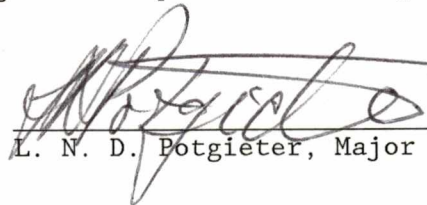


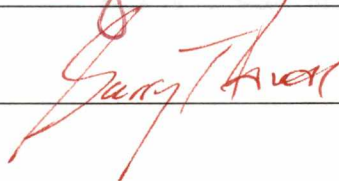
To the Graduate Council:

I am submitting herewith a dissertation written by Kenny Virgil Brock entitled "Bovine Viral Diarrhea Virus: Molecular Cloning of Genomic RNA and its Diagnostic Significance." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.


L. N. D. Potgieter, Major Professor

We have read this dissertation
and recommend its acceptance:





Accepted for the Council:


Vice Provost and
Dean of The Graduate School

BOVINE VIRAL DIARRHEA VIRUS: MOLECULAR CLONING
OF GENOMIC RNA AND ITS DIAGNOSTIC APPLICATION

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Kenny Virgil Brock

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ABSTRACT

Molecular cloning of a field isolate of bovine viral diarrhea virus (BVDV) strain 72 RNA was done in this study. The sensitivity and specificity of cloned cDNA sequences in hybridization assays with various BVDV strains were determined.

BVDV was purified from infected cell culture supernates by isopycnic density-gradient centrifugation. The density of the virions was 1.138 gm/cm^3 . Approximately 100 ug of BVDV genomic RNA was extracted from virus purified from 500 ml of infected cell culture fluids. BVDV genomic RNA then was tailed with adenine residues at the 3' end using poly-A polymerase. cDNA was synthesized from polyadenylated BVDV RNA templates with oligo-dT primers, reverse transcriptase, and DNA polymerase I. The newly synthesized double-stranded BVDV cDNA was C-tailed with terminal deoxytransferase and annealed into G-tailed, Pst-1-cut pUC9 plasmid. *Escherichia coli* was transformed with the recombinant plasmids and a library of approximately 200 BVDV specific cDNA clones varying in length from 0.5 to 2.6 kilobases were isolated.

The sensitivity and specificity of hybridization between the labelled cDNA and BVDV target sequences were determined. Cloned BVDV sequences were isolated from pUC9 plasmid DNA and labelled with ^{32}P by nick translation. The detection limit by dot blot hybridization assay was 20 pg of purified genomic BVDV RNA. cDNA

hybridization probes were specific for all strains of BVDV tested, regardless of whether they were noncytopathic and cytopathic, but did not hybridize with heterologous bovine viruses tested. Probes did not hybridize with uninfected cell culture or cellular RNA. Hybridization probes were at least as sensitive as infectivity assays in detecting homologous virus.

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I. INTRODUCTION

Bovine viral diarrhea virus (BVDV) is widespread and a major contributor to economic losses to the cattle industry. BVDV has been associated with mucosal disease and with outbreaks of diarrhea in susceptible herds. The virus also is fetopathic and causes abortions and congenital malformations. In addition, it has recently been recognized that BVDV is a major factor in the pathogenesis of respiratory tract disease in cattle. BVDV is the most common viral isolate obtained from cattle with shipping fever and infection enhances respiratory tract disease in cattle by facilitating colonization and by impairing clearance of secondary bacterial pathogens such as *Pasteurella hemolytica*.

Uncomplicated BVDV infection usually results in a trivial illness characterized by fever, leukopenia, and salivation. However, transient immunosuppression indicated by decreased blastogenic responsiveness of lymphocytes; decreased antibody production; depressed mononuclear phagocyte and neutrophil function; and decreased numbers of lymphocytes, macrophages, and neutrophils may occur. It is this immunosuppression that makes BVDV a common denominator in many diseases in cattle. The exact mechanism whereby BVDV induces immunosuppression is not understood at present. Strains of BVDV vary in antigenicity, pathogenicity and cytopathogenicity. Little is known

about the molecular mechanisms responsible for this variation.

Superinfection with certain cytopathic strains of BVDV in cattle persistently infected (as a result of *in utero* infection) with a noncytopathic strain causes acute, fatal mucosal disease. Vaccination of persistently-infected animals with modified-live BVDV and killed vaccines also appears to precipitate mucosal disease. Persistent infection with constant shedding of virus, due to an infection acquired during fetal development, appears to be the principal source of virus in the epidemiology of the disease. These infected animals remain seronegative to the homologous BVDV strain in spite of an active infection. In contrast to transient immunosuppression associated with primary post-natal infections, a permanent functional immunosuppression is present in some persistently-infected animals contributing to chronic illness and poor growth.

The molecular characteristics, replication strategy, biotype differences, and the pathogenesis of BVDV are largely unknown. This deficit in our knowledge concerning BVDV is due, in part, to difficulty in manipulation of BVDV in the laboratory and the lack of appropriate biologicals for the identification of BVDV.

Elucidation of the complex pathogenesis and epidemiology of BVDV requires sensitive and specific techniques of viral detection. At present virus isolation, serum neutralization, and the detection of viral antigen are the only methods of identifying BVDV infections. Also the existence of noncytopathic BVDV complicates pathogenesis

studies by requiring indirect methods of viral identification.

Molecular cloning techniques have recently become available and provide powerful tools for the study of viral diseases. One consequence has been the ability to construct and use cloned DNA hybridization probes which has added another dimension in diagnosis and pathogenesis studies of viral diseases. Cloning of specific viral sequences provides a genetically pure and inexhaustible supply of a specific viral DNA sequences. The detection of viral genomes is based on the principle of DNA:DNA or DNA:RNA hybridization as a result of specific base pairing. The double-stranded molecule is held together by hydrogen bonds that can be broken by heating allowing separation of the strands which, when cooled, re-hybridize. Cloned DNA have been used as hybridization probes for the detection of low levels of DNA (1-2 pg) in infected cell culture, secretions, body fluids, and in tissues either in extracted material or *in situ*. The diagnosis and pathogenesis studies of several viral diseases using hybridization probes has been reported. Other consequences of molecular cloning of viruses have been the availability of these cloned sequences for genomic sequencing which has provided a wealth of information concerning the biology of many viruses. These sequences and the information gained from their study also have led to the development of novel sub-unit vaccines.

Most of the early techniques of using DNA hybridization probes involved a detection system with labelled radionucleotides. Recently

the availability of biotin-labelled nucleotides has allowed a broader application of DNA hybridization probes. Biotinylated probes have many potential advantages over radiolabelled probes. Biotinylated probes avoid the use of radioisotopes and have a longer shelf-life. In addition assays for biotin are faster than those for radioisotopes and in some systems the sensitivity of biotinylated probes rivals that of radiolabelled probes.

There are many facets of the pathogenesis of BVDV infection that still are not clear. The mechanism by which persistence is established as a result of *in utero* infection and that by which post-natal re-infection with certain BVDV strains precipitates mucosal disease should be elucidated for the rational control of virus spread and to prevent BVDV-induced disease. It is likely that specific genomic differences among BVDV strains exist that correlate with the different consequences of BVDV infection and other differences in the natural history of these viruses. Strain-specific hybridization probes could be useful in tracking the molecular epidemiology and natural history of BVDV. Once identified, these genomic differences could be studied further by gene sequencing and identifying and characterizing gene products from these sequences.

BVDV is considered to be a major contributor to economic loss in the cattle industry as a result of disease and loss of production, primarily due to *in utero* infection and its capacity to induce immune dysfunction. Persistently-infected animals are the major

source of virus in the environment but many of these animals appear healthy and are difficult to identify. Therefore the detection of the immunotolerant, persistently-infected animals is important. The most common method of herd screening for BVDV infection is by seroconversion, but seronegative, persistently-infected animals cannot be identified in this manner. Serum virus neutralization is commonly used for identification and assaying BVDV antibodies in bovine sera. It is expensive, relatively insensitive, and laborious. Neutralizing antibody titers to homologous BVDV strains often is much higher than to heterologous strains which complicates diagnostic serology when serum virus neutralization is done (29, 61, 186). Many of these disadvantages exist also when virus isolation is done which is commonly used to identify infected animals (6). The presence of virus in persistently-infected calves may be masked by the presence of passive antibody (6).

It is important therefore to use rapid, sensitive, and specific methods of identifying the presence of BVDV infection in herds. Dot blot hybridization for BVDV in serum samples on nitrocellulose could allow simultaneous screening of many animals and may facilitate the detection of persistently-infected animals in a herd. BVDV contamination of biologicals is recognized as a major concern. The major source of adventitious contamination of biologics by BVDV is fetal bovine serum which is used universally in cell culture media. At present, it is difficult to guarantee that products are free of

BVDV since it is difficult to detect BVDV in fetal bovine serum without exhaustive virus isolation procedures. Cloned BVD viral sequences used as DNA hybridization probes could be an important method of detecting BVDV contamination in fetal bovine serum because of its sensitivity and specificity. Hybridization probes also would enable detection of BVDV contamination in other viral stocks that have been contaminated during subculture or isolation. The extent of the presence of BVDV in these materials also may be underestimated since it is difficult to detect BVDV contamination of viruses more aggressively cytopathogenic than BVDV.

It was anticipated that the cloning of BVDV would provide tools for the study of the pathogenesis and diagnosis of BVDV infections. One particular rewarding avenue may be the genetic basis which determines the biological activity of different strains of BVDV. Establishing the genetic determinants that influence cytopathogenicity, viral persistence, cross immunity, and strain interaction in disease production is necessary to adequately determine pathogenesis, prophylaxis, and control of the disease. The cloned cDNA segments provide a mechanism to completely sequence the BVDV genome. By examining sequence data, the exact coding regions can be determined and gene expression examined. The replication strategy of BVDV could be clarified with respect to its relationship to Flaviviridae and Togaviridae.

Since BVDV is included among the least characterized of the

animal viruses (152), the successful cDNA cloning of BVDV promises to have great impact on the resolution of the biology of BVDV and virus-host interactions. The study of BVDV is a major challenge, not only due to the difficulties encountered in studying the virus, but also due to the complex pathogenesis and virus-host interaction of BVDV infections. The study of BVDV has implications, not only within the field of veterinary medicine, but also in a larger scope because of the unique virus-cell and virus-host interactions (including viral persistence and recrudescence) which may lead to another dimension in the understanding of the immune response of the host to viruses and the natural history of viruses (152).

In order to examine BVDV-host interactions it first will be necessary to understand the basic biology of BVDV (152). Sequence data obtained from cloned BVDV cDNA sequences will provide direct information concerning the molecular biology of BVDV. Due to the difficulties encountered in the past with BVDV, further characterization of BVDV will be facilitated by genomic cDNA cloning. This is essentially the only route whereby conclusive information concerning the biology of BVDV can be obtained. Sequence data, leading to a BVDV genomic map would contribute specific information concerning the replication and genome organization of BVDV and the relationship among BVDV strains. Cloning of BVDV is an important step in the sequencing of the virus. BVDV strain differences now can be analyzed by cross hybridization with a variety of cloned cDNA probes

which may lead to an understanding of the complex pathogenesis of mucosal disease. Although reports giving speculative insights into the replication and organization of genomic BVDV RNA have been made by Renard *et al.*, specific data from sequenced gene sequences have not been published (182, 183).

Finally, since financial considerations are among the most important factors to consider in the cattle industry, the cDNA cloning may also have a tremendous economic impact for the livestock industry. As information concerning the molecular biology of BVDV leads to a better understanding of BVDV infections, rational control and prevention of BVDV infections can be instituted which could be reflected by increased production. Improved diagnostic testing will be available as well as the possibility of the development of a superior sub-unit or recombinant vaccine for the prevention and control of BVDV infections. The work, time, expense, and effort involved in cDNA cloning of BVDV RNA was expansive but it opens the door to an enormous wealth of information contained within the cloned BVDV sequences. Further characterization of these cloned sequences may have significant impact to the cattle industry.

II. LITERATURE SURVEY

Bovine Viral Diarrhea Virus

Historical Overview Bovine viral diarrhea virus (BVDV) has been recognized as a common pathogen and a major cause of disease in cattle (3, 52, 166). However, the most common outcome of primary postnatal BVDV infection is subclinical disease (6, 52). BVDV is responsible for three separate disease syndromes in cattle; bovine viral diarrhea, mucosal disease, and fetal disease (6, 52). In 1946, Olafson *et al.* described a disease affecting cattle that was characterized by high morbidity and low mortality (154). The disease was called bovine viral diarrhea (BVD) and its most important features were fever, excess salivation, diarrhea, and erosions of the oral and gastrointestinal mucosa (154).

Mucosal disease of cattle was described by Ramsey and Chivers in 1953 (177). It was a sporadic disease in herds with a high mortality rate (177). Mucosal disease was difficult to reproduce experimentally but it was obvious that BVDV was involved in the pathogenesis of the disease (127). It was distinguished from bovine viral diarrhea by its more sporadic occurrence, lower morbidity, longer course (up to 90 days), higher mortality, the absence of detectable antibodies, and persistent viremia (52, 159). The pathogenesis of mucosal disease and BVD differed (25, 27, 147). Mucosal disease was a late manifestation

of *in utero* infection since it occurred only in persistently-infected, viremic, antibody-negative cattle (immunotolerant as a result of *in utero* infection) after superinfection with a different strain of virus (18, 25, 26). Bovine viral diarrhea, on the other hand, resulted from a primary postnatal infection (89, 147).

Kahrs *et al.* first reported that BVDV readily crossed the placenta and resulted in fetal disease (107). Fetal death usually occurred when the fetus was infected in the first trimester of gestation (109). Fetal death as a result of BVDV infection in the second trimester was not uncommon either (109). Fetal infections also may result in various congenital malformations such as ocular, musculo-skeletal, nervous system, and skin lesions (107, 108, 109). *In utero* infection also results in the development of persistent, immunotolerant infections in the postnatal animal (45).

A major clinical consequence of BVDV infection is the enhancement of disease by various pathogens and facilitating infections by opportunistic organisms (127, 166). Enhancement of respiratory tract disease by BVDV infection was first recognized by Dinter *et al.* in 1961 (42, 44). Clinical and experimental observations of synergism of BVDV with bacterial and viral pathogens has been reported on several occasions (169, 203, 230). *In vitro* and *in vivo* experimental evidence have indicated that the synergism of BVDV infection with

other pathogens is a consequence of the immunosuppression induced by BVDV infection in cattle (17, 19).

BVDV was first isolated and identified by Underdahl, *et al.* in 1957, but its relationship with mucosal disease was not established (223). The etiological connection of BVDV was established by Gillespie *et al.* when a cytopathic strain was isolated from a case of bovine viral diarrhea (74). The virus was classified in the *Togaviridae* family as a member of the *Pestivirus* genus (95). BVDV was closely related to Border disease virus of sheep but more distantly related to hog cholera virus (39).

Pathogenesis

Prenatal infections: BVDV crossed the placenta efficiently (approaching 100 % experimentally) and therefore resulted in fetus infections when susceptible dams were infected during gestation (45, 109, 155). Disease in pregnant dams usually was subclinical, as were most postnatal infections, but fetal infection resulted in abortion; congenital malformations; weak, stunted neonates; and persistently-infected, apparently immunotolerant, calves (45, 108, 215). The response of the fetus to infection primarily depended on the stage of gestation but other determinants of disease may have been the biological variation of virus strain, immune status of the host, and breed differences (18, 19, 45, 155).

