MONTANA STATE UNIVERSITY

Bovine virus diarrhoea virus RNA synthesis
by Peter Clayton Roberts

A thesis submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
The size of the genomic RNA of the pestivirus bovine virus diarrhoea virus (BVDV) is unclear. Early estimates ranged from 8.2-10.5 kilobases (kb). More recently a size of 12.5-13 kb has been reported. Very little is known concerning the synthesis of BVDV RNA in infected cells. The goals of the project were: to accurately size the genomic RNAs of cytopathic (cBVDV) and noncytopathic (ncBVDV) BVDV strains; and to compare the synthesis of BVDV RNA in cells infected with different BVDV strains.

The BVDV genome was sized by comparison of BVDV-specific intracellular and virion RNAs to vesicular stomatitis virus mRNAs and denatured bacteriophage λ DNA restriction fragments. This resulted in a size estimate of 12.6 kb for the BVDV genome. No significant difference in genome size was detected between the strains studied. For the study of RNA synthesis, total cell RNA was prepared from infected cells at various times postinfection. The RNA was dot blotted onto duplicate nitrocellulose filters, that were probed with either plus or minus sense 32P-labelled BVDV RNA transcribed from a BVDV cDNA. Quantitation of the RNAs was achieved by scanning autoradiographs with a densitometer. The concentration of BVDV RNA was calculated using BVDV-specific RNA calibrations. The quantity of BVDV RNA varied markedly between cells infected with different strains of the virus. There was a lag of 5-10 h before BVDV RNA was detected. With cBVDV isolates the quantity of both plus and minus sense RNA increased until 22 h postinfection. In cells infected with ncBVDV, plus and minus sense RNA levels varied throughout the course of the experiment. The ratio of plus to minus sense RNA was higher in cells infected with ncBVDV as compared to cells infected with cBVDV.

The conclusions to be drawn from this work are: 1) the BVDV genome is approximately 12.6 kb in size; 2) the synthesis of BVDV RNA follows a similar time course to that observed in cells infected with flaviviruses; and 3) cBVDV isolates have lost control of minus sense RNA synthesis, possibly due to an inability to encapsidate viral plus sense RNA efficiently.

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ABSTRACT

The size of the genomic RNA of the pestivirus bovine virus diarrhoea virus (BVDV) is unclear. Early estimates ranged from 8.2-10.5 kilobases (kb). More recently a size of 12.5-13 kb has been reported. Very little is known concerning the synthesis of BVDV RNA in infected cells. The goals of the project were: to accurately size the genomic RNAs of cytopathic (cBVDV) and noncytopathic (ncBVDV) BVDV strains; and to compare the synthesis of BVDV RNA in cells infected with different BVDV strains.

The BVDV genome was sized by comparison of BVDV-specific intracellular and virion RNAs to vesicular stomatitis virus mRNAs and denatured bacteriophage λ DNA restriction fragments. This resulted in a size estimate of 12.6 kb for the BVDV genome. No significant difference in genome size was detected between the strains studied. For the study of RNA synthesis, total cell RNA was prepared from infected cells at various times postinfection. The RNA was dot blotted onto duplicate nitrocellulose filters, that were probed with either plus or minus sense ^{32}P-labelled BVDV RNA transcribed from a BVDV cDNA. Quantitation of the RNAs was achieved by scanning autoradiographs with a densitometer. The concentration of BVDV RNA was calculated using BVDV-specific RNA calibrations. The quantity of BVDV RNA varied markedly between cells infected with different strains of the virus. There was a lag of 5-10 h before BVDV RNA was detected. With cBVDV isolates the quantity of both plus and minus sense RNA increased until 22 h postinfection. In cells infected with ncBVDV, plus and minus sense RNA levels varied throughout the course of the experiment. The ratio of plus to minus sense RNA was higher in cells infected with ncBVDV as compared to cells infected with cBVDV.

