A SIMPLE AND RAPID METHOD FOR DETECTION OF BLUETONGUE VIRUS IN CELL CULTURE USING RT-PCR

By

Afra'a Tajelsir Mohamed Elata

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Supervisor:

Prof. Imadeldin Elamin Eltahir Aradaib

Department of Medicine, Pharmacology and Toxicology
Faculty of Veterinary Medicine
University of Khartoum

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DEDICATION

TO THE SOUL OF MY FATHER ..... 

TO MY MOTHER ..... 

TO MY BROTHERS AND SISTERS ..... 

WITH ALL MY LOVE
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## LIST OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>i</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>ii</td>
</tr>
<tr>
<td>List of contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>Abstract</td>
<td>vii</td>
</tr>
<tr>
<td>Arabic abstract</td>
<td>ix</td>
</tr>
</tbody>
</table>

## CHAPTER ONE: INTRODUCTION AND GENERAL LITERATURE REVIEW

1.1 Introduction ............................................ 1
1.2 The bluetongue virus ................................... 3
1.2.1 Virus classification ................................. 3
1.2.2 Virus structure ....................................... 4
1.3 Vector and Transmission ............................... 5
1.4 Epidemiology and Distribution ........................ 8
1.5 Bluetongue disease in Sudan ........................... 10
1.6 Economic Importance ..................................... 11
1.7 Pathology of bluetongue disease ....................... 12
1.7.1 Pathogenesis of BTV .................................. 12
1.7.2 Immune response to bluetongue virus ............... 13
1.7.3 Clinical signs ....................................... 14
1.7.4 Post-mortem findings ................................. 16
1.8 Diagnosis of BTV ....................................... 17
1.8.1 Virus Isolation ...................................... 17
1.8.1.1 Embryonated Chicken Eggs (ECE) ................... 18
1.8.1.2 Sheep inoculation .................................. 19
1.8.1.3 Cultured cells ..................................... 19
LIST OF FIGURES

Figure 2.1: Thermal cycler TECHNE, TC-41 ................................. 33
Figure 2.2: Electrophoresis apparatus ................................. 34
Figure 2.3: Gel documentation apparatus ................................. 35
Figure 3.1: Detection of BTV serotypes in infected cell cultures ........ 37
Figure 3.2: Specificity of RT-PCR for BTV ................................. 38
Figure 3.3: Sensitivity of RT-PCR for BTV ................................. 39
ABSTRACT

In this study, a reverse transcriptase polymerase chain reaction (RT-PCR) protocol was evaluated for detection of bluetongue virus (BTV) RNA in cell culture.

North American BTV serotypes 2, 10, 11, and 13, and Sudanese BTV serotype 2 were studied. All these serotypes were propagated in cell culture. RNAs were extracted from these serotypes and then, they were detected by the described RT-PCR assay, using primers derived from segment 6 of BTV-17 which codes for non-structural protein 1 (NS1). So, this NS1 gene was targeted for PCR amplification.

The specific 614 bp PCR product was amplified from all BTV serotypes used in the study and they were visualized on ethidium bromide-stained agarose gel.

Amplification product was not detected when the RT-PCR assay was applied to RNA from epizootic hemorrhagic disease virus (EHDV) or Palyam virus.

Also this specific 614 bp PCR product was obtained from the amounts of 1ng, 500pg, 250pg and 125pg RNA from Sudanese BTV serotype 2.
The results of this study indicated that the described RT-PCR assay, using the mentioned primers, could be applied for detection of BTV serogroups.

In conclusion, the described RT-PCR assay could be used as a simple, rapid, sensitive and specific supportive diagnostic assay to the current conventional procedures used for detection of BTV.
ملخص الأطروحة

١ الأعجن و الزيت ٢ ثغٍ ٠.٠١ ٢ ثغٍ و أكياف خلال علاج الأشخاص تجنبت النزيف.

٢ إزالة التدقيق في الأغذية ١٣ آذار ١٠ ،٢ ثغٍ و ١٥ ٢٠٢٠، ٢ ثغٍ و أكياف ٢٠٢٠ تظهر تفاعلات في الأغذية

٣ إلى أن يبلغ ٢ ثغٍ و أكياف ٢٠٢٠، ٢ ثغٍ و أكياف ٢٠٢٠ تظهر تفاعلات في الأغذية

٤ أكياف لإزالة الأكياف ٦٠ وأكياف ١٧

٥ أكياف لإزالة الأكياف ٦٠ وأكياف ١٧

٦ الأكياف ١٧ أكياف ١٧

٧ الأكياف ١٧ أكياف ١٧

٨ الأكياف ١٧ أكياف ١٧

٩ الأكياف ١٧ أكياف ١٧

١٠ الأكياف ١٧ أكياف ١٧
ختماً، الاتصال في المرآة، كيف توضح دراسة هذه النتائج بشكل انتقائي للتبادلات BASA توظيف السرعة، وحساسية وخصوصية الأزرق للأسنان، فطور الشعر NAIB triệuات في التركيب التقليدي. تذكر لاحقاً، أن الأطراف الأطقمية، الأطراف الأطقمية، الأطراف الأطقمية، الأطراف الأطقمية.
CHAPTER ONE

Introduction and General Literature Review

1.1. Introduction

Bluetongue (BT) is an infectious, non-contagious arthropod-borne disease of ruminants, caused by bluetongue virus and transmitted between vertebrate hosts via the bites of certain species of biting midges of the genus *Culicoides* (Mellor, 1990). Bluetongue was first reported in the year 1881 as a result of the introduction of European breeds of sheep into southern Africa (Howell and Verwoerd, 1971).