An increase in numbers of repeat breeders and infertility resulted from infection of cows in early gestation (45). Infection of

the conceptus occurred when BVDV-containing semen was used in artificial insemination (7). Infection before 100 days of gestation often resulted in death of the fetus (45). Hypomyelinogenesis, evident as tremors at birth, occurred also as a result of infection before 100 days of gestation (52). Hypomyelinogenesis was a major characteristic of *in utero* infections in sheep with Border disease virus (46, 121, 163).

Infection of the fetus between 100 and 150 days of gestation during the final stages of organogenesis led to destruction of differentiating cells (52). Sequelae such as retardation of tissue growth and lowered birth weights, cerebellar hypoplasia and dysplasia, cerebral cavitation, and retinal hypoplasia occurred (107, 108, 109).

Persistently-infected immunotolerant calves were a result of infection with certain biotypes of the virus before complete development of immunological competence (18, 25). Most fetuses obtained immunocompetence to BVDV at approximately 126 days of gestation (52). Therefore infection must have occurred before or during the development of self-recognition (52). Transplacental infection with a noncytopathic strain of BVDV between 60 to 120 days of gestation led to a persistent infection in fetuses and postnatal animals (52, 120, 133). Infection of pregnant cows during this time with a cytopathic virus resulted in abortion, not persistent infections, even from modified-live cytopathic strains after vaccination (37, 120). "Immunotolerance", the phenomenon of failure

of the immune response to recognize an antigen as non-self, apparently developed to the antigens of the noncytopathic strains (34, 45, 52). Animals persistently infected with BVDV excreted large amounts of virus (19). Approximately 1.7 % of animals going to slaughter in Europe were persistently infected with BVDV (52). Bolin *et al.* identified persistently-infected animals in 9 % of herds examined and 1.7 % of the total number tested (15). These animals were the primary source of virus within herds and were responsible for spreading the virus to other susceptible herd members (15). Some persistently-infected animals appeared normal but up to 50 % died prematurely due to wasting and debilitation during the first year of life (52). Lymphocytes and neutrophils were functionally impaired since neutrophil phagocytosis was reduced and the blastogenic response of lymphocytes to mitogens was attenuated (19, 196). Serum from persistently-infected animals and BVDV-infected cell culture fluids decreased the blastogenic response of lymphocytes to phytohemagglutinin and concanavalin A (129, 214). Immunosuppression in persistently-infected animals differed from the transient immunosuppression associated with primary postnatal BVDV infections in cattle since normal numbers of circulating lymphocytes were present and neutrophil dysfunction was not as severe in the former (16, 19, 145). Lesions in persistently-infected animals consisted of degeneration and satellitosis of neurons, glomerular basement membrane thickening, and antigen deposition in kidneys, neurons, lymph nodes,

spleen, intestinal epithelial cells, and endothelial cells (38, 94). The presence of immune-complex deposition indicated that there may have been some degree of specific BVDV antibody production in persistently-infected animals (38, 94). Persistently-infected animals had a high probability of developing mucosal disease (25).

Infection of the fetus after organogenesis and after the development of immunocompetance was usually of no consequence because most fetuses developed an immune response *in utero* and subsequently eliminated the virus (45).

Postnatal infections: Several consequences of primary postnatal infections in susceptible cattle; subclinical infections, bovine viral diarrhea, and transient immunosuppression; have been described (6, 52). Subclinical infections were the most common result of primary postnatal BVDV infections. BVDV infection in cattle was characterized by a transient but profound and multifactoral immunosuppression (52, 54, 179). Decreased responsiveness of lymphocytes to mitogens, depressed levels of cellular interferon production and sensitivity of exogenous interferon, decreased antibody production, depressed monocyte phagocytosis, impaired neutrophil iodination reaction, and reduced monocyte chemotaxis as a result of BVDV infection have been described (5, 16, 106, 110, 129, 144). In addition, BVDV-infected animals had reduced numbers of lymphocytes, macrophages, and neutrophils. Vaccination with some strains of modified-live BVDV vaccines were immunosuppressive (160, 195).

Modified-live BVDV vaccination resulted in decreased lymphocyte blastogenesis, decreased neutrophil chemotaxis and phagocytosis, and decreased numbers of circulating lymphocytes and neutrophils (195). This immunosuppression may have resulted in severe secondary infections after vaccination of stressed, feeder calves from virus free herds (148).

Recently it has been recognized that BVDV infections were a pivotal event in the pathogenesis of respiratory tract disease in cattle (169, 197). Several observations have led to this conclusion (42, 168, 179). BVDV was the most common virus isolated from cattle with shipping fever (179). Infection with BVDV enhanced respiratory tract disease in cattle by causing an increased susceptibility to *Pasteurella hemolytica* (169), infectious bovine rhinotracheitis virus (54, 167, 168), and malignant catarrhal fever virus (203). Edwards *et al.* reported enhanced shedding of bovine herpesvirus 1 (BHV-1) after virulent virus challenge inoculation of animals that had been vaccinated with BHV-1 while infected with BVDV (54).

Enhanced disease developed in animals after concurrent infections of BVDV and infectious bovine rhinotracheitis virus (54, 79), papular stomatitis virus (14), or malignant catarrhal fever virus (203). Wray and Roeder reported the exacerbation of *Salmonella* infections and persistence of fecal bacterial excretion in calves as a result of BVDV infection (230). Bacteremia due to impaired clearance of endogenous

bacteria was reported in calves experimentally inoculated with BVDV (180).

BVDV infections have been common since 60 % to 95 % of animals in herds were seropositive (52, 59). Serologic data indicated that fewer than 10 % of herds consisted entirely of animals with no previous exposure to BVDV (55). BVDV infection was characterized by transient fever, leucopenia, and viremia (which persisted for up to 15 days) (52). Bovine virus diarrhea occurred in some susceptible herds as an explosive outbreak of diarrhea with high morbidity and no mortality (52, 159). Affected animals had mild to severe diarrhea, erosions of the oral mucosa, and a drop in milk production. Animals developed lifelong immunity to BVDV after infection (52).

Mucosal disease: Mucosal disease was characterized by fever, anorexia, dehydration, profuse watery diarrhea, serous catarrhal exudate, and oral and nasal mucosal ulceration (177). Erosions of the coronary band and the interdigital cleft often occurred and resulted in lameness (6, 52, 89, 147). Death sometimes occurred within 2 to 3 days but more commonly the course was 2 to 3 weeks (52, 147). A chronic form occasionally developed that lasted several months before death (7). Pathological lesions in cattle with mucosal disease were extensive mucosal erosions and submucosal hemorrhages throughout the digestive tract, inflammation and necrosis of the Peyer's patches, dehydration, and lymphoid depletion from lymphoid organs (3, 147, 154, 159).

Mucosal disease occurred in persistently-infected, antibody-negative cattle (127, 147). Evidence indicated that super-infection with certain cytopathic strains of BVDV in cattle persistently infected with certain noncytopathic strains will cause mucosal disease (18, 134, 147). It may have been a function of a complex relationship of antigenic determinants between different BVDV strains (17). Persistently-infected animals do respond with neutralizing antibody production to heterologous strains but not to the homologous strain (17). A certain combination of 2 strains of BVDV were required to precipitate mucosal disease because not all combinations of noncytopathic and cytopathic BVDV caused mucosal disease (17, 18). In one study, the development of neutralizing antibody in calves from vaccination resulted in a delayed onset of mucosal disease after challenge inoculation (17). Tolerance to BVDV in persistently-infected cattle may have been terminated by an immunological response following exposure to certain strains of the virus (17). Certain combinations of virus infections result in a more chronic disease than others (17). The tolerance present in persistently-infected cattle apparently was not complete since there were microscopic lesions suggestive of immune-mediated pathology (type III hypersensitivity reaction) involving antigen-antibody complexes in the kidney glomeruli (17, 38, 94).

Vaccination of persistently-infected cattle with modified-live and killed BVDV also has been associated with the subsequent

development of mucosal disease (17, 51, 160). Most modified-live BVDV vaccines have been cytopathic and have been incriminated in the development of mucosal disease in calves persistently infected with a noncytopathic strain (17, 148). Vaccination of pregnant cows with modified-live BVDV vaccines led to birth of persistently-infected, immunotolerant calves that were chronic virus shedders (148). However, a temperature-sensitive mutant of BVDV, used as a modified-live vaccine, did not result in detectable postvaccinal sequelae in pregnant cows (123). Chronic BVD in a persistently-infected bull was associated, in a retrospective study, with inoculation of killed-BVDV vaccine (19). The exact mechanism of the pathogenesis of mucosal disease is not understood at present (152).

Epidemiology Because classical clinical signs of BVDV have been the exception rather than the rule in BVDV-infected cattle, most infections have been surreptitious which made it difficult to identify and attribute a herd problem to BVDV (159). Prevention of BVDV fetal infections, especially in early gestation, and the identification of persistently-infected animals has been identified as the major focus in prevention and control of BVDV infection (190).

Introduction of a persistently-infected animal in a susceptible herd has resulted in the occurrence of several types of clinical disease such as enteritis, respiratory disease, decreased weight gain, reduced milk production, early embryo loss, abortions, congenital

malformations, and neonatal deaths (37, 159). A variable number of progeny from dams which were infected during pregnancy would be persistently-infected and would remain a source of the virus in the herd (37, 159). Persistently-infected heifers and cows always produced persistently-infected offspring as a result of transplacental transmission (51). Virus spread among cattle was by inhalation or ingestion of materials contaminated by oculonasal discharge, saliva, urine, and feces (6, 51). Semen, uterine secretions, amniotic fluid, and placental tissues containing virus also have been responsible for transmission (52). Exposure of preimplantation embryos *in vitro* did not cause adverse effects on *in vitro* development of embryos but the *in vivo* effects of BVDV were not examined (170). Infection also has occurred by transmission from persistently-infected reservoir animals such as sheep or wild ruminants (51, 150). However the frequency of this occurrence is unknown (51). BVDV has infected sheep, goats, swine, and wild ruminants (51, 59, 114). Both BVDV and Border disease virus strains have infected cattle and sheep experimentally (51, 150).

Most modified-live vaccines contain strain NADL, Oregon C24V, or Singer strain of BVDV (148). Little substantive information has been reported on the antigenic differences among of strains of BVDV vaccines (148). It has been recommended that only inactivated vaccines be used in closed herds where BVDV has not been diagnosed (4,

148). BVDV vaccination prior to conception has been effective in protecting fetuses from *in utero* BVDV infection (51).

Contamination of fetal bovine serum is a major problem and also makes it difficult to obtain bovine cell cultures free of BVDV infection (91, 191). Toth and Hesse reported that Vero cells were refractory to BVDV infection thus allowing the elimination of BVDV from bovine respiratory syncytial virus stock (219). Heat inactivation was ineffective for eliminating BVDV from bovine fetal serum (191).

Laboratory Diagnosis BVDV infection usually has been suspected when disease such as mucosal disease, abortions, or congenital malformations were present in animals of a herd (159). Birth of unthrifty animals, excessive neonatal deaths, and episodes of enteric or respiratory tract disease (often after the introduction of new animals into the herd) have also indicated the introduction of BVDV into a herd (37, 147). Laboratory diagnosis was essential when classical signs of disease were not present (159). Seroconversion measured in paired serum samples by serum neutralization (29, 30, 75), immunofluorescence (66, 210), immunoperoxidase test (226), and ELISA indicated active BVDV infections (13, 33, 93, 98). Serum samples also have been suitable for isolation of virus from persistently-infected animals (176). The identification of viral antigen or antibody in fetal tissues or fluids from an aborted fetus was evidence of fetal infection but did not indicate BVDV fetopathy since some transplacental BVDV infections especially in late gestation were

harmless. Abortion occurred weeks after infection of the dam at which time the latter likely had developed an antibody response (52). Therefore an increase of antibody titer in paired sera taken after abortion was not likely to occur. Virus isolation, serum virus neutralization for specific antibodies, and the detection of viral antigen (ELISA and immunofluorescence) have been the primary methods of determining BVDV infections (51, 98). The separation of noncytopathic and cytopathic isolates in mixed infections required special methods such as reverse plaque titration as reported by Itoh *et al.* (36, 102). Terminal dilutions of noncytopathic BVDV grew to higher titer than cytopathic BVDV by plaque purification (104). Itoh *et al.* reported differentiation of a noncytopathic virus present in cytopathic stocks of BVDV using the exaltation of Newcastle disease virus assay (103).

Biological Characteristics Following the isolation of BVDV by Underdahl (223), the virus was further characterized by Hermodsson. It was ether sensitive, contained RNA, and virions were approximately 40 nm in diameter (92). Darbyshire (39) first reported a cross-reaction of BVDV with hog cholera virus by agar gel precipitation which was later confirmed by Dinter using cross-neutralizing studies (43) and Mengeling by immunofluorescence (138). The buoyant density of BVDV was first reported by Fernelius to be 1.15 g/cm³ (67) and in 1968, Hafez *et al.* described the morphology of BVD virions which ranged in size to be from 40 to 60 nm

depending on preparation procedure (85). These findings led to the proposal that BVDV be included in the Togaviridae family (65, 95).

As BVDV was studied and characterized further there remained some disagreement over the buoyant density of BVDV which was important for purification of the virus and also necessary in its classification. Contradictory to Fernelius' findings (67), Parks *et al.* determined that the buoyant density was approximately 1.115 g/cm^3 (158). The buoyant density was later confirmed by others to be approximately 1.12 g/cm^3 (32, 172, 173). Different estimations of these values were attributed to heterogeneity in size and buoyant density of virions (172).

Various reports have been published on the morphology of BVDV. Virion size estimates have ranged from 40 nm to 120 nm (32, 85, 95). Chu reported that BVDV particles were pleomorphic and measured $120 (+/- 30) \text{ nm}$ (32). Based on morphologic characteristics, sedimentation coefficient, buoyant density, and antigenic relationship; BVDV, hog cholera virus and Border disease virus, were taxonomically placed in the Togaviridae family as members of a separate genus, *Pestivirus* (117, 227). The Oregon C24V strain of BVDV was selected as the prototype virus for the *Pestivirus* genus. Virions from the latter BVDV strain were pleomorphic, without any discernable symmetry, and measured 50 to 60 nm in diameter (227).

BVDV RNA from infected cell culture supernate was characterized by Pritchett in 1975 (172). The major RNA component isolated had a

sedimentation rate of 38S which represents a molecular weight of 3.22×10^6 . Two minor species of genomic RNA sedimented at 31S and 24S which corresponded to molecular weights of 2.09×10^6 and 1.22×10^6 respectively (172). Purchio later identified only one species of BVDV intracellular RNA in infected cell cultures (173, 175). BVDV genome consisted of single-stranded RNA because it lacked resistance to high concentrations of RNase and because base composition analysis indicated it contained uridine (173). Viral RNA also lacked a poly-A tail (173).

Early studies identified three to four separate species of virus-specific polypeptides (72, 130). Frost identified three structural proteins in BVDV-infected cells by poly-acrylamide gel electrophoresis (72). Four BVDV proteins, with molecular weights of 110,000, 93,000, 70,000, and 23,000 daltons (110K, 93K, 70K, and 23K), were described by Pritchett (171). Matthaeus also reported finding three BVDV structural proteins by immunoprecipitation of radiolabelled polypeptides with molecular weights of 57K, 44K, and 34K. He indicated that the two larger proteins were glycosylated (gp57 and gp44) (130). Coria in 1983 detected four major proteins of the cytopathic Singer strain of BVDV with molecular weights of approximately 75K, 66K, 54K, and 26K and determined that the gp75 and gp54 were glycosylated proteins (35). Cell-free translation of BVDV RNA resulted in the *in vitro* synthesis of two species of proteins ranging from 50K to 150K (175). Protein synthesis with isolated

polysomes from infected cell cultures resulted in polypeptides of 80K and 115K (175).

