The IBR TK1 (sense) primer is located from nucleotides 975-995, and the IBR TK2 (antisense) primer originates from nucleotides 1137-1156 of the TK gene of BHV-1 strain 6660 (Mittal and Field, 1989). The nucleotide sequences of the primers were: 5'-AGA CCC CAG TTG TGA TGA ATG C-3' for IBR TK1 and 5' - ACA CGT CCA GCA CGA ACA CC-3' for IBR TK2 (Kibenge et al., 1994).

2.4.2 Standard PCR procedure

The standard PCR procedure used in this study is a modification of the accompanying protocol of Ampliwax™ PCR Gem 100 (Perkin Elmer Cetus) (Yason et al., 1995). The primers (400 μM each), dNTPs (200 μM each), 1.5 mM $MgCl_2$, 2.5

μl of 10X *Taq* polymerase buffer and enough water to make 20 μl were combined in GeneAmp™ reaction tube with one drop of AmpliWax™. The mixture was sealed by heating at 80°C for 10 min, followed by 1 min at 25°C. Then 10 μl of 10X *Taq* polymerase buffer, with 10% glycerol, 2.5 units of *Taq* polymerase and enough water to a total volume of 60 μl were added above the sealed wax layer. The viral DNA was extracted from each sample as described in section 2.3.2 and resuspended into 20μl TE buffer. Then the samples were added to the upper layer of reagents and made to a final PCR mix volume of 100 μl. The PCR was performed in DNA Thermocycler 480 (Perkin Elmer Cetus). The PCR mixture was amplified by an initial denaturation step of 95°C for 1 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 1 min, and a last cycle of 95°C for 1 min, 61°C for 1 min and 72°C for 5 min. Following the amplification, the PCR mixtures were stored at -20°C.

2.4.3 PCR using nucleic acid releasing cocktail

A proprietary nucleic acid releasing cocktail, GeneReleaser™ (Bioventures Inc., Murfreesboro, Tennessee, USA), that releases DNA from cells in a form suitable for amplification by PCR was used with a PCR thermocycle program according to the manufacturer's instructions. Instead of viral DNA extraction, one microlitre of each sample was lysed in a total volume of 20 μl of the resuspended GeneReleaser™ using the thermocycle program protocol. Then the PCR reagents were added to the same reaction tube and PCR was performed in a total volume of 100 μl containing a final

concentration of 1 X *Taq* polymerase buffer, 1.0 mM $MgCl_2$, 2.5 units of *Taq* polymerase, 0.2mM of each dNTP, 10% of glycerol, 0.4 uM of each primer, and one Ampliwax gem (Kibenge et al., 1994). The PCR cycles were performed under conditions as described in section 2.4.2.

2.4.4 Analysis of PCR products

Both agarose gel electrophoresis with EB staining and Southern blot analysis were used to detect the PCR products. Fifteen microlitres of PCR were run on 2% agarose in 1X TBE buffer for 2 h at 125V. For the Southern blot hybridization, the agarose gel was immersed in alkali (1.5 M NaCl, 0.5M NaOH) in a dish, rocking gently for 15 min and repeating once with fresh solution to denature the DNA. The gel was immersed into neutralizing solution (3M NaCl, 1.0M Tris-HCl pH 8.0) for 15 min. The DNA was then transferred from the gel to a nylon filter membrane (Amersham, Oakville, Ontario) by capillary action (Sealey and Southern, 1990). The nylon membrane was baked for 2 h at 80^0C in a vacuum oven to fix the DNA to the filter. The probe prepared as described in section 2.3.3 was added onto the filter to a final concentration of 9 X 10^6 cpm. Filters were prehybridized and hybridized with the probe and exposed to Kodak X-Omat diagnostic film at -70^0C for 72 h as described in section 2.3.4.

2.4.5 Optimization of PCR procedure

The sample preparation and reaction conditions of PCR for detection of BHV-1 were optimized for use with bovine semen. The samples with BHV-1 diluted 10^{-1} in neat semen, 1:5, 1:10, and 1:20 diluted semen, and in EMEM were tested to determine the best dilution of semen that can be used for PCR. Further optimization was done using a PCR optimization kit (PCR Optimizer™, Invitrogen Corporation, San Diego, California, USA) to determine the optimal $MgCl_2$ and pH in the reaction buffer for detection of BHV-1 by PCR. The buffers of pH 8.5, 9.0, 9.5 and 10.0 with $MgCl_2$ concentrations of 1.5 mM, 2.0 mM, 2.5 mM and 3.5 mM were tested (Table 2). In addition, since the reaction buffer used for PCR is at pH 9.0 and Mg^{2+} free (Kibenge et al., 1994, Yason et al., 1995), the optimal concentration of $MgCl_2$ was again determined in *Taq* polymerase buffer, pH 9.0, with $MgCl_2$ concentrations of 0.0 mM, 0.5 mM, 1.0 mM, 1.5mM, 2.0 mM, 3.0 mM.

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

pH	$MgCl_2$ concentration (in mM)			
	1.5	2.0	2.5	3.5
8.5	A	B	C	D
9.0	E	F	G	H
9.5	I	J	K	L
10.0	M	N	O	P

2.5 Experimental infection of bulls with BHV-1

2.5.1 Virus inoculation and sample collection

Two 18 month-old, BHV-1 seronegative bulls were experimentally infected with 2 ml of 10^5 $TCID_{50}$/100µl of BHV-1 LA strain via their prepuces. The virus was passed once in BFT cell culture and the infectivity of the virus stock was determined by titration on microtiter plates with BFT cells. Semen samples, nasal swabs, preputial swabs and blood samples were collected 7 days before infection and on day 0, 4, 10, 20, 30, and 40 after infection. The swab samples were stored in 2 ml of transport medium (EMEM with 50 µg/ml of Penicillin, 50µg/ml of Streptomycin, 10 µg/ml of Gentamicin, 4 µg/ml of Fungizone, 3.65 g/1000 ml of HEPES, 0.8 g/ 1000ml of sodium bicarbonate and 2% of FBS) at -70^{0}C. The serum was separated from the whole blood by centrifugation at 2,060 X g for 5 min at 4^{0}C, and then stored at -20^{0}C. The semen samples were stored at -70^{0}C.

2.5.2 Virus isolation

The bovine semen samples were diluted 1:20 in EMEM to eliminate the cytotoxiicity and inhibition of CPE, and virus isolation was done using the standard procedure described in section 2.2.2. The swab samples were filtered through 0.22 nm low protein binding filters (Millipore Co., Bedford, Maryland, USA). One hundred microlitres of each sample were inoculated on the BFT cell monolayers in 24-well

plates, and incubated at 37^{0}C with daily examination for CPE as described in section 2.2.2

2.5.3 Dot blot hybridization

Two mililitres of a semen sample were centrifuged at 735 X g for 30 min at 4^{0}C to remove sperm cells. The virus was pelleted by ultracentrifugation at 134,000 X g for 4 h in SW28 rotor (Beckman) at 4^{0}C. The viral DNA was extracted, dot blotted on the nylon membranes, and hybridized with radiolabeled probe as described in section 2.3.4.

2.5.4 Polymerase chain reaction (PCR)

The PCR was performed as described in section 2.4.3 with modifications as optimized in section 2.4.5. Briefly, the semen samples were diluted 1:20 in EMEM to eliminate the inhibition factor of PCR amplification. One microlitre of each sample was added in a total volume of 20 µl of the resuspended GeneReleaser^{TM} (Bioventures Inc.) using the thermocycle program protocol. Then the PCR reagents were added in the same reaction tube and PCR was performed in a total volume of 100 µl containing a final concentration of 1 X *Taq* polymerase buffer, 1.0 mM $MgCl_2$, 2.5 units of *Taq* polymerase, 0.2mM of each dNTP, 10% of glycerol, 0.4 µm of each primer, and one Ampliwax gem. Both agarose gel electrophoresis with EB staining and Southern blot hybridization analysis (section 2.4.4) were used to visualize the PCR products.

2.5.5 Virus neutralization test

The virus neutralization (VN) test was done in BFT cells and 100 $TCID_{50}$/25µl of BHV-1 as described in the protocol of the Regional Diagnostic Virology Laboratory, Atlantic Veterinary College. Briefly, all the serum samples and the positive and negative sera were inactivated at 56^0C for 30 min before testing. Twenty five microlitres of medium (EMEM with 100 u/ml Penicillin, 100 µg/ml of Streptomycin and 15% of FBS) were added to each well of the 96-well microtiter plate. Then 25 µl of each test serum sample and control sample were added in the wells using two horizontal rows. Two-fold dilutions of the serum from 1:2 to 1:2048 were made in wells from rows 2 to 12 and twenty five microlitres of BHV-1 100 $TCID_{50}$/25 µl were added into each well and the plate was incubated at 37^0C for 1 h. Fifty microlitres of BFT cells with a concentration of around 10^6/ml were added to the wells and incubated at 37^0C for 5 days. Then the CPE was identified by microscopy and crystal violet staining (Fisher Scientific). The VN titer which is the highest dilution of serum which neutralized 100 $TCID_{50}$ of BHV-1 was recorded for each sample.

CHAPTER 3: RESULTS

3.1 Optimization of virus isolation of BHV-1 in artificially infected bovine semen samples

The infectivity titer of the BHV-1 stock was determined to be 10^6 median tissue culture infective doses ($TCID_{50}$) per 100µl.

Two virus isolation procedures which were tested on different dilutions of bovine semen containing 10-fold serial dilutions of the virus suspension were used to determine the best dilution of semen which would reduce the cytotoxicity of semen in cell culture and reduce the BHV-1 CPE inhibition.

Table 3 shows the results of virus isolation in primary BFT cell cultures using the standard procedure. FA staining with conjugated BHV-1 antiserum was used to differentiate CPE from cytotoxicity, and to confirm viral replication in BFT cell cultures. All the neat and 1:5 diluted bovine semen samples were cytotoxic to the BFT cells. Cytotoxicity was not observed in the semen diluted 1:10. However, at this semen dilution, BHV-1 could only be detected by CPE and FA up to a dilution of 10^{-5}, while in 1:20 and 1:50 diluted semen samples, virus diluted up to 10^{-6} could be detected. This indicated the existence of inhibition of BHV-1 replication and CPE by bovine semen (designated R on Table 3). Consequently, 1:20 was selected as the

best semen dilution for detection of BHV-1, being free of cytotoxicity and free of inhibition of BHV-1 replication and CPE. The minimum amount of virus detected in 1:20 diluted bovine semen by the standard virus isolation procedure was determined to be 1 $TCID_{50}$/100µl.

Table 3. Isolation of BHV-1 from different dilutions of bovine semen in primary BFT cell cultures using the standard procedure followed by FA test.

Dilution of virus stock[a]	Neat[b]	1:5[b]	1:10[b]	1:20[b]	1:50[b]	EMEM[b]
10^{-1}	T-[c]	T-	+[c]	+	+	+
10^{-2}	T-	T-	+	+	+	+
10^{-3}	T-	T-	+	+	+	+
10^{-4}	T-	T-	+	+	+	+
10^{-5}	T-	T-	+	+	+	+
10^{-6}	T-	T-	-	+	+	+
10^{-7}	T-	T-	-	-	-	-
10^{-8}	T-	T-	-	-	-	-
Control[d]	T-	T-	-	-	-	-

[a] The concentration of the BHV-1 LA stock is 10^6 $TCID_{50}$/100µl.

[b] Neat = undiluted bovine semen; Other semen samples were diluted in 1:5, 1:10, 1:20, or 1:50 in EMEM; Virus diluted in EMEM was used as control for identification of cytotoxicity, and viral replication and CPE inhibitor.

[c] T denotes Cytotoxicity; - denotes negative and + denotes positive for CPE and FA.

[d] Control = negative control, i.e. no virus.

The results of the virus isolation using the modified procedure are shown in Table 4. The cytotoxicity of bovine semen to the BFT cells was found only in the neat semen samples. Cytotoxicity was not observed in any of the diluted semen samples. In addition, the inhibition factor of BHV-1 replication and CPE in bovine semen was not observed with this procedure. However, BHV-1 could only be detected up to a virus dilution of 10^{-5}, which was 10-fold less virus (i.e. 10 $TCID_{50}/100\mu l$) than that detected using the standard virus isolation procedure. The standard procedure was therefore compared with dot blot hybridization and PCR, and was used as the optimized virus isolation procedure for detection of BHV-1 in bovine semen from experimentally infected bulls.

Table 4. Isolation of BHV-1 from different dilutions of bovine semen in primary BFT cell culture using the modified procedure followed by FA test.

Dilution of virus stock[a]	Neat[b]	1:5[b]	1:10[b]	1:20[b]	1:50[b]	EMEM[b]
10^{-1}	T-[c]	+[c]	+	+	+	+
10^{-2}	T-	+	+	+	+	+
10^{-3}	T-	+	+	+	+	+
10^{-4}	T-	+	+	+	+	+
10^{-5}	T-	+	+	+	+	+
10^{-6}	T-	-	-	-	-	-
10^{-7}	T-	-	-	-	-	-
10^{-8}	T-	-	-	-	-	-
Control[d]	T-	-	-	-	-	-

[a] The concentration of the BHV-1 LA stock is 10^6 $TCID_{50}$/100µl.

[b] Neat = undiluted bovine semen; Other semen samples were diluted in 1:5, 1:10, 1:20, or 1:50 in EMEM; Virus diluted in EMEM was used as control for identification of cytotoxicity and virus replication and CPE inhibitor.

[c] T denotes Cytotoxicity; - denotes negative and + denotes positive for CPE and FA.

[d] Control = negative control, i.e. no virus.