The viral etiology of the disease was demonstrated in 1906 and its strains have been identified in many tropical and temperate areas of the world since that time. While the virus is classified antigenically and taxonomically as bluetongue virus, each serotype is unique and may not cause the disease (Callis, 1985).

Bluetongue virus (BTV) is a double stranded (ds) RNA orbivirus of the family *Reoviridae* (Borden *et al.*, 1971; Fenner *et al.*, 1974; Gould *et al.*, 1992). BTV naturally infects domestic and wild ruminants, camelids and some other herbivores such as elephants, but it is almost exclusively a disease of sheep. In cattle and goats clinical disease is rare, and, when present, is much milder than in sheep (Verwoerd and Erasmus,
Bluetongue disease was thought to be limited to sheep until 1933 when the virus was isolated in South Africa from cattle with clinical signs similar to those of foot and mouth disease (Bekker et al., 1934).

The virus has a world wide distribution and it exists in at least 25 serotypes (Davies et al., 1992). It occurs in the Americas, Africa, Asia and Australia, and it can cause an acute, sub-acute, mild or inapparent disease (Mellor and Wittmann, 2002).

Serotypes 1, 2, 4 and 16 are enzootic in the Sudan, while serotypes 2, 10, 11, 13 and 17 are enzootic in North America (Davies et al., 1992; Mohammed and Taylor, 1987).

The mortality rate of bluetongue disease vary from 0% to 30%, but can reach 75% (Mellor et al., 1983) in highly susceptible animals, but that is dependent upon the serotype involved. The real significance of bluetongue disease lies in the indirect losses sustained; these include abortion in pregnant ewes and severe loss of condition during prolonged convalescence (Tomori et al., 1991).

It has been estimated that BTV alone causes losses to international livestock trade in excess of US$ 3 billion a year (Tabachnick et al., 1996). Immunization of susceptible animals requires multivalent vaccines because bluetongue vaccines are serotype specific.

Diagnostic tests currently used for the detection of BTV involve the isolation and growth of virus isolates in eggs or sheep, followed by
passaging in tissue culture. The virus is characterized using serological tests such as the agar gel immunodiffusion test or serum neutralization test. These procedures are time-consuming and may fail to detect low levels of virus. The use of enzyme-linked immuno-sorbent assay (ELISA) for the detection of antibodies of BTV in infected animals is faster but doesn’t confirm recent infection. These traditional methods may require at least three to four weeks to provide a result. The polymerase chain reaction technique may be used not only to detect the presence of BTV but also to serogroup the virus and provide information on the serotype within a few days (Zientara et al., 2004)

The objective of the present study was to develop a simple, rapid, sensitive, specific and inexpensive method for detection of bluetongue virus serogroup using a reverse transcriptase (RT) polymerase chain reaction (RT-PCR).

1.2. The bluetongue virus

1.2.1. Virus classification

Family: Reoviridae

Contains twelve genera of multi-segmented dsRNA viruses.

Genus: Orbivirus

The members of this genus called ‘orbiviruses’. They are twenty one species which characteristically have a ten segmented
dsRNA genome that is packaged within an icosahedral protein capsid.

Species: *Bluetongue virus*

It is the prototype of twenty one different species of the genus *Orbivirus*. It has a capsid composed of three distinct protein layers: the subcore, the core-surface layer and the outer capsid layer (Mertens *et al.*, 2004).

1.2.2. Virus structure

Bluetongue virus is an icosahedral-shaped particle consisting of a segmented double-stranded RNA genome, encapsidated in a double-layered protein coat. Removal of the outer protein layer activates a viral-associated RNA polymerase which transcribes the ten genome segments into 10 mRNAs which are in turn translated into seven structural (VP1-VP7) and three non-structural (NS1,NS2 and NS3) proteins (Huismans and van Dijk, 1990). The virus particle is arranged as three concentric capsid shells surrounding the viral dsRNA (Basak *et al.*, 1997; Grimes *et al.*, 1995; Huismans and van Dijk, 1990). The outermost layer (the outer capsid) is composed of two structural proteins, VP2 and VP5, which are principally involved in virus attachment and penetration of the host cell during the initiation of infection. These are the most variable of the viral proteins and the specificity of their interactions with neutralizing
antibodies (particularly those of VP2) determines virus serotype (Eaton et al., 1990; Huismans and van Dijk, 1990; Roy et al., 1990). VP2 is coded for by genome segment 2, and VP5 is coded for by genome segment 5 (Verwoerd et al., 1970).