More recently, BVDV proteins have been further characterized. Three major proteins with molecular weights 115K, 80K, and 55K and two minor proteins, 45K and 38K, have been described by Purchio, 1984 (174). Without using immunoprecipitation, Donis identified 12 viral polypeptides with molecular weights of 165K, 135K, 118K, 80K, 75K, 62K, 56K to 58K, 48K, 37K, 35K, 32K, and 19K in infected cell cultures (47). Since the sum of the molecular weights of the polypeptides identified by Donis *et al.* was greater than the coding capacity of the BVDV genome some of the polypeptides must have been precursor polypeptides (47). The polypeptides were identified from infected cell cultures by radiolabelling in the presence of a hypertonic initiation block and SDS-polyacrylamide gel electrophoresis analysis (47). Six specific glycoproteins were identified by Donis including two major species of 48K and 56K to 58K and less abundant species of 118K, 75K, 65K and 25K (48).

The replication strategy of BVDV has not been described (97). The organization of BVDV genomic RNA differed from other members of the Togaviridae family (96). BVDV genomic RNA was not poly-adenylated as were most members of the Togaviridae family (172). From preliminary unpublished sequence data, it was speculated that the genome contained not one but two open reading frames (183). Subgenomic species of viral RNA have not been detected in cells

infected with BVDV which differs from the life cycle of members of the Togaviridae family (173). Also speculative arguments have been presented that the structural proteins probably were encoded from the 5' end of the genome RNA whereas the 3' end encoded the nonstructural proteins which was opposite to that of members of the Togaviridae family (183).

It has been proposed that either the *Pestivirus* genus belongs in a separate family or that its members belong in the Flaviviridae family (68, 97, 183). Flaviviruses lacked a 3'poly-A tail, had similar genome organization as BVDV (5' structural and 3' non-structural), and produced viral proteins by post-translational cleavage of one polyprotein (185). Evidence suggested that BVDV replication was similar to that of the Flaviviridae and Picornaviridae in that the genome was translated into a polyprotein with subsequent post-translational processing (48, 183). Because BVDV genomic RNA possibly contained two open reading frames, it may deserve separate family status (97, 183).

BVDV strains were antigenically related but some cytopathic and noncytopathic strains were differentiated antigenically by virus serum virus neutralization (36, 68) and indirect immunofluorescence (161). Gillespie *et al.* however, reported that in a study of three strains, neutralizing antibody to heterologous as well as the homologous strain of BVDV virus was produced which was later confirmed by Castrucci *et al.* (29, 75). Grouping according to serotype has

not existed, but strains were distinguished by their ability to produce cytopathic effects in cell culture. Grouping of strains within noncytopathic or cytopathic biotypes has been the major means of biological differentiation (130). Noncytopathic strains did not cause microscopically visible cytopathic changes in cell culture and were identified by indirect methods such as immunofluorescence or viral interference (66, 103). Analysis of glycoproteins induced by various isolates of BVDV suggested marked heterogeneity between virus strains (49, 50, 164). Cytopathic BVDV strains synthesized an 80K polypeptide that was absent in noncytopathic BVDV-infected cells. It has been postulated that the 118K polypeptide present in cells infected with both biotypes of BVDV may be a precursor protein of the 80K polypeptide and the proteolytic cleavage site may not be present in the 118K polypeptide of noncytopathic strains (49). This molecular difference may be a factor determining the biological differences that exist between different biotypes of BVDV (49). Donis used monoclonal antibodies to distinguish between noncytopathic and cytopathic BVDV by their recognition of the presence or absence of epitopes between biotypes (50). The observations of Donis confirmed an earlier report by Peters who indicated that differences between cytopathic and noncytopathic strains were recognized by certain monoclonal antibodies (161). Peters was able to identify epitopes present on polypeptides of cytopathic virus that were not present in cell cultures infected with noncytopathic virus (161). This

monoclonal antibody reacted with an epitope present on a 79K polypeptide present in virions of cytopathic strains of BVDV (161).

Renard was first to report the successful cloning of BVDV sequences in 1985 (182). BVDV genomic RNA was 12.5 kilobases (kb) long and was a single-stranded molecule (182). BVDV genomic RNA was purified from bovine embryonic kidney cells infected with a high passage Osloss strain of BVDV. Complementary DNA (cDNA) was synthesized after denaturation of BVDV RNA with methylmercury hydroxide and addition of a poly-A tail with poly-A polymerase. First and second-strand synthesis was done according to the techniques of Gubler and Hoffman. A library of clones was obtained ranging in size from 0.5 to 1.5 kb representing five overlapping groups of clones (182). No further information of the BVDV has been published by Renard.

cDNA Cloning

DNA molecular cloning techniques, developed over the past decade, have been invaluable in the development of improved diagnostic methods, novel vaccine production, and investigation of the biology and pathogenesis of many viruses (61). Cloning of viral gene sequences have provided inexhaustible and pure supplies of gene sequences and gene products (61). This has been especially useful important for viruses that are not adapted to growth in cell cultures or grow to low titers in cell culture (61, 202).

The development of DNA cloning technology has been possible

because of the burgeoning knowledge of enzyme function in DNA synthesis and modification (61). These enzymes include restriction endonucleases, DNA polymerases, ligases, and transferases (61). Enquist, *et al.* were among the first to clone viral sequences when they ligated DNA fragments of HSV-1, produced by digestion with restriction endonucleases, into restriction endonuclease sites within phage lambda DNA (62). The annealed hybrid of herpesvirus DNA and phage DNA was used to transform *Escherichia coli* (*E. coli*) by transfection (61, 62). The cloned sequences were replicated along with vector DNA during host replication. Vector (phage) DNA was extracted with phenol from the phage lysates following proteinase K and sodium dodecyl sulfate digestion and the cloned sequences were removed from the phage DNA by restriction endonuclease digestion (62). These techniques amplified production of specific cloned DNA sequences for further study (62).

Cloning of RNA sequences was based on the discovery, by Temin, of reverse transcriptase, since RNA itself can not be directly cloned into DNA vectors (128, 184). The enzyme reverse transcriptase, in the presence of the four deoxynucleosides, synthesized complementary DNA (cDNA) from a RNA template (128). After its discovery, the application and use of reverse transcriptase was dependent on the development in the mid 1970's of optimal enzymatic reaction conditions for transcription of large [>1000 to 2000 base pairs (bp)] RNA templates (71, 184). Successful cloning of cDNA from RNA sequences

was first reported by Rougeon and Mach, and by Efstratiadis *et al.* in 1976 (56, 57, 198). After synthesis of the first-strand DNA product from RNA by reverse transcriptase, the enzyme DNA polymerase I was used to synthesize the second-strand utilizing the newly-synthesized first-strand as the template. To initiate DNA synthesis from an RNA template the polymerase molecule of reverse transcriptase or DNA polymerase I must originate from a free 3' end to proceed in a 5' to 3' direction. A primer was essential to begin DNA synthesis in this manner for first and second-strand synthesis (128).

Two different approaches have been used for cDNA synthesis, both of which include the use of reverse transcriptase. The methods of Okayama and Berg were dependent on the formation of a hair-pin loop structure at the 3' end of the single-stranded cDNA by reverse transcriptase "turning the corner" and copying itself (153). The RNA template then was removed by alkali treatment and the second-strand DNA was produced with DNA polymerase I (153). The closed hair-pin loop was removed by S1 nuclease which resulted in the final double-stranded product complementary and equivalent to template RNA (153).

The methods developed by Gubler and Hoffman were very similar to those of Okayama and Berg but differed by utilizing RNase H-DNA dependent second-strand synthesis (80). Hair-pin loop formation and S1 nuclease digestion were unnecessary which reduced the steps for

synthesis of cDNA (80). RNase H was used to digest the RNA template after first-strand synthesis and, by including it and DNA polymerase in the same reaction mixture, the fragmented RNA template served as a primer for the latter while the RNA template was degraded (80).

Various investigators have obtained cDNA clones of up to 2.4 kb in size by using the methods of Gubler and Hoffman which are comparable to results obtained by Okayama and Berg (80, 116, 153).

Cloning of viral sequences has had a profound scientific impact and has provided a powerful investigative tool in diverse disciplines (61). The sequencing of cloned segments has given great impetus to study of the biology of various viruses (116, 151, 232). Sequence data has led to knowledge of the genome organization such as location of open reading frames (determined by initiation and stop codons) and the probable amino acid sequences of gene products (151). Other pertinent information on viral proteins such as signal sequences, hydrophobic and hydrophilic regions, membrane insertion sites, and proteolytic cleavage sites also have been determined from sequence data (116, 151). This information has provided insight into the replication strategy and gene function of several viruses (116, 151). One example is that the DNA cloning of hepatitis B virus, which allowed sequencing of the genome and mapping of amino acids of gene products resulting in the production of synthetic peptides which stimulate antibodies active against virus surface antigens (119, 224, 232).

Specific cloned sequences have been identified and placed into appropriate expression vectors to obtain correct translation of the protein(s) encoded by the gene sequences (119). Gene sequences have been expressed in yeast and various species of bacteria (119). Cloned gene products from expression vectors in yeast or bacteria have been used in candidate sub-unit vaccines or as diagnostic reagents (61, 78).

Gene sequences; cloned into viral vectors such as adenovirus, SV40 virus, retrovirus, baculovirus, and vaccinia virus; have been used for transfection of eucaryotic cells to obtain cellular expression of various gene products (78, 83, 105, 119, 124, 157, 229). Vaccinia virus has become popular as an expression vector for candidate vaccine development (10, 24, 58, 76, 125, 126, 143, 207, 208).

Hybridization Probes

The diagnosis, pathogenesis, and epidemiology of viral disease classically has been based primarily on virus isolation (or antigen detection) and serologic assays (31, 186). Recently laboratory diagnosis of viral diseases using monoclonal reagents have improved antigen detection techniques such as enzyme-linked immunoassays, immunofluorescence, and immunoperoxidase techniques (186). Diagnostic methods must be sensitive, specific, rapid, inexpensive, and simple (31, 186). The application of recombinant DNA biotechnology to viral diagnostics has encompassed three methods; molecular cloning,

restriction endonuclease digestion, and nucleic acid hybridization (112).

The basis for the development of DNA hybridization technology was the nature of the reassociation kinetics and "zippering" effect of complementary DNA strands (86). DNA and RNA were bound covalently by hydrogen bonding in a specific manner which was determined by nucleotide base pairing (86). When the double-stranded molecule was heated to a certain temperature the strands dissociated (melting point) and when the mixture was gradually cooled the strands reannealed in the exact manner as before by specific pairing of bases (86). Therefore the specific nucleotide sequence determined the capacity of DNA and/or RNA strands to bind to one another which is a highly specific phenomenon (86).

Cloned gene sequences have proven valuable as diagnostic hybridization probes to detect specific viral DNA or RNA sequences in a variety of samples (186, 187, 202). Hybridization probes have been most important when viruses were not detectable by infectivity such as during viral latency and after defective or incomplete viral replication (100). Hybridization probes also have been used to distinguish between viral DNA and viral mRNA to estimate the relative level of viral genome and genome transcripts present in the cell (100).

Hybridization probes have been prepared by specific labelling of appropriate DNA or RNA sequences with radionucleotides or a

nonradioactive reporter substance (112). Cloned double-stranded DNA in plasmids, bacteriophages, and cosmids have been suitable for constructing hybridization probes (100). However the M13 phage vector system provided single-stranded cloned DNA sequences which has allowed synthesis of single-stranded DNA probes (112, 187). Hybridization probes also have been prepared from RNA which hybridizes with either RNA or DNA (187). The labelling of hybridization probes with reporter substances can be done *in vivo* during virus replication or by *in vitro* DNA synthesis. Viral sequences have been uniformly labelled with radioisotopes *in vivo* during the replication cycle in cell culture to levels of 10^6 to 10^7 cpm/ug (100). However this method of labelling required efficient virus replication, easy virus purification, and efficient replication-dependent labelled nucleotide incorporation (100).

Several methods for the *in vitro* labelling of polynucleotides have been reported and include nick translation, DNA synthesis with random primers, and RNA transcription of cloned genes under the control of promoters (such as SP6 phage) (137). However, the most common method used for uniformly labelling polynucleotides with radionucleotides has been nick translation as reported by Rigby, *et al.* (100, 188).

DNA synthesis with random primers in the presence of isotopically-labelled nucleotides primarily has been useful for labelling crude DNA preparations such as restriction fragments that

have been purified by gel electrophoresis (137). Random-primer labelling of approximately 25 ug of substrate has yielded approximately 50 ng of labelled DNA (5×10^9 cpm/ug), but the resulting probes varied greatly in length (137). Labelling of DNA using the phage polymerase system required subcloning of the appropriate DNA fragment into a SP6 vector immediately downstream from a SP6 phage promoter. Using bacteriophage SP6 RNA polymerase, transcription of the SP6 plasmid DNA was initiated at this promoter and labelled RNA transcripts of the cloned DNA with a high degree of specific activity was obtained (137). The RNA probe had several advantages because RNA:RNA and DNA:RNA hybrids were more stable than DNA:DNA hybrids and nonspecifically bound probe was removed by ribonuclease A. In addition after subcloning of sequences there was no need to purify cloned DNA fragments by electrophoresis because transcription was done from cloned plasmid DNA (137). Single-stranded RNA probes have been suitable for solution hybridizations with shortened hybridization times (137, 201).

Nick translation with DNA polymerase I allowed the sequential incorporation of nucleotides to the 3' hydroxyl terminus of nicks (formed by the action of DNAase I) as it removed nucleotides from the 5' phosphoryl end of the nicks. Consequently, labelled nucleotides replaced the unlabelled nucleotides of the double-stranded DNA template (188). Double-stranded DNA was labelled with ^{32}P -labelled nucleotides to levels greater than 10^8 cpm/ug of

DNA which was ten-fold higher than *in vivo* labelling during replication (188). The labelling of the double-stranded DNA was evenly distributed and 40 % to 50 % of nucleotides incorporated during nick translation were labelled (188). Rigby reported that an equivalent of one genome per cell could be detected with a nick-translated probe labelled to 10^8 cpm/ug of S40 DNA (188). Use of nonradioactive reporter substances such as biotin has allowed an alternative to radioisotopes for labelling of hybridization probes (23). Langer *et al.* reported the synthesis of biotinylated hybridization probes using biotin-labelled derivatives of UTP and dUTP (115). The detection of biotin-labelled DNA hybridization was based on the unique and high degree of affinity of avidin for biotin (111). Avidin binds four molecules of biotin with a dissociation constant (Kd) of 10^{-15} M (111). Leary *et al.* reported the detection of biotinylated DNA with streptavidin which was used to sandwich the hybridized probe to biotinylated alkaline phosphate for colorimetric identification of hybridization (118). Biotinylated hybridization have been used for the identification of viral sequences by dot blot and by *in situ* methods (23, 53, 70, 146, 149, 199). Although these probes were 10- to 100-fold less sensitive than radiolabelled probes but have the advantages of a longer shelf-life (up to 1 year), greater safety, easier disposal, and faster detection (less than 24 hours).

