Validation of a reverse transcriptase multiplex PCR test for the serotype determination of U.S. isolates of bluetongue virus

by

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LIST OF ABBREVIATIONS AND ACRONYMS

AGID	agar gel immunodiffusion
BHK	baby hamster kidney
bp	base pairs
BT	bluetongue
BTV	bluetongue virus
C-ELISA	competitive enzyme-linked immunosorbent assay
cDNA	complementary deoxyribonucleic acid
CPE	cytopathic effect
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
EHD	epizootic hemorrhagic disease
EHDV	epizootic hemorrhagic disease virus
IFA	indirect fluorescent antibody
IV	intravenous
MAb	monoclonal antibody
mRNA	messenger ribonucleic acid
mRT-PCR	multiplex reverse transcriptase polymerase chain reaction
PCR	polymerase chain reaction
RNA	ribonucleic acid
spp.	species
RT-PCR	reverse transcriptase polymerase chain reaction
sRT-PCR	serotype-specific reverse transcriptase polymerase chain reaction
ssRNA	single-stranded ribonucleic acid
$TCID_{50}$	tissue culture infected dose (50% infected)
VIB	virus inclusion body
VN	virus neutralization

GENERAL INTRODUCTION

Introduction

Bluetongue (BT) is an infectious, noncontagious, arthropod-borne, viral disease of wild and domestic ruminants. Clinical symptoms in sheep include pyrexia, leukopenia, facial edema, mucosal erosions and ulcerations, and hyperemia of the oral mucous membranes and coronary band (Spreull, 1905; Parsonson, 1990; MacLachlan, 1994). Wild ruminants, such as white-tailed deer, may develop a fatal hemorrhagic disease. Often, however, infected animals are asymptomatic or only mildly affected. This is particularly true of cattle, which are considered the host reservoir of the virus due to the prolonged viremia and inapparent infections that are characteristic of cattle infections (MacLachlan et al., 1992a; Brewer and MacLachlan, 1994; MacLachlan, 1994).

Bluetongue virus (BTV) is the prototype member of the *Orbivirus* genus in the family *Reoviridae*. The virus is characterized as a nonenveloped, double icosahedral capsid enclosing ten segments of double-stranded RNA (dsRNA) (Mertens et al., 1984; Roy, 1989, 1996). Each RNA segment encodes a separate viral protein with the exception of segment 10, which encodes two small proteins. Structural proteins are VP2 and VP5, comprising the outer capsid, VP3 and VP7 which form the inner core, and VP1, VP4, and VP6, that together with the segmented genome, are enclosed within the core. Four nonstructural proteins are also encoded by the virus. The gene segments encoding the viral proteins are all highly conserved with the exception of L2 and M5 which encode the outer capsid proteins VP2 and VP5 (Kowalik and Li, 1987). The serotype determinants are located on the VP2 protein, accounting for its variability (Huismans and Erasmus, 1981).

Twenty-four serotypes of BTV have been identified; five have been isolated in the United States: BTV-2, BTV-10, BTV-11, BTV-13, and BTV-17. The geographic distribution of BTV is

dependent upon the range of biting midges, *Culicoides* spp., which are the only known vector of the virus (Gorman, 1990; Gibbs and Greiner, 1994; MacLachlan, 1994; Osburn, 1994a). The virus is considered to be endemic in tropical or subtropical regions of the world where the vector is present year-round. In more temperate areas where susceptible *Culicoides* spp. are present seasonally, epidemics of BT disease usually coincide with large populations of the vector from midsummer until frost.

The economic impact of BTV can be observed directly in the loss or reduced vigor of infected sheep or other ruminants to the disease. However, the greatest financial burdens have been as the result of restrictions that have been placed on the export of susceptible animals or their germplasm from regions where BT occurs (MacLachlan et al., 1989). As a consequence, most countries require that serological and/or isolation procedures be performed on the serum, blood, semen or embryo fluids of ruminants before the animals or animal products can enter their country.

Diagnosis of BT involves serological procedures, polymerase chain reaction (PCR) tests, and/or virus isolation (Pearson et al., 1992a; Afshar, 1994). As an aid to epidemiological studies, serotype identification is done on viral isolates. Several serotyping procedures have been used over the years including virus neutralization, plaque neutralization, plaque inhibition, and fluorescence inhibition (Howell et al., 1970; Davies and Blackburn, 1971; Afshar, 1994; Blacksell and Lunt, 1996). Common features of these tests are that they can be cumbersome, sometimes provide inconclusive results, and often need to be repeated. More recently, PCR tests have been developed for serotyping BTV isolates in Australia and the United States (McColl and Gould, 1991; Wilson and Chase, 1993). The PCR procedure, which detects a unique segment within the serotype-specific segment L2 of the viral genome, is considered to be very specific. These tests have the potential of providing a more rapid and reliable method for the serotype determination of BTV isolates. The

purpose of this study is to validate a multiplex PCR test that determines the serotype of U.S. BTV isolates (Wilson and Chase, 1993).

Thesis Organization

This thesis consists of a general introduction, a review of the literature, a separate manuscript, a general conclusion, and a list of literature cited in the nonmanuscript chapters. The master's candidate, Donna S. Johnson, is the senior author and principal investigator for the manuscript.

LITERATURE REVIEW

Bluetongue Disease

History

Bluetongue disease in sheep was first reported in South Africa in 1902 (Hutcheon, 1902). Initially referred to as "malarial catarrhal fever," the condition was described as causing high fever and inflammation of the mucous membranes of the oral cavity, often accompanied by inflammation of the coronary band. The origin of the disease, while unknown, was assumed to be "micro-parasitic." In 1905, Spreull suggested that the name of the syndrome should be "bluetongue," as that term was more applicable to the disease (Spreull, 1905). In an extensive study of BT, he determined that the causative agent could not be transmitted by contact, but that blood from afflicted animals was highly infectious. Spreull also determined that the agent of the disease was filterable and could be transmitted to goats and cattle, in which infection was inapparent. He also noted the seasonal nature of the disease as coinciding with large populations of biting insects. However, it wasn't until the 1940s that du Toit (1944) actually isolated the virus from *Culicoides* spp. and determined that the insect was capable of transmitting the disease to sheep. The first vaccine for BT was developed in 1906 (Theiler, 1908), and this attenuated strain was used for over 40 years in South Africa despite evidence of severe reactions and inadequate immunity. Suspecting that the vaccine problems were in part caused by the existence of different strains of the virus, Neitz did immunological studies and was the first to recognize the existence of multiple serotypes of the virus; subsequently, multivalent vaccines were developed (Neitz, 1948). Serum neutralization tests were later developed as a method of serotyping BTV isolates (Howell, 1960).

Bluetongue was considered to occur only in Africa until 1943 when an outbreak occurred in Cyprus (Gorman, 1990). According to reports, similar outbreaks had occurred in Cyprus for many years, but the causative agent had not been previously identified. Within the next few years, BTV was also isolated from outbreaks throughout the Middle East, and in 1956 a major epizootic occurred in Spain and Portugal. The first report of a bluetongue-like disease in the United States was in Texas in 1948, but it wasn't until 1952 that BTV was actually isolated from sheep in California (McKercher et al., 1953). As a consequence of the apparent spread of the disease, in the early 1960s BT was considered to be an "emerging" disease and restrictions were placed on the movement of ruminants and their germplasm (Howell, 1963). Australia, in particular, was most threatened by the disease, which many thought could devastate their large sheep population. However, in 1975, BTV was isolated from biting midges that had been collected in the Northern Territory (St. George et al., 1978). To date, eight serotypes of BTV have been identified in northern Australia; however, there has never been any evidence of clinical disease in ruminants. It is now recognized that the classification of BT as an emerging disease was erroneous since BTV had been present for many years in areas that had been previously considered free of the virus.

World distribution and the vector

BTV is currently recognized to infect ruminants in tropical, subtropical, and temperate regions of the world, generally within a belt with boundaries of 40°N and 35°S (Parsonson, 1992). Approximately 70% of the world's sheep population is found within this area. The geographic distribution of BT coincides with the range of susceptible *Culicoides* spp. In tropical and subtropical regions, the vector is present year-round; BT infection is common, but clinical disease is rare (Gibbs, 1992; Oviedo et al., 1992; Mo et al., 1994). In more temperate climates, such as southern and western United States, large populations of the vector appear seasonally and coincide

with disease outbreaks. If the trend towards global warming continues, there could be a profound effect on the geographic distribution of many arthropod-borne diseases, including BT (Gibbs, 1992; St. George, 1992; Standfast and Maywald, 1992).

Twenty-four serotypes of BTV have been isolated worldwide (Table 1). In the United States, the common serotypes are BTV-10, BTV-11, BTV-13, and BTV-17. In 1982, BTV-2 was isolated in southern Florida from infected cattle and, subsequently, from pools of *C. insignis* (Collisson and Barber, 1985). However, since 1983 there have not been any other isolates, nor has there been any serological evidence of BTV-2 since 1986 (Pearson et al., 1992b).