3.2 Optimization of dot blot hybridization in artificially infected bovine semen samples.

In order to verify the sensitivity of the dot blot hybridization method for detection of BHV-1 in semen samples and to determine if there are factors which may influence virus detection in bovine semen by dot blot hybridization, serial dilutions of the BHV-1 stock (10^{-1} to 10^{-4}) were made in neat bovine semen, semen diluted 1:20 and in EMEM. For easy comparison to virus isolation, 0.1 ml of each sample was used to prepare viral DNA for dot blotting. All dot blots were probe-negative at this sample volume. When the sample volume was increased to 2 ml, BHV-1 viral DNA could be detected, the neat semen samples giving the strongest reaction of all the sample dilutions tested (Fig. 1). No signal was observed in the negative control samples of the neat semen and of semen diluted 1:20. As shown in Figure 1, dot blot hybridization detected 10^{-3} dilution of BHV-1 stock in neat semen, 10^{-2} dilution of the virus in semen diluted 1:20 and 10^{-1} dilution of the virus in EMEM. Therefore, the minimum amount of virus detected by dot blot hybridization was with neat bovine semen, and was determined to be 20,000 $TCID_{50}/100\mu l$.

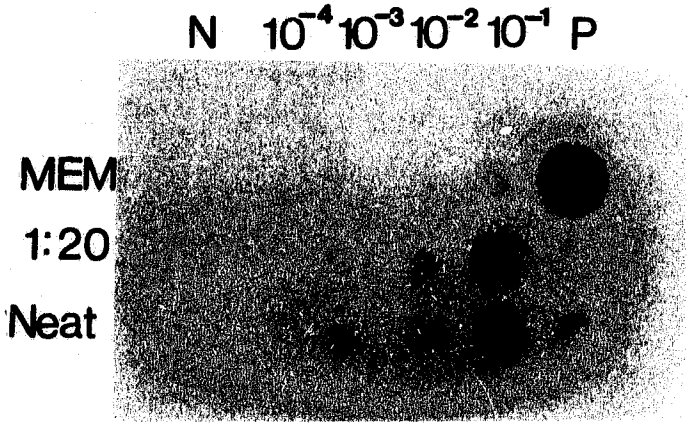

Figure 1. Detection of BHV-1 DNA in different dilutions of bovine semen by dot blot hybridization. Lane N denotes no BHV-1 in EMEM, bovine semen diluted 1:20 or in neat bovine semen. Lanes 10^{-4} to 10^{-1} denote decreasing 10-fold dilutions of BHV-1 in EMEM, bovine semen diluted 1:20 or in neat bovine semen. Lane P denotes BHV-1 stock (10^6 $TCID_{50}$/100µl) as positive control. The neat bovine semen samples gave the best results and could detect 10^{-3} of BHV-1 stock.

3.2.1 Artificially infected salmon sperm DNA samples

The neat bovine semen was determined to contain 40 μg/ml of sperm DNA. When BHV-1 was resuspended in salmon sperm DNA solution, the dot blot hybridization method detected 10^{-4} dilution of BHV-1 stock in 40 μg/ml salmon sperm DNA and 10^{-2} dilution of the virus in the 2 μg/ml salmon sperm DNA (Fig. 2). These results demonstrated that dot blot hybridization of samples with higher concentrations of total DNA gave better sensitivity, confirming the initial observation that higher dilutions of BHV-1 could be detected in neat bovine semen than in diluted semen. The 10-fold more virus detected with salmon sperm DNA (10^{-4} dilution of BHV-1 stock) than with bovine sperm DNA (10^{-3} dilution of BHV-1 stock) may be due to the fact that pure salmon sperm DNA was used compared to bovine sperm DNA which was extracted from bovine semen.

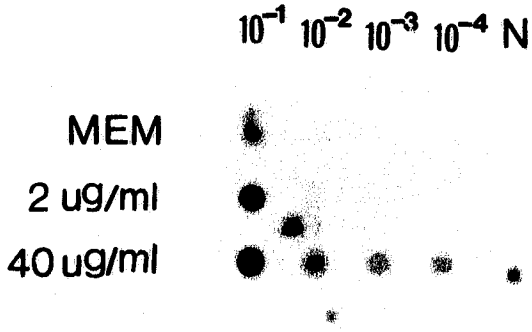

Figure 2. Detection of BHV-1 DNA in different dilutions of salmon sperm DNA by dot blot hybridization. Lane N denotes no BHV-1 in EMEM, 2 μg/ml salmon sperm DNA solution or in 40 μg/ml salmon sperm DNA solution. Lanes 10^{-4} to 10^{-1} denote decreasing 10-fold dilutions of BHV-1 in EMEM, 2 μg/ml salmon sperm DNA solution or in 40 μg/ml salmon sperm DNA solution. Some non-specific binding of the probe occurred on the filter and can be differentiated from the specific dot blot hybridization signals by the shape and the position of the dots. The samples containing 40μg/ml of salmon sperm DNA gave the best result and could detect 10^{-4} of BHV-1 stock.

3.3 Optimization of polymerase chain reaction in artificially infected bovine semen samples

3.3.1 PCR using standard procedure

When the PCR with standard procedure was used for detection of BHV-1 in different dilutions of bovine semen, the PCR product of 183 bp long could be detected on the agarose gel by EB staining. However, since this method involved a lot of sample manipulations, it was associated with cross contamination. In addition, the sensitivity of virus detection was influenced by the DNA purification and extraction procedures, and therefore could not be determined accurately.

3.3.2 Optimization of sample preparation for PCR using nucleic acid releasing cocktail

When the established reaction conditions of PCR with nucleic acid releasing cocktail (GeneReleaser^{TM}) for detection of BHV-1 in tissue culture media were used on the infected bovine semen samples, no virus was detected in neat bovine semen indicating the existence of a PCR inhibitor (Fig. 3). This inhibitior could be eliminated by dilution of bovine semen. When different dilutions of bovine semen (1:5, 1:10, 1:20 and EMEM) with equal amounts of BHV-1 were used, the semen diluted 1:20 gave the best amplification result. The band on the agarose gel using this dilution was as intense as the band in the EMEM sample (Fig. 4). Therefore, the 1:20 dilution of

bovine semen was selected for use in PCR amplification from bovine semen with GeneReleaser™.

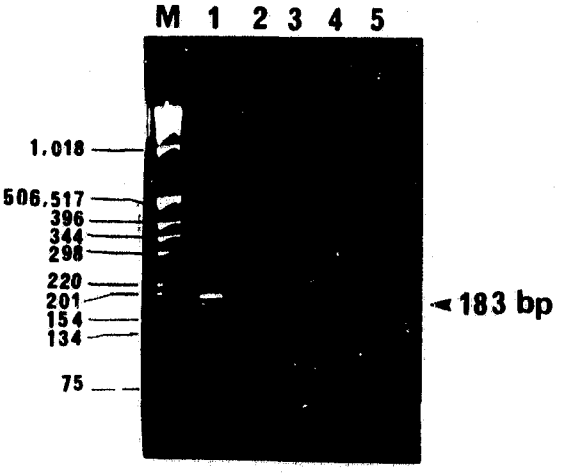

Figure 3. Detection of BHV-1 by PCR with GeneReleaser™ in EMEM and in neat bovine semen. Lane M denotes molecular weight markers (GIBCO BRL Canada); Lane 1 denotes 10^{-1} of BHV-1 in EMEM; Lane 2 denotes 10^{-1} of BHV-1 in neat bovine semen; Lanes 3 and 4 denote EMEM and neat bovine semen without BHV-1 as negative controls; Lane 5 denotes carryover control in which the distilled water was used instead of sample, respectively. The PCR product was detected in the EMEM sample in Lane 1 but was not detected in the bovine semen sample which contained the same amount of virus in Lane 2. This indicated the presence of a PCR inhibitor in neat bovine semen.

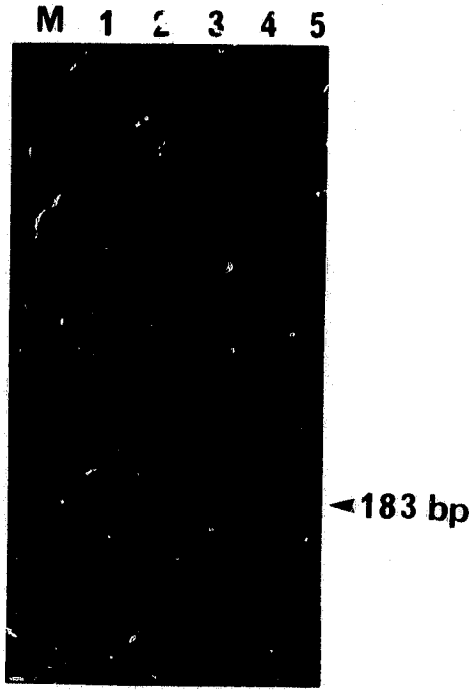

Figure 4. Detection of BHV-1 by PCR with GeneReleaser™ in different dilutions of bovine semen. Lane M denotes molecular weight markers (as in Figure 3); Lanes 1-4 denote 10^{-1} of BHV-1 in EMEM, semen diluted 1:5, semen diluted 1:10, and semen diluted 1:20, respectively. Lane 5 denotes carryover control (as in Figure 3). The 1:20 diluted bovine semen sample gave the best result.

3.3.3 Optimization of reaction conditions for PCR using nucleic acid releasing cocktail

It was decided to optimize the pH and $MgCl_2$ concentration of the PCR buffer for use with nucleic acid releasing cocktail (GeneReleaser™) and bovine semen. The best amplification results with the primer pair IBR TK1/IBR TK2 were obtained when the buffer contained 1.5 mM of $MgCl_2$ at pH 8.5 (Fig. 5), or 2.5 mM of $MgCl_2$ at pH 9.5 (Fig. 6). When the concentration of $MgCl_2$ was further optimized for the commercially available *Taq* polymerase buffer at pH 9.0, it was determined that the optimal $MgCl_2$ concentration was 1.0 mM for detection of BHV-1 in bovine semen diluted 1:20 (Fig. 7), while for the tissue culture media samples it was 1.5 mM (Fig. 8).

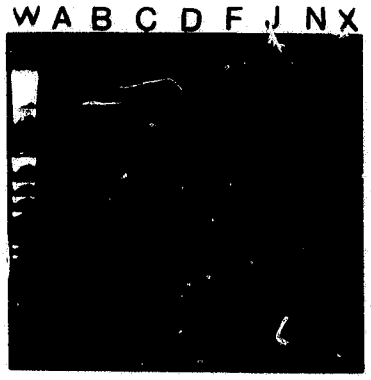

Figure 5. Optimization of pH and $MgCl_2$ concentration for detection of BHV-1 by PCR with GeneReleaser™ in 1:20 diluted bovine semen (step 1). Lanes A, B, C, D, F, J, N denote reaction buffers containing 1.5 mM of $MgCl_2$ at pH 8.5, 2.0 $MgCl_2$ at pH 8.5, 2.5 mM of $MgCl_2$ at pH 8.5, 3.5 mM $MgCl_2$ at pH 8.5, 2.0 mM of $MgCl_2$ at pH 9.0, 2.0 mM of $MgCl_2$ at pH 9.5, and 2.0 mM of $MgCl_2$ at pH 10.0, respectively (See also Table 2). Lane W denotes molecular weight markers (GIBCO BRL Canada). Lane X denotes carryover control (as in Fig. 3). The reaction mixture containing 1.5 mM $MgCl_2$ at pH 8.5 (Lane A) was the best condition for detection of BHV-1 in 1:20 diluted bovine semen

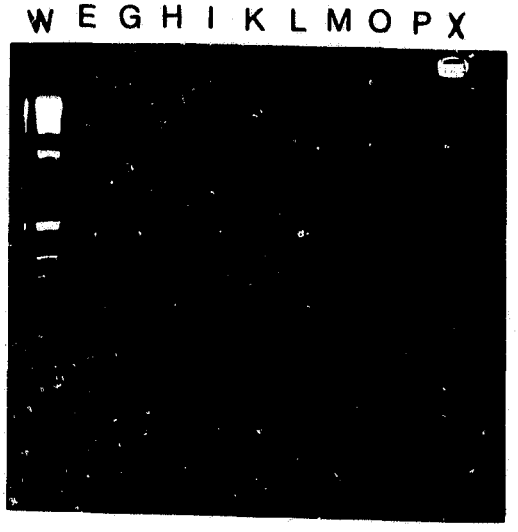

Figure 6. Optimization of pH and $MgCl_2$ concentration for detection of BHV-1 by PCR with GeneReleaser™ in 1:20 diluted bovine (step 2). Lane E, G, H, I, K, L, M, O, and P denote reaction buffers containing 1.5 mM of $MgCl_2$ at pH 9.0, 2.5 $MgCl_2$ at pH 9.0, 3.5 mM of $MgCl_2$ at pH 9.0, 1.5 mM $MgCl_2$ at pH 9.5, 2.5 mM of $MgCl_2$ at pH 9.5, 3.5 mM of $MgCl_2$ at pH 9.5, 1.5 mM of $MgCl_2$ at pH 10.0, 2.5 mM of $MgCl_2$ at pH 10.0, and 3.5 mM of $MgCl_2$ at pH 10.0 respectively (See also Table 2). Lane W denotes molecular weight markers (GIBCO BRL Canada). Lane X denotes carryover control (as in Fig. 3). The reaction mixture containing 2.5 mM $MgCl_2$ at pH 9.5 (Lane K) was the best condition for detection of BHV-1 in 1:20 diluted bovine semen.

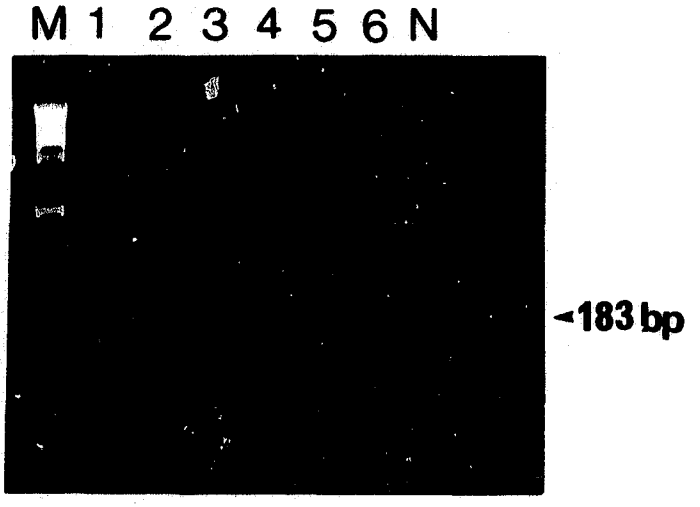

Figure 7. Optimization of $MgCl_2$ concentration for detection of BHV-1 in bovine semen diluted 1:20 by PCR with GeneReleaser™ in Taq polymerase buffer, pH 9.0. Lane M denotes molecular weight markers (GIBCO BRL Canada); Lanes 1 - 6 denote the $MgCl_2$ concentrations (in mM) of 0, 0.5, 1.0, 1.5, 2.0, and 2.5; Lane N denotes carryover control (as in Fig. 3). The reaction mixture containing 1.0 mM $MgCl_2$ (Lane 3) was the best condition for detection of BHV-1 in 1:20 diluted bovine semen.