The conclusions to be drawn from this work are: 1) the BVDV genome is approximately 12.6 kb in size; 2) the synthesis of BVDV RNA follows a similar time course to that observed in cells infected with flaviviruses; and 3) cBVDV isolates have lost control of minus sense RNA synthesis, possibly due to an inability to encapsidate viral plus sense RNA efficiently.

INTRODUCTION

Bovine virus diarrhoea virus (BVDV) is the etiological agent of one of the most complex disease syndromes in veterinary medicine (78). The virus appears to be ubiquitous, since there is a high incidence of seropositive animals throughout the world (147,197). Only recently, through a better understanding of its epidemiology, has the full economic impact of BVDV become appreciated (78). The infection of animals may not be diagnosed for several months after the introduction of the virus into a susceptible herd and losses may continue for several years (6,79). The virus is now considered to be one of the major causes of economic loss to the cattle industry (130).

Field isolates of BVDV are initially characterised by their cytopathic effect in cell culture. They are usually described as either cytopathic (cBVDV) or noncytopathic (ncBVDV) (93); intermediate cytopathologies also occur (93,94,115,186). Fernelius (93) felt that these differences were genetic in nature and labelled them biotypes. They appear to be genetically stable traits in bovine cell lines, but can be altered by passage in non-bovine cell lines (93) or in rabbits (94). The virus occurs as a single serotype (55), though subtle but distinct antigenic differences are detected between BVDV strains (40-42,55,58,62,81,92,114,115,117,136,142, 163,173,296). These antigenic differences extend to presumed identical strains from different laboratories (42,55).

No definite correlation has been found between a specific strain of BVDV and specific clinical symptoms, though such a correlation has been described for the closely related Border disease virus (BDV) (215).

The Pestiviruses

Bovine virus diarrhoea virus is a small, spherical, enveloped virus with a single stranded, positive polarity RNA genome. It is the type species of the genus *Pestivirus*, at present classified in the family *Togaviridae* (299) but it is likely that the genus will be reclassified as being in the family *Flaviviridae* (52,55). Other members of the *Pestivirus* genus are hog cholera virus of swine (HCV) (also referred to as classical or European swine fever virus) and BDV of sheep. There is some evidence that a human pestivirus exists (101,221,303,305) that may cause infantile gastroenteritis associated with respiratory inflammation (305). The cBVDV strain Oregon C24V has been designated the type strain of the genus (299).

The pestiviruses are serologically related (211,216), share similarities in their pathogenicity, especially for the foetus (125), and have a lower sedimentation coefficient and buoyant density than members of other togavirus genera (157). The pestiviruses are generally species specific but interspecies infections do occur (81,273). The three pestiviruses can be differentiated by panels of monoclonal antibodies (42,81,111,129,190,213,273,293-295,307). Monoclonal antibodies specific for pestiviruses in general, HCV alone or BVDV alone are available; however, a monoclonal antibody that is specific for BDV has not yet been described.

BVDV and BDV seem to be very similar and are increasingly being referred to as the ruminant pestiviruses. They are antigenically diverse, have a wide host range (Table 1) and a similar range of biotypes (125,134,158). In contrast, HCV has a limited host range (Table 1), strains form a compact antigenic group and all field isolates are noncytopathic (120,134). There is about 85% homology at the amino acid level between BVDV and HCV (193). HCV also differs from the

Table 1. Members of the *Pestivirus* genus and their host species.

Virus	Host species
Bovine virus diarrhoea virus	Cattle, sheep, goats, swine, wild ruminants, rabbits[a] (5,76,78,94,119)
Border disease virus	Sheep, goats, cattle, swine (125)
Hog cholera virus	Swine (120)

a. Rabbits have not been shown to be infected outside the laboratory.

other pestiviruses in that virulent and avirulent strains occur (120). Virulent HCV strains are antigenically different from BVDV and BDV (120); however, avirulent HCV strains have been described that are antigenically more diverse: they appear to be more closely related serologically to BVDV than to virulent HCV (120,158). BVDV, BDV and avirulent HCV may prove to be the same virus, since they are only truly differentiated on the basis of their host species.