Occasionally, outbreaks of BT disease have occurred in regions outside the normal range of the vector, such as in Spain and Portugal during 1956 - 1957, in the Greek Isles during 1979, and in the Okanagan Valley of British Columbia, Canada, during 1987 (Dulac et al., 1992; Papadopoulos,

Table 1[a]. Geographic distribution of bluetongue

Region	Affected areas	Outbreaks of disease	Serotypes isolated
Africa	Probably endemic in all areas except northwest Africa	Yes	1 - 16, 18, 19, 22, 24
Asia	Probably endemic in all areas east of Turkey through Indian sub-continent to Indonesia; northern extent beyond Nepal not determined	Yes	1 - 4, 6, 7, 9, 10, 12, 16, 17, 18, 20, 21, 23
Australia	Endemic in northern areas	No	1, 3, 9, 15, 16, 20, 21, 23
Europe	Epidemic in Spain and Portugal (1956 - 1957); Greece (1979); not considered endemic	Yes	4, 10
North America	Endemic in southern and western U. S. states and Mexico	Yes	2, 10, 11, 13, 17
South America, Central America, Caribbean Basin	Endemic but southern extent not determined	No	1, 3, 4, 6, 8, 12, 17

[a]Table adapted from Gibbs and Greiner, 1994

1992; Gibbs and Greiner, 1994). These incursions may likely be the result of infected insects being carried into nonnative areas by prevailing winds. In all instances, BTV did not perpetuate in the area. Wind-borne vectors are also considered responsible for introduction of different BTV serotypes between islands of the Caribbean (Thompson et al., 1992), and into Indonesia (St. George, 1992), and possibly for the introduction of BTV-2 into southern Florida in 1982 (Sellers and Maarouf, 1989).

Of over one thousand known species of *Culicoides*, only eight are known to transmit BTV (Mellor, 1990; Gibbs and Greiner, 1994). Molecular studies have suggested that the susceptibility of certain *Culicoides* spp. to BTV is linked to a single locus in the vector genome. In the Americas, *C. insignis* is the BTV vector for Central America, the Caribbean basin, and the southern tip of Florida. *C. variipennis* is the vector for the remainder of North America. The insect, which is most active in warm and humid weather conditions, becomes infected with BTV only after imbibing a viremic blood meal from an infected animal (Mellor, 1990). The virus replicates in the midgut of the insect and reaches maximum concentration at 7 - 9 days postinfection. There has been no evidence of transovarial transmission of BTV in the *Culicoides* vector.

Bluetongue virus infection

Sheep from temperate regions of the world are generally most affected by BT disease. The severity of the disease is dependent upon animal age, breed, and physiological state, as well as, pathogenicity of the virus and environmental conditions (Parsonson, 1992; MacLachlan, 1994; Ward, 1994). Older sheep are more susceptible to infection than younger sheep, and breeds from colder climates will succumb to disease far more often than tropical breeds (Gibbs and Greiner, 1994). In many cases, the symptoms are mild or subclinical and the animal completely recovers in a few weeks. However, some animals exhibit a more severe response to the virus (Spreull, 1905;

Parsonson, 1990; MacLachlan, 1994). Symptoms developed by severely affected sheep may include: high fever, nasal discharge, drooling, facial edema, hyperemia and swelling of the buccal and nasal mucosa, erosions around the lips, hyperemia of the coronary bands, hoof lesions, and muscle degeneration. Occasionally, the tongue may become swollen and cyanotic. Vomiting may also occur as a result of smooth muscle lesions in the esophagus and pharyngeal area. This may lead to a fatal pneumonia. Mortality rates in Africa are reported to vary from 2 - 30%; in the United States, it is estimated to be around 5% (Parsonson, 1992).

Cattle can become readily infected with BTV but rarely exhibit any clinical symptoms (du Toit, 1962; Luedke et al., 1969; Parsonson, 1990). Cattle do, however, develop a high titered and prolonged cell-associated viremia and, subsequently, are considered the reservoir host for the virus (Luedke et al., 1969; MacLachlan et al., 1992a; Brewer and MacLachlan, 1994; MacLachlan, 1994). Infection of cattle is also significant to the epidemiology of the disease since *Culicoides* spp. have been shown to readily transmit the virus from cattle to other ruminants (du Toit, 1962). Peak viremic titers in cattle generally occur at around 7 days after infection, and BTV is usually present in the blood for 35 - 45 days, and possibly for up to 90 days; in some areas, this may facilitate overwintering of the virus when vector populations are low or absent (Nevill, 1971). Bluetongue virus infection has been reported to elicit a slightly different viremic response in sheep than in cattle (Luedke, 1969; Richards et al., 1988). While cattle commonly have a viremia that can extend beyond 40 days, the viremic phase in sheep is somewhat shorter and generally lower titered.

Studies done on cattle indicate that the virus initially replicates in the lymph node and then is transported to the blood via efferent lymph cells. Secondary viral replication then occurs in the spleen followed by a high titered cell-associated viremia (MacLachlan et al., 1992a; Barratt-Boyes and MacLachlan, 1994). Erythrocytes, which have a half-life of 160 days and do not support virus replication, are critical to the maintenance of the prolonged viremia. The virus associates with these

red blood cells by becoming embedded into invaginations on the cell surface, which also serves to protect the virus from neutralization by circulating antibody. (Brewer and MacLachlan, 1994). During the later stage of viremia, infected erythrocytes are eventually removed from the blood by mononuclear phagocytic cells and virus is cleared from the animal through the humoral and possibly the cell-mediated immune system (Barratt-Boyes and MacLachlan, 1994). Interestingly, viral genetic material can be detected in the blood by PCR tests for up to 140 days, which is very close to the half-life of the erythrocyte (MacLachlan et al., 1994).

Other ruminants may also be infected with BTV. Acute or peracute fatal hemorrhagic disease often occurs in infected white-tailed deer, similar to that seen with an infection of epizootic hemorrhagic disease virus (EHDV), a closely related orbivirus. In Africa, large populations of susceptible wild ruminants have antibodies to BTV, but few show any clinical symptoms (Parsonson, 1990). Like cattle, these wild bovids serve as host reservoirs for the virus. Goats are also susceptible to BTV, but infection is usually inapparent or mild (Spreull, 1905; Parsonson, 1990).

Effect on reproduction

Bluetongue virus infection can have various impacts on reproduction. In most cases, infected cows or ewes will deliver normal offspring (Parsonson, 1992; MacLachlan, 1994). However, there have been instances when fetuses exposed to BTV early in gestation are born with teratogenic defects (MacLachlan et al., 1992a; Osburn, 1994b). The use of attenuated or modified live vaccines during early gestation can also result in mummified fetuses and stillbirths (S. J. Johnson, 1992; Parsonson, 1992; Walton, 1992; MacLachlan, 1994; Murray and Eaton, 1996).

Rarely, BTV has been isolated from the semen of infected bulls (Bowen et al., 1983; Howard et al., 1985; Melville and Gard, 1992). However, studies have shown that BTV is

sporadically found only in the semen of viremic bulls, which is likely a consequence of the presence of contaminating blood (Bowen et al., 1983; Osburn, 1994b). The venereal infection or transfer of BTV by artificial insemination is not considered epidemiologically significant (Monath and Guirakhoo, 1996).

Bluetongue has also been reported to cause abortions, stillbirths, and maternal deaths in bitches that had been vaccinated in late pregnancy with a BTV-contaminated canine vaccine (Wilbur et al., 1992; Akita et al., 1994). The source of the contamination is thought to be either BTV-infected cell culture or a BTV-containing fetal bovine serum used in the maintenance of the cell cultures in which the vaccine was produced.

Vaccines

The most common type of vaccine used in controlling BT is an attenuated live virus vaccine. Bluetongue vaccines are produced against the serotype-specific neutralizing antigen located on the outer capsid of the virus (Parsonson, 1992; Walton, 1992; MacLachlan, 1994; Murray and Eaton, 1996). Consequently, the vaccines will protect only against certain serotypes of the virus. Several risks are associated with the use of attenuated vaccines. The virus may revert to a virulent form causing clinical disease, and these BT vaccines have also been shown to be teratogenic when used in early pregnancy. It has also been shown that attenuated virus strains present in vaccinated animals can be transmitted by *Culicoides* vectors to other animals (Foster et al., 1968). This could lead to attenuated viruses and wild-type viruses reassorting to create new viruses of unpredictable properties (Murray and Eaton, 1996; Wade-Evans et al., 1996).

Vaccines for BT have been widely used in South Africa where three different attenuated live virus vaccines, representing 15 serotypes, are given a month apart (Roy et al., 1994; Wade-Evans et al., 1996). This method has proven effective in controlling outbreaks in that region. In the United

States, only one BT vaccine is, at present, commercially available which is an attenuated live virus vaccine produced against BTV-10. Since BTV-10 is not frequently encountered, the vaccine is generally ineffective and not widely used (Parsonson, 1992; Walton, 1992).

Current research in vaccine technology has lead to the preparation of serotype-specific subunit vaccines. In one study, the outer capsid proteins and two core proteins were expressed in a baculovirus vector. The resulting virus-like particle was found to be very immunogenic when used in conjunction with an appropriate adjuvant (Roy et al., 1990b, 1994). Since a vaccine of this type contains no genetic material, there is no possibility of viral replication in vaccinated animals or transmission to vectors. However, large-scale commercial production of a recombinant vaccine may be difficult due to its complex nature.