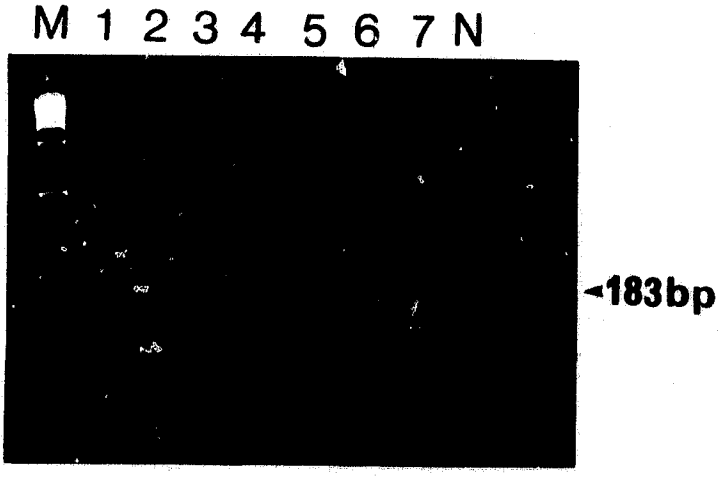

Figure 8. Optimization of $MgCl_2$ concentration for detection of BHV-1 in EMEM by PCR with GeneReleaser™ in Taq polymerase buffer, pH 9.0. Lane M denotes molecular weight markers (GIBCO BRL Canada); Lanes 1 - 7 denote the $MgCl_2$ concentrations (in mM) of 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0; Lane N denotes carryover control (as in Fig. 3). The reaction mixture containing 1.5 mM $MgCl_2$ (Lane 4) was the best condition for detection of BHV-1 in EMEM.

Under these optimized conditions, 1 $TCID_{50}$/100μl of BHV-1 could be detected in 1:20 diluted bovine semen by PCR with EB staining (Fig. 9). Prior to this optimization of $MgCl_2$, the minimum of BHV-1 in 1:20 diluted bovine semen that could be detected by PCR using the PCR buffer at pH 9.0 which contains 1.5 mM of $MgCl_2$, with EB staining was 100 $TCID_{50}$/100 μl of sample (Fig. 10). Therefore, the sensitivity of BHV-1 PCR for bovine semen samples diluted at 1:20 was increased 100-fold following optimization for $MgCl_2$ concentration. This sensitivity was further increased when EB staining was replaced by Southern blot hybridization, detecting 0.01 $TCID_{50}$/100 μl of sample (Fig. 11). Thus, PCR with Southern blot hybridizaztion was the most sensitive of the methods studied for detection of BHV-1 in bovine semen (Table 5).

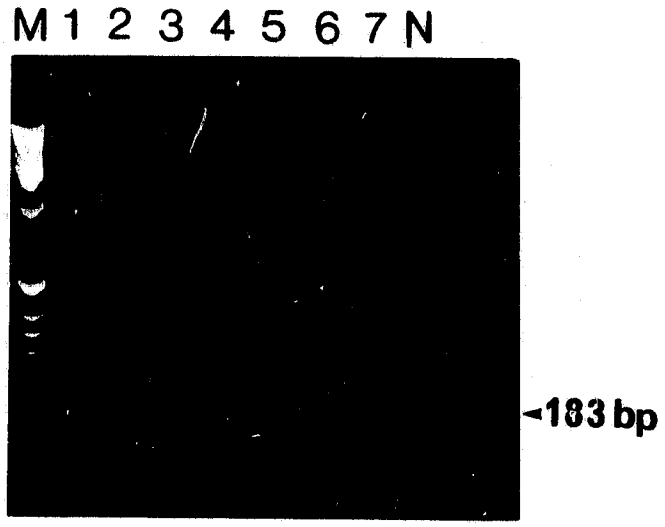

Figure 9. Sensitivity of PCR with EB staining for detection of BHV-1 in bovine semen diluted 1:20 after optimization for $MgCl_2$ concentration. Lane M denotes molecular weight markers (GIBCO BRL Canada); Lanes 1 to 7 denote 10^{-1} to 10^{-7} of BHV-1 in bovine semen diluted 1:20 in EMEM; Lane N denotes carry over control (as in Fig. 3). Under the optimized reaction conditions, the PCR could detect 10^{-4} of BHV-1 stock in 1 µl of 1:20 diluted bovine semen.

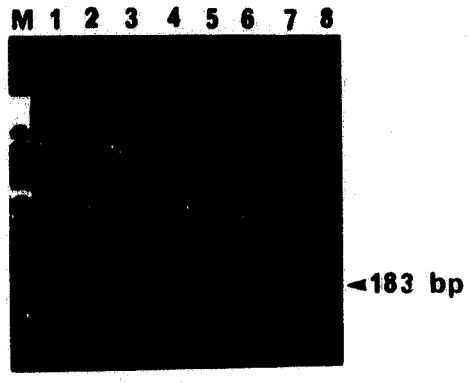

Figure 10. Sensitivity of PCR for detection of BHV-1 in bovine semen diluted 1:20 before optimization for $MgCl_2$ concentration. Lane M denotes molecular weight makers (GIBCO BRL Canada); Lanes 1 to 7 denote 10^{-7} to 10^{-1} of BHV-1 in bovine semen diluted 1:20 in EMEM; Lane 8 denotes carryover control (as in Fig. 3). Before optimization of reaction conditions, the PCR could only detect 10^{-2} of BHV-1 stock in 1 µl of 1:20 diluted bovine semen.

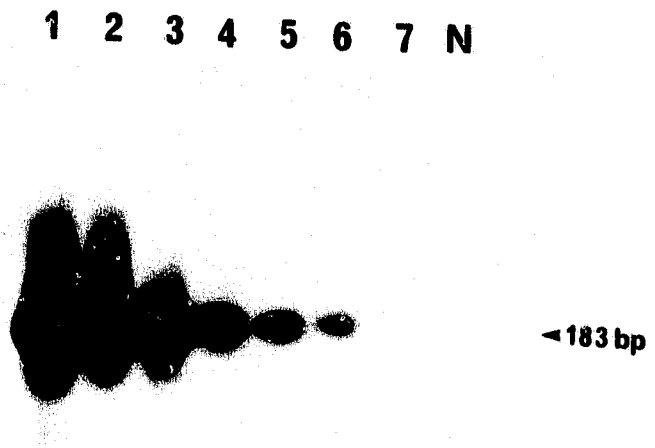

Figure 11. Southern blot hybridization of the agarose gel from Fig. 9. Lanes 1 to 7 denote 10^{-1} to 10^{-7} of BHV-1 in bovine semen diluted 1:20 in EMEM; Lane N denotes carryover control (as in Fig. 3). Under the optimized reaction conditions, the PCR with Southern blot hybridization could detect 10^{-6} of BHV-1 stock in 1 µl of 1:20 diluted bovine semen.

3.4 Comparison of the sensitivity of dot blot hybridization, PCR amplification and virus isolation for detection of BHV-1 in bovine semen

As shown in Table 5, dot blot hybridization could detect 20,000 $TCID_{50}$/100 µl of BHV-1 in neat bovine semen samples, while virus isolation, and PCR with EB staining could detect 1 $TCID_{50}$/100 µl of BHV-1 in bovine semen samples diluted 1:20. Since the semen samples have to be diluted 1:20 before being tested by virus isolation or PCR to eliminate the cytotoxicity, viral replication and CPE inhibitor and PCR inhibitor, the minimum amount of BHV-1 that can be detected in a semen sample by virus isolation or by PCR with EB staining is therefore 20 $TCID_{50}$/100 µl. When corrected for the dilution factor, these two methods are still more sensitive (1000-fold more) than the dot blot hybridization method. PCR with Southern blot hybridization was the most sensitive method, detecting 0.01 $TCID_{50}$/100µl of BHV-1 in 1:20 diluted bovine semen samples, which is equivalent to 0.2 $TCID_{50}$/100 µl of BHV-1 when corrected for the dilution factor for semen samples.

Table 5. Comparison of the sensitivity of dot blot hybridization, PCR amplification and virus isolation for detection of BHV-1 in artificially infected bovine semen

Amount of virus ($TCID_{50}$/100µl)	Dot blot hybridization[a]	PCR amplification[a]		Virus isolation[a]
		EB staining	Southern blot	
2X 10^6	+[b]	+	+	+
2X 10^5	+	+	+	+
2X 10^4	+	+	+	+
2X 10^3	-	+	+	+
2X 10^2	-	+	+	+
2X 10^1	-	+	+	+
2X 10^0	-	-	+	-
2X 10^{-1}	-	-	+	-
2X 10^{-2}	-	-	-	-

[a] Dot blot hybridization was done on neat bovine semen; PCR amplification and virus isolation were done on bovine semen samples diluted 1:20 and corrected for the dilution factor.

[b] + denotes positive; - denotes negative for CPE (virus isolation) or 183 bp product (PCR amplification) or hybridization signal (dot blot or Southern blot).

3.5 Experimental infection of bulls

3.5.1 Clinical signs

Following virus inoculation, balanoposthitis was observed in Bull 1 at day 10 and in Bull 2 at day 20. This was manifested as a small amount of creamy exudate on the preputial hairs, swelling of prepuce, hyperaemia of the penis with small pustules disseminated over the preputial and penile mucosa accompanied by blood and mucopurulent discharge (Figs 12 A and B). These signs were still visible on the preputial and penile mucosa 40 days after virus inoculation. Bull 2 also developed a fever at day 10. No typical clinical signs of the respiratory form of IBR were observed in any of the bulls.

Figure 12 A. Balanitis following experimental infection of Bull 1; There are pustules on penile mucosa.

B. Posthitis following experimental infection of Bull 2. The figure shows the swollen prepuce and creamy exudate.

3.5.2 Virus isolation

Bovine semen samples collected at day -7, 0, 4, 10, 20, 30, 40, and nasal swabs and preputial swabs collected at day -7, 4, 10, 40 from both bulls, were screened for BHV-1 by virus isolation. As shown in Table 6, two semen samples and two preputial swab samples collected 4 days after virus inoculation were positive by virus isolation. The semen and preputial swab of Bull 1 had CPE in all the 4 test wells, while the semen and preputial swab of Bull 2 had CPE only in 3 out of the 4 test wells. No virus was isolated from any of the nasal swab samples.

Table 6. Results of virus isolation for detection of BHV-1 from samples collected from experimentally infected bulls.

Time of sample collection[a]	Semen		Preputial swabs		Nasal swabs	
	Bull 1	Bull 2	Bull 1	Bull 2	Bull 1	Bull 2
Day -7	-[b]	-	-	-	-	-
Day 0	-	-	ND[c]	ND	ND	ND
Day 4	+	±[b]	+	±	-	-
Day 10	-	-	-	-	-	-
Day 20	-	-	ND	ND	ND	ND
Day 30	-	-	ND	ND	ND	ND
Day 40	-	-	-	-	-	-

[a] Day -7: 7 days before inoculation, and days after inoculation.

[b] +: CPE in all 4 wells; -: No CPE in any of the 4 wells; ±: CPE in less than 4 wells.

[c] ND denotes not done

3.5.3 Dot blot hybridization

All bovine semen samples collected were processed to obtain BHV-1 DNA, and were subjected to dot blot hybridization. Only the day 4 semen sample collected from Bull 1 showed a hybridization signal (Fig. 13).

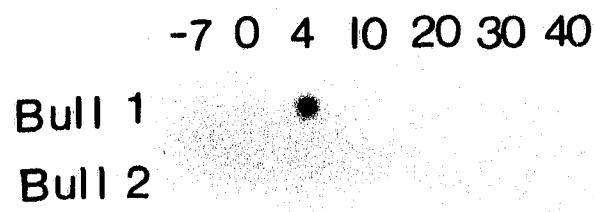

Figure 13. Detection of BHV-1 in bovine semen by dot blot hybridization. Lanes -7, 0, 4, 10, 20, 30, 40 denote samples collected 7 days before inoculation, on the inoculation day, and 4, 10, 20, 30, and 40 days after inoculation. Only the sample collected from Bull 1 at day 4 was positive by this method.

3.5.4 Polymerase chain reaction

All the semen samples were subjected to PCR. The two samples collected at day 4 from both bulls yielded the 183 bp PCR product detectable by EB staining of agarose gels (Figs 14 and 15). No DNA band was seen with the negative control samples. When the same gels were analyzed by Southern blot hybridization, all the semen samples of Bull 1 collected after inoculation had the 183 bp PCR product, while 4 samples of Bull 2 collected at days 4, 10, 20, 30 had the PCR product (Figs 16 and 17).