The two innermost protein shells that make up the transcriptionally active virus core, are composed of VP3 and VP7, respectively. These are more highly conserved proteins, showing serological cross reactions within the BTV species (Grimes et al., 1995; Mertens, 1999; Mertens et al., 1987; Verwoerd et al., 1972). VP3 is coded for by genome segment 3 (Huismans and van Dijk, 1990), and VP7 is coded for by genome segment 7 (Huismans et al., 1987).

The non-structural proteins NS1, NS2 and NS3 are coded for by genome segments 6, 8 and 10, respectively (Roy, 1992).

1.3. Vector and Transmission

Midges of the genus Culicoides act as biological vectors of bluetongue virus. Of the approximately 1400 species of Culicoides worldwide, less than 20 are considered actual or possible vectors (OIE, 1998; Mellor, 1990).

The most well-studied vector species are C. varipennis and C. insignis in the United States of America, C. fulvus, C. wadai, C. actoni
and *C. brevitarsis* in Australia, and *C. imicola* in Africa and the Middle East (Erasmus, 1990).

The insect vectors of BTV breed in moist conditions in a variety of habitats, particularly damp, muddy areas and in faecal and plant matter. They have nocturnal feeding habits, preferring still, warm conditions, pastures and open pens.

Females take a blood meal prior to egg laying, feed at roughly 4-day intervals and live for about 2 to 3 weeks. The eggs hatch in 2 to 3 days, and, depending on the temperature, the larval stage lasts 12 to 16 days. Adults emerge 2 to 3 days after pupation and take a blood meal 1 day later and they also mate during this time (Roberts, 1990). The activities of the midge are influenced by temperature and the optimum lies between 13°C and 35°C (Sellers, 1981).

BTV has evolved a life cycle where alternate cycles of virus replication in vertebrate and invertebrate hosts are essential for virus persistence (Roberts, 1990).

The midges may be infected when biting viraemic vertebrates. The chance of infection depends in part on the genotype of the midge, the strain of virus, the level of viraemia, and environmental factors. The incubation period (between feeding on infected blood and the appearance of virus in the saliva of the midge) is 1-2 weeks (Mellor *et al.*, 2000). Viraemia must be of the order of 10⁴ infectious units of virus per 1ml of
blood or greater for feeding midges to have much chance of infection. The peak levels of viraemia, in virus infectious units per 1ml of blood, were reported as 104.4 to 106.3 for cattle, 106.4 to 108 for sheep and 106 for goats. Viraemia peaks in the first two weeks after infection, before the appearance of serum antibodies. Virus titres then drop rapidly and are very low if infection persists for a month or more (OIE, 1998). The duration of viraemia in the infected vertebrates is an important factor in the transmission of BTV to biting midges (Mac Lachlan, 1994). The duration of viraemia of most cattle is less than 4 weeks with less than 1% exceeding 8 weeks (OIE, 1998). The maximum viraemia reported for sheep is 54 days (Koumbati et al., 1999). Singer et al. (2001) analyzed a large volume of existing data on the length of bluetongue viraemia of cattle and concluded that this was equal to or less than 9 weeks in >99% of adults.

There is no evidence of vertical transmission of the virus in the invertebrate hosts. Any vertical transmission in vertebrates is considered to be of no consequence to virus ecology because observations on the placental transmission of virus in the vertebrate hosts are contradictory (Roberts, 1990). There is a little evidence of direct or indirect contact transmission in either host, other than rare instances of seminal transmission in vertebrates (OIE, 1998). The virus can not be spread by meat, milk or dairy products (Erasmus, 1990).
1.4. Epidemiology and Distribution

Bluetongue is a common, generally sub-clinical infection of ruminants throughout the tropics and subtropics, within a number of separate ecosystems. Seasonal incursions of the virus into more temperate latitudes, sometimes accompanied by disease, may occur under favorable climatic conditions at certain key locations (Gibbs and Greiner, 1994). Bluetongue disease is the result of a complex interaction between the animal, the virus and the environment. It is almost exclusively a disease of sheep, with European breeds most susceptible. Most breeds of sheep, especially in regions where the virus is endemic, are resistant to disease though there is increasing information that native breeds in India and China can be clinically affected. Outbreaks of disease typically occur either when susceptible sheep are introduced to endemic areas, or when infected midges carry the virus from endemic regions to adjacent areas containing populations of immunologically-naive susceptible sheep (Erasmus, 1990).

There is evidence that infected midges are carried on the wind for long distances (Sellers, 1981). It has been postulated that the major epidemics of bluetongue, in regions where disease occurs only sporadically can often be traced to windborne carriage of infected Culicoides from distant areas (Gibbs and Greiner, 1988).
Over the past 30 years, evidence of regular virus activity, but not necessarily disease, has been found in most countries in the tropics and subtropics with substantial populations of ruminants. The virus may be found in a geographic band between latitudes 40° N and 35° S. The presence of BTV within this band, whether year round or seasonal, depends on the climatic zone type. Genetic studies (topotyping) indicate that the virus existence in discrete, stable ecosystems, probably the result of co-evolution of different strains of virus and vectors (OIE, 1998). Numerous countries in the tropics and subtropics have bluetongue virus unknowingly circulating subclinically in cattle and other ruminants.