The Bluetongue Virus

Orbiviruses

Orbiviruses constitute one genus within the family *Reoviridae*. The *Orbivirus* genus was proposed by Borden et al. (1971) for a group of formerly unclassified arboviruses, including BTV and African horse sickness. The name is derived from "orbis," which is Latin for circle or ring, and reflects the doughnut-shaped capsomeres seen on the virus surface when negative stained particles are viewed with an electron microscope. A characteristic common to all the members of *Reoviridae* is the segmented genome of dsRNA which is enclosed in a nonenveloped, icosahedral capsid shell. Unlike other *Reoviridae*, however, orbiviruses are smaller, lack a defined structure to the outer capsid, are acid labile, and are transmitted by arthropod vectors (Borden et al., 1971; Eaton et al., 1990; Monath and Guirakhoo, 1996; Roy, 1996).

Orbiviruses are primarily of importance as agents of animal disease rather than human disease (Monath and Guirakhoo, 1996). The genus is divided into 14 serogroups or species based on serological tests (Gould and Hyatt, 1994; Roy, 1996). The *Orbivirus* serogroups are outlined in Table 2. Serogroups are further divided into distinct serotypes based on neutralization tests. The prototype virus of the genus is BTV, which has been extensively studied.

Bluetongue virus structure and genome organization

The BT virion consists of a nonenveloped, double-shelled capsid enclosing ten segments of dsRNA (Mertens et al., 1984; Van Dijk and Huismans, 1988; Roy, 1989, 1996; Huismans and Van Dijk, 1990; Gould and Hyatt, 1994). The genome segments are identified according to their size as determined by polyacrylamide gel electrophoresis and referred to as L1 - L3 (large segments), M4 - M6 (medium segments), and S7 - S10 (small segments). Seven viral proteins (VP1 - VP7) and four nonstructural proteins (NS1, NS2, NS3, and NS3a) are encoded by the genome. The coding assignments and functions of the viral proteins are summarized in Table 3.

The virion core is formed by five structural proteins. The minor proteins, VP1, VP4, and VP6, form the inner area of the core of the virion (Roy, 1989). Preliminary data suggest that VP1, the largest viral protein, is the viral RNA polymerase. The VP4 protein is considered to be the guanylyl transferase which catalyzes the capping of the 5' ends of mRNA during transcription. The third minor protein, VP6, is thought to be involved in the unwinding of dsRNA prior to synthesis of mRNA (Stäuber et al., 1997). The largest major core protein, VP3, surrounds the three minor proteins and forms a scaffold for the second major core protein, VP7. Trimers of VP7 form the icosahedral structure of the core. The VP7 layer is not completely shielded by the outer capsid and has been identified as the group-specific antigen recognized by VP7-specific antibodies in serological

Table 2[a]. The orbiviruses

Serogroups	Serotypes	Vector	Host
African horse sickness	9	Culicoides	Equids, dogs
Bluetongue	24	Culicoides	Sheep, cattle, wild ruminants, goats
Changuinola	12	Phlebotomines, Culicine mosquitoes	Humans, rodents
Corriparta	3	Phlebotomines, Culicine mosquitoes	
Epizootic hemorrhagic disease	9	Culicoides	Deer, cattle
Equine encephalosis	7	Culicoides	Equids
Eubenangee	4	Culicoides, Anopheline and Culicine mosquitoes	Humans, cattle, kangaroo
Lebombo	1	Culicine mosquitoes	Humans, rodents
Orungo	4	Culicine mosquitoes	Humans, primates, sheep, cows
Palyam	11	Culicoides, Culicine mosquitoes	Cattle, buffalo, sheep, deer
Umatilla	3	Culicine mosquitoes	Humans, birds
Wallal	2	Culicoides	Wallabys, kangaroos
Warrego	2	Culicoides, Anopheline and Culicine mosquitoes	Cattle, wallabys, kangaroos
Kemerovo complex	40	Ixodes, Argas, Ornithodoros, Boophilus, Rhipicephalus, Hyalomma	Birds, camels, buffalo, pigs, dogs, donkeys, rodents, domestic animals

[a]Table adapted from Roy, 1996

Table 3[a]. BTV genome coding and proteins

Segment	Protein	Size (daltons)	Location	Function
L1	VP1	149,588	Inner core	RNA polymerase
L2	VP2	111,112	Outer capsid	Serotype specific, virus attachment to cell
L3	VP3	103,344	Core	Forms scaffold for VP7 trimers
M4	VP4	76,433	Inner core	Capping enzyme
M5	VP5	59,163	Outer capsid	May be associated with VP2 conformation
M6	NS1	64,445	Nonstructural	Tubules, may be associated with virus translocation to cell membrane
S7	VP7	38,548	Core	Serogroup specific
S8	NS2	40,999	Nonstructural	Inclusion bodies, binds ssRNA
S9	VP6	35,750	Inner core	Viral helicase, binds ssRNA and dsRNA
S10	NS3 NS3a	25,572 24,020	Nonstructural	May aid virus release from cell

[a]Table adapted from Roy, 1996

tests (Wang et al., 1996).

The outer capsid of the BT virion, which encloses the core structure, is composed of VP2 and VP5. The genomic sequences, L2 and M5, which encode these proteins are the least conserved of the BTV genome (Fukusho et al., 1987; Ghiasi et al., 1987; Yamaguchi et al., 1988; Heidner et al., 1991; Roy, 1996). The VP2 protein is the hemagglutinin and is responsible for the attachment of the virus to cell receptors (Cowley and Gorman, 1987; Huismans and Van Dijk, 1990). The protein is also the antigen that determines the virus serotype, and the serotype-specific neutralizing antibody elicited in response to VP2 will confer protection to an animal against reinfection with the same serotype (Huismans et al., 1987; Roy, 1989). The function of the second outer capsid protein, VP5, is not known. The protein is not as variable as VP2 among serotypes, and does not appear to

be involved in binding neutralizing antibody. Some studies indicate that VP5 may be involved in maintaining the conformation of VP2 (Roy, 1996).

Four nonstructural proteins have been identified in BTV-infected cells (Van Dijk and Huismans, 1988). NS1 has been associated with the formation of tubules in BTV-infected cells (Urakawa and Roy, 1988). The tubules, which are characteristic of BTV-infected cells, are found throughout the cytoplasm. Their function is not known. The tubules may be merely a by-product of BTV morphogenesis and/or a storage form of the protein (Eaton et al., 1988). It has also been proposed that the tubules may be involved in the translocation of newly formed virus particles to the plasma membrane (Roy, 1996). The other major nonstructural protein, NS2, has been shown to bind single-stranded RNA (ssRNA) but not dsRNA (Roy, 1996). NS2 is associated with the virus inclusion bodies (VIBs), which are the site of mRNA translation and viral core assembly. The protein may be involved in recruiting specific mRNAs for translation, as well as, mediating the interaction of the core components with the mRNAs that serve as templates for the dsRNA genome during virus assembly (Theron and Nel, 1997). The two smallest nonstructural proteins, NS3 and NS3a, are both encoded by segment 10 and produced in minute amounts (Van Dijk and Huismans, 1988). Some studies indicate that the NS3 proteins may be integral components of the cellular membrane and possibly involved in the transport of virus particles out of the cell (Roy, 1996).

Replication

The binding of BTV to susceptible cells is mediated by the outer capsid protein, VP2 (Eaton et al., 1990; Roy, 1996). The attached virus then enters the cell by receptor-mediated endocytosis forming a coated vesicle. As the virions are transported through the cell, the virus-containing vesicles can fuse to form endosomes (Eaton et al., 1990; Gould and Hyatt, 1994; Roy, 1996). VP2 is removed in the acidic (pH 5.0) environment of the endosome, and the partially uncoated particle is

then released into the cytoplasm near the nucleus. The other outer shell protein, VP5, is then removed forming a core particle. Removal of the two outer shell proteins activates the virion transcriptase. The core particles then bind to the cell cytoskeleton and begin to transcribe the viral mRNA which, in turn, either directs the synthesis of viral proteins or acts as templates for the synthesis of the progeny genomic dsRNAs. A matrix, consisting of mRNA and translated virus-specific proteins, develops around the core particles forming VIBs. As the new core particles exit the VIB, they acquire the outer shell proteins VP2 and VP5, inhibiting viral transcriptase activity. The intact virions are then transported toward the plasma membrane through interactions with the cytoskeleton, and possibly the tubules, and generally exit the cell through a lesion on the plasma membrane, which may be associated with the NS3 proteins.

Genetic diversity

The natural replication cycle of BTV, which includes an insect vector and a ruminant host, offers a significant opportunity for the generation of diverse virus populations (C. A. de Mattos et al., 1994; C. C. P. de Mattos et al., 1994). Changes in the biological environment can also generate a shift in the predominant viral variant resulting in changes, both between serotypes and within a serotype, on the antigenic properties, tissue tropism, and virulence of different BTVs (Neitz, 1948; Bernard et al., 1994; C. A. de Mattos et al., 1994). This diverse nature is probably the result of an accumulation of point mutations and natural reassortment (Roy, 1989; C. C. P. de Mattos et al., 1991, 1994; Heidner et al., 1991; MacLachlan et al., 1992b; Monath and Guirakhoo, 1996). Another significant factor in the generation of genetic diversity is the lack of a proofreading mechanism in the polymerase of RNA viruses which results in the high mutation frequency associated with these viruses (Holland et al., 1992).