Hybridization of probes with target sequences have been done in

solution, on solid media such as nitrocellulose filters, and *in situ* (112). Optimal hybridization kinetics were obtained when hybridization was done in solution. Following hybridization in solution, DNA:DNA or RNA:DNA duplexes were distinguished from single-stranded, unhybridized probe by adsorption of the duplexes onto hydroxyappetite or by S1 nuclease digestion of unhybridized probe sequences (20). However, this technique required fairly large amounts of probe DNA to achieve high levels of sensitivity (112).

Membrane filter hybridization has been used extensively for the identification of viral sequences (20, 99, 112, 136, 213, 225). Single-stranded DNA and RNA (under the proper salt conditions) from sample were immobilized onto nitrocellulose or nylon membrane filters (20). Stalhandske and Pettersson first reported the identification and differentiation of type 1 and type 2 herpesvirus DNA in infected cell culture by membrane filter hybridization (213). Southern transfer to nitrocellulose of restriction endonuclease digestion fragments after separation by gel electrophoresis was one method for immobilizing single-stranded DNA onto membrane filters for subsequent hybridization (128, 225). This technique allowed a more rational analysis of hybridization signals than dot blot assays because specific sequences were separated according to their electrophoretic migration pattern and thereby specific sequences were distinguished from contaminating sequences (225).

Membrane filter hybridization has been done most commonly by dot

blot hybridization on membranes in a filter support with 96 wells in which specimens were applied by passing the material through the filter with negative pressure. (82, 86, 156). Samples were denatured by heat or treatment with NaOH prior to their application to the membrane filter (206, 213, 228). DNA or RNA extracted from infected cell cultures, tissue samples, body fluids, and excreted materials (such as feces and urine) have been screened by dot blot hybridization for target sequences (1, 20, 112, 187, 201, 206, 213). Dot blot hybridization allowed fast, simultaneous, and relatively simple testing of a large number of samples immobilized on the filter (86, 112, 187). Disadvantages of dot blot or membrane filter hybridization were that differentiation of nonspecifically bound probe from that bound to target sequences was difficult and cellular localization of viral sequences within infected cells was not possible (20, 112). Dot blot hybridization has been used for the detection of bluetongue virus (212), herpesviruses (213), rotaviruses (122), coronaviruses (206), enteroviruses (101), adenoviruses (216), and hepatitis B virus (141, 204).

In contrast to membrane or solution hybridization, *in situ* hybridization provided information on the location of viral sequences in particular cell types and within the cell (81, 83, 100). Brahic and Haase reported the detection of as few as 20 visna virus genomes per cell by *in situ* hybridization (21, 83). This procedure allowed detection of latent viral infections at levels of one to two

genome copies per cell when no detectable expression or replication of the viral genome occurred (as may occur with retroviruses) (82, 83, 100, 181, 211). *In situ* hybridization has been done on impression smears or in frozen tissues sections (83). Brahic *et al.* reported the simultaneous detection of Theiler's murine encephalomyelitis virus RNA, by hybridization, and viral antigens, by immunocytochemistry, in cell cultures and paraffin embedded sections (22). Hybridization sensitivity in this assay was at a level of 10 genome copies per cell (22). *In situ* hybridization probes have provided useful information on mechanisms and pathogenesis of viral infections (11, 139). Disadvantages of *in situ* hybridization included extended time required for detection of radiolabelled probes by autoradiography and a decreased sensitivity (10 to 100 fold less than membrane and solution hybridization) (83, 100, 112).

The sensitivity and specificity of hybridization probes for the presence of viruses were equal or greater than other laboratory methods such as immunoassays, virus isolation, and electron microscopy (186). The sensitivity of hybridization of probes with target sequences was dependent on the methods of labelling, hybridization conditions, detection system, and hybridization system (20). Hybridization probes were as sensitive and specific as immunofluorescence with monoclonal reagents for herpes simplex virus detection in clinical specimens (186). Teramoto compared ELISA, hemmagglutination, electron microscopy, and DNA hybridization for the

detection of canine parvovirus in fecal samples (73, 217). The ELISA was specific and sensitive for canine parvovirus hemagglutinating protein but DNA hybridization was ten-fold more sensitive. DNA hybridization indicated the presence of virus in 7 of 11 ELISA-negative samples (217). The detection limit of DNA hybridization in this study was approximately 20 pg of virus DNA (217).

The specificity and sensitivity of hybridization probes was demonstrated by Lin *et al.* who differentiated known subgroups and serotypes of rotavirus with hybridization probes prepared from different viral genome segments (122). Genomic RNA was detected at threshold levels of approximately 20 to 50 ng (122). Shockley *et al.* differentiated TGE and BCoV coronaviruses with hybridization probes prepared from cloned cDNA sequences (206). They reported a sensitivity level of 25 pg of genomic RNA by dot blot hybridization (206). Schuster *et al.* reported that DNA and RNA hybridization probes had an 83 % correlation in sensitivity and a 92 % correlation in specificity compared to virus isolation for the detection of cytomegalovirus in 95 urine samples (201). The DNA hybridization probes were prepared from specific cloned sequences whereas the RNA probes were from *in vitro* synthesized transcripts of specific DNA sequences and were equally sensitive (detecting 3.2 to 10 pg of DNA) (201).

The degree of homology among genomic nucleic acids from different viruses has been determined by hybridization using conditions of low and high stringency obtained by adjusting hybridization temperature and formamide concentration (99). The relatedness of virus strains has been determined by high stringency hybridization with corresponding sequences from various locations within their genomes (99, 162, 192, 198, 220). Peterson *et al.*, using a synthetic probe of 22 bp in length, identified and differentiated herpes simplex type 1 and 2 (162).

Several factors have been considered in the application and use of hybridization probes (8, 41, 140, 193, 201). False-positive hybridization signals occurred when large amounts of *Escherichia coli* DNA was present in samples (201). This factor was considered when samples were contaminated with large numbers of bacteria (e.g. feces or urine) (201). It has been difficult to prevent contamination of cloned sequences with vector DNA during isolation of the former and probes made often contained some vector-specific sequences (201). Therefore samples containing bacteria with certain plasmids have given false-positive results (201). False-positive hybridization assays for hepatitis virus due to contamination by residual vector sequences in the hybridization probe was reported by Diegutis *et al.* (41). Clinical samples contaminated with bacteria have reacted with probes prepared from plasmid vector sequences (such as pBR322) which indicated that hybridization between plasmid vectors and probes could

contribute false-positive results (2). The importance of using plasmid vector DNA as a negative control has been emphasized by several investigators (2, 41). False-positive results also have been reported from homologous hybridization of probe and cellular RNA sequences (131).

Another factor that has been considered in using hybridization probes was probe size (8, 140). A stable double-stranded nucleic acid duplex required a minimum of 15 to 20 consecutive base pairs between of homologous sequences (86, 162). The construction of small specific probes resulted in a high degree of specificity and a reduced sensitivity (86, 162). In most hybridization assays, a probe length of 100 to 200 bp was optimal for obtaining both a high level of specificity and sensitivity (8). Moench *et al.* reported that small probes (approximately 70 bp) gave improved hybridization signals used for *in situ* hybridization (140). By using a short probe length of 25 bp, the length of hybridization was shortened to 90 minutes without an appreciable loss in sensitivity (30 to 150 fold less sensitive) (162, 178).

III. MATERIALS AND METHODS

Cell Cultures and Medium

Dulbecco's MEM (Kansas City Biologicals, Kansas City, KS) supplemented with 10 % fetal bovine serum (FBS) was used for cell growth. Prior to its use, FBS was heat inactivated at 56 C for 30 minutes then cooled to room temperature. Forty percent (w/v) polyethylene glycol (6000-8000 mw; 4 ml per 100 ml) and beta-propiolactone (Sigma Chemical Co., St. Louis, MO; 25 ul per 100 ml) was added to FBS and mixed (69). The FBS then was incubated for 12 hours at 22 C, heated for 1 hour at 37 C and stored at -20 C. Secondary bovine turbinate cell cultures (BTU) that had been passaged 5 to 12 times were used for propagating BVDV. Cells were negative for BVDV antigen by indirect immunofluorescence (IFAT).

Viruses

Two field strains of BVDV; strain 72 (168), a cytopathic strain, and strain 2724 (167), a non-cytopathic strain; were used in this study. Other strains of BVDV used included strain 7443, New York-1 strain, Singer strain, and NADL strain (Steve Bolin, NADC, Ames, IA). Other viruses included in this study were bluetongue virus (209), bovine coronavirus (135), and bovine adenovirus (Veterinary Medical Research Institute, Iowa State University, Ames, Iowa).

Ribonucleases

All buffers, except Tris-HCl buffers, contained 0.1 % diethyl pyrocarbonate (DEPC); were prepared in clean, autoclaved glassware; and were autoclaved. Tris-HCl buffers were prepared with autoclaved 0.1 % DEPC-treated water.

Virus Purification

Differential centrifugation Two-day-old BTU cell cultures were inoculated with BVDV strain 72 at a multiplicity of infection of 0.05 median cell culture infectious doses (CCID₅₀) per cell. The cell cultures were incubated at 37 C in a 5 % CO₂ atmosphere for 2 to 3 days or until the cytopathic effect (CPE) reached 80 to 100 %. The virus was collected from cell culture supernatant fluids which were clarified by centrifugation first at 4000 g for 30 minutes at 4 C and again at 15000 g for 20 minutes at 4 C. The clarified supernate was collected and centrifuged at 100,000 g for 2 hours at 4 C to pellet the virus. The viral pellet was resuspended in 0.5 ml of NTE buffer (0.05 M tris-HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.6).

Isopyknic density gradient centrifugation In some instances the virus was purified by isopyknic density gradient centrifugation. Twenty-five ml of clarified cell culture fluids were placed onto 20 %-60 % sucrose cushions. The latter were prepared by layering 5.0 ml of 20 % sucrose onto 5.0 ml 60 % sucrose in NTE buffer. The cushions, overlaid with cell culture fluids, were centrifuged at 100,000 g for 3 hours at 4 C. The supernate was removed by needle aspiration from the

top of the tube to 1.0 cm above the fluid-sucrose interface and then replaced with fresh fluids until all the clarified cell culture fluid was processed. After final centrifugation, the light scattering band at the sucrose-sucrose interface was collected in a syringe after stabbing the tube with an 18 gauge needle. The fraction collected was diluted 1:2 with NTE buffer, layered onto a continuous gradient of 20 to 60 % (w/w) sucrose in NTE buffer, and centrifuged at 100,000 g at 4 C for 14 hours. Following centrifugation, 0.5 ml fractions were collected in a fraction collector and the refractive index of each fraction was determined using a bench top refractometer (Bausch and Lomb, Rochester, NY). The absorbance of fractions with a specific gravity of 1.150 to 1.115 was determined in a spectrophotometer set at a wavelength of 260_{nm}. The absorbance peak of the fractions was determined and fractions corresponding to that peak were pooled. The pooled material was diluted in NTE buffer to a volume of 36.0 ml, and centrifuged at 100,000 g for 5 hours at 4 C. The virus pellet then was resuspended in 0.5 ml NTE buffer and placed in a siliconized glass tube.

Extraction of RNA from Purified Virus

Sufficient 20 % SDS was added to 0.5 ml of purified virus to obtain a final concentration of 1.0 %. Approximately 0.5 mg of proteinase K then was added and incubated at 37 C for 30 minutes. The solution was extracted twice with phenol:chloroform:isoamyl alcohol (24:24:2) and the aqueous layer was collected to which 2.0 M sodium

acetate was added to obtain a concentration of 0.2 M followed by two volumes of ethanol to precipitate the viral RNA. The RNA was stored in ethanol at -80 C.

Poly-A tailing of BVDV RNA

The concentration of BVDV RNA was determined by spotting 1 ul of purified RNA onto DE-81 fiber filters adjacent to known concentrations of yeast transfer RNA (Bethesda Research Laboratories, Gaithersburg, MD) followed by staining with ethidium bromide and examination under UV light (128). The tailing reaction volume of 100 ul consisted of 50 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 2.5 mM MnCl₂, 250 mM NaCl, 0.25 mM ATP, 50 ug BSA, 10 ug of BVDV RNA, and 6 units of poly-A polymerase (BRL, Bethesda, MD). The reaction was continued for 12 minutes at 37 C to add approximately 100 adenine bases to the 3' end of BVDV RNA. The enzymatic reaction was stopped by the addition of 0.5 M EDTA to obtain a concentration of 10 mM and by heat inactivation at 60 C for 5 minutes. Na acetate (3.0 M) was added to a final concentration of 0.3 M and then 3 volumes of 100 % ethanol were added to the mixture which was placed at -80 C for storage.

cDNA Synthesis

cDNA synthesis was done by a method adapted from Maniatis and Gubler and Hoffman using reagents supplied by Amersham, Arlington Heights, IL (80, 128). BVDV RNA was heated to 90 C for 5 minutes prior to first strand synthesis. The latter was done in a volume of

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20 ul containing 10 mM Tris-HCl (pH 8.3), 10 mM MgCl₂, 10 mM dithiothreitol, 2 mM dCTP, 2 mM dTTP, 2mM dATP, 2 mM dGTP, 50 pmoles oligo-dT₁₂₋₁₈, 30 units RNasin, 20 units of reverse transcriptase, and 10 ug of poly-A tailed BVDV RNA. The mixture was incubated at 42 C for 1 hour and placed on ice. Second strand cDNA synthesis was done in a volume of 100 ul containing the products of first-strand synthesis, 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 10 mM (NH₄)₂SO₄, 100 mM KCl, 0.15 mM NAD, 50 mg/ml BSA, 40 uM dNTP's, 10 units ribonuclease H, and 10 units of DNA polymerase I. This mixture was incubated at 12 C for 60 minutes and at 70 C for 10 minutes. Next, 2 units of T4 DNA polymerase were added and incubated at 37 C for 10 minutes and the reaction was stopped by adding 10 ul of 0.25 M EDTA per 100 ul of reaction mixture and 10 ul of 10 % SDS per 100 ul of reaction mixture. The cDNA product was extracted once with phenol and chloroform (as previously described), precipitated in ethanol, and placed at -20 C.

Poly-dCTP Tailing of BVDV cDNA

Homopolymer dCTP tailing of the BVDV cDNA was done according to the methods of Roychoudhury and Wu (200). This procedure was followed in detail and the addition of 15-20 dCTP's was obtained by incubation for 1.5 minutes at 37 C. The reaction volume of 50 ul consisted of 5 ul of tenfold concentrated (10X) tailing buffer (1.0 M potassium cacodylate, 0.25 M Tris base, 10 mM CoCl₂, and 2 mM DTT) 10 mM dCTP, and 30 units of terminal deoxy-transferase (Pharmacia,

Piscataway, NJ). The reaction was stopped by adding 5 ul of 0.5 M EDTA to the mixture followed by incubation at 68 C for 5 minutes and the product was precipitated in ethanol as described before.

Annealing of Tailed cDNA to G-tailed pUC9 Plasmid

A mixture of 30 ul of annealing buffer (10 mM Tris-HCl, 1 mM EDTA, and 0.15 M NaCl), 1-5 ng of C-tailed BVDV cDNA, and 15 ng of dGTP-tailed, Pst I-cut pUC9 plasmid (Pharmacia, Piscataway, NJ) was incubated at 58 C for 3 hours and then gradually cooled to 4 C over 1 hour.