A properly designed serological survey would reveal the presence of the virus. The virus is endemic in areas of some countries, being more or less continuously active. Depending on climatic factors affecting the vector, in most years the virus will seasonally extend to adjacent areas (Gibbs and Greiner, 1988).

Many strains of bluetongue virus appear incapable of causing significant disease following natural or experimental infection of sheep known to be susceptible to disease. Experimental reproduction of disease can be inconsistent, except with the most virulent strains of virus. This could be because exposure of sunlight can have a marked influence on the severity of disease (Erasmus, 1990).
1.5. Bluetongue disease in Sudan

In Sudan, bluetongue disease was first reported in 1953 when samples from the Blue Nile Province were confirmed by the Veterinary Research Laboratory at Onderste poort, South Africa, to contain bluetongue virus (Anon, 1953).

Infection of sheep with BTV at Khartoum University Farm, Shambat, was suspected on the basis of clinical symptoms but was not confirmed by virus isolation (Pillai, 1961).

Bluetongue virus group specific antibodies have been detected throughout the Sudan in the sera of many species of ruminants, which suggested a wide distribution of the virus. Although the high levels of bluetongue antibodies and antigens that have been found among cattle, sheep, goats and camels in Sudan, no virus isolation was made in earlier years (Eisa et al., 1979, 1983; Abu Elzein, 1983, 1985a, 1986; Abu Elzein et al., 1987; Herniman et al., 1980).

An outbreak, from which BTV was isolated, involved indigenous sheep in Western Sudan. The stress which these animals have been exposed to while being driven over long distances enhanced the severity of the disease (Eisa et al., 1980).

An outbreak of bluetongue disease in 3-6 months old indigenous lambs was reported in Khartoum Province in 1982. The BTV was isolated
and the disease was experimentally induced (Abu Elzein and Tag Eldin, 1985).

Bluetongue virus was suggested as the cause of death of a Frezian cross-herd calf at the Khartoum University Farm at Shambat, but the virus was not isolated (Mohamed et al., 1980).

BTV serotype 5 was isolated from *Culicoides* species in Sudan (Mellor *et al.*, 1984).

### 1.6. Economic Importance

Bluetongue can be a costly infection for several reasons. The clinical disease in sheep can be severe, resulting in wool break, weight loss and death. In some countries where disease is endemic such as Sudan, South Africa and USA, vaccination is a recurring cost. However, the greater cost of bluetongue is to infected countries which export live animals, germplasm and some animal products such as fetal calf serum. Here the presence of bluetongue virus, even if wholly sub clinical, causes loss of trade due to restriction on the source of animals, and the cost of health testing. It has been estimated that in the late 1970s, the ban on US cattle semen exports resulted in an annual loss of $24 million (Gibbs and Greiner, 1988).

Bluetongue is included in the OIE list A diseases, largely because of dramatic outbreaks of disease in Cyprus in 1943 and Portugal and
Spain in 1956. The Cyprus outbreak was due to a particularly virulent strain of the virus causing between 60-70% losses in some flocks (Gambles, 1949). Within the first 4 months, 46,000 sheep had died in Portugal and 133,000 in Spain (Roberts, 1990). This listing of bluetongue in the most serious list of animal diseases exacerbates the trade sensitivity and associated costs to countries with the infection (Gibbs and Greiner, 1994).

1.7. Pathology of bluetongue disease

1.7.1. Pathogenesis of BTV

After introduction by the bite of an infected midge, bluetongue virus first replicates in the local lymph node and subsequently induces a primary viraemia which seeds other lymph nodes, spleen, lung and vascular endothelium (Gibbs and Greiner, 1988). Circulating virus associates with blood cells, mostly with erythrocytes and platelets, though virus associated with mononuclear cells is critical for dissemination of virus throughout the animal. Later in viraemia, the virus is exclusively associated with erythrocytes (Mac Lachlan, 1994). Virus particles appear to be sequestered in invaginations of the erythrocyte membrane, allowing prolonged viraemia in the presence of neutralizing antibodies (OIE, 1998).
All of the pathology of bluetongue can be assigned to vascular endothelial damage resulting in changes to capillary permeability and fragility, with subsequent disseminated intravascular coagulation and necrosis of tissues supplied by damaged capillaries. These changes result in oedema, congestion, hemorrhage, inflammation and necrosis (Erasmus, 1990).

1.7.2. Immune response to bluetongue virus

The mechanism of immunity to BTV infection, and whether this immunity is mediated by the humoral or the cellular components of the immune system, is not fully understood.

Evidence of the role of cell-mediated immunity (CMI) in BTV infection has been demonstrated in sheep (Stott et al., 1979). Sheep which had been vaccinated with an inactivated BTV vaccine, were refractory to challenge with homologous virus in the absence of neutralizing antibodies. Bluetongue virus specific cytotoxic T-Lymphocytes (CTLs) have been induced in sheep (Jeggo et al., 1985) and their important role in clearing BTV in sheep was indicated (Ghalib et al., 1985). CTLs also have been induced in mice infected with live BTV (Jeggo and Wardley, 1982).