The segmented genome of orbiviruses makes them particularly susceptible to reassortment during coinfection of more than one serotype or different strains of the same serotype. This has been demonstrated experimentally in insects and vertebrates (Oberst et al., 1987; Samal et al., 1987; Stott et al., 1987). Natural occurrence of probable genetic reassortment has also been reported (Sugiyama et al., 1981; Oberst et al., 1985). Studies of two field isolates of BTV-11 from California, one virulent and the other avirulent, indicated that the virulent isolate had a gene segment M5 that was homologous to the BTV-10 M5 segment, while the L2 segment was homologous to BTV-11 (C. C. P. de Mattos et al., 1991, 1992). The enhanced virulence of the reassortant strain may have been due to a modification in the outer capsid conformation brought on by the interaction between VP2 and VP5 of different serotypes. While reassortment within an *Orbivirus* serogroup can occur, there has been no evidence of reassortment between serogroups, such as BTV and EHDV, despite the fact that such viruses have been isolated from the same naturally infected animal (Gould and Hyatt, 1994; Monath and Guirakhoo, 1996).

A comparison of BTV isolates from different continents suggests that the virus gene pools have diverged significantly (Knudson et al., 1982; Gould, 1988). Using a recombinant DNA probe at high stringency to the L3 gene, a $\geq 95\%$ of nucleotide homology was observed among all Australian serotypes; that was distinctive when compared to North American or South African isolates (Gould and Pritchard, 1990; Gould and Hyatt, 1994). Gould and his co-workers further defined three major geographic regions, Australiasian, African/American, and a single unknown geographic isolate, BTV-15, using hybridization analyses, and proposed the term "topotype" to define geographic relatedness.

Similar studies of L2 sequences showed that certain serotypes are more closely related, regardless of geographic origin (Gould and Pritchard, 1990; Gould et al., 1992). The amino acid sequences of these genotypes indicate they evolved from a common ancestral pool. Two major

groups have so far been identified (Gould and Hyatt, 1994). The first is the BTV-4-related serotypes BTV-4, BTV-10, BTV-11, BTV-17, and BTV-20; and the second is the BTV-3-related serotypes BTV-3, BTV-13, and BTV-16.

Diagnosis

Serology

Serological tests are used to determine the presence of BTV-specific antibody in serum (Pearson et al., 1992a; Afshar, 1994). These tests are routinely used to certify animals for import or export, and each country has specific requirements as to the tests it will accept. For diagnostic purposes, a single serum sample may show evidence of specific antibody to BTV, but tests cannot ascertain when the animal was exposed to the virus. Determination of recent exposure to BTV is best accomplished by testing paired sera, acute and convalescent, on a quantitative test such as virus neutralization (VN) or competitive enzyme-linked immunosorbent assay (C-ELISA). A significant increase in titer in the convalescent serum is indicative of a recent exposure to virus. A third test commonly used for detection of BTV antibody is the agar gel immunodiffusion test (AGID).

The AGID test is based on the reaction of a purified soluble antigen and a positive BT reference serum producing a line of precipitation in an agar gel matrix (Pearson et al., 1992a; Afshar, 1994). Positive sera and test sera are dispensed into alternating wells located around a center antigen well. The plates are incubated for 24 hours, and the location or absence of the precipitation lines at the test serum well determines if the sample is positive or negative. Advantages of the AGID test are that it is economical and easy to perform. However, the test is not quantitative, is not as sensitive as the C-ELISA, has cross-reactions with EHDV-positive sera, and requires one day to complete.

The C-ELISA assay, on the other hand, requires less than three hours to complete. Testing is done in wells of a microtiter plate that have previously been coated with a BTV group-specific antigen that is derived from the major core protein, VP7 (Stott and Osburn, 1990; Pearson et al., 1992a; Afshar, 1994). Test serum and VP7-specific monoclonal antibody (MAb) are added to each well and allowed to incubate. Samples with BTV antibody will compete with the MAb for the antigen attached to the well. Subsequent addition of a horseradish peroxidase labelled anti-mouse conjugate and chromagen substrate will provide a color reaction. C-ELISA plates are generally best evaluated with a plate reader which can determine the exact optical density of each well. Samples with greater than 50% reduction in optical density compared to the negative control are considered positive. The C-ELISA test is considered to be very specific and sensitive.

The VN test is the only serological test that is serotype-specific (Pearson et al., 1992a; Afshar, 1994). Test samples are diluted and allowed to react with each serotype of BTV for one hour in a microtiter plate. A suspension of BTV-sensitive cell culture is then added to each well, and the plate is incubated for three days. Plates are evaluated by the presence or absence of cytopathic effect (CPE). The VN test is the most labor-intensive of the three assays and relies on the availability of reference BT viruses and antisera of each serotype. Often sera from older animals will show cross-reactions among the different serotypes making the test difficult to interpret.

Virus isolation

Until the late 1960s, the most reliable method of isolating BTV was by inoculation of sheep with infected blood or tissues (Goldsmit et al., 1975; Afshar, 1994). There are many obvious disadvantages to this method including the need for sheep and their care, as well as, the requirement of an insect-free facility in which to house them. In 1968, intravenous (IV) inoculation of embryonating chicken eggs was proposed as an alternative to sheep inoculation for BTV isolation,

and subsequent studies showed that the method was nearly, if not equally, as sensitive as sheep inoculation (Goldsmit and Barzilai, 1968; Foster et al., 1972; Goldsmit et al., 1975). While the IV method is cumbersome and does require up to four weeks to complete, it is still considered the best method for isolation of BTV (Howell et al., 1970; Pearson et al., 1992a; Afshar, 1994). Attempts to isolate BTV directly in cell culture have all shown that BTV requires amplification in embryonating chicken eggs before it is detectable in cell culture.

For the IV procedure, lysed red blood cells, tissue suspensions from suspect animals, or sonicated semen are inoculated intravenously into 10- to 11-day-old embryonating chicken eggs which are then incubated for seven days at 33°C (Jochim et al., 1975; Pearson et al., 1992a; Afshar, 1994). Bluetongue virus present in the sample will generally kill the embryos between two and five days. Dead embryos and those alive at seven days are harvested, macerated, and passaged into embryonating chicken eggs by the yolk sac route and onto baby hamster kidney (BHK-21) or Vero cell cultures. Infected cell cultures will display distinct CPE, and the isolate is then identified as BTV using the indirect fluorescent antibody (IFA) procedure using a BTV serogroup-specific monoclonal antibody.

Nucleic acid detection

With the expanding knowledge of BT viral genetics in the last 15 years, new procedures have been developed to detect specific genomic sequences. Initially, most of the new tests were based on hybridization techniques where complementary DNA (cDNA) or RNA probes were used to detect viral nucleic acid of BTV (Roy et al., 1985; Huismans and Cloete, 1987; Kowalik and Li, 1987; Brown et al., 1988; Gould, 1988; Pedley et al., 1988; Ritter and Roy, 1988; C. A. de Mattos et al., 1989; Dunn and Stott, 1989). These tests are generally highly specific and results can be obtained much faster than with virus isolation procedures. However, the tests are complicated and

sometimes don't consistently detect low levels of virus present in the sample; consequently, they are not generally used in diagnostic laboratories (Dangler et al, 1990; Afshar, 1994). Hybridization tests have been more commonly used to determine the genetic relationship between serotypes or strains of BTV (Roy et al., 1990a).

More recently, PCR procedures that detect specific genomic sequences have become more common in diagnostic laboratories. Several of the tests which have been developed for the detection of BT viral RNA have been shown to be highly specific and sensitive (Dangler et al, 1990; Wade-Evans et al., 1990; McColl and Gould, 1991; Akita et al, 1992; Katz et al, 1993; Wilson and Chase, 1993; Harding et al., 1995). These tests have been used to detect BTV in whole blood, animal tissues, and in *Culicoides* spp. Prior to the procedure, RNA is extracted from the sample, followed by synthesis of cDNA using primers specific for a target sequence on the BTV genome. The cDNA is then amplified exponentially through repeated heat cycles of denaturation, annealing of primers, and extension of the sequence. In the most sensitive PCR assays, a small amount of the PCR product is further amplified using nested primers (Katz et al, 1993; McColl and Gould, 1994; Roux, 1995). The final PCR product can be identified by size after agar gel electrophoresis, gene sequencing, restriction enzyme analysis, or hybridization techniques (Afshar, 1994).

The PCR procedures have proven to be a valuable tool in the detection of viral sequences, but these tests do not necessarily reflect the viremic state of the animal. Studies have shown that BT viral nucleic acid can be detected by PCR tests in blood for up to 140 days after infection in animals whose blood was negative by virus isolation after 56 days (Katz et al., 1993; MacLachlan et al., 1994). Such PCR+/VI- blood has been shown to be noninfectious when inoculated into susceptible sheep or *Culicoides* spp. (MacLachlan et al., 1994; Tabachnick et al., 1996).

Serotype identification

Cell culture adapted isolates of BTV are further characterized as to their serotype primarily for epidemiological information. For the past 30 years, several approaches to serotype identification have been used, most of which are based on virus neutralization procedures, including: virus neutralization in embryonating chicken eggs (Goldsmit and Barzilai, 1968), plaque neutralization (Howell et al., 1970; Barber and Jochim, 1976; Jochim and Jones, 1976; Thomas et al., 1979), plaque inhibition (Davies and Blackburn, 1971; Stott et al., 1978), microtiter virus neutralization (Eaton, 1996), and fluorescence inhibition (Blacksell and Lunt, 1996). For these procedures, dilutions of the BTV isolate are allowed to react with an optimal dilution of serotype-specific reference antisera. The antiserum homologous to the isolate inhibits virus growth, and the absence of pathological effect indicates the serotype (Afshar, 1994). Currently, the microtiter VN test is probably the most widely used because it is the least labor-intensive and requires commonly available laboratory equipment and supplies. However, there are drawbacks to all of these tests (Thomas et al., 1979; Afshar, 1994). Cross-reactions among serotypes are common, and identification of actual mixed infections in an animal can be difficult, if not impossible. Complications can also arise in determining the relationship between extent of neutralization and antiserum dilutions. Another disadvantage is the inherent differences displayed by different isolates, such as the ability to propagate to high titer in cell culture or unexpected reactions to the antisera. Most of these tests also require 4 - 6 days to complete.