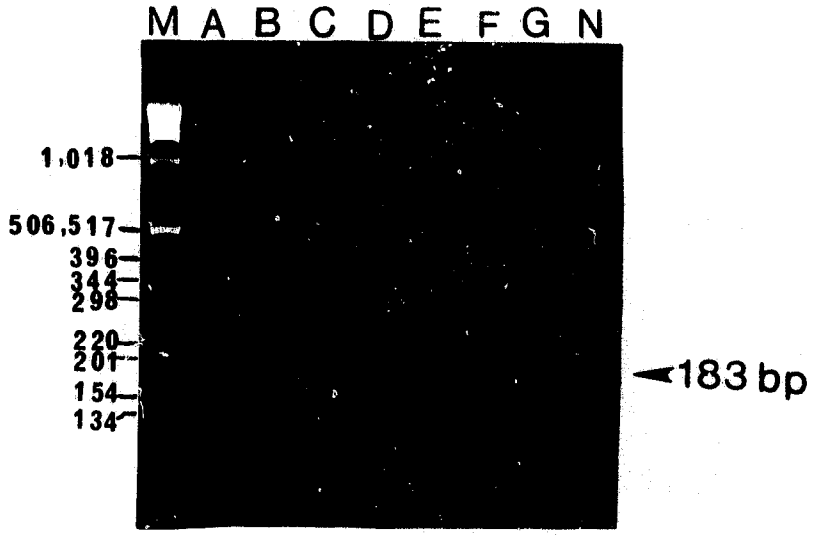

Figure 14. Detection of BHV-1 in semen from experimentally infected Bull 1 by PCR with EB staining. Lane M denotes molecular weight markers (GIBCO BRL Canada); Lanes A to G denote semen collected 7 days before inoculation, and 0, 4, 10, 20, 30, and 40 days after inoculation. Lane N denotes carryover control (as in Fig. 3). The sample in Lane C, which was collected at day 4, was positive.

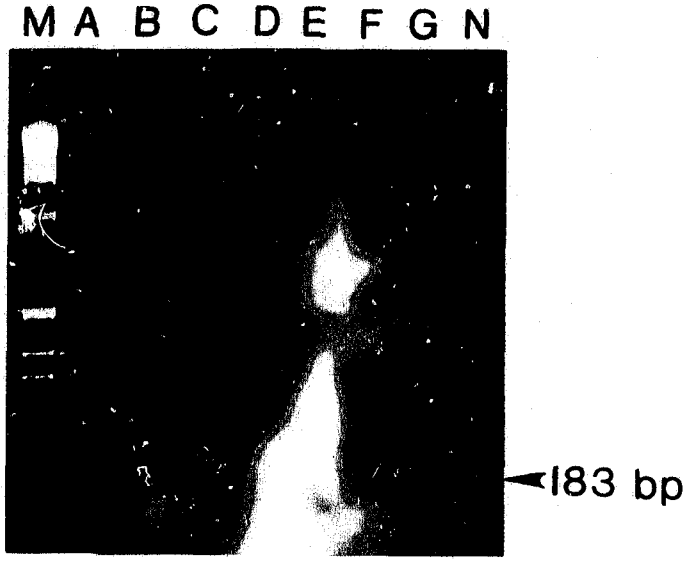

Figure 15. Detection of BHV-1 i nen from experimentally infected Bull 2 by PCR with EB staining. Lane M (tes molecular weight markers (GIBCO BRL Canada); Lanes A to G denote semen collected 7 days before inoculation, and 0, 4, 10, 20, 30, and 40 days after inoc ion. Lane N denotes carryover control (as in Fig. 4). The sample in Lane C, which was collected at day 4, was positive.

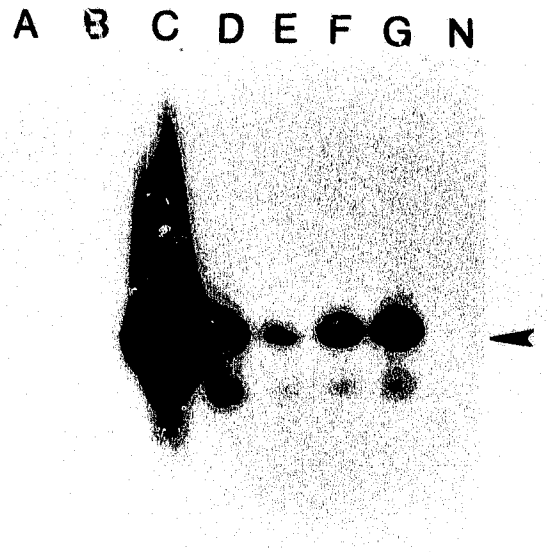

Figure 16. Detection of BHV-1 in semen from experimentally infected Bull 1 by PCR with Southern blot hybridization of gel in Fig. 14. Lanes A to G denote semen collected 7 days before inoculation, and 0, 4, 10, 20, 30, and 40 days after inoculation. Lane N denotes carryover control (as in Fig. 4). The weak bands on the filter were as a result of the X-ray film being accidentally moved during exposure. The samples collected before infection (Lanes A and B) were negative, and all the samples collected after infection (Lanes C to G) were positive.

Figure 17. Detection of BHV-1 in semen from experimentally infected Bull 2 by PCR with Southern blot hybridization of gel in Fig. 15. Lanes A to G denote semen collected 7 days before inoculation, and 0, 4, 10, 20, 30, and 40 days after inoculation. Lane N denotes carryover control (as in Fig. 4). The samples collected before infection (Lanes A and B) were negative, and the samples collected at day 4, 10, 20, and 30 (Lanes C to F) were positive.

3.5.5 Virus neutralization test

The experimental bulls did not have a BHV-1 antibody titre until 10 days after virus inoculation when titres of 1/12 for Bull 1 and 1/6 for Bull 2 were detected . Bull 1 had antibody titres of 1/24, 1/24, and 1/48, while Bull 2 had titres of 1/12, 1/16, and 1/16 on days 20, 30, and 40, respectively (Fig. 18).

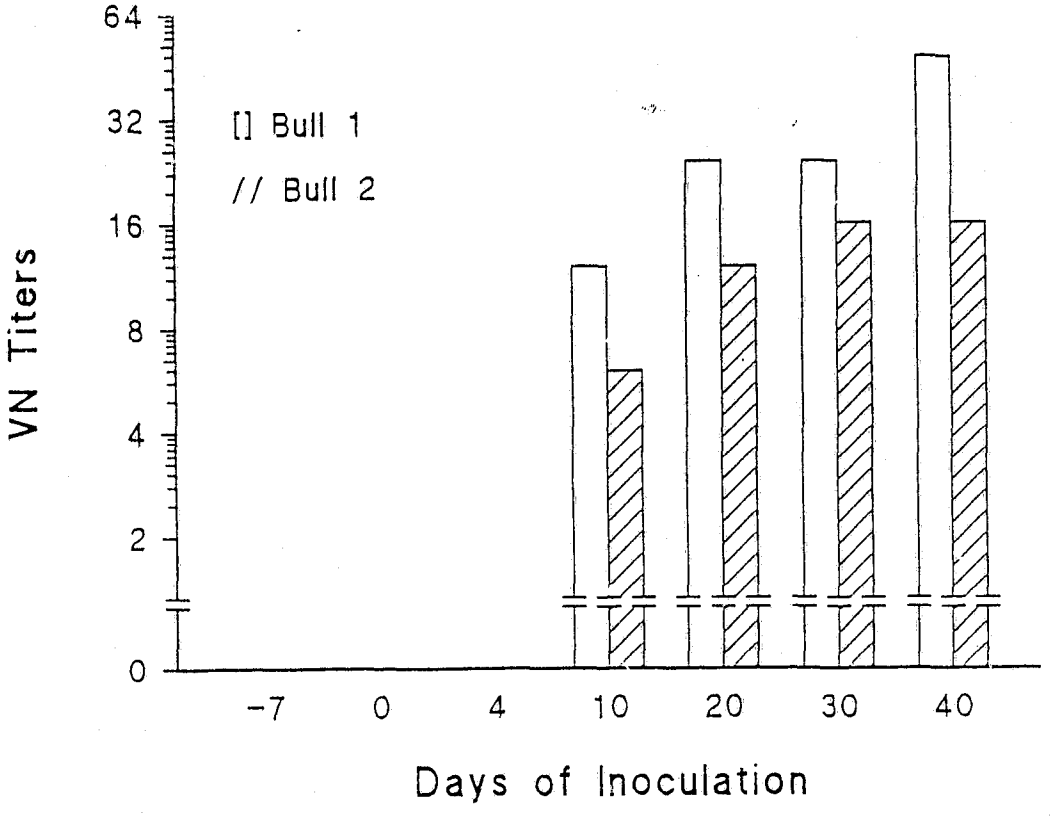

Figure 18. Virus Neutralization (VN) antibody response of Bulls infected with BHV-1. The VN antibody titre is expressed as a mean of positive values following $\log_2$ transformation of reciprocal titres. Both bulls started to develop a detectable antibody titre at day 10. The antibody titres were increasing till the last day of sample collection. Bull 1 developed a higher antibody titre than Bull 2.

3.6 Comparison of the sensitivity and specificity of dot blot hybridization, polymerase chain reaction and virus isolation for detection of BHV-1 in bovine semen samples from experimentally infected bulls

All semen samples collected before inoculation were negative for BHV-1 by dot blot hybridization, PCR, and virus isolation. Thus the specificity of all the methods used in this study was 100% within the limits of the study.

As shown in Table 7, nine positive results were detected by PCR with Southern blot hybridization, while 2 positive results were each obtained by virus isolation and by PCR with EB staining. Only one positive result was detected by dot blot hybridization. All these methods detected virus 4 days after virus inoculation, which was the earliest sample collected post infection. In contrast PCR with Southern blot hybridization detected virus also 10 days after virus inoculation, and this test remained positive for both bulls 30 days after virus inoculation and for Bull 1 even 40 days after virus inoculation, which was the last sample collected in this study. These results confirmed the initial observation that PCR with Southern blot hybridization was the most sensitive method for detection of BHV-1 in bovine semen.

Table 7: Comparison of dot blot hybridization, PCR and virus isolation for detection of BHV-1 in semen from experimentally inoculated bulls.

Days PI[a]	Dot blot hybridization		PCR				Virus isolation	
			EB staining		Southern blot			
	Bull 1	Bull 2	Bull 1	Bull 2	Bull 1	Bull 2	Bull 1	Bull 2
-7	-[b]	-	-	-	-	-	-	-
0	-	-	-	-	-	-	-	-
4	+	-	+	+	+	+	+	+
10	-	-	-	-	+	+	-	-
20	-	-	-	-	+	+	-	-
30	-	-	-	-	+	+	-	-
40	-	-	-	-	+	-	-	-

a PI denotes post-inoculation; -7 denotes seven days before inoculation.

[b] - denotes negative; + denotes positive for CPE (virus isolation) or 183 bp product (PCR amplification) or hybridization signal (Dot blot or Southern blot hybridization).

CHAPTER 4: DISCUSSION AND CONCLUSION

Bovine herpesvirus 1 (BHV-1) is one of the major infectious agents which can be transmitted between cattle by natural service or artificial insemination (Kahrs et al., 1980; Drew et al., 1987; Orischot et al., 1993; Philpott, 1993). The current methods for detection of BHV-1 in bovine semen have limitations with regard to sensitivity, time and cost (Wyler et al., 1989). Recent molecular biotechniques, such as nucleic acid hybridization and PCR have stimulated strong interest in the area of BHV-1 detection (Brunner et al., 1988; Pacciarini et al., 1988; Vilček et al., 1993; Vilček, 1993; Engelenburg et al., 1993; Wiedmann et al., 1993; Kibenge et al., 1994; Yason et al., 1995). In this thesis, dot blot hybridization and PCR, targeting the BHV-1 thymidine kinase (TK) gene region (Kibenge et al., 1994; Yason et al., 1995), were compared with virus isolation in BFT cell culture for detection of BHV-1 in bovine semen. Virus isolation in cell culture is the "gold standard" for detection of BHV-1 infections.

4.1 Virus isolation

Currently, virus isolation, the routine method for detection of BHV-1 in bovine semen, has been variously modified. For example, a pelleting method, a dilution method and an extensive washing method (Brunner et al., 1988) are frequently used to reduce the cytotoxicity of bovine semen. In the dilution method, several dilutions (1:10, 1:20, 1:40, 1:64, or 1:128) of bovine semen have been used for virus isolation

(Bielanski et al., 1988; Weiblen et al., 1992; Orischot et al., 1993). Clearly, there is no uniform standard procedure for virus isolation from bovine semen samples. In the present study, the semen dilution for BHV-1 isolation was optimized by modifying the standard procedure used for BHV-1 isolation at the Atlantic Veterinary College Regional Diagnostic Virology Laboratory and a modified washing procedure described by Darcell et al. (1977). Based on the results obtained, the best way to detect BHV-1 in bovine semen by virus isolation on primary BFT cell cultures was to use semen diluted 1:20 by the standard procedure. This dilution completely eliminated the cytotoxicity and inhibition of BHV-1 replication and CPE by bovine semen. The procedure could detect 1 $TCID_{50}$/100μl of BHV-1 in 1:20 diluted bovine semen. In the modified washing procedure, although the cytotoxicity was eliminated at 1:5 dilution of bovine semen and the inhibition of BHV-1 replicaton and CPE were never observed, a minimum of 10 $TCID_{50}$/100μl of BHV-1 could be detected in the diluted bovine semen samples. Thus, 4 times less diluted sample could be used with the modified washing procedure which had 10 times lower sensitivity than the modified standard procedure. Consequently, the modified washing procedure was 2.5 times less sensitive than the modified standard procedure. It was also more laborious than the modified standard procedure since it required an extra washing step. Therefore, the modified standard procedure was selected for use in the subsequent experiments of this study.

4.2 Dot blot hybridization

Different dilutions of semen were evaluated for detection of BHV-1 in bovine semen by dot blot hybridization to determine if there were factors that may influence the result. It was observed that virus in the undiluted bovine semen samples gave the strongest hybridization signal, followed by virus in semen diluted 1:20 and last by virus in EMEM samples. The same phenomenon was observed when the equivalent amount of virus in different dilutions of salmon sperm DNA was examined, suggesting that the sperm DNA may act as a carrier in the BHV-1 DNA extraction procedure, thereby increasing the sensitivity of the dot blot hybridization method. The effectiveness of salmon sperm DNA also indicated that use of pure foreign DNA as a carrier can increase the sensitivity of the dot blot hybridization method.