The immune response to BTV infection in cattle is complex when compared to that of sheep. Whereas the appearance of neutralizing
antibodies in the serum of infected sheep coincides with a subsequent
decline in circulating virus, this does not seem to be the case in cattle
(Luedke et al., 1977). Even in the presence of high titre of neutralizing
antibodies, the viraemia in cattle persists for months. The failure of cattle
to display clinical disease in the face of prolonged viraemia suggests an
impaired immune response (Osburn, 1985). Immunological tolerance and
viral persistence have been reported in congenitally infected calves
(Luedke, et al., 1977). Congenital infection has also been demonstrated in
bovine fetuses infected between 85 and 125 days of age. The virus was
not recovered from these calves at birth, but virus specific antibodies
were detected in precoolostral serum samples (Mac Lachlan et al., 1985).
Clinical bluetongue disease in cattle is mediated by IgE antibodies, and
the role of CMI in bluetongue immunology in cattle is not fully
understood (Jochim, 1985).

1.7.3. Clinical signs

Fever is usual but not invariable. Other common clinical signs
include oedema (of lips, nose, face, submandibulum, eyelids and
sometimes ears), congestion (of mouth, nose, nasal cavity, conjunctiva,
skin and coronary bands), lameness and depression. The oedema of lips
and nose can give the sheep a ‘monkey-face’ appearance. There is
frequently a serous nasal discharge, later becoming mucopurulent. The
congestion of the nose and nasal cavity produces a ‘sore muzzle’ effect, the term used to describe the disease seen in sheep in the USA before its bluetongue virus etiology was realized. The mouth is sore and the sheep may champ to produce a frothy oral discharge. Sheep are not strictly anorexic, but eat less because of oral soreness and will hold food in their mouths to soften it before chewing. Affected sheep occasionally have swollen, congested, cyanotic tongues. Lameness, due to coronary band congestion, may occur early in the disease, and lameness, as a result of skeletal muscle damage, may occur later.

If fever occurs, sheep are first pyretic 4-10 days after infection. The other clinical signs, soon followed with acute deaths, occurring during the second week following infection. Many of these deaths are the result of pulmonary oedema and/or cardiac insufficiency. Further sheep may die from chronic disease 3 to 5 weeks after infection with bacterial complications, especially Pasteurellosis. The production loss due to bluetongue may be the result of deaths, unthriftiness during prolonged convalescence and possibly reproductive wastage (OIE, 1998).

Although the frequency of infection of cattle with BTV is generally higher than in sheep, disease in cattle is rare. Clinical infection is actually a hypersensitivity reaction including fever, stiffness or lameness and increased salivation. The skin of the muzzle is often inflamed, and may crack and peel. The lips and tongue may be swollen, with ulcers on the
oral mucosa. Similarly, the skin of the neck, flanks, perineum and teats may be affected (Erasmus, 1990).

Hydranencephaly and congenital deformities may develop in bovine and sheep fetuses of bluetongue virus-infected dams. The severity of lesions is depending on the stage of gestation. Fetuses seem to be most susceptible during the period of active brain development (Erasmus, 1990).

It is clear that cell culture-adapted virus more readily crosses the placenta than unadapted virus, suggesting that the occasionally instances of natural virus-induced teratogenesis may be due to strains of virus derived from live virus vaccines (Mac Lachlan, 1994).

Bluetongue in dogs associated with use of a contaminated vaccine was reported by Akita et al. (1994). Only pregnant bitches were affected.

1.7.4. Post-mortem findings

In animals dying acutely, the oral mucosa is hyperemic and petechiae or ecchymoses may be present. Excoriations may be in areas subject to mechanical abrasion; the edges of lips, dental pad, tongue and cheeks opposite to molar teeth. There may be hyperemia in the fore-stomach. The lungs may be hyperemic with severe alveolar and interstitial oedema, froth in the bronchi, and excess fluids in the thoracic cavity. The pericardial sac may have petechiae and excess fluids.
A variable sized hemorrhage in the tunica media near the base of pulmonary artery is almost pathognomonic. Sub-epicardial and sub-endothelial hemorrhages, particularly those involving the left ventricle are common. Generalized damage to the cardiovascular system is evidenced by widespread hyperemia, oedema and hemorrhage (Erasmus, 1990).

Animals that die later than 14 days after infection often show dramatic degeneration and necrosis of the skeletal musculature. Muscles lose pigmentation and the inter-muscular fasciae are infiltrated with clear gelatinous fluids (Erasmus, 1990).

Microscopic examination of mucosal lesions shows mononuclear cells infiltration, degeneration and necrosis of epithelial cells in which large acidophilic intra-cytoplasmic masses accumulate. Affected muscles have oedema, hemorrhage, hyaline degeneration and necrosis. Infiltration by neutrophils, macrophages and lymphocytes is present in acute cases (Verwoerd and Erasmus, 1994).

1.8. Diagnosis of BTV

1.8.1. Virus Isolation

A number of methods for the isolation of bluetongue virus have been developed over the past fifty years in an attempt to increase the efficiency with which virus in field materials can be amplified to
facilitate identification. Favored methods include replication in embryonated chicken eggs (ECE), sheep, and a wide variety of cultured cells (Clavigo et al., 2000; Gard et al., 1988; Gard et al., 1992; Gould et al., 1989; Wechsler and McHolland, 1988).