History

It is likely that the first description of the bovine virus diarrhoea-mucosal disease complex was that of Simonds and Brown (268) in England in 1871. Their description probably went unnoticed by many later researchers, as they attributed the syndrome they observed to acorn poisoning. In 1946, Olafson *et al.* (208) described a highly contagious and transmissible disease of cattle in New York, of high morbidity but low mortality. They called the disease virus diarrhoea (VD), as no bacteria could be isolated from infectious material and diarrhoea was a common clinical symptom (209). A similar but more severe disease syndrome, X disease, was described by Childs (44) in Saskatchewan the same year. Both

acute and chronic forms of X disease were described. The low morbidity and high mortality of X disease differentiated it from VD. In 1953, Ramsey and Chivers (232) described a syndrome in Iowa and surrounding states they called mucosal disease (MD). They believed that the syndrome had not previously been reported but their description is very similar to that of Child's X disease, a fact Ramsey later acknowledged (cited in reference (223)). These early descriptions led to a belief that, although they were similar in general symptoms, VD and MD were separate disease entities.

At that time the nature of the etiological agent of VD was still in question. In 1954, Baker *et al.* (4) conducted an extensive study of infectious material from two cases of VD. The results concurred with the earlier studies of Olafson *et al.* (208,209) i.e., the etiological agent of VD was a virus as no other agent was detected. This conclusion was supported the following year by the filtration studies of Pritchard *et al.* (224) and Schipper *et al.* (261). In 1957, Lee and Gillespie (160) successfully propagated *in vitro* one of Baker's isolates, New York-1. The virus was found to be noncytopathic. The same year, Underdahl *et al.* (282) propagated in tissue culture two cytopathic viruses isolated from the tissues of animals from separate outbreaks of MD. Cross-neutralisation studies showed that the two viruses were related. In addition, cattle from herds with no history of MD were found to have serum neutralising antibody to the two viruses. Kniazeff (cited in reference (223)) later showed that Underdahl's viruses were related to New York-1. In 1960, Gillespie *et al.* (103) described a cytopathic virus that was isolated from the spleen of a cow reported to have VD (a later report indicates that the cow actually had MD (197)). This isolate, Oregon C24V, has been designated the *Pestivirus* type strain (299). Cross-neutralisation studies, performed both *in vivo* and *in vitro*, showed that Oregon C24V was antigenically

related to New York-1. The following year the same group showed that viruses isolated from cases of VD and MD were antigenically related (104). This finding was supported by comparisons of VD and MD virus isolates from Europe and the United States (152,223).

The ability to grow BVDV in tissue culture greatly facilitated studies of the bovine virus diarrhoea-mucosal disease complex. It provided irrefutable evidence that BVDV was the etiological agent of VD. Although virus neutralisation studies implied that VD and MD were caused by the same virus, proof of this remained elusive. A major problem was the inability to reproduce MD experimentally. In their original description of MD, Ramsey and Chivers (232) described attempts to transmit the disease to healthy calves. The only response they detected was a transient pyrexia. Similar results were obtained in other studies (102,224,261). Tyler and Ramsey (281) failed to detect any significant differences in the pathologic, immunologic and clinical responses to VD and MD virus infections in healthy calves. The shroud of mystery surrounding the pathogenesis of MD began to be lifted in 1964 when Thomson and Savan (276) observed that only animals without an antibody titre to BVDV succumbed to MD. In 1968, Malmquist (177) published a landmark paper. He observed that animals suffering from MD had a persistent BVDV viraemia but failed to develop neutralising antibodies to the virus. He hypothesised that the affected animals had been infected *in utero* prior to the attainment of immunocompetence and were subsequently born immunotolerant to the virus. Although there was circumstantial evidence that BVDV could infect the foetus, direct proof was provided in 1969 by Ward *et al.* (289). They demonstrated that BVDV could cross the placenta and that it was a teratogenic agent. In 1974, Liess *et al.* (161) reproduced MD experimentally. Having also observed that only seronegative animals died of MD, they selected four