Several laboratories have described the detection of BHV-1 genomic DNA in different cell cultures and clinical samples by various hybridization techniques (Dorman et al., 1985; Dunn et al., 1986; Rock et al., 1986; Andino et al., 1987 ; Pacciarini et al., 1988; Belák et al., 1988). Although different BHV-1 genomic DNA fragments were used as probes in these studies, all were cloned in plasmid or bacteriophage vectors. Another method of producing probe sequences is by manufacturing synthetic oligonucleotides. Although these probes are single stranded and commercially available, it requires a detailed knowledge of the target sequence to produce them and they are limited to short pieces of 15-30 nucleotides long (Wolcott, 1992). This method was used to synthesize the probes used to detect PCR

products by dot blot hybridization (Vilček, 1993; Wiedmann et al., 1993). In the present study, the PCR product was first used as the DNA probe for detection of BHV-1. In comparison with other methods, PCR can produce a large amount of DNA for use as a probe more quickly and conveniently (Wright and Wynford-Thomas, 1990). A disadvantage of this method is that the *Taq* polymerase used in PCR does not have the 3'-5' exonuclease "proof-reading" function, and as a result, it has a relatively high error rate in PCR (Cha and Thilly, 1993). Each *Taq* polymerase-induced error, once introduced, will be amplified exponentially during subsequent cycles. When these products are used as probes in dot blot hybridization, they can be associated with decreased sensitivity and specificity. Consequently, it is essential to sequence PCR products. The error rate in PCR could be reduced by optimization of the reaction conditions, careful selection of target sequences and by using freshly made PCR products rather than cloned PCR products for making probes (Kocher and Wilson, 1991).

4.3 Polymerase Chain Reaction

A standard PCR protocol was developed by Kibenge et al. (1994) for detection of BHV-1 using the primer pair selected in the coding region of the BHV-1 TK gene (Kibenge et al., 1994; Yason et al., 1995). This primer pair was used because of the following reasons: (i) the TK region has the most sequence information available on BHV-1; (ii) BHV-1 can be differentiated from BHV-5 in the TK gene sequences (Smith et al., 1991); and (iii) BHV-1 with a deleted TK gene can potentially be used

as vaccine strains (Kit et al., 1986), and therefore this primer pair would be able to differentiate the BHV-1 field strains from the vaccine strains (Kibenge et al., 1994). The specificity of the primers for the BHV-1 TK gene was confirmed by failure to amplify DNA in a BHV-1 TK deletion mutant strain, 2 strains of BHV-4, a feline herpesvirus, an equine herpesvirus, a bovine adenovirus, and noninoculated bovine cell cultures (Kibenge et al., 1994).

When the PCR method, using the standard DNA purification procedure of proteinase K and RNase A digestion, followed by phenol-chloroform extraction (Kibenge et al., 1994; Yason et al., 1995) was used on bovine semen, BHV-1 DNA could be amplified and the PCR inhibitor in semen was not evident. However, since this method involved excessive sample manipulation, it was associated with cross contamination, and took at least 2 days to complete. Moreover, the sensitivity of this procedure could not be established because of loss of varying amounts of DNA during the purification and extraction procedure.

The high sensitivity of PCR makes it prone to false-positive results, since a single contaminant molecule may be amplified to a large amount. Cross contamination by previously amplified nucleic acids, which give false-positive results, is currently the greatest impediment for using PCR as a diagnostic tool (Wright and Wynford-Thomas, 1990; Rys and Persing, 1993). In order to minimize the chances of false positive reaction in this study, the following precautions were observed: (i) separate

rooms were assigned for aliqoting and preparation of clean reaction mixes, amplification and analysis of clean PCR products; (ii) use of aerosol resistant tips throughout the procedure; (iii) separate freezer for storage of clean reagents, specimen and PCR products; (iv) separate laminar flow hoods, tube openers and pipettes for clean reagents, unprocessed specimens and PCR products; (v) using undiluted 5.25% sodium hypochlorite (Javex) to clean the tube openers and the working area; (vi) wearing disposable gloves and changing them frequently; (vii) autoclaving reagents whenever possible and storing them in small aliquots; and (viii) last and most important, incorporation of a carryover control, in which distilled water was used instead of the sample, in each test or after every five samples (Yason et al., 1995). However, these procedures still could not prevent cross contamination when the BHV-1 DNA was amplified by the standard PCR protocol, since the tubes had to be opened several times during sample preparation. It was therefore desirable to develop a protocol which involved less sample manipulation.

When the GeneReleaser™ PCR method (Kibenge et al., 1994) was used in the present study, the DNA purification and extraction protocol was replaced by a one step sample preparation and 10 min thermocycle program. The GeneReleaser™ procedure efficiently minimized the cross contamination, and reduced the sample preparation time from 2 days to 10 min. It also increased the sensitivity of the PCR method by requiring less specimen volume to give a positive result. However, BHV-1 in neat bovine semen could not be detected by the GeneReleaser™ procedure, which

indicated the presence of a PCR inhibition factor in bovine semen. A PCR inhibition factor in bovine semen was also reported by Wiedmann et al (1993), and these authors used Chelex in the DNA preparation to eliminate this factor. However, this still involved a lot of preparation steps.

In the present study, the PCR inhibition factor was eliminated by diluting bovine semen 1:20 in EMEM, as was done for the virus isolation procedure to eliminate the cytotoxicity, and BHV-1 replication and CPE inhibitor. This dilution protocol with the GeneReleaser™ procedure could detect a minimum of 100 $TCID_{50}$/100µl of BHV-1 in 1:20 diluted bovine semen. Thus compared to virus isolation which could detect at least 1 $TCID_{50}$/100µl of the virus, the sensitivity of this protocol was not satisfactory, hence the optimization of the reaction conditions.

Since the Mg^{2+} concentration affects many reaction parameters in PCR, such as primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity, it is strongly recommended to optimize the Mg^{2+} concentration for each individual PCR protocol (Williams, 1989; Innis and Gelfand, 1990). Therefore, a commercial PCR Optimizer Kit (Invitrogen) was used to optimize the pH and Mg^{2+} concentration of the PCR with GeneReleaser™ for bovine semen. The reaction buffers which contained 1.5 mM of $MgCl_2$ at pH 8.5 and 2.5 mM of $MgCl_2$ at pH 9.5, gave the best amplification results. However, the commercially available *Taq*

polymerase buffer which was used in this study has a pH of 9.0. One millimolar of $MgCl_2$ was determined to be the optimal concentration at pH 9.0 for detection of BHV-1 in bovine semen diluted 1:20, while 1.5 mM $MgCl_2$ was the optimal concentration for tissue culture media samples. Thus bovine semen samples required less Mg^{2+} than tissue culture media samples. This would be expected since bovine semen contains 3.5 mM of Mg^{2+} and has a pH of 6.4-7.8 (Hafez and Garner, 1993), while tissue culture medium (EMEM, Sigma) contains only 0.8 mM of Mg^{2+} and has a pH of 7.2. Perhaps high Mg^{2+} concentrations such as in neat bovine semen inhibited the PCR at low pH buffers, since the 1:20 semen dilution in EMEM which effectively reduced the Mg^{2+} concentration also eliminated the inhibitor. Some other components in bovine semen, such as K^+, Ca^{2+} (Hafez and Garner, 1993), may also influence the yield of the PCR product (Innis and Gelfand, 1990). The sensitivity of BHV-1 PCR in bovine semen samples was increased 100-fold following the optimization of $MgCl_2$ concentration. The GeneReleaser™ procedure on bovine semen diluted 1:20 could detect 1 $TCID_{50}$/100µl of BHV-1 by EB staining, which was equivalent to the sensitivity of the optimized virus isolation procedure.

The sensitivity of PCR can be further increased by the method used to detect the PCR product (Kawai et al., 1993; Holmstrom et al., 1993). Generally, PCR-amplified DNA is identified by gel electrophoresis followed by EB staining. This detection method reveals the size of the amplified product and is a good and simple confirmatory test. In the present study, Southern blot hybridization was used to

increase the sensitivity of PCR for detection of BHV-1 in bovine semen. The GeneReleaser™ PCR method followed by Southern blot hybridization could detect a minimum of 0.01 $TCID_{50}$/100µl of BHV-1 in bovine semen diluted 1:20, which is 100-fold less virus than that which could be detected with EB staining.

4.4 Comparison of dot blot hybridization, PCR and virus isolation for detection of BHV-1 in artificially infected bovine semen

In the present study, both virus isolation and PCR with EB staining method were about 20,000-fold more sensitive than dot blot hybridization for detection of BHV-1 in the experimentally infected bovine semen samples. In semen from the naturally infected bulls, since the semen had to be diluted 1:20 prior to testing either by virus isolation or by PCR amplification, the difference between the sensitivity of either virus isolation, or PCR, and dot blot hybridization would be reduced to 1000-fold. However, PCR with Southern blot hybridization was the most sensitive method for detection of BHV-1 in bovine semen, being 100 times more sensitive than either virus isolation or PCR with EB staining. Although the sensitivity of dot blot hybridization was not satisfactory, the technique does not need special expensive equipment as required for PCR, and as such has the potential for use in resource-poor clinical settings.

4.5 Detection of BHV-1 in bovine semen of experimentally infected bulls

In the present study, the virus neutralization (VN) test was used to monitor the infections in the bulls after experimental inoculation with BHV-1 via the prepuce. The production of detectable antibodies by VN test begins approximately 8-12 days post infection and may persist for at least 5 years (Gibbs and Rweyemamu, 1977). We could not detect any VN antibody from both bulls until 10 days after inoculation. This was almost at the same time as when the clinical signs of balanoposthitis were observed. These observations indicated that the virus was released into semen before the bulls developed detectable antibody and clinical signs.

The antibody titers of both bulls kept increasing and reached the highest level on day 40, which was the last day of sample collection. Bull 1 had more severe clinical signs of infection and developed a higher VN titer than Bull 2. In addition, more virus was detected by virus isolation, dot blot hybridization and PCR in bovine semen samples collected from Bull 1 than from Bull 2.

The PCR protocol can be completed in a few hours, while it takes at least 7 days to obtain a result by virus isolation. Even PCR with Southern blot hybridization takes only 5 days, and it is much more sensitive than virus isolation. The earliest time of virus detection was 4 days post infection when the first sample was taken post-inoculation. It is possible that virus could have been detected by PCR with Southern blot hybridization as early as 24 hours after infection via the prepuce although results

would not have been known until 5 days post infection. This would still be sooner than a serological positive result. The detection of virus in semen samples collected 4 days after infection but before detectable VN antibody emphasizes the need for sensitive and early diagnostic methods for BHV-1 infection.

The interval and the duration after inoculation when the virus can be detected in different samples is variable depending upon the method used for virus detection (MacLachlan et al., 1994). This is the first report on the use of PCR and dot blot hybridization to study the duration of BHV-1 detection after animal inoculation. The data obtained in this study are consistent with the pathogenesis of BHV-1 infection in the reproductive tracts of bulls (Snowdon, 1965). The weak signal of PCR with Southern blot hybridization of the semen sample from Bull 1 on day 20 and the negative result of the semen from Bull 2 on day 40 in the present study suggested these could be intervals during which there is little or no virus shedding in semen. The similarity of the virus isolation results of the preputial swabs and semen samples in the present study supports the suggestion by Straub (1991) that natural infection of the semen usually occurs during ejaculation which results in free virus in the seminal plasma. No virus was detected in the genital tracts of cattle inoculated intranasally (Snowdon, 1965; Straub, 1990). Our data showed that virus could not be recovered in the nasal swabs of bulls inoculated with BHV-1 via the prepuce. These observations lead us to speculate that in adult animals, BHV-1 replicates and causes lesions at the primary site of infection, rather than from a viremia phase.

4.6 General discussion and conclusion

In the present study, dot blot hybridization, PCR and virus isolation for detection of BHV-1 in bovine semen were optimized, and the sensitivity, specificity, and time spent in the diagnostic laboratory were compared for detection of BHV-1 in bovine semen. Artificially infected bovine semen samples were used to optimize the four methods examined in this study. The cytotoxicity, inhibition of viral replication and CPE and the PCR inhibition factors in bovine semen could be eliminated by dilution of the bovine semen 1:20. The presence of bovine sperm DNA or salmon sperm DNA increased the sensitivity of BHV-1 detection by dot blot hybridization. After the optimization of $MgCl_2$ concentration in diluted semen samples, the sensitivity of the PCR method followed by EB staining was equivalent to that of the optimized virus isolation method. Both virus isolation and PCR with EB staining were 1000 times more sensitive than dot blot hybridization. The PCR followed with Southern blot hybridization was 100 times more sensitive than either virus isolation or PCR followed by EB staining. In the semen samples collected from experimentally infected bulls, only one semen sample was positive by dot blot hybridization, and two semen samples were positive by either virus isolation or PCR with EB staining, while 9 semen samples were positive by PCR with Southern blot hybridization. PCR with Southern blot hybridization was the most sensitive method detecting the virus for the longest period. Both bulls developed detectable antibody by day 10, which was later than the virus shedding detected by day 4. This observation confirmed the BHV-1

infection of bulls and emphasized the need for early and sensitive diagnostic methods of BHV-1 in bovine semen.

This study showed that PCR with Southern blot hybridization is the most sensitive method for detection of BHV-1 both in artificially infected semen samples and in semen collected from experimentally infected bulls, followed by both virus isolation and PCR with EB staining. The dot blot hybridization protocol used in this study was the least sensitive of all the methods studied. However, the requirement of dot blot hybridization for no special equipment makes it a potential method to be developed for BHV-1 detection in resource-poor clinical settings.

REFERENCES

ACKERMANN M, PETERHANS E, WYLER R. DNA of bovine herpes virus type 1 in the trigeminal ganglia of latently infected calves. Am J Vet Res 1982; 43: 36-40.

ACKERMANN M, BELAK S, BITSCH V. Round table on infectious bovine rhinotracheitis/infectious pustular vulovaginitis virus infection diagnosis and control. Vet Micro 1990;23: 361-363.

ANDINO RH, TORRES HN, POLACINO PS, SCHUDEL A, PALMA EL. Detection of bovine herpesvirus -1 nucleic acid sequences, using a dot-blot hybridization procedure. Am J Vet Res 1987; 48: 984-987.