1.8.1.1. Embryonated Chicken Eggs (ECE)

Mason, Coles and Alexander (1940) first reported the growth of BTV in chicken embryos following inoculation into the yolk sac of ECE (Mason et al., 1940). Over a quarter of a century later, Goldsmit and Barzilai (Goldsmit and Barzilai, 1968) and Foster and Luedke (Foster et al., 1972) showed that intravenous inoculation of ECE was 100-1000 times more sensitive than yolk sac administration. Since then, intravenous inoculation of 10-13-day-old ECE has been widely used as the method of choice in the isolation of BTV from clinical samples (Clavigo et al., 2000). The preferred tissues for isolation include washed, unseparated blood cells, spleen, lung and lymph nodes (Pearson et al., 1992). Preparation of washed blood cells for inoculation into ECE is straightforward, whereas tissues must be homogenized by grinding with sand in a mortar and pestle or in tissue grinder. The number of ECE inoculated per sample varies but is usually 10, the incubation temperature 33-34°C and the inoculum dose 0.01ml. Although dead embryos are usually the source of virus for identification, embryo deaths are neither an
indication of BTV replication nor are surviving embryos indicative of virus absence (Eaton and White, 2004).

### 1.8.1.2. Sheep inoculation

Sheep have been variously described to be as efficient as ECE (Foster et al., 1972; Goldsmit et al., 1975), less efficient than ECE (Breckon et al., 1980) and more efficient than ECE (Luedke, 1969; Parsonson et al., 1981). The latter suggested that the larger sample volume that can be administered to sheep might account for the enhanced efficiency of isolation compared with ECE. However, sheep inoculation is often an impracticable option because of the requirement to maintain the sheep for at least 30 days after inoculation to permit development of the antibody response that provide evidence of virus infection (Eaton and White, 2004).

### 1.8.1.3. Cultured cells

The first successful attempt to grow BTV in cultured cells was in 1956. BTV adapted for growth in eggs by serial passage in ECE was shown to replicate in primary lamb kidneys (Haig et al., 1956). The first successful isolation in tissue culture of wild-type non-egg adapted virus from the blood of infected sheep was in 1959 (Fernandes, 1959). Shortly
thereafter, direct isolation of BTV in cultured cells was confirmed
(Livingston and Moore, 1962; Pini et al., 1966).

Among the large number of mammalian cell lines that have been
evaluated for their sensitivity to BTV, baby hamster kidney (BHK),
African green monkey (Vero) and calf pulmonary artery endothelium
(CPAE) are most frequently used (Pearson et al., 1992).

1.8.2. Virus Identification

Identification of BTV is an essential part of the laboratory
confirmation of BTV infection. This may be achieved in three different
ways:

a. Identification of antibodies by serological assay

b. Identification of the virus antigens by virological assay

c. Identification of the specific nucleic acids of BTV by reverse
   transcriptase-polymerase chain reaction (RT-PCR) and sequence
   analysis (Zientara et al., 2004).

1.8.2.1. Serological techniques

The outer capsid, structural viral proteins VP2 and VP5 of BTV are
the serotype determinants and are responsible for generation of serotype-
specific neutralizing antibodies (Roy et al., 1990).
Testing sera for the presence of BTV antibodies may be required for serotype identification of field strains, for monitoring vaccination campaigns, for serological surveillance, and to facilitate safe international trade in live animals, animal products and germplasm (Hamblin, 2004).

Two prescribed tests were outlined by the OIE Manual (OIE, 2000) for international trade, namely, the agar gel immunodiffusion (AGID) (Pearson et al., 1979) and competitive enzyme-linked immunosorbent assay (c-ELISA) (Jeggo et al., 1992).

1.8.2.1.1. Agar gel immunodiffusion (AGID)

The AGID test (Pearson et al., 1979) is well documented as a serogroup-specific test for the detection of BTV antibodies. Although the AGID test may still be used in some laboratories, the lack of sensitivity (Gustafson et al., 1992; Pearson, et al., 1992) and documented cross-reactions that can occur with other orbivirus serogroups (Pearson, et al., 1992) makes the continued use of this assay questionable when more rapid, sensitive and specific tests are readily available.

1.8.2.1.2. Competitive enzyme-linked immunosorbent assay

(c-ELISA)

The ELISA has been used for approximately 40 years (Voller et al., 1979) and has provided a valuable means of studying numerous
antigens and their antibodies. ELISA is a serogroup-specific test, identifying primarily the highly conserved BTV VP7 of all known serotypes. Using the c-ELISA as a spot test will only provide a qualitative measurement of positivity (Hamblin, 2004).

Competitive ELISA (cELISA) is probably the most widely used and validated method (Jeggo et al., 1992).

1.8.2.2. Virological techniques

Several virus/antibody-based methodologies for the identification of BTV have been described and they fall into two categories, being either serogroup-specific such as ELISA, or serotype-specific such as virus neutralization test (Hamblin, 2004).

1.8.2.2.1. Virus neutralization test

It is a serotype-specific test which can be used to identify all antigenically distinct serotypes of BTV. The sensitivity of this assay is dependent on the titer of virus in the test sample (Hamblin, 2004).