More recently, BTV serotypes have been identified by PCR procedures. Two primers are developed for each serotype based on the sequence of L2, the genome segment encoding the serotype-specific outer capsid of the virus. In Australia, eight primer pairs are used to differentiate serotypes present in that area (McColl and Gould, 1991). In the United States, a multiplex PCR has been developed in which a pool of five different pairs of primers, each pair specific to one U.S.

serotype, is used to identify domestic isolates of BTV (Wilson and Chase, 1993). Advantages of the PCR procedures are that these tests are very specific and results are usually obtained in 1 - 2 days. However, the materials and equipment required for the tests are expensive, and such tests may only be practical in laboratories where PCR procedures are already routinely performed.

VALIDATION OF A REVERSE TRANSCRIPTASE MULTIPLEX PCR TEST FOR THE SEROTYPE DETERMINATION OF U.S. ISOLATES OF BLUETONGUE VIRUS

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Abstract

Bluetongue (BT) is an arthropod-borne viral disease affecting ruminants primarily in tropical and temperate regions of the world. Sheep are clinically the most severely affected, whereas cattle are considered the host reservoir of the virus and generally exhibit no clinical symptoms. Twenty-four serotypes of BT virus (BTV) have been identified worldwide, of which five serotypes have been found in the United States. Serotype identification of BTV isolates is considered important to the epidemiology of the virus but current methods are cumbersome. A multiplex reverse transcriptase polymerase chain reaction (mRT-PCR) assay, previously developed for the serotype determination of U.S. BTV isolates, was evaluated. Five primer pairs, each pair specific for one U.S. serotype, were used in a single-tube reaction for reverse transcription and PCR. The determination of serotype was based on the size of the resultant amplified product. The procedure was evaluated using all 24 serotypes of BTV and nine serotypes of epizootic hemorrhagic disease virus (EHDV), a closely related orbivirus. Only the U.S. serotypes of BTV were detected by the mRT-PCR. The assay was further tested using 132 BTV isolates originating from 24 western and southern U.S. states, from several different host species, spanning a period of 24 years. The serotypes of the isolates were determined by both a virus neutralization (VN) procedure and the mRT-PCR. Comparison of the two procedures showed that the mRT-PCR successfully identified

the serotypes of 130 of the isolates and was shown to be more reliable and specific than the VN assay.

Introduction

Bluetongue is an infectious, noncontagious, arthropod-borne, viral disease of wild and domestic ruminants. The disease occurs primarily in tropical and temperate areas of the world where large populations of the vector, *Culicoides* spp., are present (du Toit, 1944; Gorman, 1990; Gibbs and Greiner, 1994; Osburn, 1994a). Sheep are the most severely affected domestic animal and may exhibit clinical symptoms of pyrexia, leukopenia, facial edema, mucosal erosions and ulcerations, and hyperemia of the oral mucous membranes and coronary band (Spruell, 1905; Parsonson, 1990; MacLachlan, 1994). In the United States, mortality is about 5% in BTV-infected sheep. Other manifestations of the disease include reproductive losses in cattle and sheep, usually as a result of dam vaccination with an attenuated strain of BTV during early gestation (Parsonson, 1990; Osburn, 1994b). Also, infected white-tailed deer develop a fatal hemorrhagic disease (Parsonson, 1990). However, many infected animals are asymptomatic or only mildly affected. This is particularly true of cattle, which are considered the host reservoir of the virus due to the prolonged viremia and absence of clinical disease that are characteristic of cattle infections (MacLachlan et al., 1992; Brewer and MacLachlan, 1994; MacLachlan, 1994). The impact of BT on the livestock industry is directly apparent in production losses; however, the greatest economic impact is due to restrictions placed on the export of animals or animal products from regions where BTV is known to exist (MacLachlan et al., 1989).

Bluetongue virus is the prototype member of the *Orbivirus* genus within the family *Reoviridae*. Twenty-four serotypes of BTV have been defined, of which five (BTV-2, BTV-10,

BTV-11, BTV-13, and BTV-17) have been identified in the United States (Gorman, 1990; Gibbs, 1992; Gibbs and Greiner, 1994). The virus is characterized as a nonenveloped, double-shelled capsid enclosing ten segments of double-stranded RNA (dsRNA) (Mertens et al., 1984; Roy, 1989, 1996). Each segment encodes a single viral protein, with the exception of segment 10, which encodes two small proteins. The virus structure consists of the core component made up of three minor proteins, VP1, VP4, and VP6, that are enclosed by an icosahedral inner capsid, which is composed of two major proteins, VP3 and VP7. Surrounding the inner capsid is an amorphous outer layer which consists of the proteins VP2 and VP5. Several nonstructural viral proteins are also produced in BTV-infected cells. The BTV genome is highly conserved among the serotypes, with the exception of segment L2, which encodes the serotype-specific outer capsid protein, VP2, and segment M5, which encodes the second outer capsid protein, VP5 (Kowalik and Li, 1987; Heidner et al., 1991).

Diagnosis of BT involves serological procedures, PCR tests, and/or virus isolation (Pearson et al, 1992; Afshar, 1994). Serotype identification of viral isolates is considered epidemiologically important. Several serotyping procedures have been used over the years, including virus neutralization, plaque neutralization, plaque inhibition, and fluorescence inhibition (Howell et al., 1970; Davies and Blackburn, 1971; Afshar, 1994; Blacksell and Lunt, 1996). Common features among these tests are that they can be cumbersome, sometimes provide inconclusive results, and often need to be repeated. More recently, PCR tests have been developed for differentiating BTV serotypes in Australia and the United States (McColl and Gould, 1991; Wilson and Chase, 1993). These PCR procedures, which detect unique regions on the serotype-specific segment, L2, are considered to be very specific and have the potential of providing rapid and reliable serotype identification. The purpose of this study was to validate a multiplex reverse transcriptase PCR (mRT-PCR) test previously developed to determine the serotype of U.S. BTV isolates (Wilson and

Chase, 1993). The procedure was originally tested with the five U.S. serotypes of BTV and two U.S. serotypes of EHDV, and has been used primarily for serotype determination on pools of *Culicoides variipennis*. This study further evaluated the procedure on the exotic strains of BTV and EHDV and determined the sensitivity and specificity of the test using a large number of BTV isolates.

Materials and Methods

Viruses

Stock viruses of all 24 serotypes of BTV (BTV-1 through BTV-24) and nine serotypes of EHDV (EHDV-1 through EHDV-8 and Ibaraki virus) were propagated in BHK-21 or Vero cell cultures.

One hundred thirty-two BTV isolates that originated from U.S. samples submitted to the National Veterinary Services Laboratories from 1974 - 1997 were propagated and endpoint titers were determined on BHK-21 cell monolayers. The serotype identification of all the isolates was determined using a microtiter VN assay (Eaton, 1996).

Preparation of nucleic acid carrier and nucleic acid extraction

A nucleic acid carrier was used during the nucleic acid extraction process to increase the sensitivity of the mRT-PCR. Lysed bovine red blood cells were used as the carrier for this assay. BTV-negative bovine blood collected in ethylenediaminetetraacetic acid (EDTA) was washed once with phosphate buffered saline, centrifuged at 1300 x g for 10 minutes, and the red blood cells were lysed with an equal volume of RNase-free water. A 200 µl volume of the lysed cells was centrifuged at 12000 x g and the supernatant discarded. A 100 µl volume of the cell culture virus to be

extracted was added to the red blood cell pellet and the total RNA was extracted with guanidine thiocyanate using a commercial kit (Isoquick® Nucleic Acid Extraction Kit, Microprobe Corp., Bothell, WA) as per manufacturer's instructions. Extracted RNA pellets were stored in 100% ethanol at -70°C until the mRT-PCR was performed.

Primers

Five primer pairs were used, each of which hybridizes to a specific region of the BTV genome segment L2 (Table 1). The primer sequences were designed from published sequences of the five U.S. prototypes of BTV (Purdy et al., 1985; Fukusho et al., 1987; Ghiasi et al., 1987; Yamaguchi et al., 1988). The primers, with the exception of primer 17a, were originally described by Wilson and Chase (1993). The original 17a primer was redesigned when multiple bands were visualized on several BTV-17 isolates. Equimolar amounts of each of the ten primers were combined into a multiplex primer mixture with a final concentration of 50 pmol/µl or 5 pmol/µl of

Table 1. Oligonucleotide primers for BTV mRT-PCR.