ARMSTRONG JA, PLOWRIGHT W, MACADAM RF. Observations on the virus of infectious bovine rhinotracheitis, and its affinity with the herpesvirus group. Virology 1961; 14: 276.

BABIUK LA, ACRES SD, MISRA V, STOCDALE PHG, CLERCQ E. Susceptibility of bovine herpesvirus 1 to antiviral drugs: *in vitro* versus *in vivo* effect of (E)-5-(2-bromovinyl)-2'-deoxyuridine. Antimicrob Agen Chemoth 1983; 5: 715-720.

BABIUK LA, OHMANN HB, GIFFORD G, CZARNIECKI CW, SCIALLI VT, HAMILTON EB. Effect of bovine α1 interferon on bovine herpesvirus type 1 induced respiratory disease. J Gen Virol 1985; 66: 2383-2394.

BABIUK LA, L'ITALIEN J, VAN DRUNEN LITTEL-VAN DEN HURK S, ZAMB T, LAWMAN MJP, HUGHES G, GIFFORD GA. Protection of cattle from bovine herpesvirus (BHV-1) infection by immunization with individual viral glycoproteins. Virology 1987; 159: 57-66.

BARANOWSKI E, DUBUISSON J, PASTORET PP, THIRY E. Identification of 108 K, 93 K, and 42 K glycoproteins of bovine herpesvirus-1 by monoclonal antibodies. Arch Virol 1993; 133: 97-111.

BELÁK S, LINNE T, MAGYAR G, HARRACH B, BENKÖ M, KLINGEBORN B, KLINTEVALL K, BARTHA A. Bovine herpesvirus 1 : rapid diagnosis of infection by direct filter hybridization. Mol Cell Probe 1988; 2: 147-156.

BELLO LJ, WHITBECK JC, LAWRENCE WC. Map location of the thymidine kinase gene of bovine herpesvirus 1. J Gen Virol 1987; 61: 4023-4025.

BELLO LJ, WHITBECK C, LAWRENCE WC. Sequence and transcript analysis of the bovine herpesvirus 1 thymidine kinase locus. Virology 1992; 189: 407-414.

BIELANSKI A, LOEWEN KG, HARE WCD. Inactivation of bovine herpesvirus-1 (BHV-1) from in vitro infected bovine semen. Theriogeno 1988; 30: 649-657.

BIELEFELDT OHMANN H, BABIUK LA. Viral infections in domestic animals as models for studies of viral immunology and pathogenesis. J Gen Virol 1986; 66: 1-25.

BORDT DE, THOMAS PC, MARSHALL RF. Early protection against infectious bovine rhinotracheitis with intramuscularly administered vaccine. Proc Annu Meet US Anim Health Assoc 1976; 79: 50-60.

BOUTERS R, VANDEPLASSCHE M,, FLORENT A, LEUNEN J, DEVOS A. De ulcereuse balanoposthitis bij fokstieren, Vlaams diergeneesk Tijdschr 1960; 29: 171-186.

BRAKE F, STUDDERT MJ. Molecular epidemiology and pathogenesis of ruminant herpesviruses including bovine, buffalo, and caprine hepresvirus 1 and bovine encephalitis herpesvirus. Aust Vet J 1985; 62: 331-334.

BRATANICH AC, JONES CJ. Localization of cis-acting sequences in the latency-related promoter of bovine herpesvirus 1 which are regulated by neuronal cell type factors and immediate-early genes. J Virol 1992; 66: 6099-6106.

BRATANICH AC, HANSON ND, JONES CJ. The latency-related gene of bovine herpesvirus 1 inhibits the activity of immediate-early transcription unit 1. Virology 1992;191: 988-991.

BRANTANICH AC, SARDI SI, SMITSAART EN, SCHUDEL AA. Comparative studies of bovine herpesviru-1 variants by in vitro tests. J Vet Med 1991; 38: 41-48.

BRUNNER D, ENGELS M, SCHWYZER M, WYLER R. Acomparison of three techniques for detecting bovine herpesvirus type 1 (BHV-1) in naturally and experimentally contaminated bovine semen. Zuchthyg 1988; 23: 1-9.

CARPENTER CE, MISRA V. The most abundant protein in bovine hepres 1 virions is a homologue of herpes simplex virus type 1 UL47. J Gen Virol 1991; 72: 3077-3084.

CHA RS, THILLY WG. Specificity, efficiency and fidelity of PCR. PCR Meth Appli 1993; 3: 518-529.

CHAPMAN MS, LUCAS MH, HERBERT CN, GOODEY RG. Survival of infectious bovine rhinotracheitis in stored frozen semen. Vet Sci Comm 1979; 3: 137-139.

CHOW TL, PALOTAY JL, DEEM WA. Infectious rhinotracheitis in feedlot cattle. III. An epizootiological study in a feedlot. J Am Vet Med Assoc 1956; 128: 348-351.

CLERCQ E, DESCAMPS J, VERHELST G, WALKER RT, JONES AS, TOURRENCE PF, SHUGAR D. Comparative efficacy of different herpes drugs against different strains of herpes simplex virus. J Infect Dis 1980; 141: 563-574.

CLERCQ E, VERHELST G, DESCAMPS J, BERGSTROM DE. Differential inhibition of herpes simplex viruses, type 1(HSV-1) and type 2 (HSV-2) by (E)-5-(2-X-vinyl-2-deoxyuridine). Acta Microbiol Acad Hung 1981; 28: 307-312.

COLLINS JK, BUTCHER AC, RIEGEL CA. Immune response to bovine herpesvirus type-1 infections: Virus-specific antibodies in sera from infected animals. J Clin Microbiol 1985; 21: 546-552.

CURTIS RA, ANGULO A. A field trial to evaluate an intranasal infectious bovine rhinotracheitis virus vaccine. Can Vet J 1974; 15 : 327-330.

DARCEL C, DORWARD QJ. Skin reactivity and infectious bovine rhinotracheitis. Can Vet J 1972; 13: 100-101.

DARCEL C, YATES WDC, MITCHELL D. A technique for the isolation of bovine herpesvirus 1 from bull semen using microfilter plates. Proc Amer Assoc Vet Lab Diagn 1977; 20: 209-214.

DARCEL C. Pseudopositive ELISA results for infectious bovine rhinotracheitis IgG reactors in three artificial insemination studies. Can Vet J 1992; 33: 407.

DEREGT D, CHO HJ, KOZUB GC. A comparative evaluation of two sensitive serum neutralization tests for bovine herpevirus-1 antibodies. Can J Vet Res 1993; 57: 56-59.

DONKERSGOED JV, BABIUK LA. Diagnosing and managing the respiratory form of infectious bovine rhinotracheitis. Vet Med 1991; : 86-94.

DORMAN MA, BLAIR CD, COLLINS JK, BEATY BJ. Detection of bovine herpesvirus 1 DNA immobilized on nitrocellulose by hybridization with biotinylated DNA probe. J Clin Microbiol 1985; 22: 990-995.

DREW TW, HEWITT-TAYLOR C, WATSON L, EDWARDS S. Effect of storage conditions and culture technique on the isolation of infectious bovine rhinotracheitis virus form bovine semen. Vet Rec 1987; 121: 547-548.

DRUNEN LITTEL-VAN DEN HURK S, VAN DEN HURK JV, GILCHRIST JE, MISRA V, BABIUK LA. Interactions of monoclonal antibodies and bovine herpesvirus type 1 (BHV-1) glycoproteins: characterization of their biochemical and immunological properties. Virology 1984; 135: 466-479.

DRUNEN LITTEL-VAN DEN HURK S, BABIUK LA. Antigenic and immunogenic characteristics of bovine herpesvirus type-1 glycoproteins GVP 3/9 and GVP 6/11/16, purified by immunoadsorbent chromatography. Virology 1985; 144: 204-215.

DRUNEN LITTEL-VAN DEN HURK S, BABIUK LA. Synthesis and processing of bovine herpesvirus 1 glycoproteins. J Virol 1986; 59: 401-410.

DRUNEN LITTEL-VAN DEN HURK S, GIFFORD GA, BABIUK LA. Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. Vaccine 1990; 8: 358-368.

DRUNEN LITTEL-VAN DEN HURK S, VAN DEN HURK JV, GILCHRIST JE, MISRA V, BABIUK LA. Interactions of monoclonal antibodies and bovine herpesvirus (BHV-1) glycoproteins: characterization of their biochemical and immunological properities. Virology 1984; 135: 466-479.

DRUNEN LITTEL-VAN DEN HURK S, PARKER MD, FITZPATRICK DR, VAN DEN HURK JV, CAMPOS M, BABIUK LA, ZAMB T. Structural, functional, and immunological characterization of bovine herpesvirus-1 glycoprotein gI expressed by recombinant baculovirus. Virology 1992a; 190: 378-392.

DRUNEN LITTEL-VAN DEN HURK S, PARKER MD, MASSIE B, VAN DEN HERK JV, BABIUK LA, ZAMB TJ. Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. Vaccine 1992b; 11: 25-35.

DRUNEN LITTEL-VAN DEN HURK S, TIKOO SK, LIANG X, BABIUK LA. Bovine herpesvirus-1 vaccines. Immunol Cell Biol 1993; 71: 405-420.

DUNGWORTH DL. The respiratory system. In: Jubb KVF, Kennedy PC, Palmer N, eds. Pathology of domestic animals (Vol 2). San Diego: Academic Press, Inc. 1993: 556-558.

DUNN DC, BLAIR CD, WARD DC, BEATY BJ. Detection of bovine herpesvirus specific nucleic acids by in situ hybridization with biotinylated DNA probe. Am J Vet Res 1986; 47 : 740-746.

EDWARDS S, CHASEY D, WHITE H. Experimental infectious bovine rhinotracheitis: comparison of four antigen detection methods. Res Vet Sci 1983; 34: 42-45.

ELION GB. Mechanism of action and selectivity of acyclovir. Am J Med 1982; 73: 7-13.

ENGELENBURG FAC, KAASHOEK MJ, RIJSEWIJK FAM, VAN DEN BURGB, MOERMAN A, GIELKENS ALJ, VAN OIRSCHOT JT. Deletion of the glycoprotein E gene or thymidine kinase gene of bovine herpesvirus type 1 produces attenuated vaccine strains. 17th International Herpesvirus Workshop. Abstract No. 379. 1992: Edinburgh, Scotland, UK. .

ENGELENBURG FAC, MAES RK, VAN OIRSCHOT JT, RIJSEWIJK FAM. Development of a rapid and sensitive polymerase chain reaction assay for detection of bovine herpesvirus type 1 in bovine semen. J Clin Microbiol 1993; 31: 3129-3135.

ENGELS M, STECK F, WYLER R. Comparison of the genomes of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis virus strains by restriction endonuclease analysis. Arch Virol 1981; 67: 169-174.

ENGELS M, STECK F, WYLER R. Comparison of the genomes of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis virus strains by restriction endonuclease analysis. Arch Virol 1981; 67: 169-174.

ENGELS M, GIULIANI C, WILD P, BECK TM, LOEPFE E, WYLER R. The genome of BHV-1 strains exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross hybridization. Virus Res 1986;6: 57-73.

EUGSTER AK, ANGULOS AB, JONES LP. Herpesvirus encephalitis in range calves. Pro Ann Mtg Am Assoc Vet Lab Diagn 1974;17: 267-290.

FALSON P. Improved phenol-based method for the isolation of DNA fragments from low melting temperature agarose gels. BioTechniques 1992; 13: 25-27.

FEHLER F, HERRMANN J, SAALMULLER A, METTENLEITER TC, KEIL GM. Glycoprotein IV of bovine herpesvirus 1 expressing cell line complements and rescues a conditionally lethal viral mutant. J Virol 1992; 66: 831-839.

FENNER FJ, GIBBS EPJ, MURPHY FA, ROTT R, STUDDERT MJ, WHITE DO. Immune responses to viral infection. In: Veterinary virology. San Diego: Academic Press Inc, 1993: 137-159.

FIELD HJ. Resistance and latency. Brit Med Bul 1985;41: 345-350.

FIELD HJ, OWEN L. The problem of virus drug resistance in antiviral drug development. In: Clercq ED edts Antiviral drug development: A multidisciplinary approach, New York and London: Plenum Press, 1988: 203-236.

FITZPATRICK DR, ZAMB T, PARKER MD, VAN DRUNEN LITTEL-VAN DEN HURK S, BABIUK LA, LAWMAN MJP. Expression of bovine herpesvirus-1 glycoproteins gI and gIII in transfected murine cells. J Virol 1988; 62: 4239-4248.

FITZPATRICK DR, BABIUK LA, ZAMB TJ. Nucleotide sequence of bovine herpesvirus type 1 glycoprotein gIII, a structure model for gIII as a new member of the immunoglobulin superfamily, and implications for the homologous glycoproteins of other herpesviruses. Virology 1989; 173: 46-57.

FITZPATRICK DR, ZAMB TJ, BABIUK LA. Expression of bovine herpesvirus type 1 glycoprotein gI in transfected cells induces spontaneous cell fusion. J Gen Virol 1990; 71: 1215-1219.

FORMAN A, BABIUK LA, MISRA V, BALDWIN F. Susceptibility of bovine macrophages to infectious bovine rhinotracheitis virus infection. Infect Immunol 1982; 35: 1048-1057.

FRAEFEL C, WIRTH UV, VOGT B, SCHWYZER M. Immediate-early transcription over covalently joined genome ends of bovine herpesvirus 1: the *circ* gene. J Virol 1993; 67: 1328-1333.

FRAEFEL C, ZENG J, CHOFFAT Y, ENGELS M, SCHWYZER M, ACKERMANN M. Identification and zinc dependence of the bovine herpesvirus 1 transactivator protein BICP0. J Virol 1994; 68: 3154-3162.

FRASER NW, VALYI-NAGY T. Viral, neuronal and immune factors which may influence herpes simplex virus (HSV) latency and reactivation. Micro. Patho. 1993; 15: 83-91.

FRIEDLI K, METZLER AE. Reactivity of monoclonal antibodies to proteins of a neurotropic bovine herpesvirus (BHV-1) strain and to proteins of representative BHV-1 strains. Arch Virol 1987; 94: 109-122.