seronegative cattle from herds where the majority of animals were immune to BVDV. The four animals were challenged by intranasal inoculation with a cBVDV strain and two subsequently died of MD. In 1978, Coria and McClurkin (59) described an apparently healthy bull with a persistent ncBVDV viraemia, proving the presence of persistent BVDV infections in animals other than those with MD. In recent years, several groups have shown that MD only occurs in persistently infected animals (25,34,99,248). The syndrome has been reproduced by superinfection of persistently infected animals with cBVDV but not ncBVDV (25,34). Brownlie *et al.* (34) proposed the hypothesis that MD is produced by superinfection of persistently infected cattle with cBVDV. This was later modified when it became obvious that the cytopathic virus infecting animals with MD was always antigenically homologous to the original persisting ncBVDV (36). The hypothesis is now that MD arises in persistently infected animals either through mutation of ncBVDV to cBVDV or superinfection with an antigenically homologous cBVDV. Current research is aimed at elucidating the mechanism of induction of MD.

The Disease

Acute Infection

Seronegative and immunocompetent animals postnatally infected with BVDV undergo an acute infection. The primary site of infection is probably the respiratory tract, in particular the bronchiolar epithelia and tonsils (10). From there the virus is disseminated to epithelial and lymphoid tissues throughout the animal, especially those of the alimentary tract and skin. This dissemination may be achieved by transport of the virus in cells of the reticuloendothelial system (10).

Most acute infections are subclinical. They are characterised by a transient pyrexia that is often biphasic, a profound leucopenia and a viraemia that may persist for 2 weeks (78,223). During the period of viraemia, virus is shed at low levels in body secretions and excretions; however, transmission to other animals is not considered to be very efficient (78). Two weeks after infection animals start to produce serum neutralising antibody (38). This response peaks after 10-12 weeks and is generally considered to protect the animal for life (38,78); in two cases, however, immunity was shown to only last for 3-4 months (127,224).

Clinical acute infections are rarely encountered. They generally occur in animals 6 months to 2 years old. Prior to six months of age, colostrum-derived neutralising antibody protects the calf from BVDV infection (267). Severe, clinical epizootics are diagnosed as bovine virus diarrhoea (BVD). Affected animals may present a variety of symptoms other than those described above: inappetence, rapid respiration, depression, oculonasal discharges and occasional ulceration and erosion of the oral mucosa (5). Mucus is sometimes present in the faeces, often as strands that can be nearly an inch thick and several feet long (223). Diarrhoea, when present, appears 1-7 days after the febrile period (223). It may be explosive in character and present continuously or intermittently (78,223). In the later stages large quantities of bright red blood may be present in the faeces (223). Diarrhoeic animals are often severely dehydrated and can lose as much as 25% of their total bodyweight (223). Affected dairy cows have a marked reduction in milk yield (6,78).

The lesions of BVD are less severe than would be expected from the symptoms (208). There are diffuse reddened areas in the mucosa of the entire alimentary tract but they are more prevalent in the upper regions. These areas may develop into erosions and shallow, punched-out ulcers, especially in the oral

cavity, oesophagus, abomasum and caecum (208,223). In nonfatal cases the ulcers heal very rapidly (208).

The morbidity rate in infected herds is generally close to 100% of the susceptible animals present when based on seroconversion. The mortality rate ranges from 0-20% (223). Death is due to the effects of either the primary infection, especially dehydration and emaciation, or secondary infections by opportunistic pathogens (212).