Primers	Sequence	Nucleotide position[a]	Product size (bp)
BTV-2 a	GTTAAAACAGGATCGCGATGGATG	1	
BTV-2 b	CCGGCTATGTTCCTCATCAATCGA	401	400
BTV-10 a	ACGTCCGGTGCCGTATCGATTAGAT	2141	
BTV-10 b	GGAATGCACCTTCAGTTGTCCACC	2788	647
BTV-11 a	CCTGGAAGTCGGTGATCAAGTAGT	1267	
BTV-11 b	GCCTCTCTCATATCGCGCAATTGA	2085	818
BTV-13 a	CTCGAGGATGGAAGAGCTTGTGAT	15	
BTV-13 b	CTATCTGAAGCGGCTATCACCATCAGCACA	1070	1055
BTV-17 a	TATGGACAGCACGAGCGACAGTTA	1196	
BTV-17 b	ATAGAAGCCGCAGATCGTAGTAGG	1733	537

[a] Numbering is based on nucleotide positions relative to the 5'-terminal nucleotide of the positive-sense strand of genome segment L2, which encodes the serotype specific outer capsid protein.

each primer.

mRT-PCR

The extracted nucleic acid samples were centrifuged briefly (7500 x g) and the ethanol removed. The RNA pellets were resuspended in 15 µl of RNase-free water. Denaturation of the RNA was achieved by adding 3 µl of the RNA suspension to a denaturation-primer mixture consisting of 3 µl of the multiplex primer mixture and 2 µl of 50 mM methyl mercury hydroxide (Crescent Chemical, Hauppage, NY). After incubation at room temperature for ten minutes, 3 µl of 240 mM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) was added to neutralize the methyl mercury hydroxide. Nine microliters of a reverse transcription mixture was added to each tube for a total volume of 20 µl with a composition of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 10 mM dithiothreitol, 2.5 mM $MgCl_2$, 4 mM dNTP pool, and 10 units/µl M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD). The reverse transcription mixture was incubated at 42°C for 60 minutes followed by four minutes at 98°C. Two units of *E. coli* RNase H (Life Technologies, Gaithersburg, MD) was added to each tube along with a wax bead (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ). Tubes were incubated at 37°C for 20 minutes followed by a 98°C incubation for 4 minutes. A PCR mixture was prepared with a concentration of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.9 mM $MgCl_2$, 0.7 mM dNTP pool, and 5 units *Taq* DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), and 80 µl of the mixture was added to each tube. The amplification program consisted of one cycle of 3 minutes denaturation at 95°C, 45 seconds primer annealing at 58°C, and 70 seconds primer extension at 72°C; followed by 35 cycles of 25 seconds denaturation at 95°C, 45 seconds primer annealing at 58°C, and 70 seconds primer extension at 72°C. The final cycle had similar conditions except for a five minute primer

extension period. All incubation and amplification procedures were performed using a Perkin-Elmer 9600 PCR system. Following the PCR, 15% of the amplification product was analyzed on a 2% NuSieve® agarose gel (FMC BioProducts, Rockland, ME) containing .5 µg/ml ethidium bromide (Sigma Chemical Co., St. Louis, MO). Electrophoresis was performed at 70 V for 1 - $1\frac{1}{2}$ hours in a Tris-borate buffer containing .5 µg/ml ethidium bromide. The gels were photographed under ultraviolet light and the size of the DNA products were compared visually with the BTV controls and a 100 base pair DNA ladder (Life Technologies, Gaithersburg, MD).

Experimental design

The sensitivity of the mRT-PCR was determined by comparing the endpoint dilution of each serotype in cell culture with the endpoint dilution of each serotype by mRT-PCR. The sensitivity of the RT-PCR method using only the serotype-specific primer pair was similarly evaluated.

To ascertain that the mRT-PCR was capable of detecting multiple serotypes that may be present in one isolate, mixtures of two to five serotypes were pooled. The nucleic acid of each pool was then extracted and amplified by mRT-PCR.

Evaluation of the specificity of the mRT-PCR assay included testing each of the 19 exotic serotypes of BTV and all serotypes of EHDV. The nucleic acid of each virus was extracted and PCR amplification was performed concurrently using both the mRT-PCR assay and either a BTV serogroup-specific PCR assay on the BT viruses (Katz et al., 1993), or an EHDV serogroup-specific PCR assay on the EHD viruses (Wilson, 1994). The serogroup-specific PCR tests, which detect all serotypes of their respective virus, were performed to determine that viral nucleic acid was present in each extracted sample. Nineteen negative samples were also evaluated by the mRT-PCR.

A panel of 132 BTV isolates collected over a 24-year period, originating from 24 southern and western states and several different ruminant or insect species, was used to evaluate the mRT-

PCR. Each isolate was determined to have a virus titer sufficient for mRT-PCR detection. The serotype of each isolate was determined by a microtiter VN assay (Eaton, 1996). Each isolate was then amplified by mRT-PCR, and VN and PCR results were compared.

Results

Prototypes of the five U.S. serotypes of BTV were evaluated using the mRT-PCR procedure. Following reverse transcription, amplification, and electrophoresis, a single, discrete band was produced from each serotype when visualized under ultraviolet light (Figure 1, lanes 2 - 6). Serotype identification was easily determined visually according to the size of the amplification product with BTV-2, -10, -11, -13, and -17 forming bands of 400 base pairs (bp), 647 bp, 818 bp, 1055 bp, and 537 bp, respectively. In order to determine if multiple serotypes could be detected in a single sample, two to five serotypes of BTV were pooled, nucleic acid extracted, and amplified by mRT-PCR. The amplified DNA pools of two to three serotypes formed easily distinguishable bands for each serotype contained within the pool (Figure 2). The bands formed from pools of four or five serotypes were fainter and more difficult to identify.

The sensitivity of the PCR was assessed for each serotype using both the multiplex primer mixture and the individual serotype-specific primer pair. The mRT-PCR was able to detect a minimum of 1.86×10^1 to 1.86×10^4 $TCID_{50}$ per ml of virus, depending upon the serotype (Table 2). The sensitivity of the PCR could be increased for three of the serotypes when a serotype-specific primer pair was used instead of the multiplex primer mixture (Figure 3).

The mRT-PCR was further evaluated against the 19 serotypes of BTV not found in the United States. Each serotype was previously propagated and titrated on BHK-21 or Vero cell cultures and determined to contain the minimum detectable titer of virus for the mRT-PCR assay.

Figure 1. Agarose gel electrophoresis of mRT-PCR amplification products for the determination of BTV serotype: BTV-2, 400 bp; BTV-10, 647 bp; BTV-11, 818 bp; BTV-13, 1055 bp; and BTV-17, 537 bp. Lanes 1 and 20: 100 bp DNA ladder; lane 2 - 6: BTV controls, serotypes 2, 10, 11, 13, and 17, respectively; lane 7: original BTV-2 prototype virus from 1983; lane 8: 1977 BTV-10 ovine isolate (OK); lane 9: 1994 BTV-10 bovine isolate (GA); lane 10: 1974 BTV-11 bovine isolate (OR); lane 11: 1996 BTV-11 deer isolate (TX); lane 12: 1982 BTV-13 bovine isolate (NE); lane 13: 1996 BTV-13 bovine isolate (NM); lane 14: 1977 BTV-17 ovine isolate (MS); lane 15: 1996 BTV-17 bovine isolate (NM); lane 16: 1977 mixed BTV-10 and BTV-17 ovine isolate (MS); lane 17: EHDV-1; lane 18: BTV-20; lane 19: uninfected BHK-21 cells.

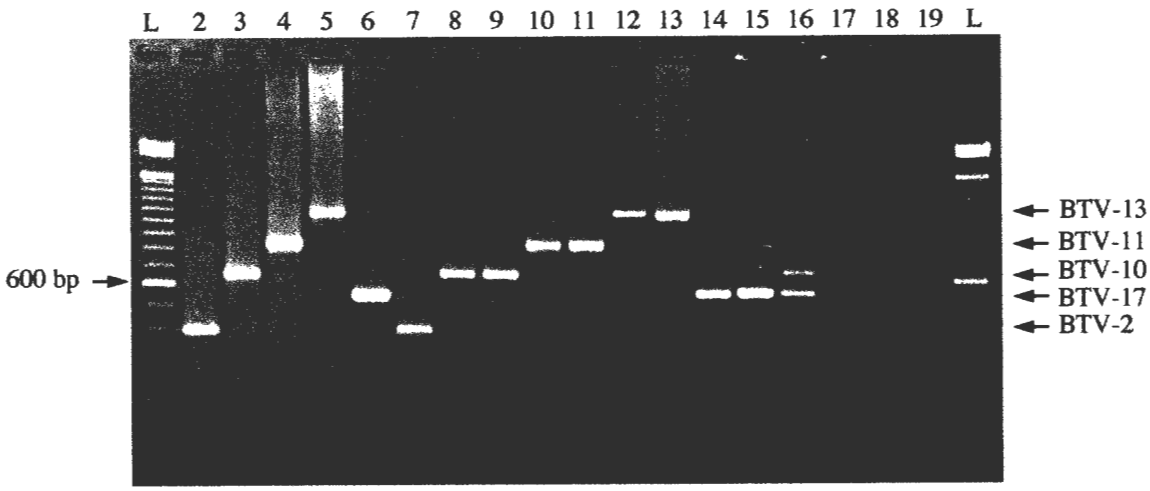
L 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 L
600 bp
BTV-13
BTV-11
BTV-10
BTV-17
BTV-2

Figure 2. mRT-PCR amplification of pools of two to three serotypes of BTV. Lane L: 100 bp DNA ladder; lane 2: BTV-2, -10, and -11; lane 3: BTV-2, -17, and -13; lane 4: BTV-10, -11, and -13; lane 5: BTV-17, -11, and -13; lane 6: BTV-10 and -11; lane 7: BTV-10 and -13; lane 8: BTV-17 and -11.