GILLESPIE JH, MCENTEE K, KENDRICK JW, WAGNER WC. Comparison of infectious pustular vulvovaginitis virus with infectious bovine rhinotracheitis virus. Cornell Vet 1959; 49: 288.

GIBBS EPJ, RWEYEMAMU MM. Bovine herpesviruses. Part I. Bovine herpesvirus 1. Vet Bull 1977; 47: 317-343.

GODING CR, O'HARE P. Herpes simplex virus Vmw65-Octamer binding protein interaction: A paradigm for combinatorial control of transcription. Virology 1989; 173: 363-367.

GUERIN B. IBR/IPV effects on reproduction and control in AI stud. Pro Meet AI Vet EEC, Luxembourg 1989;1.

HAFEZ ESE, GARNER DL. Spermatozoa and seminal plasma. In: Hafez E.S.E. ed. Reproduction in farm animals. Philadelphia: Lea and Febiger, 1993: 165-187.

HAMMERSCHMIDT W, LUDWIG H, BUHK H. Specificity of cleavage in replicative-form DNA of bovine herpesvirus 1. J Virol 1988; 62: 1355-1363.

HJERPE CA. Bovine vaccines and herd vaccination programs. Vet Clin N Am Food Anim Prac 1990; 6: 188-194.

HOLMSTROM K, ROSSEN L, RASMUSSEN OF. A highly sensitive and fast nontadioactive method for detectin of polymerase chain reaction products. Analy Biochem 1993; 209: 278-283.

HOMAN EJ, EASTERDAY BC. Experimental latent and recrudescent bovine herpesvirus 1 infections in calves. Am J Vet REs 1983;44: 309-313.

HUCK RA, MILLAR PG, EVANS DH, STABLES JW, ROSS A. Penoposthitis associated with infectious bovine rhinotracheitis / infectious pustular vulvovaginitis (IBR/IPV) virus in a stud of bulls. Vet Rec 1961; 88: 292-297.

HUGHES G, BABIUK LA, VAN DRUNEN LITTEL-VAN DEN HURK S. Functional and topographical analysis of epitopes on bovine herpesvirus type 1 glycoprotein IV. Arch Virol 1988; 103: 47-60.

HYLAND SJ, EASTERDAY BC, PAWLISCH R. Antibody levels and immunity to infectious bovine rhinotracheitis (IBR) infections in Wisconsin dairy cattle. Dev Biol Stand 1975; 28: 510-525.

INNIS MA, GELFAND DH. Optimization of PCRs. In: PCR protocols: A guide to methods and applications. Academic Press, Inc, 1990: 3-12.

ISRAEL BA, MARSHALL RL, LETCHWORTH GL, LETCHWORTH GJ. Epitope specificity and protective efficacy of the bovine immune response to bovine herpesvirus-1 glycoprotein vaccines. Vaccine 1988; 6: 249-356.

JENSEN R, PIERSON RE, BRADDY PM. Shipping fever pneumonia in yearling feedlot cattle. J Am Vet Med Assoc 1976; 169: 500-506.

JERICHO KWF, YATES WDG, BABIUK LA. Bovine herpesvirus 1 vaccination against experimental bovine herpsvirus-1 and *Pasteurella haemolytica* respiratory tract infection: onset of protection. Am J Vet Res 1982; 43: 1776-1780.

JERICHO KWF, CARTER GR. Pneumonia in calves produced with aerosols of *Pasteurella multocida* alone and in combination with bovine herpesvirus 1. Can J Comp Med 1985; 49: 138-144.

JONES C, DELHON G, BRATANICH A, KUTISH G, ROCK D. Analysis of the trascriptional promoter which regulates the latency-related transcript of bovine herpesvirus 1. J Virol 1990; 64: 1164-1170.

KAHRS RF. Infectious bovine rhinotracheitis. A review and update. J Am Vet Med Assoc 1977; 171: 1055-1064.

KAHRS RF, SCHULTZ RD, BEAN BH, Epidemiologic considerations regarding the detection of infectious bovine rhinotracheitis virus in bovine semen. Proc Amer Assoc Vet Lab Diag 1976;19: 385-394.

KAHRS RF, JOHNSON ME, BENDER GM. Studies on the detection of infectious bovine rhinotracheitis virus in bovine semen. Proc Amer Assoc Vet Lab Diag 1977; 20: 187-208.

KAHRS RF, GIBBS EPJ, JARSEN RE. The search for viruses in bovine semen, a review. Theriogenol 1980; 14: 151-165.

KAWAI S, MAEKAWAJIRI S, YAMANE A. A simple method of detecting amplified DNA with immobilized probes on microtiter wells. Analy Biochem 1993; 209: 63-69.

KENDRICK JW, GILLESPIE JH, MCENTEE K. Infectious pustular vulvovaginitis of cattle. Cornell Vet 1958; 48: 458-495.

KENDRICK JW. Effects of the infectious bovine rhinotracheitis virus on the fetus. J Am Vet Med Assn 1973; 163:852-854.

KIBENGE FSB. Differential detection of infectious bursal disease virus serotypes, using cDNA probes to VP2 coding region. Am J Vet Res 1992; 53: 1337-1342.

KIBENGE FSB, HARRIS LM, MCKENNA PK, WADOWSKA D, YASON CV. Amplification of strains of bovine herpesvirus 1 by use of polymerase chain reaction with primers in the thymidine kinase region. Am J Vet Res 1994; 55: 1206-1212.

KIT S. Thymidine kinase. Microbiol Sci 1985;2: 369-375.

KIT S, QAVI H. Thymidine kinase (TK) induction after infection of TK-deficient rabbit cell mutants with bovine herpesvirus type 1(BHV-1): isolation of TK- BHV-1 mutants. Virology 1985; 130: 381-389.

KIT M, KIT S. Thymidine kinase deletion mutants of bovine herpesvirus 1, vaccines against infectious bovine rhinotracheitis containing same and methods for the production and use of the same. USA patent application Number 796.840, 1986.

KIT S, KIT M, MCCONNELL S. Intramuscular and intravaginal vaccination of pregnant cows with thymidine kinase-negative, temperature resistant infectious bovine rhinotracheitis virus (bovine herpes virus 1). Vaccine 1986; 4: 55-61.

KIT S, KIT M, PIRTLE EC. Attenuated properties of thymidine kinase-negative deletion mutant of pseudorabies virus. Am J Vet Res 1985; 6: 1359-1367.

KIT S, OTSUKA H, KIT M. Gene-deleted IBRV marker vaccine. Vet Rec 1990; 127: 363-364.

KOCHER TD, WILSON AC. DNA amplification by the polymerase chain reaction. In: Brown, T.A. ed. Essential molecular biology- a practical approach (Vol II). Oxford: IRL Press, 1991: 202.

KOHLER H, KUBIN G. The virology, serology and paramorphology of the male genitalia after natural and experimental infection with IBR/IPV virus. Dtsch Tierartzl Wschr 1972; 79: 209-214.

KUCERA CJ, WHITE RG, BECKENHAUER WH. Evaluation of the safety and efficacy of an intranasal vaccine containing a temperature-sensitive strain of infectious bovine rhinotracheitis virus. Am J Vet Res 1978; 39: 607-610.

KUPFERSCHMIED HU, KIHM U, BACHMANN P, MULLER K, ACKERMANN M. Transmission of IBR/IPV virus in bovine semen: a case report. Theriogen 1986; 25: 439-443.

KUTISH G, MAINPRIZE T, ROCK D. Characterization of the latency-related transcriptionally active region of the bovine herpesvirus 1 genome. J Virol 1990; 64: 5730-5737.

LABOISSIERE S, TRUDEL M, SIMARD C. Characterization and transcript mapping of a bovine herpesvirus type 1 gene encoding a polypeptide homologous to the herpes simplex virus type 1 major tegument proteins VP13/VP14. J Gen Virol 1992; 73: 2941-2947.

LAWRENCE WC, D'URSO RC, KUNDEL CA, WHITBECK JC, BELLO LJ. Map location of the gene for a 130,000-Dalton glycoprotein of bovine herpesvirus 1. J Virol 1986; 60: 405-414.

LEARY TP, SPLITTER GA. Infectious bovine rhinotracheitis. In: Castro AE and Heuschele WP eds. Veterinary Diagnostic Virology. St. Louis: Mosby-Year Book Inc, 1992: 103-106.

LEUNG-TACK P, AVDONNET J, RIVIERE M. The complete DNA sequence and the genetic organization of the short unique region of the bovine herpesvirus type 1 (ST strain). Virol 1994; 199: 409-421.

LIANG X, BABIUK LA, VAN DRUNEN LITTEL-VAN DEN HURK S, FITZPATRICK DR, ZAMB TJ. Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. J Virol 1991a; 65: 1124-1132.

LIANG XP, BABIUK LA, ZAMB TJ. An *in vivo* study of a glycoprotein gIII-negative bovine herpesvirus 1 (BHV-1) mutant expressing β galactosidase: evaluation of the role of gIII in virus infectivity and its use as a vector for mucosal immunization. Virology 1991b; 189: 629-639.

LIANG XP, BABIUK LA, ZAMB TJ. Pseudorabies virus gIII and bovine herpesvirus 1 gIII share complementary functions. J Virol 1991c; 65: 5553-5557.

LIANG XP, BABIUK LA, ZAMB TJ. An *in vitro* study of glycoprotein gIII-negative bovine herpesvirus 1(BHV-1) mutant expressing β-galactosidase: evaluation of the role of gIII in virus infectivity and its use as a vector for mucosal immunization. Virology 1992; 189: 629-639.

LIANG XP, TANG M, MANNS B, BABIUK LA, ZAMB TJ. Identifiction and deletion mutagenesis of the bovine herpesvirus 1 dUTPase gene and a gene homologous to herpes simplex viurs UL49.5. Virology 1993; 195: 42-50.

LORETU K, MARINOV P, GENOV I, BOHNEL H. Virus isolations from cases of infectius bovine pustular vulvovaginitis and posthitis (IPV/IPB) in cattle in Tanzania. Bull epizoot Dis Afr 1974; 22: 303-309.

LUDWIG H. Bovine herpesviruses. In: Roizman B eds The herpesviruses. New York: Plenum Press, 1983: 135-214.

MACLACHLAN NJ, NUNAMAKER RA, KATZ JB, SAWYER MM, AKITA GY, OSBURN BI, TABACHNICK WJ. Detection of bluetongue virus in the blood of inoculated calves: comparison of virus isolation, PCR assay, and *in vitro* feeding of *Culicoides variipennis*. Arch Virol 1994; 136: 1-8.

MADIN SH, YORK CJ, MCKERCHER DG. Isolation of the infectious bovine rhinotracheitis virus. Science 1956; 124: 721-722.

MANSERVIGI RP, SPEAR PG, BUCHAN A. Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc Natl Acad Sci USA 1977; 74: 3913-3917.

MARSHALL RL, RODRIGUEZ LL, LETCHWORTH GJ III. Characterization of envelope proteins of infectious bovine rhinotracheitis virus (Bovine herpesvirus 1) by biochemical and immunological methods. J Virol 1986; 57: 745-753.

MAYFIELD JE, GOOD PJ, VANOORT HJ, CAMPBELL AR, REED DE. Cloning and cleavage site mapping of DNA from bovine herpesvirus 1 (Cooper strain). J Virol 1983; 47: 259-264.

MCKERCHER DG, MOULTON JE, KENDRICK JW, SAITO J. Recent development on upper respiratory disease of cattle. Proc Annu Meet US Amin Health Assoc 1955; 59: 151-172.

MCKERCHER DG. Studies of the etiologic agents of infectious bovine rhinotracheitis and Bläschenausschlag (coital vesicular exanthema). Am J Vet Res 1963;24: 508-510.

MCKERCHER DG, CRENSHAW GL. Comparative efficacy of intranasally and parenterally administered infectious bovine tracheitis vaccine. J Am Vet Med Assoc 1971; 159: 1362-1369.

MCKERCHER DG. Viruses of other vertebrates. In: Kaplan AS, ed. The Herpesvirus. New York: Academic Press, 1973: 427-493.

METZLER AE, MATILE H, GASSMANN U, ENGELS M, WYLER R. European isolates of bovine herpesvirus 1: a comparison of restriction endonuclease sites, polypeptides, and reactivity with monoclonal antibodies. Arch Virol 1985; 85: 57-69.

METZLER AE, SCHUDEL AA, ENGELS M. Bovine herpesvirus 1: Molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease. Arch Virol 1986; 87: 205-217.

MEYER AL, PETROVSKIS EA, DUFFUS WPH, THOMSEN DR, POST LE. Cloning and sequencing of an infectious bovine rhinotracheitis (BHV-1) gene homologous to glycoprotein gH of herpes simplex virus. Biochim Biophys Acta 1991; 1090: 267-269.

MILLER JM. Infectious necrotic rhinotracheitis in cattle. J Am Vet Med Assoc 1955; 126: 463-467.

MILLER JM. The effects of IBR virus infection on reproductive function of cattle. Vet Med 1991;86: 95-98.

MILLER JM, WHETSTONE CA, MAATEN VAN DER MJ. Abortifacient property of bovine herpesvirus type 1 isolates that represent three subtypes determined by restriction endonuclease analysis of viral DNA. Am J Vet Res 1991a; 52: 458-461.

MILLER JM, WHETSTONE CA, BELLO LJ, LAWRENCE WC. Determination of ability of a thymidine kinase-negative deletion mutant of bovine herpesvirus-1 to cause abortion in cattle. Am J Vet Res 1991b: 52: 1038-1043.

MISRA V, BLUMENTHAL RM, BABIUK LA. Proteins specified by bovine herpesvirus 1 (infectious bovine rhinotracheitis virus). J Virol 1981; 40: 367-378.

MISRA V, BABIUK LA, DARCEL C. Analysis of bovine herpes virus-type 1 isolates by restriction endonuclease fingerprinting. Arch Virol 1983; 76: 341-345.

MITCHELL D. An outbreak of abortion in a dairy herd following inoculation with an intramuscular infectious bovine rhinotracheitis vaccine. Can Vet J 1974; 15: 148-150.