Transformation

E. coli strain JM-83 cells were prepared for transformation as described by Hanahan (87). Bacteria were grown in broth culture in SOB medium (2.0 % Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄) at 37 C in a shaking incubator at 225 revolutions/minute until a density of 5×10^7 colony forming units/ml was reached. After growth, 2.5 ml of bacterial culture was placed on ice for 10-15 minutes and then pelleted by centrifugation at 1000 g for 12 minutes at 4 C. The bacterial pellet was resuspended in 0.8 ml of transformation buffer (TFB) [10 mM potassium-2-[N-morpholino]ethanesulfonic acid (pH 6.2), 0.1 M RbCl, 45 mM MnCl₂-4H₂O, 10 mM CaCl₂-2H₂O, 3 mM H₃CoCl₃] and placed on ice for 10-15 minutes. The suspension was centrifuged at 1000 g for 10 minutes at 4 C and the pellet was

resuspended in 200 ul of TFB and DMSO was added to obtain a final concentration of 3.5 %. The mixture was placed on ice for 5 minutes and then dithiothreitol was added to obtain a concentration of 75 mM and placed on ice for 10 minutes. Next, the same volume of DMSO was added and the mixture was held on ice for 5 minutes. The mixture then was placed in a 17 mm x 100 mm polypropylene tube (Falcon #2059, Becton Dickinson, Rutherford, NJ) and 7 ng of DNA was mixed in which was followed by incubation on ice for 30 minutes. The mixture was heat shocked for 90 seconds at 42 C and placed on ice for 2 minutes after which 800 ul of SOC medium (2.0 % Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) at 20 C was added. The mixture then was incubated at 37 C in a shaker incubator at 37 C for 1 hour. The transformed bacteria were plated on selective medium [YT agarose: 1.5 % agarose, 0.8 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 0.008 % X-gal, and 0.0001 % sodium ampicillin (the latter two ingredients were added after cooling to 50 C)] 150 mm petri plates after placing 100 ul of the bacterial suspension into 100-200 ul of SOB medium. The plates were incubated for 12-24 hours at 37 C. White colonies were subcultured on selective medium and incubated for 12-24 hours at 37 C.

Colony Screening

Transformed bacterial colonies were screened according to the methods of Maniatis *et al.* (128). A nitrocellulose filter was placed on the agar surface after bacterial growth and removed when

moistened. The bacteria transferred to the nitrocellulose filter were lysed in alkali by placing the filter with bacteria onto a series of 3MM Whatman paper saturated with various solutions. In this way the bacteria were exposed sequentially to 10 % SDS for 3 minutes, 0.5 M NaOH and 1.5 M NaCl (Southern #1) for 5 minutes, 3.0 M NaCl and 0.5 M Tris-HCl pH 7 (Southern #2) 5 minutes, 2X SSC (diluted from 20X solution containing 3.0 M NaCl and 0.3 M trisodium citrate, pH 7.0) for 5 minutes, and 2X SSC for 5 minutes. The nitrocellulose filter was allowed to dry at 22 C for 30 minutes and then baked for 3 hours at 80 C in a vacuum oven.

Preparation of Hybridization Probe for Colony Screening

BVDV cDNA was labelled with ^{32}P by first strand synthesis, as described earlier, in the presence of alpha dCT ^{32}P , dAT ^{32}P , and dGT ^{32}P . Density gradient-purified BVDV RNA (2 ug) was used as the template and random primers prepared from calf thymus DNA according to Maniatis *et al.* (128) were used to initiate the reaction. The latter was used at 5 times the concentration of BVDV RNA in the reaction (9).

Hybridization

DNA hybridization was done according to the methods of Maniatis *et al* (128). Nitrocellulose filters with colonies of lysed bacteria were placed in plastic bags (Scotch-Pak bags, Fisher Scientific, Atlanta, GA) and wetted with 6X SSC followed by treatment with 10 ml

of pre-hybridization solution [5X Denhardt's solution (50X contains 1.0 % Ficoll, 1.0 % polyvinylpyrrolidone, 1.0 % BSA fraction V), 5X SSC, 0.1 % SDS, and 100 ug/ml salmon sperm DNA (denatured by sonication and then heating to 100 C and rapid cooling)] per 100 cm² of filter at 68 C for 4-6 hours with gentle agitation. The first strand cDNA probe (20 ul) was treated with NaOH (final concentration of 0.3 N) at 22 C for 1 hour to denature RNA template attached to radioactive complementary DNA. After pre-hybridization of the filters, the probe was added to the pre-hybridization solution and incubated at 68 C for 12-16 hours with gentle agitation. After hybridization, the solution was removed and nitrocellulose filters were washed in a specific sequence; 2x SSC and 0.5 % SDS at 22 C for 5 minutes, 2x SSC and 0.1 % SDS at 22 C for 5 minutes, 0.1x SSC and 0.5 % SDS at 68 C for 2 hours, and 0.1x SSC and 0.5 % SDS at 68 C for 30 minutes. The filters then were dried on Whatman paper at 22 C and were placed in radiographic film cassettes containing XAR-5 Kodak film (Eastman Kodak Co., Rochester, NY) and an intensifying screen. The film was exposed to radiation for 24 to 72 hours.

Hybridization of RNA dot blots was done as described for screening bacterial colony blots with the following exceptions. The pre-hybridization solution consisted of 50 % formamide, 5X SSC, 0.2 % SDS, 1X Denhardt's solution, and 250 ug sheared salmon sperm DNA. Hybridization was done at 42 C for 16 to 24 hours. Following hybridization the nitrocellulose filter was washed 4 times for 5

minutes at 22 C with 2X SSC and 0.1 % SDS and twice for 15 minutes at 50 C with 0.1X SSC and 0.1 % SDS.

Plasmid Characterization

Bacterial colonies which had a hybridization signal on radiographic film were subcultured and grown in 6.0 ml of YT broth supplemented with ampicillin (0.05 mg per ml). Mini-plasmid preparations were prepared to further characterize plasmids containing inserts (128). Bacteria from 5.0 ml of broth culture were pelleted by repeated centrifugation at 2,000 g for 10 minutes in a 2.0 ml microcentrifuge tube and removal of supernatant. The final bacterial pellet was suspended in 0.1 ml of lysozyme solution [2.0 mg lysozyme in 1.0 ml of 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA] for 5 minutes at 22 C and then 0.2 ml of 0.2 N NaOH in 1.0 % SDS was added and incubated for 15 minutes on ice. Then 0.15 ml of 5 M Na acetate (pH 4.8) was added and the mixture placed on ice for 5 minutes. The solution then was centrifuged for 15 minutes at 13,000 g at 4 C and the supernate transferred to a 2.0 ml microcentrifuge tube. The DNA was precipitated with ethanol and placed at -80 C for 1 hour.

The precipitated plasmid DNA then was centrifuged for 20 minutes at 13,000 g at 4 C and the pellet was dried in a vacuum centrifuge. The DNA was dissolved in 50 ul of TE buffer (10 mM Tris-HCl pH 7.8 and 1.0 mM EDTA). A horizontal gel of 1.0 % agarose in TAE buffer (50 X solution contained 1.0 M Tris-HCl, 5.0 M Na acetate, 0.1 M EDTA) containing 0.005 % ethidium bromide was prepared in which 5 ul of the

plasmid preparation was electrophoresed and examined under ultraviolet light to determine the size of the plasmid DNA by the migration pattern (128). Cloned plasmids migrating slower than parental supercoiled pUC9 plasmid were digested with Pst-I restriction endonuclease according to the techniques of Maniatis et al (128).

Storage of Bacteria with Cloned Sequences

The transformed bacteria was stored for future use at - 80 C. Sixteen hour broth cultures of bacteria containing the plasmids were diluted 1:1 in YT medium containing 40 % glycerol. The bacterial stocks were frozen in liquid nitrogen before storage at -80 C. ?

Plasmid and cDNA Insert Purification

Broth cultures (500 ml) of bacteria with plasmids were grown in YT medium supplemented with ampicillin (0.05 mg per ml) for maxi-plasmid preparations as described by Maniatis et al. (128). Bacteria were pelleted at 4000 g for 10 minutes at 4 C and washed with 100 ml of STE buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 7.8], and 1.0 mM EDTA) by centrifugation. The washed bacterial pellet was resuspended in 8.0 ml of solution I [50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 5.0 mg/ml of lysozyme (added prior to use) (Sigma Chemical Co., St. Louis, MO)] and incubated at 22 C for 5 minutes. To the latter mixture, 16.0 ml of solution II (0.2 N NaOH and 1.0 % SDS) were added, mixed gently and placed on ice for 10 minutes. Next, 12.0 ml of 5.0 M potassium acetate (pH 4.8), chilled to 4 C, were added, mixed

vigorously, placed on ice for 10 minutes and centrifuged at 55,000 g for 20 minutes at 4 C (Beckman SW27 rotor, Beckman, Fullerton, CA). The supernate was collected, mixed with 0.6 volumes of isopropanol, incubated at 22 C for 15 minutes, and centrifuged at 12,000 g for 30 minutes at 20 C. The DNA pellet obtained was washed with 70 % ethanol, dried, and stored at - 20 C. Equilibrium density gradient centrifugation was done for purification of supercoiled plasmid DNA by cesium chloride. The washed DNA was dissolved in 8.0 ml of TE buffer, CsCl (1.0 g/ml) was added to the mixture, and then transferred to a heat-sealable tube (Beckman). Ethidium bromide was added to the mixture to obtain a concentration of 1.0 mg/ml and the tubes were filled with mineral oil, sealed and centrifuged at 132,000 g in a Beckman Ti 70 rotor for 36 hours at 20 C. The lower of two bands visualized by ultraviolet light was collected by aspiration into a 10 ml syringe using a 21 gauge needle. Ethidium bromide was removed from the aspirated solution of plasmid DNA by alternate extractions with sec-butanol and water-saturated sec-butanol followed by dialysis for 12-24 hours against TE buffer (pH 8.0). Following dialysis, the plasmid DNA was precipitated by the addition of 3 M sodium acetate to obtain a final concentration of 0.3 M and 3 volumes of ethanol and then stored at -20 C. Insert cDNA was separated from plasmid DNA by restriction endonuclease digestion using *Pst* I or *Hind* III and *Bam*H I enzymes followed by gel electrophoresis and electroelution (128).

Dot blot procedure

DNA and RNA dot blots were prepared as described by Maniatis et al (128). RNA dot blots were prepared from infected cell culture fluids on nitrocellulose (228, 128). Cell culture fluids were clarified by centrifugation at 2000 g for 10 minutes, mixed with 0.1 volume of 5.0 % Nonidet P-40 (Sigma Chemical Co., St. Louis, MO), placed on ice for 10 minutes and centrifuged at 15000 g for 2.5 minutes. The supernate was collected and an equal volume of freshly made denaturing solution [2 parts 37 % (w/w) formaldehyde and 3 parts 20 X SSC] was added. The processed specimens were incubated at 60 C for 15 minutes and then stored at -80 C.

Nitrocellulose was soaked in DEPC-treated water until wet and then in 20 X SSC. The filter was placed in a dot-blot apparatus (Bio-Rad, Richman, CA). Serial tenfold dilutions of processed specimens were made in a siliconized 96 well microtiter plate and 100 ul of each dilution was applied with vacuum (25 psi). The filters were air dried and baked at 80 C for 90 minutes.

Preparation of Hybridization Probe for RNA Blots

Cloned BVDV cDNA sequences, purified as described previously, were nick translated in the presence of alpha dCT³²P for use as hybridization probes. The reaction mixture of 50 ul contained 1 ug of purified insert DNA; 100 uCi alpha dCT³²P; 0.02 mM of dATP, dGTP, and dTTP in 0.5 M Tris-HCl (pH 7.8); 50 mM Tris-HCl (pH 7.8); 5 mM MgCl₂; 10 mM 2-mercaptoethanol; 10 ug/ml BSA; 2 units of DNA

polymerase I; and 200 pg DNase I. The mixture was incubated for 60 minutes at 15 C and then 5 ul of 300 mM EDTA was added. The labelled DNA was separated from unincorporated nucleotides by centrifugation through a Sephadex G-50 column (128). The DNA was labelled to a specific activity of $1-4 \times 10^8$ cpm per ug. Prior to hybridization, the probe was heated for 5 minutes at 100 C and chilled on ice.

Preparation of Southern Blots

For cross hybridization mapping of clones, Southern blots of agarose gels containing electrophoresed restriction digests of specific clones were done according to the methods of Maniatis *et al.* (128). Specific BVDV clones were digested with *Pst* I restriction endonucleas and electrophoresed in 1.0 % agarose as previously described. After electrophoresis the agarose gel was trimmed to remove excess agarose and exposed to UV light (UV light box Fischer Scientific, Pittsburgh, PA) for 10 minutes. The gel was denatured by washing with Southern no. 1 (0.5 N NaOH, 1.5 M NaCl) for 1 hour with constant shaking followed by neutralization in Southern no. 2 (3.0 M NaCl, 0.5 M Tris-HCl; pH 7.0) for 1 hour with constant shaking. The electrophoresed DNA then was transferred from the agarose gel to nitrocellulose by osmosis for 24 hours using a transfer buffer (10 X SSC). After transfer of DNA the nitrocellulose filter was baked for 2-3 hours at 80 C.

IV. RESULTS

BVDV Genomic RNA Purification

The titer obtained when BVDV strain 72 was grown in BTU cell cultures was approximately 10^7 CCID₅₀ per ml. After isopyknic density gradient centrifugation, a light scattering band was present in the gradients and corresponded to a density ranging from 1.118 to 1.161 g/cm³. Specific gravity of density gradient centrifugation fractions with maximum absorbance values at 260 nm ranged from 1.151 to 1.127 in the 11 batches of purified virus prepared. The peak of absorbance of the BVDV band fractions was at a specific gravity of 1.138. Approximately 250 ug of RNA was extracted from BVDV purified from 500 ml of infected cell culture fluids and precipitated in ethanol.

cDNA Cloning

Only 10 ug of BVDV RNA was used in the poly-A tailing reaction for cDNA synthesis. By comparing the number of picamoles of adenine (as AT³²P) incorporated with time during poly-adenylation of a 7.5 kb yeast transfer RNA (Bethesda Research Laboratories, Gaithersburg, MD), it was determined that a reaction time of 12.5 minutes would result in the addition, on average, of 100 adenines at the 3'-end of BVDV genomic RNA. All the poly-A tailed RNA produced in the reaction

in 12.5 minutes was used for cDNA synthesis. The C-tailing of cDNA was successful only when the procedure of Roychoudhury and Wu was followed strictly (200). The reaction time was determined by comparing the incorporation of dCT³²P with time in the C-tailing reaction to allow the addition of 15-20 cytosine bases at the 3' end of *Pst* I-cut pBR322 DNA. Following annealing of C-tailed BVDV cDNA sequences with G-tailed, *Pst* I-cut pUC9 plasmid DNA and transformation of *E. coli*, approximately 5,000-6,000 white *E. coli* colonies grew on selective medium. The transformation efficiency of the bacteria with pUC9 plasmid DNA was 5.0×10^7 per μ g of pUC9 plasmid. Approximately 1 white colony for every 4 blue colonies grew on selective medium following transformation. All white colonies were subcultured onto a grid plate so that approximately 500 colonies were grown per plate. Approximately 10 % of the white colonies were unstable (deleted the newly acquired insert sequences) and turned blue after subculturing for colony screening.

Twelve nitrocellulose grid filters of bacterial colony blots were screened by DNA hybridization with a random primed, ³²P-labelled, first strand cDNA probe and autoradiographed as described in Figure 1. Approximately 200 bacterial colonies had strong signals with this probe. These colonies were subcultured for further analysis.