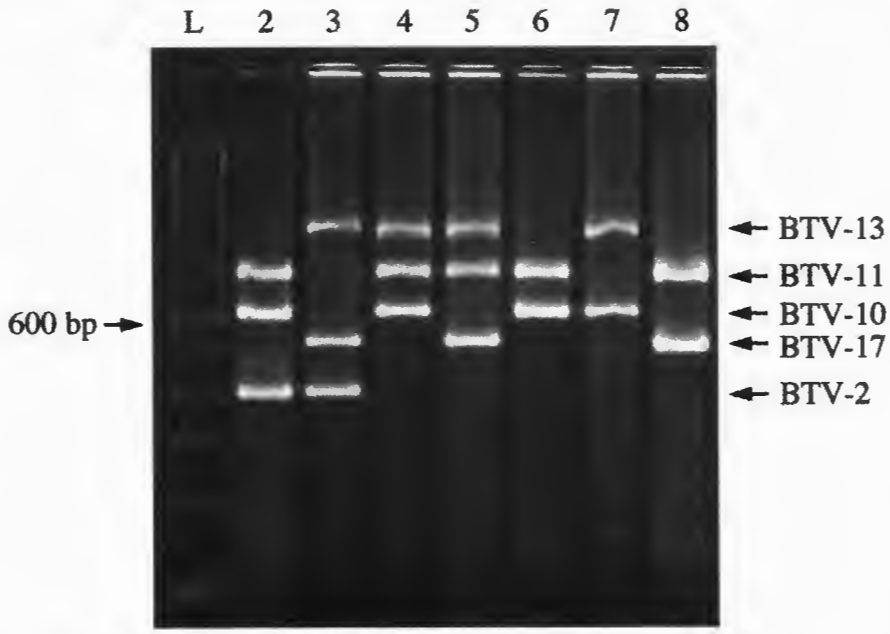
L
2
3
4
5
6
7
8
600 bp
BTV-13
BTV-11
BTV-10
BTV-17
BTV-2

Table 2. Comparison of the sensitivity of the PCR assay for each serotype using the serotype-specific (sRT-PCR) primers and the multiplex (mRT-PCR) primers.

	Minimum $TCID_{50}$ of virus per microliter necessary for determination of serotype	
Serotype	sRT-PCR	mRT-PCR
2	1×10^{1}	1×10^{2}
10	1.86×10^{1}	1.86×10^{2}
11	1.86×10^{3}	1.86×10^{4}
13	1.86×10^{1}	1.86×10^{1}
17	1.86×10^{3}	1.86×10^{3}

The mRT-PCR and a BTV serogroup-specific PCR were concurrently performed on each of the exotic serotypes. The mRT-PCR test resulted in no visible amplified product from any of the exotic serotypes, although BTV nucleic acid was detected in all 19 using the serogroup-specific test. Similar testing was also done with the nine serotypes of EHDV using an EHDV serogroup-specific PCR. No amplified product was visually detected using the mRT-PCR, however each EHDV sample was amplified by the EHDV serogroup-specific PCR. The mRT-PCR was also evaluated with 19 other known BTV-negative cell cultures and viruses, including two other *Reoviridae* family members: porcine reovirus (type 3) and bovine rotavirus. All viruses and cell cultures tested resulted in no visible amplified product using the mRT-PCR test.

A panel of 132 U.S. BTV isolates collected over a 24-year period was used to evaluate the mRT-PCR (Table 3). No isolates of BTV-2 were available for testing as that serotype was infrequently isolated in the southeastern United States only in 1982 and 1983 (Collisson and Barber, 1985). Serotype identification was achieved by PCR for 130 of the isolates. A single isolate of BTV-10 was not identified by the mRT-PCR; however, serotype determination was made after using the BTV-10-specific primer pair for PCR amplification. Another isolate showed amplification products for both BTV-10 and BTV-17 (Figure 1, lane 16) indicating a mixed infection that had not

Figure 3. Visual comparison of results of the mRT-PCR with the serotype-specific PCR. The viruses amplifed here represent the last 10-fold dilution that was detectable by mRT-PCR. Lanes L: 100 bp DNA ladder; lane 2: BTV-2 amplified with BTV-2 serotype-specific primers; lane 3: BTV-2 amplified by mRT-PCR; lane 4: BTV-10 amplified with BTV-10 serotype-specific primers; lane 5: BTV-10 amplified by mRT-PCR; lane 6: BTV-11 amplified with BTV-11 serotype-specific primers; Lane 7: BTV-11 amplified by mRT-PCR; lane 8: BTV-13 amplified with BTV-13 serotype-specific primers; lane 9: BTV-13 amplified by mRT-PCR; lane 10: BTV-17 amplified with BTV-17 serotype-specific primers; lane 11: BTV-17 amplified by mRT-PCR. The reason for the band artifacts located in lanes 5, 8, 10, and 11 is not known, but they may be due to less-than-optimum cycling parameters for those particular primers or viruses.

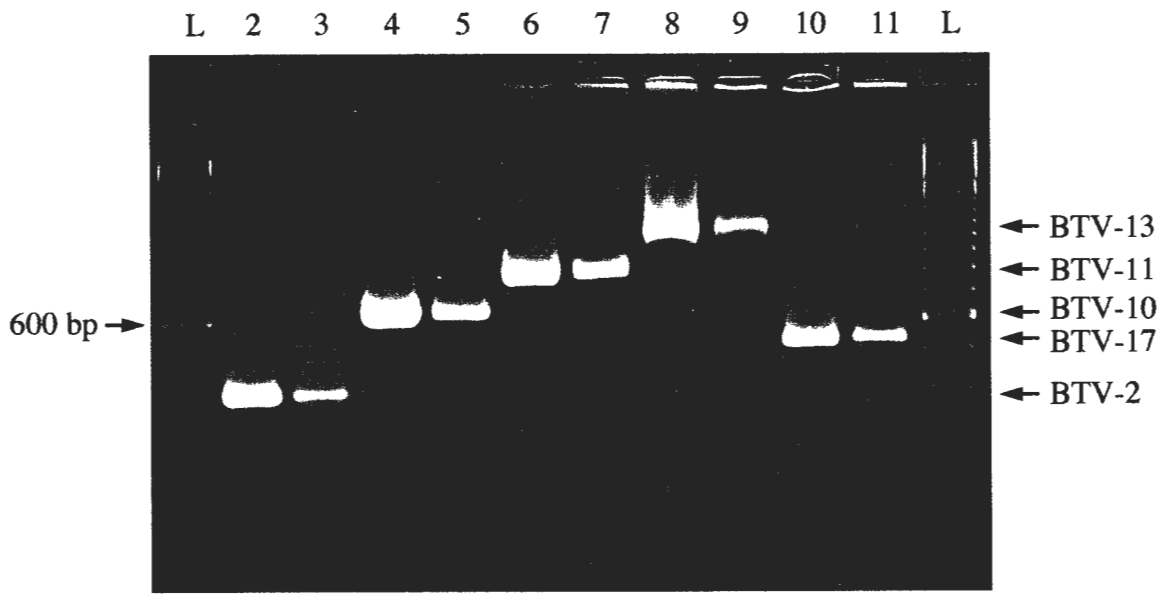
L 2 3 4 5 6 7 8 9 10 11 L
600 bp
BTV-13
BTV-11
BTV-10
BTV-17
BTV-2

Table 3. Numbers of serotype identifications with the mRT-PCR test as they compare with the VN assay for 132 U.S. BTV isolates made from 1974 - 1997. No isolates of BTV-2 were available as that serotype was infrequently isolated only in 1982 and 1983.

BTV serotype by VN	BTV serotype by mRT-PCR					
	10	11	13	17	10 & 17	ND[a]
10	27	-	-	-	1	-
11	-	36	-	-	-	1
13	-	-	22	-	-	1
17	-	-	-	35	-	-
inconclusive	5[b]	3	-	1	-	-
total	32	39	22	36	1	2

[a] Serotype not determined by mRT-PCR.

[b] The serotype of one of the five isolates was identified only after PCR amplification using the BTV-10-specific primers.

been previously detected by the VN test. The remaining isolates showed a single amplification product specific for one serotype. Nine of the isolates had shown inconclusive VN results making serotype identification difficult, if not impossible. The serotypes of those isolates were subsequently identified by PCR. The serotypes of two isolates were not identified by the PCR assay. One isolate was from a white-tailed deer from Oklahoma in 1981 which was identified as BTV-13 by VN. The second isolate originated from a bighorn sheep from New Mexico in 1991 and was identified as BTV-11 by VN. Other white-tailed deer and bighorn sheep isolates were successfully identified by the mRT-PCR method.

Discussion

The mRT-PCR method described here was shown to amplify and differentiate only the U.S. serotypes of BTV-2, BTV-10, BTV-11, BTV-13, and BTV-17. The BTV isolates used for this study originated from 24 western and southern states and were from several different types of animals, including sheep, cattle, deer, antelope, bison, and *Culicoides*. Despite the broad geographic range, variety of hosts, and large time span (24 years), the PCR procedure successfully determined the serotype of 130 of the 132 isolates that were tested (98.5%).