There is some evidence that acute BVDV infection of neonatal calves can result in a severe, sometimes fatal, enteritis (5). This has been reproduced experimentally, with both colostrum-fed and colostrum-deprived animals (153). The importance of the virus in neonatal calf diarrhoea is still an open question (5). Older, colostrum-fed calves undergo acute infections, as described above (207). Another syndrome that has been associated with neonatal infection with BVDV is hyena disease, a disorder of skeletal development (86).

Haematological Changes

The most obvious haematological change after BVDV infection is the transient leucopenia. Bolin *et al.* (23) showed that 4 days after infection the total white blood cell count is significantly reduced. The most marked reduction is that of T lymphocytes. Most cell populations return to or exceed pre-exposure levels within 3 days but lymphocyte numbers do not recover until 2 weeks postinfection. Flow cytofluorimetric analysis showed that helper T cells, cytotoxic/suppressor T cells, B lymphocytes and neutrophils are depleted (83). The numbers of other lymphocytes and monocytes are not affected. There are some indications that leucopenia, involving both neutropenia and lymphopenia, correlates with a more severe clinical course than lymphopenia alone (212).

Several recent reports have described the association of thrombocytopenia, with resultant haemorrhagic conditions, with severe outbreaks of BVD (57,212, 233). The thrombocytopenia has been reproduced experimentally (56,57). Platelet destruction appears to be due to direct interaction with the virus (56). As the incidence of thrombocytopenia is low (233) and can be reproduced experimentally with specific strains of ncBVDV (56,57) it is likely that it is induced by a limited number of BVDV strains.

Immunosuppression

Although acute infections with BVDV are generally innocuous, there is strong evidence that they involve a profound, transient immunosuppression. The most obvious manifestation of this is the transient leucopenia. BVDV infections also result in the destruction of lymphoid tissues (2,10). The most severely affected lymphoid tissues are those in the submucosa of the intestine, especially the Peyer's patches and the area distal to the ileocaecal valve. The tonsils and lymph nodes exhibit a loss of differentiation between the cortex and the medulla, with general loss of lymphocytes, especially from the germinal centres (2,10). There is often a depletion of thymocytes in the thymus (2,10). In the spleen, the periarteriolar lymphoid sheath is severely affected (10). In both lymphoid and nonlymphoid tissues BVDV antigen is present predominantly in cells of the reticuloendothelial system (10).

In vitro studies have shown that the virus can replicate in several different lymphoid cell populations: B and T lymphocytes (2,3,18,257,279); neutrophils (257,258); and monocytes and macrophages (2,18,278,279). The virus has been isolated from, and can replicate in, the leucocytes of actively immunised animals (2,196,279). BVDV infection can result in the suppression of cellular functions.

The B lymphocyte response to pokeweed mitogen is suppressed (2,3,155). This results in a significant reduction in plasma cell development and IgG and IgM synthesis. Antibody secretion but not synthesis is affected in persistently infected animals (202). The mitogenic response of T lymphocytes is depressed by BVDV infection (2,28,100,143,154,155,201,256,257). A more marked depression of the blastogenic response to phytohemagglutinin compared with that to concanavalin A has been noted (28,257). The blastogenic response can be partially restored by addition of isoprinosine (100). In two cases, however, BVDV infection did not affect the mitogenic response of lymphocytes (39,269). Interleukin-2 receptor expression may be compromised in BVDV infected lymphocytes (100). Infection of monocytes results in a significant decrease in their random locomotion and chemotaxis (151). Infected neutrophils exhibit a defect in the myeloperoxidase, hydrogen peroxide, halide system and antibody dependent cell-mediated cytotoxicity (257,258). Active BVDV infection of cells is required to produce the defects in cellular response and function; heat-killed or ultraviolet-irradiated virus has no effect (2,3,83,151,201,257,258).