Insert Characterization

Mini-plasmid preparations were analyzed after electrophoresis to determine the presence and size of BVDV cDNA inserts contained in the

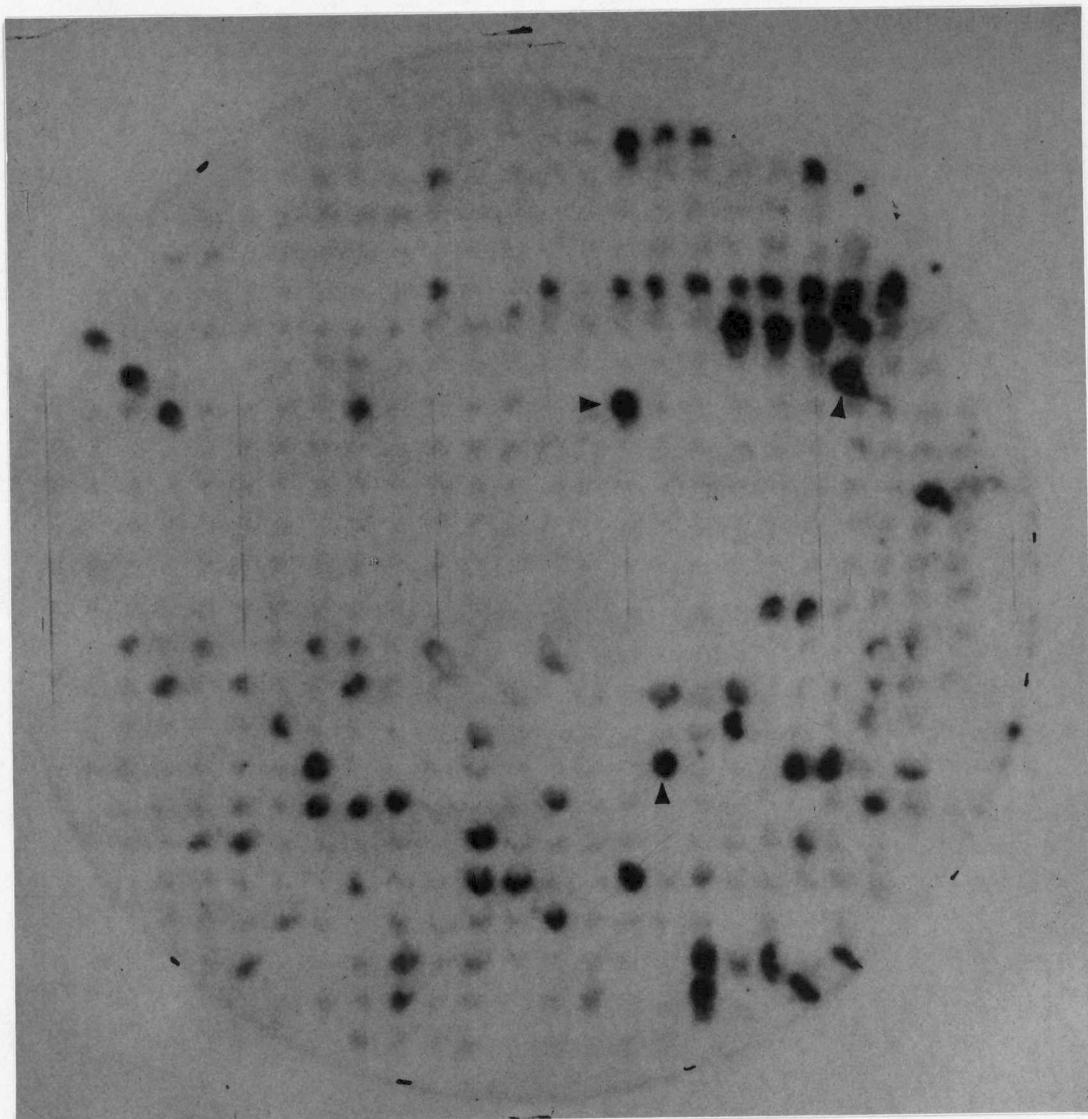


Figure 1. Autoradiograph of bacterial colony blots hybridized with BVDV genomic first-strand cDNA probe. Radiographic film was exposed for 24 hours following hybridization as described in Materials and Methods. Arrows indicate some of the positive reactions with bacterial colonies representing bacteria with plasmids containing specific BVDV cDNA inserts.

plasmids (Figure 2). The distance of migration during electrophoresis of plasmids containing cDNA sequences compared to native supercoiled plasmid DNA and was indirectly proportional to insert size.

Forty-seven recombinant plasmids appeared to have inserts larger than 0.5 kilobases (kb) in length and sixty-seven recombinant plasmids had inserts smaller than 0.5 kb in length as listed in the Appendix.

Twenty-two recombinant plasmids were selected for further analysis.

Pst I restriction endonuclease digestion of recombinant plasmids and subsequent electrophoresis in 1.0 % agarose gels was done to examine the size of the cDNA inserts. Inserts were identified that ranged from 1.0 kb to 2.6 kb in size as listed Table 1.

A cloned sequence of cDNA 1.1 kb in length, identified as clone no. 5, was obtained from a recombinant plasmid that had one of the strongest signals by colony hybridization with random-primed cDNA probe. When it was removed from the vector and used as a nick-translated probe, it hybridized under conditions of high stringency with 7 of the 17 largest clones. Furthermore, 8 clones (#5, #4.3, #85.3, #126, #197.3, #243, and #310) contained 1 or 2 internal *Pst* I restriction endonuclease sites since digestion with *Pst* I generated two to three distinct fragments as shown in Figure 3 and 4 and Table 1. Clone no. 5 did not hybridize with non-infected cell culture blots but strong reactions occurred with BVDV-infected cell culture blots (Figure 5).

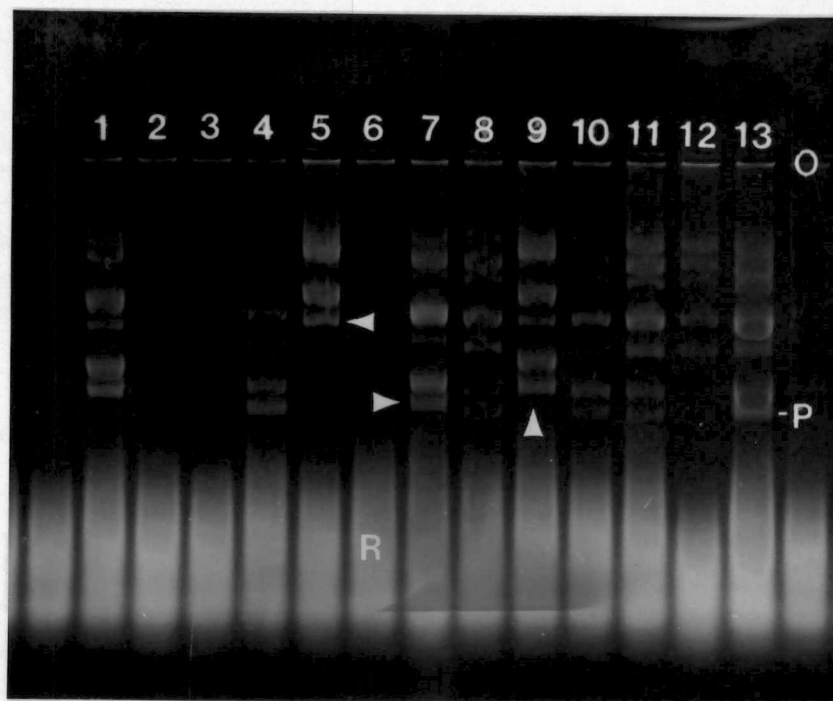


Figure 2. Agarose gel electrophoresis of DNA from mini-plasmid preparations of various recombinant plasmids which hybridized with BVDV first-strand cDNA probe. P - indicates migration of supercoiled native control plasmid (no insert). O - indicates origin of migration. Arrow in lane 7 indicates band of native supercoiled plasmid. Arrow pointing to band in lane 5 indicates supercoiled plasmid containing a significant insert (>1.0 kb). Arrow pointing to band in lane 9 indicates migration of plasmid containing smaller insert (<1.0 kb). R - migration pattern of contaminating bacterial RNA.

Table 1. Characterization of various cDNA clones identified by gel electrophoresis after digestion of recombinant plasmid DNA with restriction enzyme *Pst* I as described in Materials and Methods. More than one fragment was produced when inserts had internal *Pst* I restriction endonuclease sites. The order of DNA fragments within inserts has not been determined.

clone #	size of inserts in kilobases ^a			total kb ^b
1	0.5	0.5		1.0
4.3	0.55	0.65		1.2
5	0.5	0.6		1.1
71.2	2.0			2.0
74.2	0.7			0.7
85.3	0.3	0.5	1.8	2.6
102	1.3			1.3
126	0.5	0.6	0.8	1.9
170	1.4			1.4
194.2	1.3			1.3
197.3	0.35	0.5	1.8	2.65
227	1.35			1.35
243	0.7	1.6		2.3
246	0.6			0.6
310	0.3	0.6	1.0	1.9
315	0.5			0.5

^aFragments produced by *Pst* I digestion.

^bTotal size of insert in kilobases.

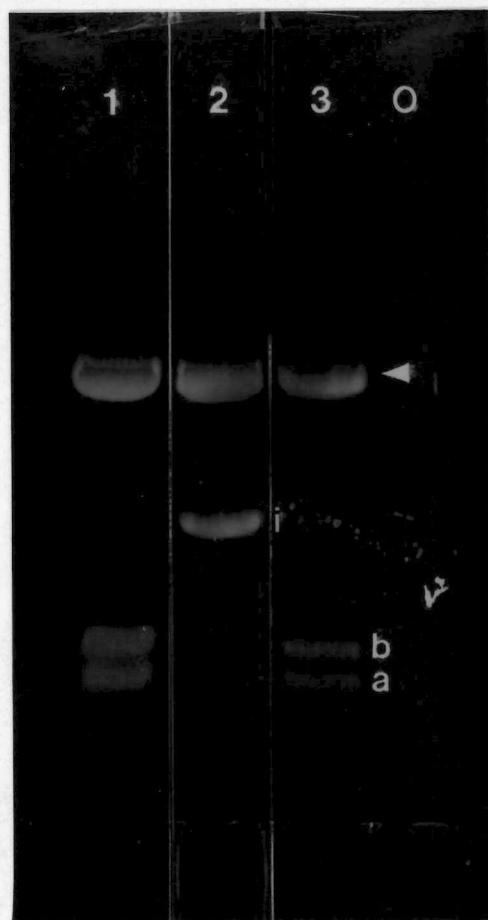


Figure 3. Agarose gel electrophoresis of plasmid DNA digested with restriction endonucleases to release insert cDNA fragments. Arrow indicates migration of linearized plasmid alone with insert cDNA removed (2.7 kb). 0 - indicates origin of migration. Lane 1 contains clone #4.3 digested with *Pst* I. Lane 2 contains clone #5 digested with *Bam*H I and *Hind* III, (i) indicates a single 1.1 kb insert fragment. Lane 3 contains clone #5 digested with *Pst* I, (a) and (b) indicate two insert fragments of 0.5 and 0.6 kb in size respectively. Note the two corresponding fragments in lane 1 from digested insert sequences of clone #4.3.



Figure 4. Agarose gel electrophoresis of recombinant plasmid DNA digested with restriction endonucleases to release insert cDNA fragments. Arrow indicates migration of linearized plasmid alone (2.7 kb) with insert removed. 0 - indicates origin of migration. Lane 1 contains clone #126 digested with Bam HI and HindIII. Lane 2 contains clone #126 digested with *Pst* I; (a), (b), and (c) indicate separate insert fragments of 0.5, 0.6, and 0.8 kb respectively. (i) indicates a single 1.9 kb insert fragment.

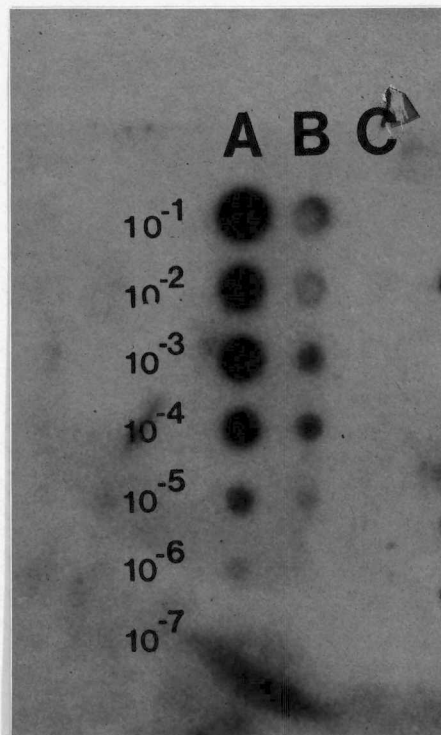


Figure 5. Dot blots of ten-fold dilutions of cytopathic BVDV strain 72 (column A), noncytopathic BVDV strain 2724 (column B), and uninfected cell culture (column C). Dot blots were hybridized with clone #5 as described in Materials and Methods. Both strains were detected to a dilution of 1.0×10^{-7} .

Cross hybridization mapping identified three separate non-overlapping groups of BVDV cDNA sequences within the 17 largest clones (Figure 6). Table 2 contains the results of cross hybridization mapping. Recombinant plasmids no. 5, 243, and 126 designated the three non-overlapping sequences. All hybridization probes for cross hybridization (no. 5, 243, and 126) contained 1 internal *Pst* I site with the exception of no. 126 which contained two. Internal *Pst* I restriction sites were present in 2 cDNA sequences (no. 245 and 4.3) hybridizing with recombinant plasmid no. 5 and one cDNA sequence (no. 74.2) hybridizing with recombinant plasmid no. 243.

Hybridization

Sensitivity The sensitivity of a nick-translated, ^{32}P -labelled DNA hybridization probe prepared from cDNA clone #5 in a hybridization assay with dot blots of known amounts of BVDV RNA was determined. As little as 20 pg of specific BVDV RNA resulted in a detectable hybridization signal as described in Figure 7. Twenty pg of BVDV genomic RNA represents the genomic content of approximately 3.8×10^6 virions assuming a genomic molecular weight of 3.2×10^6 daltons. When hybridization was done with 0.1 ml volumes of BVDV-infected cell cultures, the detection limit of the assay for BVDV RNA was the 1.0×10^{-7} dilution of the cell culture fluids whereas the infectivity titer of this material was 10^5 CCID₅₀ / 0.1 ml.