MITTAL SK, FIELD HJ. Analysis of the bovine herpesvirus type 1 thymidine kinase (TK) gene from wild-type virus and TK-deficient mutants. J Gen Virol 1989; 70: 901-918.

MOHANTY SB. Bovine respiratory viruses. Adv Vet Sci Comp Med 1978; 22: 83-109.

MOREIN B, SIMONS K. Subunit vaccines against enveloped viruses: Virosomes, micelles and other protein complexes. Vaccine 1985; 3: 83-93.

MSOLLA PM, ALLAN EM, SELMAN IE. WISEMAN A. Reactivation and shedding of bovine herpesvirus 1 following *Dictyocaulus viviparus* infection. J Comp Pathol 1983; 93: 271-274.

MYERS MW, GLASGOW LA, GALASSO GJ. Summary of a workshop on antiviral agents for genital herpesvirus infection. J Infect Dis 1982; 145: 774-482.

NARITA M, INUI S, MURAKAMI Y, NAMBA K, SHIZU Y. Pathological changes in young and adult cattle after intranasal inoculation with infectious bovine rhinotracheitis virus. J Comp Pathol 1982; 92: 41-49.

NARITA M, INUI S, NAMBA K, SHIMIZU Y. Recrudescence of infectious bovine rhinotracheitis virus and associated neural changes in calves treated with dexamethasone. Am J Vet Res 1981; 42: 1192-1197.

NORRILD B, SHORE SL, CROMEANS TL, NAHMIAS AJ. Participation of three major glycoprotein antigens of herpes simplex virus type 1 early in the infectious cycle as determined by antibody-dependent cell-mediated cytotoxicity. Infect Immun 1980; 28: 38-44.

NYAGA P, MCKERCHER DG. Pathogenesis of bovine herpesviru-1 (BHV-1) infection: interactions of the virus with peripheral bovine blood cellular components. Comp Immunol Microbiol Infect Dis 1979; 2: 587-602.

OKAZAKI K, HONDA E, MINETOMA T, KUMAGAI T. Mechanisms of neutralization by monoclonal antibodies to different antigenic sites on the bovine herpesvirus type 1 glycoproteins. Virology 1986; 150: 260-264.

ORISCHOT JT, STRAVER PJ, VAN LIESHOUT AH, QUAK J, WESTENBRINK F, VAN EXSEL ACA. A subclinical infection of bulls with bovine herpesvirus type 1 at an artificial insemination centre. Vet Rec 1993; 132: 32-35.

OWEN LJ, FIELD HJ. Genomic localization and sequence analysis of the putative bovine herpesvirus-1 DNA polymerase gene. Arch Virol 1988; 98: 27-38.

PACCIARINI M, AGRESTI A, DESIMONE F, POLI G, TORRETTA E, SICCARDI AG, MENEVERI R, GINELLI E. Detection of bovine herpes virus 1 (BHV-1) semen infections by a dot blot hybridization assay. Brit Vet J ; 1988; 144: 55-63.

PARSONSON IM, SNOWDON WA. The effect of natural breeding and artificial breeding using bulls infected with a semen contaminated with IBR virus. Austr Vet J 1975; 51: 365-369.

PASTORET PP, BABIUK LA, MISRA V, GRIEBEL P. Reactivation of temperature-sensitive and non-temperature-sensitive infectious bovien rhinotracheitis with cexamethasone. Infect Immun 1980; 29: 483-488.

PAUL PS. Applications of nucleic acid probes in veterinary infectious diseases. Vet Microb 1990; 24 : 409-417.

PETER CP, GRATZEK JB, RAMSEY FK. Isolation and characterization of a strain of infectious bovine rhinotracheitis virus associated with enteritis in cattle. pathogenesis studies by fluorescent antibody tracing. Am J Vet Res 1966; 27: 1583-1590.

PHILPOTT M. The dangers of disease transmission by artificial insemination and embryo transfer. Br Vet J 1993; 149: 339-369.

RAMSHAW IA, ANDREW ME, PHILIPS SM, BOYLE DV, COUPAR BEH. Recovery of immunodeficient mice from a vaccinia virus/IL-2 recombinant infection. Nature (London) 1987; 317: 813-815.

RICHARDS JT, KERN ER, OVERALL JC, GLASGOW LA. Anti-herpesvirus activity of adenine arabinoside analogues in tissue culture and a genital infection of mice and guinea pigs. Antiviral Res 1982; 2: 27-39.

RIJSEWIJK FAM, MAGDALENA J, MOEDT J, KAASHOEK MJ, MARIS-VELDHUIS MA, GIELKENS J, OIRSCHOT. Identification and functional analysis of glycoprotein E of bovine herpesvirus type 1. 17th International herpesvirus workshop,1992. Abstract No. 245. Edinburgh, Scotland, UK..

ROCK DL, HAGEMOSER W, OSORIO F, REED DE. Detection of bovine herpesvirus type 1 RNA in trigeminal ganglia of latently infected rabbits by in sity hybridization. J Gen Virol 1986; 67: 2515-2520.

ROCK DL, REED DE. Persistent infection with bovine herpesvirus 1: rabbit model. Infect Immun 1982; 35: 371-373.

ROIZMAN B, SEARS AE. Herpes simplex viruses and their replication. In: Fields Bh, Knipe DM et al., eds. Fields' viriology, vol 2, 2nd edn. New York: Raven Press, 1990: 1795-1894.

ROIZMAN B, DESROSIERS DC, FLECKENSTEIN B, LOPEZ C, MINSON AC, STUDDERT MJ. The family *Herpesviridae*: An update. Arch Virol 1992; 123: 425-449.

ROIZMAN B, CARMICHAEL LE, DEINHARDT F, DE-THE G, NAHMIAS AJ, PLOWRIGHT W, RAPP F, SHELDRICK P, TAKAHASHI M, WOLF K. Herpesviridae. Definition, provisional nomenclature, and taxonomy. Intervirology 1981; 16: 201-217.

RYS PN, PERSING DH. Preventing false positives: quantitative evaluation of three protocols for inactiviation of polymerase chain reaction amplification products. J Clin Microbiol 1993; 31: 2356-2360.

SARMIENTO M, SPEAR PG. Membrane proteins specified by herpes simplex viruses. Conformation of the virion glycoprotein designated VP7 (B2). J Virol 1979; 29: 1159-1167.

SAXEGAARD F. Problems connected with the diagnosis of subclinical infection with infectious pustular vulvovaginitis virus (IPV virus) in bulls. Mord Vet Med 1966; 18: 452-459.

SAXEGAARD F. Infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis (IBR/IPV) virus infection of cattle with particular reference to genital infectious. Vet Bull 1970; 40: 605-611.

SCHROEDER RJ, MOYS MD. An acute upper respiratory infectious of dairy cattle. J Am Vet Med Assoc 1954; 125: 471-472.

SCHULTZ RD, ADAMS LS, LETCHWORTH G, SHEFFY BE, MANNING T, BEAN B. A method to test large numbers of bovine semen samples for viral contamination and results of a study using this method. Theriogenol 1982; 17: 115-123.

SEAL BS, IRVING JM, WHETSTONE CA. Transcriptional analysis of the bovine herpesvirus 1 Cooper isolate temporal analysis and characterization of immediate-early, early, and late RNA. Arch Virol 1991; 121: 55-73.

SEALEY PG, SOUTHERN EM. Gel electrophoresis of DNA. In: Rickwood D, Hames ED, 2nd eds. Edl electrophoresis of nucleic acids. A practical approach series. Oxford University: IRL Press, 1990: 51-99.

SIMARD G, NADON F, SEGIUN C, LABOISSIERE S, TRUDEL M. Gene mapping of infectious bovine rhinotracheitis viral DNA genome. Arch Virol 1990; 110: 63-75.

SHEFFY EB, DAVIES DH. Reactivation of a bovine herpesvirus after corticosteroid treatment. Proc Soc Exp Biol Med 1972; 140: 974-976.

SMITH GA, YOUNG PL MATTICK JS. The location and nucleotide sequence of the thymidine kniase gene of bovine herpesvirus type 1.2. J Gen Virol 1990; 71: 2417-2424.

SNOWDON WA. The IBR-IPV virus: reaction to infection and intermittent recovery of virus from experimentally infected cattle. Austr Vet J 1965; 41: 135-141.

SPRADBROW PB. Isolation of infectious bovine rhinotracheitis virus from bovine semen. Austr Vet J 1968; 44:410-412.

STRAUB OC. Infectious bovine rhinotracheitis virus. In: Dinter Z, Morein B, eds. Virus infections of ruminants. New York: Elservier Science, 1990: 71-108.

STRAUB OC. BHV-1 infections: relevance and spread in Europe. Comp Immunol Microbiol Infect Dis 1991; 14: 175-186.

SUTTON ML. Rapid onset of immunity in cattle after intramuscular injection of a modified-live-virus IBR vaccine. Vet Med 1980; 75 :1447-1456.

THIRY E, SALIKI J, BUBLOT M, PASTORET PP. Reactivation of infectious bovine rhinotracheitis virus by transport. Comp Immun Microbiol Infect Dis 1987; 10: 59-63.

THIRY E, SALIKI J, SCHWERS A, PASTORET PP. Parturition as a stimulus of IBR virus reactivation. Vet Rec 1985; 116: 599-600.

TIKOO SK, FITZPATRICK DR, BABIUK LA, ZAMB TJ. Molecular cloning, sequencing, and expression of functional bovine herpesvirus glycoprotein gIV in transfected bovine cells. J Virol 1990; 64: 5132-5142.

TIZARD I. Secondary immunological defects. In: Veterinary Immunology: an introduction. Philadelphia: W.B Saunders Company, 1992: 434-447.

TODD JD, VOLENEC FJ, PATON IM. Intranasal vaccination against infectious bovine rhinotracheitis: studies on early onset of protection and use of the vaccine in pregnant cows. Am Vet Med Assoc 1971; 159: 1370-1374.

TRUDEL M, BOULAY G, SEGUIN C, NADON F, LUSSER G. Gontrol of infectious bovine rhinotracheitis in calves with a BHv-1 subunit ISCOM vaccine. Vaccine 1988; 6: 525-529.

VILČEK S, DELIOVÁ I, STROJNÝ L, FORGÁČ O, HARVAN M. The effect of the mode of sampling on BHV-1 detection in infected cattle by dot-blot hybridization. Vet Microbiol 1993; 36: 355-358.

VILČEK S. Detection of the bovine herpesvirus -1 (BVH-1) genome by PCR. J Virol Meth 1993; 41 : 245-248.

WEIBLEN R, KREUTZ LC, CANABARRO TF, SCHUCH LF, REBELATTO MC. Isolation of bovine herpesvirus 1 from preputial swabs and semen of bulls with balanoposthitis. J Vet Diagn Invest 1992; 4 : 341-343.

WEINMASTER GA, MISRA V, MCGUIRE R, BABIUK LA, DECLERCQ E. Bovine herpesvirus type-1 (infectious bovine rhinotracheitis virus)-induced thymidine kinase. Virology 1982; 118: 191-201.

WHETSTONE CA, MILLER JM, BORTNER DM, MAATEN VAN DER MJ. Changes in the bovine hepresvirus 1 genome during acute infection, after reactivation, from latency, and after superinfection in host animal. Arch Virol 1989; 106: 261-279.

WHETSTONE CA, MILLER JM, SEAL BS, BELLO LJ, LAWRENCE WC. Latency and reactivation of a thymidine kinase-negative bovine herpes 1 deletion mutant. Arch Virol 1992; 122: 207-214

WIEDMANN M, BRANDON R, WAGNER P, DUBOVI EJ, BATT CA. Detection of bovine herpesvirus 1 in bovine semen by a nested PCR assay. J Virol Meth 1993; 44: 129-140.

WILLIAMS JF. Optimization strategies for the polymerase chain reaction. BioTechniques 1989; 7: 762-766.

WILLIAMS SD, KWOK S. Polymerase chain reaction: applications for viral detection. In: Lenette EH, ed. Laboratory diagnosis of viral infections. Second edition. New York : Marcel Dekker Inc, 1992 : 147-174.

WIRTH UV, GUNKEL K, ENGELS M, SCHWYZER M. Spatial and temporal distribution of bovine herpesvirus-1 arise from two divergent and spliced transcription units. J Virol 1989; 63: 4882-4889.

WIRTH UV, VOGT B, SCHWYZER M. The three major immediate-early transcripts of bovine herpesvirus-1 arise from two divergent and spliced transcription units. J Virol 1991; 65: 195 - 205.

WIRTH UV, FRAEFEL C, VOGT B, VLCEK C, PACES V, SCHWYZER M. Immediate-early RNA 2.9 and early RNA 2.6 of bovine herpesvirus 1 are 3' coterminal and encode a putative zinc finger transactivation protein. J Virol 1992; 66: 2763-2772.

WOLCOTT MJ. Advances in nucleic acid based detection methods. Clin Microbiol Rev 1992; 5: 370-386.

WRIGHT PA, WYNFORD-THOMAS D. The polymerase chain reaction: miracle or mirage? a critical review of its uses and limitations in diagnosis and research. J Pathol 1990; 162: 99-117.

WYLER R, ENGELS M, SCHWYZER M. Infectious bovine rhinotracheitis/ vulvovaginitis (BHV 1). In: Wittmann G. eds. Herpesvirus diseases of cattle, horses, and pigs. Boston/Dordrecht/London: Kluwer Academic Publishers, 1989: 1-72.

YASON CV, HARRIS LM, MCKENNA PK, WADOWSKA D, KIBENGE FSB. Establishment of conditions for the detection of bovine herpesvirus -1 (BHV-1) by polymerase chain reaction using primers in the thymidine kinase region. Can J Vet Res, 1995; in press..

YATES WDG. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can J Comp Med 1982; 46: 225-263.

ZHANG Y, SIRKO DA, MCKNIGHT JL. Role of herpes simplex virus type 1 UL46 and UL47 in alpha TIF-mediated transcriptional induction characterization of three viral deletion mutants. J Virol 1991; 65: 829-841.