Comparison of the mRT-PCR results with those of serotype identification by VN revealed that the serotype identities of 120 of the samples were the same using both procedures. Of the remaining ten samples, nine had inconclusive results with the VN, indicating possible cross-reactions of two or more serotypes. The mRT-PCR assay identified each of these nine isolates as a single serotype of BTV. It is possible that these isolates may have indeed been a result of coinfection with multiple serotypes and that the mRT-PCR was able to identify only the predominant serotype. However, in serological tests cross-reactions between BTV-10, BTV-11, and BTV-17, which are genetically closely related, are common (Ghiasi et al., 1987; Gould and Pritchard, 1990; Gould and Eaton, 1992), and the reactions observed with these nine isolates may likely be the result of deficiencies in the VN procedure which can make differentiation of genetically similar isolates in samples difficult.

The tenth isolate that had differing results with the mRT-PCR and VN procedure, was a sample that had been identified as BTV-10 by VN. However, mRT-PCR results clearly indicated a mixed infection of BTV-10 and BTV-17. This was the only natural infection of a multiple serotype detected by mRT-PCR that was observed in the study. Natural infections of an animal with more than one serotype have been reported (Sugiyama et al., 1981; Oberst et al., 1985). However, the

frequency of such occurrences is not known. These mixed infections can be very difficult to detect since one serotype may be predominant (Samal et al., 1987; Stott et al., 1987). In order for any procedure to be able to successfully detect a mixed infection, both serotypes would need to be amplified to high titer during the isolation procedure. In many cases, only one serotype grows to a sufficient titer, so mixed infections are easily missed. The true capability of the mRT-PCR for the detection of natural mixed infections is not known. However, when combinations of different serotypes were made *in vitro*, each serotype in the combined pool was amplified by the mRT-PCR, indicating that the assay is able to detect any combination of serotypes if sufficient virus of each serotype is present.

Analysis of the sensitivity of the mRT-PCR procedure revealed that, depending upon serotype, from 1.86×10^1 to 1.86×10^4 $TCID_{50}$ of virus per ml was necessary for successful PCR amplification. One reason for the low sensitivity of the procedure is that this multiplex assay uses only one set of primers in a single stage amplification process, and such procedures are less sensitive than nested procedures, in which a second set of primers with additional rounds of amplification are performed (Roux, 1995). The lack of sensitivity may be further attributed to the fact that multiplex procedures are often inherently less sensitive. Optimization of a multiplex reaction requires compromises in concentrations of reagents, annealing temperature, and/or cycling conditions in order to attain the best overall strategy for amplification of more than one target sequence. Such compromises may contribute to less than optimal conditions for some primers (Henegariu et al, 1997). This situation may also be a factor in the variation in sensitivity displayed by the mRT-PCR for the different serotypes. However, the observed serotype variability is more likely a result of primer design. One study suggests that different primers can alter the sensitivity of a PCR significantly, and in some instances, moving a primer by as little as a few base pairs can have a dramatic effect on the sensitivity (Wang et al., 1994). To find five primer pairs for a multiplex

assay that would exhibit equal sensitivity for their target sequences would require extensive testing. Problems with primer sensitivity in multiplex reactions can sometimes be overcome by adjusting the relative concentrations of the different primer pairs to maximize the sensitivity for each primer pair to its target sequence (Henegariu et al., 1997). Adjustment of primer concentrations was not done for this study since the sensitivity of the assay was sufficient for the isolates tested.

In this study, all 132 of the isolates that were tested had the minimum concentration of virus that was required for the mRT-PCR assay. This would indicate that for most isolates of BTV the lack of sensitivity exhibited by the PCR procedure should not inhibit successful serotype determination. In instances where the mRT-PCR procedure is not successful, the PCR procedure can be repeated using the serotype-specific primer pairs, which is generally more sensitive than the multiplex procedure. Indeed, for one BTV-10 isolate in this study, serotype determination was accomplished only when the BTV-10 primer pair was used.

Two of the isolates could not be typed in this study, the reason for which is not known. It's possible that the L2 sequence of these two isolates had undergone significant genetic drift, which could have prevented annealing of the primers. Other studies have suggested that such mutations in the L2 genome do occur and may be the result of environmental pressures which can affect the vector/host relationship and could allow viral variants to occur (C. A. de Mattos et al., 1994; C. C. P. de Mattos et al., 1994). Sequencing of the L2 segment of these two isolates is being done to more accurately define their serotype.

Occasionally, after agar gel electrophoresis band artifacts were present in some of the lanes (Figure 3). These extra bands were more noticeable after amplification with the individual serotype-specific primers. This may be due to less-than-optimum cycling parameters, such as annealing temperature or concentration of some reactants, which could cause improper annealing of the

primers. The bands may also represent incomplete viral genome fragments originating from defective interfering particles.

The multiplex procedure described here was shown to be very useful for the identification of U.S. serotypes of BTV. The assay was also more reliable and consistent than the VN assay. When comparing the PCR results with the VN results, it was found that the tests disagreed only when the VN test was difficult to interpret. By combining the detection of the five serotypes into a single amplification reaction, the assay provides a practical and cost-effective PCR method for serotype determination.

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GENERAL CONCLUSIONS

Summary

PCR procedures have been widely used in research laboratories for many years with great success, but their use in veterinary diagnostic laboratories has been somewhat limited, in part due to the initial expense, lack of trained personnel and a low demand for such tests. However, an increasing number of PCR procedures have been developed which show a marked improvement in sensitivity and specificity for the identification of pathogens. Consequently, more laboratories are routinely performing these types of tests in order to provide more rapid, and sometimes more reliable diagnoses.

The mRT-PCR procedure described here was shown to be remarkably specific for the U.S. serotypes of BTV. Valid identification was achieved on 98.5% of the isolates tested, and there was no evidence of any false reactions. In comparing the PCR results with the VN results, it was found that the only instances when the two tests didn't agree was when the VN results were difficult to interpret.

One aspect of the mRT-PCR procedure which could be a problem is that a relatively large amount of virus is required for the test to be successful. Most isolates of BTV have a virus titer sufficient for serotype determination. However, the possibility always exists that if a second or third serotype of BTV is also present in low amounts, it may not be detected. This situation would be the same for any serotyping procedure.

In conclusion, the mRT-PCR procedure described here is more rapid, accurate, and easier to interpret than any of the other serotyping procedures. Results could be completed in two days

compared to a minimum of five days for most neutralization tests. Disadvantages of the test would include the expense involved in performing PCR procedures and the need for personnel that are trained in PCR techniques, however, many laboratories are already routinely doing PCR procedures, and the extra expenses may be offset by the reduced time required to make a serotype identification.

Future Studies

During the 1960s and 1970s when BT was considered to be an emerging disease, extensive studies were done on the virus with the hope of containing the spread of the disease. Reports that BTV could be transmitted via the semen of infected bulls also prompted further restrictions on trade. When, after years of study, it was determined that the range of the disease was not expanding and that the risk of venereal infection with BTV was extremely rare, most of the trade restrictions were eased so that currently most countries require only that the blood or serum of the imported animal be tested for BTV or BT antibodies. This more relaxed attitude toward BT has also resulted in a reduction of the amount of research being done on the virus. However, BT is still a major focus in some laboratories, such as the Arthropod-borne Animal Diseases Research Laboratory in Laramie, WY. Ongoing studies there include the determination of factors affecting the virulence of different isolates and the interactions of the vector, virus, and host involved in the infection cycle. This research is considered important not only for the study of BT, but also to gain insight into the epidemiology of other vector-borne diseases, such as vesicular stomatitis.

While the incidence of BT in the United States has remained relatively stable over the last several years, the possible insurgence of exotic strains into this country could have a devastating impact on U.S. livestock or wildlife populations. One area of the United States that would be

particularly susceptible to an incursion of exotic strains would be southern Florida with its close proximity to the Caribbean where BTV infection of ruminant livestock is common (Gibbs and Greiner, 1994; Mo et al., 1994). A study of the susceptibility of the North American *Culicoides* species to the exotic BT viruses would be valuable in understanding the epidemiology of the disease. Determination that different *Culicoides* species are refractory for some serotypes of the virus would help explain why the distribution of serotypes worldwide has remained somewhat constant over the years. A second, related research topic would be pathogenicity studies using the exotic serotypes on U.S. livestock.

Changing weather patterns worldwide could also have a profound effect upon the distribution of many vector-borne diseases including BT (Gibbs, 1992; Gibbs and Greiner, 1994). It has been proposed that an additional 15 states could become endemic for BTV in the next 50 years if the trend toward global warming continues. The range of susceptible vectors and the insurgence of exotic vectors into the United States will need to be closely monitored.

One subject that has perplexed many of those involved with the disease is the method by which the virus survives the winter in temperate regions when the vector is absent. Some have suggested that the virus survives in persistently infected animals, but such animals have never been identified, nor has persistence ever been successfully induced experimentally (MacLachlan et al., 1989). Others have proposed that the prolonged viremia exhibited in cattle could provide a method of maintaining the virus between vector cycles (Nevill, 1971). However, the length of the observed viremic episodes in cattle is not sufficient in many areas to span the gap when the vector is absent.

Another area in the future study of BTV would be in the development of new diagnostic tests. While serological testing has become rapid and very reliable with the advent of the C-ELISA, the virus isolation procedure involving the intravenous inoculation of embryonating chicken eggs

remains tedious and time consuming. The development of a new method for the isolation of virus from blood and tissues could offer considerable savings in time and resources. Despite the fact that a significant amount of research has previously been done in this area, the search for new cell cultures and methods for isolation of BTV should continue.

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