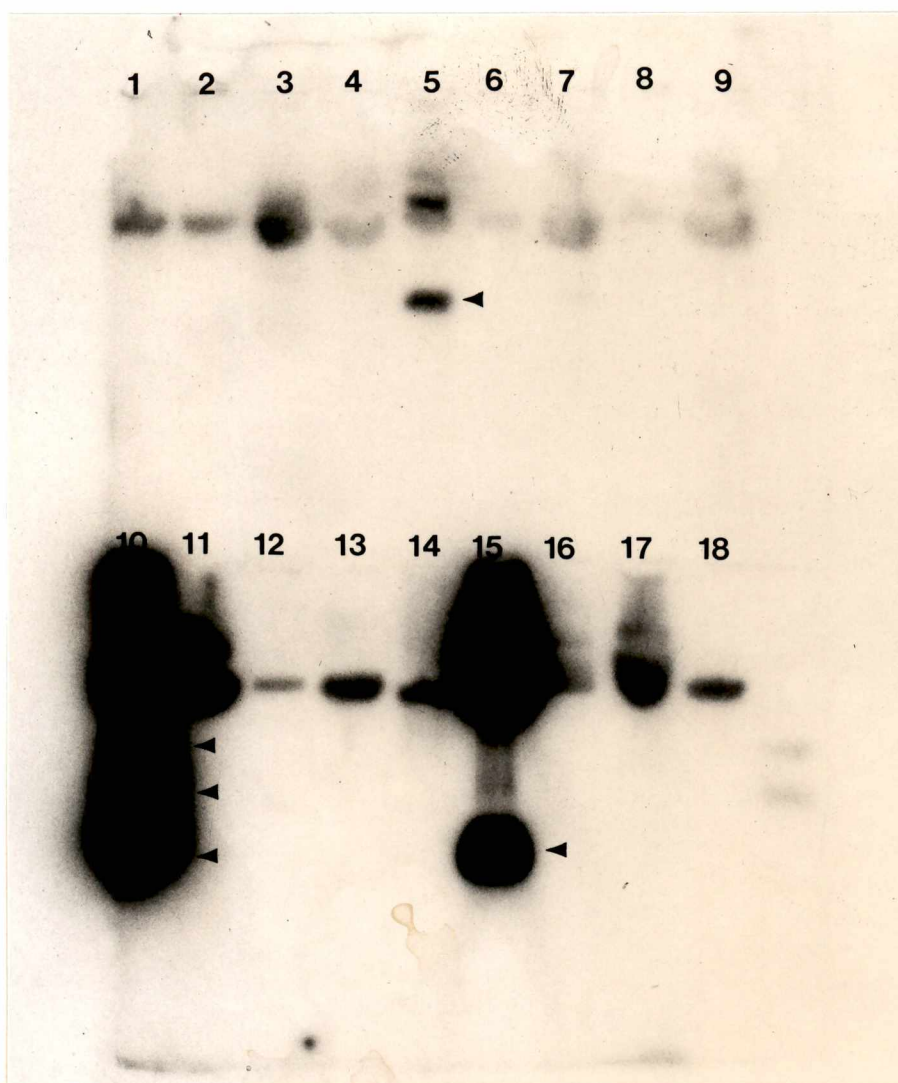


Figure 6. Autoradiograph of Southern blot of various recombinant plasmids for cross hybridization mapping with clone #126. Plasmid DNA was digested with *Pst* I restriction endonuclease, electrophoresed, and transferred to nitrocellulose as described in Materials and Methods. Lane 5 arrow indicates hybridization with cDNA of clone #74.2. Lane 10 arrow indicates hybridization of probe to itself (*Pst* I digestion of clone #126 results in three insert fragments indicated by three arrows). Lane 15 arrow indicates hybridization with one insert fragment from clone #170. Hybridization present in all lanes with bands closer to the origin than cDNA fragments represent hybridization of native plasmid sequences as a result of contaminating plasmid DNA in the probe.

Table 2. Results of cross hybridization mapping of 17 BVDV cDNA clones with probes prepared from three BVDV cDNA clones labelled with dCT³²P by nick translation as described in Materials and Methods.

Hybridization probe prepared from clone #				
clones	(kb)	5	243	126
5	(1.1)	+		
4.3	(1.2)	+		
246	(0.6)	+	+	
245	(1.6)	+	+	
165	(1.2)	+	+	
243	(2.3)		+	
102	(1.3)		+	
85.3	(2.6)		+	
74.2	(0.7)		+	+
170	(1.4)		+	+
126	(1.9)			+
194.2	(0.8)			
37.11	(1.4)			
200.12	(0)			
310	(1.9)			
227	(1.4)			
248	(0)			
kilobases		1.2	2.3	1.9
- 5.5 total kb				

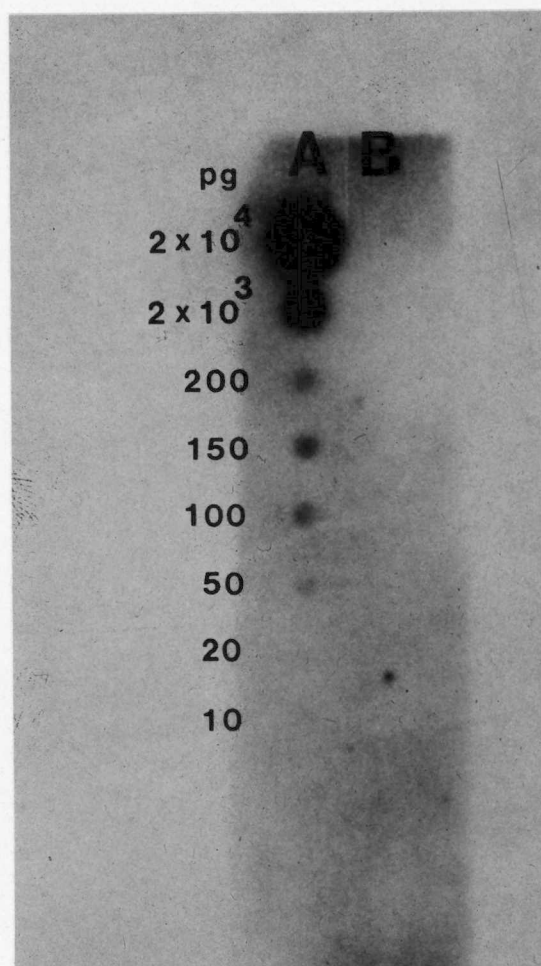


Figure 7. Dot blots of known concentrations of BVDV genomic RNA (column A) and transfer RNA (column B). BVDV genomic RNA was detected to a level of 20 pg. Blots were hybridized with clone #5 as described in Materials and Methods.

Specificity The specificity of the ^{32}P -labelled DNA hybridization probe prepared from clone #5 was determined by hybridization with six different strains of BVDV and other bovine viruses as described in Table 3 and Figure 8. Hybridization was obtained with all cytopathic and noncytopathic BVDV strains tested but not with viruses from any other family tested. There was no hybridization reaction between this probe and with uninfected cell culture, cellular ribosomal RNA, or transfer RNA by dot blot assay (Figure 4). Probe prepared from pUC9 plasmid alone did not hybridize at any time with the samples tested.

Table 3. Hybridization results of dot blots of cell culture fluids infected with various viruses. ^aCell culture fluids were prepared as described in Materials and Methods. ^bCloned cDNA sequences from plasmid clone #5 was nick translated and used in dot blot hybridization assays. (*) indicates cytopathic BVDV strains. (+) indicates hybridization detection levels of 20 to 50 pg of RNA. (-) indicates no detectable hybridization.

Virus ^a	Hybridization results ^b
BVDV strain 72 *	+
BVDV strain 2724	+
BVDV strain 7443	+
BVDV NADL strain *	+
BVDV New York strain	+
BVDV Singer strain *	+
bovine coronavirus	-
bluetongue virus	-
bovine adenovirus	-
non-infected BTU cells	-

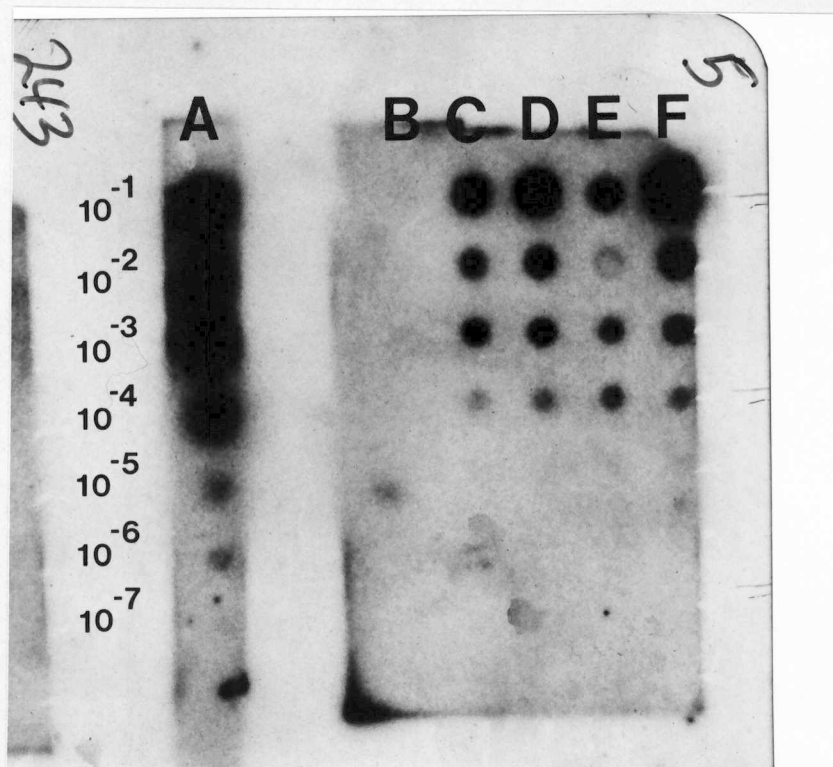


Figure 8. Dot blot hybridization of BVDV cDNA with cell culture fluids infected with various strains of BVDV. Dilutions of BVDV strain 72 (column A), uninfected cell culture (column B), NADL strain (column C), strain 7443* (column D), Singer strain (column E), and New York-1 strain* (column F) were diluted tenfold and blotted on nitrocellulose and hybridized with probe #5 as described in Materials and Methods. (*) indicates noncytopathic strains of BVDV.

V. DISCUSSION

Although standard techniques were used for molecular cloning of BVDV several major impediments particular to this virus were encountered. The major difficulty encountered in the cloning of BVDV was the difficulty in obtaining a sufficient amount of purified BVDV RNA to use as a template for first-strand cDNA synthesis. This difficulty was compounded by the lack of information on the molecular biology of BVDV. Several methods of BVDV purification have been reported in the literature but, without exception, great difficulties were encountered in obtaining adequately purified virus (35, 64, 142, 171, 173, 182, 183). Initially, in the present study, the methods of Felmingham and Brown were used for BVD virus purification to obtain genomic RNA for cDNA synthesis (64). BVD virions seem to be highly cell associated (117) and, when virus is collected and purified from infected cell cultures, virus particles tend to co-purify with cell vesicles and membranes (64, 183). Consequently it is very difficult to visualize virions in negative-stained preparations by electron microscopy and to purify virions. Due to this fact, the methods reported by Felmingham and Brown which included the use of Tween 80, a nonionic detergent, to dissociate virions from cellular membranes and improve yields of purified infectious virions were used (64). Initial attempts with these procedures seemed successful because characteristics (location virus band in centrifuged gradients, specific gravity of fractions, and infectivity titer) of virus

preparations obtained from density gradient purification corresponded to that reported by Felmingham and Brown (64). However, purified virus preparations yielded less than 3.0 ug of genomic BVDV RNA per liter of infected cell culture fluid and, although cDNA synthesis was attempted with this material, the amount of cDNA product obtained was not adequate for cloning.

The lack of information in the literature on BVDV molecular biology also contributed to other difficulties encountered in virion purification. Various values for the density of BVD virions have been reported which ranged from 1.12 to 1.174 g/cm³ (32, 158, 172, 174, 227). It was necessary to rely on tedious study of fractions of infectious cell-culture materials after centrifugation in sucrose gradients. Infectivity, density, and absorbance of fractions collected were determined and compared to obtain the true density of BVD virions. In this study, gradient purified virus had a density of 1.128 to 1.139 g/cm³ which corresponded to some previously-reported values (158, 172, 174). The dearth of information on the biology of BVDV may explain also that only one group (associated with a commercial company) has reported successful cloning of the virus (182).

Unlike the other Togaviridae members, which are polyadenylated, BVDV does not contain a 3' polyadenylated sequence (173, 182). The flaviviruses, which have been removed from the Togaviridae family and given separate Family status (Flaviviridae), like BVDV, do not contain

a 3' polyadenylated tail (185). Therefore, since BVDV is not poly-adenylated, random primers were used initially to initiate first-strand synthesis. Random primers were selected also for cDNA synthesis because a greater possibility existed for obtaining a library of clones that represented the entire genome than by using an oligo-dT primer. However, sufficient number of transcripts for cloning were not obtained with random primers for cDNA synthesis. The inadequacy of the latter transcripts for cloning was determined by agarose-gel electrophoresis of the products of attempted first-strand cDNA synthesis (in the presence of dCT³²P) followed by autoradiography. These products consisted only of short (less than 100 bp) pieces of cDNA. Since random primer-initiated cDNA synthesis was inadequate, genomic RNA then was polyadenylated prior to first-strand synthesis to allow the use of oligo-dT primers. A high degree of secondary structure of BVDV viral RNA may have been responsible for the inadequate results with random primers due to physical constraints placed on the polymerase during transcription. Due to this possibility, BVDV genomic RNA template was heated to denature the template prior to first-strand synthesis. However, in retrospect, the failure of random-primed cDNA synthesis also could have been the result of the purification methods used (Felmingham and Brown) which yielded too little viral RNA or perhaps yielded RNA that was fragmented (or denatured) by Tween 80 and not due to the nature of transcription initiation. Tessler *et al.* reported that Tween-80

destroyed BVDV virions and used it to differentiate hog cholera virus from BVDV (218). BVD virion lysis by Tween-80 could have resulted in degradation by exposure of genomic RNA to ribonucleases. This conclusion was based, in part, on the fact that, for colony screening, hybridization probes were successfully prepared with random-primed, first-strand cDNA synthesis from BVDV RNA purified by isopyknic density gradient purification as described in Materials and Methods.

After these initial failures to purify virus and to obtain sufficient BVDV RNA for cDNA synthesis, conventional methods of differential centrifugation were used for virus purification (as described in the Materials and Methods). Initially, due to the reported cell-associated nature of BVDV virions, three cycles of freezing (-80 C) and thawing of infected cell culture fluids were done prior to virus purification in an attempt to release virions from cell debris. However, freezing and thawing reduced the yield of subsequently purified virus and reduced the infectivity titer ten-fold. The most satisfactory procedure for virion purification was by clarifying and pelleting virus from infected cell culture supernates without freezing. Using standard methods of virus purification, viral RNA extraction, addition of a 3' poly-A tail with poly-A polymerase, and cDNA synthesis with oligo-dT primers, adequate amount of double-stranded cDNA was obtained to proceed further with cDNA cloning of BVDV.

The C-tailing of double-stranded BVDV cDNA was another critical

step in the cloning procedure. Several parameters in the methods used for C-tailing (such as incubation temperature and reaction time) may affect the efficiency of tailing. (200). However one factor, the tailing buffer as described by Roychoudhury and Wu, was important for consistent C-tailing of DNA (200). In addition the quality of terminal deoxytransferase used was important (200). These factors influenced the rate and success of the reaction and therefore the number of CTP's added to the 3' end of the double-stranded DNA which must be precisely controlled. The optimal number of CTP's added to a DNA sequence is 15 to 20 residues to ensure proper annealing and repair of the C-tailed DNA fragment and G-tailed vector DNA (128, 200).

BVDV cDNA synthesis and tailing (poly-A and C-) was done "cold" (without any radionucleotide labelling) to maintain optimal transformation efficiency of *E. coli* with recombinant plasmids. The transformation efficiency obtained with C-tailed BVDV cDNA annealed into G-tailed pUC9 [5.0×10^7 colony forming units (CFU)/ug of pUC9 DNA] was near the average range desired of 2 to 7×10^8 CFU/ ug of plasmid DNA which indicated that a high proportion of cells were susceptible to transfection (competent) with the recombinant vector DNA (77, 87).