1.8.2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The PCR is a method for \textit{in vitro} amplification of DNA. It is a series of multiple rounds of primer extension reactions in which complementary strands of a defined region of a DNA molecule are
simultaneously synthesized by a thermo stable DNA polymerase (Zientara, et al., 2004).

This primer-directed amplification of viral nucleic acid has revolutionized BT diagnosis. Results to date indicate that PCR technique may be used, not only to detect the presence of viral nucleic acid, but also to ‘serogroup’ orbiviruses and provide information on the serotype and possible geographic source of BTV isolates within a few days of receipt of a clinical sample, such as infected sheep blood.

Oligonucleotide primers used to date have been derived from RNA7 (VP7 gene), RNA6 (NS1 gene), RNA3 (VP3 gene) and RNA2 (VP2 gene).

The PCR assay involves three separate procedures. In the first, BTV RNA is extracted. The second procedure is the denaturation of viral ds-RNA and reverse transcription (RT) to generate DNA, which is amplified by PCR. The final step of the process is the analysis of the PCR product by electrophoresis (OIE, 2000; Dadhich, 2004).

1.9. Prevention and control

Bluetongue is a disease of sheep, but cattle are the principal vertebrate reservoirs of the virus. Once established, it is impossible to actively eradicate bluetongue virus. The virus will circulate, generally sub clinically, in cattle and other ruminants, and in midges. In countries
marginally suitable for virus persistence, the virus may be maintained for several years before dying out (Roberts, 1990).

In seasonally infected areas, the onset of cold weather will reduce midge populations to ineffective levels and cause the virus to retreat to regions of year-round activity.

The bluetongue virus cycle could be interrupted by the immunization of vertebrate hosts, especially cattle, removal of vectors, or prevention of vector attack. Understandably, the immunization of animals that will not suffer from the disease is not acceptable to farmers. The control of midges by the application of insecticides and larvicides to insect resting and breeding sites, or systemically to cattle, has not been fully investigated but is likely to have local success only. Protecting sheep from exposure to midges is a more practical approach and can be achieved by moving sheep from insect resting and breeding sites, stabling animals overnight, or the use of insect repellents. Mixing cattle with sheep will draw vectors with a host preference for cattle from sheep, but may raise the virus infection level of the midge population. Prophylactic immunization of sheep is the most practical and effective control measure, especially when the threat is from an epidemic due to a single serotype. Multiple serotypes of virus are usual in endemic situations (Hawkes, 1996), requiring multivalent vaccines because bluetongue vaccines are serotype specific.
The first method of immunization against bluetongue developed around 1900 in South Africa with inoculation of immune serum and infective blood. The attenuation of a strain of virus was achieved after limited serial passages in sheep. This was referred to as Theiler's vaccine, and over a period of 40 years more than 50 million doses were used. This vaccine was inadequate because of the plurality of virus strains occurring in nature (Howell and Verwoerd, 1971). The use of embryonated chicken egg-attenuated BTV in the production of polyvalent vaccine for sheep was found to be effective and the virus did not regain its virulence (Alexander and Haig, 1951; McKercher et al., 1957).

However, multivalent vaccines have attendant problems resulting from interference between virus strains, differences in immunogenicity and growth rates between various strains, as well as differences in the response of individual animals to the components of such vaccines (Verwoerd and Erasmus, 1994).

Additionally, there is growing concern by some scientists about the use of live attenuated bluetongue vaccines. Murray and Eaton (1996) summarized these concerns into four areas. These areas are: the known teratogenicity of attenuated virus for the developing fetus; the propensity for vaccine virus to be excreted in the semen of bulls and rams; the possibility that vaccine virus will infect vectors and establish in the environment; and the generation of recombinant progeny virus with novel
genetic and biological properties after the reassortment of genes from wild and vaccine virus in the vaccinated animal or the vector.

Alternatives to live attenuated vaccines are described by Murray and Eaton (1996). Vaccines based on inactivated whole virus, recombinant virus-like particles or recombinant core-like particles all show promise, but require more research. If a commercial product of any of these achieved, it will likely cost considerably more than a live attenuated vaccine.

Live attenuated bluetongue vaccines have wide use in South Africa, and more limited use in USA and a few other countries. The vaccines are compromises between attenuation and immunogenicity and may have residual pathogenicity for some vaccinated sheep. The application of the vaccines has to be well managed. Colostral immunity in young sheep can interfere with the development of active immunity to the vaccine, and breeding ewes and rams should be vaccinated before mating.
CHAPTER TWO

Materials and Methods

2.1. Bluetongue virus

The North American Bluetongue virus prototypes serotypes 2, 10, 11 and 13 were obtained from Arthropod-Borne Animal Disease Research Laboratory, Laramie, WY. The Sudanese isolates of BTV serotypes were recovered from Khartoum University farm at Shamba (Mohammed and Mellor; 1990).