Apart from the direct action of BVDV on lymphoid cell populations it also impacts on the immune system in other ways. Bovine foetal lung cells infected with BVDV release substances that suppress concanavalin A stimulation of bovine leucocytes (180). The agent responsible has been tentatively characterised as a prostaglandin. The serum of animals suffering from MD has been shown to suppress the mitogenic response of lymphocytes (155,269). BVDV also affects the production and action of interferon (IFN). Cells infected with BVDV do not produce IFN (66) but can be induced to produce IFN by treatment with polyriboinosinic-polyribocytidylic acid (253). The production of IFN by phytohemagglutinin stimulated lymphocytes infected with BVDV is reduced (234). In contrast,

animals infected both *in utero* and postnatally have been shown to produce IFN in response to BVDV infection (234,240). BVDV-infected tissue culture cells are refractory to IFN action (66,255); however, pretreatment of cells with IFN prior to infection results in reduced virus yields (15,66,97). Recombinant bovine IFN-$\alpha_1$1, IFN-γ and tumour necrosis factor alpha, when added to the growth medium of BVDV-infected cells, cause enhanced cBVDV cytopathogenesis and a cytopathic effect in ncBVDV infected cells (15).

A major effect of BVDV-induced immunosuppression is the potentiation of opportunistic pathogens (5). Evidence is accumulating that the virus plays a major role in bovine respiratory tract disease (5,181,270). It is believed to act synergistically with several organisms, the most important of which is *Pasteurella haemolytica* (5,218,220,235,280). Though BVDV infection can impair bacterial clearance from the lungs (220), the clearance of *P. haemolytica* may actually be enhanced (280). It has been suggested that the more severe pulmonary lesions observed in dual infections of BVDV and *P. haemolytica* are a product of neutrophil degranulation (280). Bacterial blood clearance mechanisms are also impaired, resulting in an endogenous bacteraemia in 85% of animals with acute BVDV infections (219). Other pathogens that BVDV may act synergistically with are infectious bovine rhinotracheitis virus (82,235), parainfluenza-3 virus (33), respiratory syncytial virus (33), bovine leukosis virus (244), *Coxiella burnetii* (222), *Leptospira interrogans* serovar *hardjo* (222) and salmonella (304).

Transplacental Infection

BVDV infection of pregnant, seronegative, immunocompetent cattle generally results in transplacental transmission of the virus to the foetus (reviewed in reference (285)). Such infections are the major source of economic losses due to

BVDV (13,121,249); 78-90% of total losses (13). Embryonic death has been produced early in gestation after intrauterine infections with BVDV; however, this is unlikely to be a common occurrence in the field (285). Exposure of bovine embryos, with or without the *zona pellucida*, to BVDV *in vitro* did not result in embryonic death (9). The virus has also been shown to impair fertilisation (109). Placental attachment, which occurs after about 35 days of gestation, appears to be necessary for transfer of BVDV from the dam to the foetus (150). After this early refractive period, the virus is thought to cross the placenta by sequential infection of adjacent cells (285). Transplacental infections do not occur in seropositive animals, limiting the number of foetuses at risk.

The outcome of foetal infection with BVDV is dependent primarily on the developmental age of the foetus, though the genotype of the host and the virus strain may also play a role (12,285). Congenital infections can result in prenatal death, stillbirth, malformations, lesions or the birth of calves with a persistent viraemia (Figure 1) (285). Abortions are common early in gestation, though they can occur as a consequence of foetal infection and death at any time (70,285). Foetal mummification may also occur (38,70). During the second trimester of pregnancy, congenital malformations are the most frequent sequelae to foetal infection. Common lesions are cerebellar hypoplasia, dysmyelination of the central nervous system, ocular defects such as retinopathy and cataracts, hydranencephaly, intrauterine growth retardation of the whole animal or specific organs (especially the brain, thymus and lungs) and skeletal defects (19,285). The outcome of BVDV teratism is the birth of calves that may be stillborn, stunted, ataxic, blind or that manifest various neuromuscular disorders.

In sheep and cattle the generation of malformations appears to coincide with the initial development of the immune system (164,250,251). The bovine foetus