A ratio of one white colony (with disrupted lac-Z gene) to four blue colonies (intact lac-Z gene) and the total number of white colonies (6,000) obtained in this study was optimal. It was an

indication of the integrity of the recombinant plasmids produced by annealing BVDV cDNA sequences into plasmids and that the concentration of recombinant vector DNA relative to numbers of competent log-phase *E. coli* organisms was below the saturation level (77). Near the saturation point of DNA relative to bacterial cells, at plasmid to cell ratios of greater than 2 to 1, the transformation efficiency begins to decrease (87). In addition, the ratio of white to blue colonies indicated a low degree of transformation with native pUC9 plasmid (background). A low background is desired because a relatively high concentration of native supercoiled plasmid DNA present in the transformation mixture would decrease the uptake of circular recombinant plasmids. Relaxed circular plasmid DNA transforms at 75 % the efficiency of supercoiled plasmid DNA and transforms considerably more efficiently than linear plasmid DNA (87, 88)

The library of cloned BVDV sequences obtained, which ranged from less than 1.0 kb to 2.65 kb in length, was consistent with cDNA cloning results of others using the methods of Gubler and Hoffman (200). These methods of cDNA cloning usually produced clones averaging approximately 2.0 kb in length (80, 116).

Hybridization of cloned sequences with BVDV-infected cell cultures, the absence of hybridization of these sequences with noninfected cell cultures and cellular RNA, and the colony hybridization results with first-strand cDNA from random-primed BVDV RNA indicated that the cloned DNA sequences identified were indeed

complementary to BVDV RNA. It was necessary to characterize the recombinant plasmids by several parameters to ensure that the cDNA clones obtained represented BVDV genomic sequences and not contaminating cellular RNA or DNA. This was especially important since the method of virus purification by differential centrifugation (used for producing recombinant plasmids) may have allowed more cellular contamination than in virus preparations obtained by isopyknic density gradient centrifugation (used for probe preparation for colony screening).

Since virus used for obtaining RNA to initiate cloning was purified by differential centrifugation (and possibly contained a small degree of cellular contamination), it was mandatory that colonies of bacteria with recombinant plasmids were screened with a first-strand cDNA probe synthesized by using, as a template, highly purified RNA (extracted from virus purified by isopyknic density gradient centrifugation). An initial indication was obtained with such a probe that 200 of 5,000 colonies of transformed of *E. coli* contained recombinant plasmids with BVDV cDNA inserts. This was not incontrovertible evidence that the identified colonies contained BVDV cDNA sequences because traces of cellular DNA contaminating the purified virus preparation used to prepare the first-strand cDNA probe for colony screening still could have hybridized with plasmids containing cellular DNA. However, it is probable that if cellular sequences were identified by colony

hybridization, a higher percentage of colonies would have hybridized because of the relative abundance of cellular DNA and RNA compared with that of viral RNA present in infected cell culture fluids.

Hybridization of probes prepared by nick translation of insert cDNA from recombinant plasmids #5 and #1 with BVDV-infected cell culture and with RNA extracted from isopyknic density gradient-purified BVDV, and the lack of hybridization with uninfected cell culture extracts confirmed that the DNA sequences identified by colony screening were indeed BVDV cDNA sequences. The lack of hybridization with uninfected cell cultures; which included dot blots of cell culture supernates of frozen and unfrozen whole cells scraped from flasks, and of phenol-extracted formalin-denatured whole cells; was convincing evidence indicated that the identified cDNA clones were not from cellular DNA or RNA. It was important to establish that the cloned cDNA sequences were virus-specific since some putative viral cDNA probes have hybridized with cellular nucleic acids (131). Additional proof that the cloned cDNA sequences were BVD-specific was that probe prepared from native pUC9 plasmid did not hybridize with uninfected cell culture or BVDV-infected cell cultures.

Further evidence of the specificity of the recombinant BVDV cDNA sequences was to determine the extent of cross hybridization of these sequences with other bovine RNA and DNA viruses. However, cross hybridization with any of the viruses tested did not occur indicating that the cDNA sequences tested were specific for BVDV RNA. Some virus

stocks (bovine adenovirus, bluetongue virus, and parainfluenza virus) were contaminated with BVDV since initially they contained sequences that hybridized with cloned BVDV probe. However, the hybridization signal disappeared after 0.5 % chloroform treatment of the viruses that were resistant to chloroform (bluetongue virus and bovine adenovirus) indicating that the original virus stocks had been contaminated with BVDV.

Theoretically, the addition of a 3' poly-A tail to the BVDV RNA template and use of an oligo-dT primer for first-strand cDNA synthesis, all the transcripts should have had common sequences representing the 3' end of the genome. Mapping by cross hybridization of a limited number of the largest clones unexpectedly revealed three separate non-overlapping groups of clones. Eight out of 17 clones tested cross hybridized with probe ^{2.3 Kb} #243, which constituted the largest group of overlapping clones. Although only a limited number of clones have been examined in this manner since only the largest clones were selected, it appeared that most belonged to one of two groups and a few to one small group. This suggested that many cDNA sequences cloned had a common point of origin in the BVDV genome. Because BVDV does not contain a polyadenylated genome, the 3'-end-specific polyadenylated tail cannot be used as a marker to establish orientation of the cloned cDNA sequences for a physical map of the genome. The distribution of cDNA sequences characterized by cross hybridization revealed that 47 % of the clones examined represented

one overlapping group, 29 % represented another group, and 18 % in a third group. All the cDNA sequences which hybridized with the 2.3 kb probe #243 shared some sequences with the latter which indicated that 47 % of cDNA sequences cloned originated within this 2.3 kb genomic segment. Therefore the cDNA clones obtained had an uneven distribution since an even distribution along a 12.5 kb genome would not have 47 % of the clones arising from 17 % [or 2.3 kb (clone #243)] of the genome sequence (Table 3, page 70). It is likely that the largest group of clones originated at the 3' end of the genome since an oligo-dT primer was used with the polyadenylated genomic template. Therefore, assuming that a majority of genome molecules were intact prior to polyadenylation during cDNA cloning, it can be tentatively concluded that the related clones of the largest groups originated at the 3' end of the BVDV genome. Considering the relationship of the overlapping sequences identified to the non-overlapping sequences, the mapping region of cDNA from clone no. 243 lies between that of no. 5 and no. 126. If the latter is correct, it can be tentatively concluded that the group of recombinant cDNA plasmids identified by clone no. 5 originated from the most distal 3' end of the BVDV genomic RNA.

Obviously not all the cDNA sequences cloned originated from the 3' end of the genome since some cDNA probes did not cross hybridize with the majority of the cloned sequences. If the BVDV RNA was degraded by contaminating nucleases into smaller segments prior to

polyadenylation and cDNA synthesis, other regions of the genome also could be represented in the cDNA library. This is entirely possible because RNA is extreme susceptibility to various ribonucleases. Also, newly synthesized cDNA sequences could have re-hybridized with available regions of BVDV RNA template during first-strand cDNA synthesis and served as primers at sites closer to the 5' end of the genome (71). It is known also that reverse transcriptase can initiate first-strand synthesis at random sites on the template RNA in the absence of a primer. These arguments support the premise that the majority of transcripts originated from the 3' end but that some transcripts may have originated at random sites.

Another factor supporting the concept that most of the cDNA clones had a common origin on the RNA genome was the presence of an internal *Pst* I restriction endonuclease site in 50 % of the clones (8/17) characterized and represented in each of the two largest groups identified by cross hybridization mapping. In addition, 25 % of the 17 cDNA clones contained two internal *Pst* I sites. *Pst* I restriction endonuclease cleavage of cDNA clones containing internal *Pst* I restriction endonuclease sites always resulted in one fragment no greater than 600 bp in length. Perhaps numerous *Pst* I restriction endonuclease sites in the BVDV genome exist but it is tempting to speculate that the sequences cloned had a similar origin on the BVDV genomic RNA 600 bp from a *Pst* I site. This possibility along with the

cross hybridization results supported the homologous relationship among the majority of the clones.

The potential usefulness of cloned BVDV cDNA sequences as hybridization probes evaluated in the present study may rival that reported for other viruses (12, 23, 28, 101, 122, 141, 213, 216). The sensitivity threshold for detection of hybridization signals was 10 to 20 pg of BVDV genomic RNA. This was determined by dot blot hybridization with known concentrations of RNA extracted from density gradient-purified BVD virions. This level of sensitivity was obtained with a hybridization time of 16 hours and after stringent washing (low salt concentrations) of the hybridized dot blots before they were exposed to radiographic film for 24 to 48 hours. Standard methods of measuring hybridization by autoradiography has had a detection limit of 10 to 25 pg of genomic RNA or DNA in other systems (122, 141, 206). Morace et al. reported the detection of as little as 10 pg of hepatitis B viral DNA under similar conditions employed in the present study (141).

Although the sensitivity of hybridization between BVDV cDNA probes and genomic RNA target sequences was high in the present study, several parameters still could be manipulated to improve the limits of detection encountered. Detectable signals between hybridization probes and as little as 2-5 pg of viral RNA in a sample have been observed (83). Herpesvirus has been detected by hybridization at a level of 1-4 infected cells per sample (178). Increasing the exposure

time for autoradiography of hybridized dot blots to greater than 48 hours decreased, by 10 fold, the level of sensitivity (1 pg of hepatitis B virus DNA) (141). Use of a single-stranded probe labelled by transcription in the presence of radionucleotides eliminates competitive binding which occurs when double-stranded probe (labelled by nick translation) reassociates instead of hybridization with target sequences. The SP6 phage polymerase probe system could have increased the level of sensitivity encountered in the present study since it is a single-stranded RNA probe. In addition, use of the latter RNA probe may increase sensitivity because of the increased stability of RNA:DNA compared to DNA:DNA hybrids. However since BVDV is an RNA virus, the use of a RNA rather than a DNA probe is of little consequence since the stability of RNA:DNA hybrids are equal to that of RNA:RNA hybrids (86). Single-stranded DNA probes produced by primer extension using a Messing M13 vector system, which allows the synthesis of probes containing a higher percentage of radionucleotide incorporation, would have the dual advantage of higher specific activity and superior hybridization kinetics of a single-stranded DNA probe (231).

In the present study, detection of BVDV RNA in cell culture fluids by hybridization with cDNA was at least as sensitive as infectivity assays. In fact, in most assays hybridization was 10 to 100 times more sensitive than infectivity assays when serial dilutions of BVDV-infected cell culture fluids were examined. However, it was determined that, after freezing of samples, a 10-fold drop in

sensitivity in the hybridization assay for BVDV occurred. This may have been due to damage of virus particles during freezing thereby allowing penetration of ribonucleases and the subsequent destruction of BVDV RNA. This observation emphasizes the need for rapid and appropriate processing of samples that contain ribonuclease-susceptible RNA for hybridization. It may be that the use of methods less destructive than formalin for denaturation of proteins in samples destined for dot blot hybridization assays for RNA (such as heating) may increase the sensitivity of the procedure (86). In addition, it may be necessary, for optimum sensitivity, to include RNAase inhibitors in sample preparations (128).

Few markers which characterize BVDV strains exist with the exception of *in vitro* cytopathogenicity. Therefore hybridization studies with specific esoteric sequences from different strains may be of great value in genetic classification of BVDV strains. Hybridization probes prepared from clone #5 and also #126 were specific for BVDV strain 72. In the present study cDNA clone #5 used as a hybridization probe reacted with all the BVDV strains tested which included cytopathic and noncytopathic viruses. However, the hybridization assays for noncytopathic virus indicated approximately 100-fold lower concentrations than cytopathic BVDV. This difference apparently reflected differences in the replication of the viruses since it was reflected also in the infectivity titers of the viruses

and thus was not the result of differences of specificity of the probe.

Use of small specific sequences as probes could result in greater specificity which may allow discrimination among closely-related viruses (60). The ideal probe would consist of sequences that are not highly conserved among strains of BVDV thereby allowing strain differentiation. Optimal specificity has been obtained by decreasing probe length to the minimal number of base pairs needed for stable hydrogen bonding (15-20 bp) but, unfortunately, probes of this are 100-fold less sensitive than larger probes (60, 86).

The molecular cloning of BVDV genomic RNA has provided the basis for further characterization and understanding of this unique viral pathogen. Genomic sequencing of cDNA sequences followed by genomic mapping of these sequences and further cDNA cloning (if needed) of identified missing regions of the genome should be given high priority in working toward the complete sequencing of the BVDV genome. The 5' region has particular significance since it may encode the structural genes. Characterization of genomic organization, open reading frames, replication strategy, and genome products can result from these efforts. Once the genome has been sequenced and open reading frames have been identified, the expression of specific sequences in expression vectors such as vaccinia can be done. Further cDNA cloning and subcloning may lead to construction of single recombinant vectors containing the complete BVDV genome. This would

make possible the production of infectious RNA and subsequently the effects of deletion of specific regions on pathogenicity, persistence, and viral protein expression can be determined.

Another avenue to pursue would be the further application and development of novel diagnostic hybridization probes. The identification of specific sequences allowing biotype differentiation by hybridization assays should be done. The ability to differentiate biotypes may require further cDNA cloning of noncytopathic strains of BVDV. Hybridization probes and hybridization conditions should be refined to improve sensitivity and specificity, and parameters required for *in situ* hybridization should be established.

In conclusion, the molecular cloning of BVDV genomic sequences is significant in many respects, as has been mentioned previously, and will provide an avenue for future study and characterization of bovine viral diarrhea virus. The scientific information gained from the molecular cloning of BVDV and the benefits to veterinary medicine and the cattle industry will be determined by fully taking advantage of the wealth of information that judicious use of these cloned cDNA sequences could reveal.

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APPENDIX

clones > 0.5 kb

1	213
4	214
4.3	221
5	227
7	233
17	243
35.1	245
36	246
37.11	248
43.3	283
47	303
49.2	310
54	315
59.1	
71	
71.2	
74.2	
85.5	
96	
102	
106	
107	
110	
119	
126	
165	
170	
197.3	
201.12	
207	
207.3	
209.3	
210	
213	
214	
221	
227	

clones < 0.5 kb

12	197.2
27	200.12
28	202
68.2	205.3
74	209
75	212
75.2	223
82	225
85	228
104	228.2
111	229
114	233
116	234
117	237
121	240
123	241.1
125	241.2
127	247
135	248
144	252
147	261
150	264
155	268
166	270
168	271
171	285
172	293
176	295
191.2	300
191.3	301
193	309
194	313
194.2	
197.2	
200.12	
202	
205.3	

List of recombinant plasmids grouped by approximate size of cDNA inserts as determined by gel electrophoresis of DNA extracted from mini-plasmid preparations. See Figure 2 (page 60) for more detailed explanation.

VITA

Kenny Virgil Brock was born in Gadsden, Alabama on September 17, 1958. He graduated from Monroe County High School in June 1976. The following September he entered Harding College and after 3 semesters transferred to Auburn University. After completing prerequisites for entrance to Veterinary School he entered Veterinary School at Auburn University School of Veterinary Medicine. During the Winter of his sophomore year he began work toward a Master's degree in Veterinary Microbiology. He was awarded the degree of Doctor of Veterinary Medicine in June of 1983 and was awarded a Master's degree in December of 1983.

After one year of private veterinary practice, he entered The Graduate School of The University of Tennessee, Knoxville, in July 1984. He received the Doctor of Philosophy Degree with a major in Comparative and Experimental Medicine in December 1987.

Dr. Brock will be employed by The Ohio State University at the Ohio Agricultural Research Development Center after graduation.