2.2. Virus propagation in tissue culture

Vero cells were cultured in 25 ml tissue culture flasks containing minimal essential medium (MEM). Fetal Bovine Serum (FBS) was used at a concentration of 10% for growth and maintenance of cells, and they were incubated at 37 °C for 2-3 days. All viruses were propagated on confluent monolayers of Vero cells. The infectious materials were harvested when 80% cytopathic effect (CPE) was observed (Usually 3-5 days after infection). The virus-infected cell culture was then kept at 4°C till used for the dsRNA extraction.
2.3. Viral nucleic acid extraction from infected cell monolayers

Viral nucleic acid was extracted by the QIAamp viral RNA extraction kit following the mini spin protocol (QIAGEN GmbH, Hilden, Germany). The carrier RNA was dissolved in 1ml AVL buffer and transferred to the AVL bottle and mixed thoroughly. 560 microlitre (µl) from this preparation were dispensed in each 1.5ml micro-centrifuge tube and kept at 4°C. All samples and reagents were equilibrated to room temperature (about 25°C). 140 µl from the infected cell culture were added to the Buffer AVL/Carrier RNA in the micro-centrifuge tube, and mixed by pulse-vortexing for 15 seconds. The mixture incubated at room temperature (25°C) for 10 minutes. The tube was briefly centrifuged to remove drops from inside of the lid. 560 µl of ethanol (100%) were added and mixed by pulse vortexing for 15 seconds followed by brief centrifugation. 630 µl from the mixture were applied to the QIAamp spin column (in a 2ml collection tube) and centrifuged at 8000 rpm for 1 minute. The column was placed into a clean 2ml collection tube, the remaining 630 µl of the mixture were applied to it and the previous centrifugation step was repeated. 500 µl of buffer AW1 were added to the column, after placing it into a clean 2ml collection tube, and centrifuged at 8000 rpm for 1 minute. 500 µl of wash buffer AW2 were added to the column, after placing it into a clean 2ml collection tube, and centrifuged at 14000 rpm for 3 minutes. The spin column was placed into
1.5ml micro-centrifuge tube and 60 µl of Buffer AVE were added. After it had been left at room temperature for 1 minute, the micro-centrifuge tube was centrifuged at 8000 rpm for 1 minute. Finally the micro-centrifuge tubes containing RNA extracts were labeled and kept at -20ºC till used in RT-PCR. The dsRNA concentration was determined by spectrophotometer at 260 nm wave length.

### 2.4. Primers selection

Primers (P1 and P2) were selected from the published sequence of genome segment 6, which codes for non structural protein 1 (NS1) of BTV-17 (Hwang et al., 1993). P1 included bases 648-667 of the positive sense strand of genome segment 6: 5´-GCC CTT ACA CTG GAT ACA GA-3´. P2 was designed from the complementary strand of the above sequence between bases 1242-1261: 5´-CCT CGC TCC AGT GTA ACA AT-3´. PCR amplification using P1 and P2 would be expected to produce a 614 base pair (bp) PCR product.

Primers were synthesized on a DNA synthesizer (Milligen iosearch/Millipore, Burlington, MA) and purified using Oligo-Pak oligonucleotide purification columns (Glen Research, Sterling, VA) as per manufacturer’s instructions.
2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

For each PCR amplification, 1.0 µl methyl mercuric hydroxide of 80 mM concentration was used to denature a mixture of 5 µl of viral RNA and 2 µl of primers (P1 and P2). The primers were used at a concentration of 20µg/µl. The mixture was then incubated at 25ºC for 10 minutes. The mixture was neutralized by 10 µl of neutralizing solution containing 1 µl of β-mercapto ethanol, 8 µl of dNTPs (2 µl of each dATP, dTTP, dGTP and dCTP) and 1 µl of enzyme RNAse inhibitor.

A reverse transcription step was performed to synthesize a complementary DNA (cDNA) from RNA templates using a reverse transcriptase mixture composed of 2.7 µl of 10X PCR buffer, 5 µl MgCl2 of 1.5 mM concentration and 1.1 µl of the enzyme reverse transcriptase. 8.8 µl of the reverse transcriptase mixture was added to each PCR tube. The PCR tubes were placed in the thermal cycler at 40ºC for 30 minutes.

For amplification, a mixture of 7.3 µl of 10X PCR buffer, 8 µl of MgCl2 and 1 µl of Taq DNA polymerase at a concentration of 5 units/µl was added to each PCR tube. The total volume of the PCR mixture was brought to 100 µl using double distilled water.

The RT-PCR was performed in (TECHNE, TC-412, USA) thermal cycler (figure 2.1) following a program of a 2 minute incubation at 95ºC, followed by 40 cycles of (95ºC for 1 minute as denaturing temperature; 56 ºC for 30 seconds as annealing temperature and 72 °C for 45 seconds
for extension of the predicted amplified PCR product). A final incubation at 60°C for 5 minutes was performed to complete the extension of uncompleted fragments of the PCR products.

2.6. Agarose gel electrophoresis

Following RT-PCR assay, the amplified products of cDNA transcribed from viral RNA molecules by RT-PCR were analyzed in agarose gel (SeaKem, agarose FMC Bioproducts, Rockland, ME). 1% agarose gel was prepared by suspending 1 gram of agarose powder in 100ml of 1X Tris-boric acid-EDTA (TBE) buffer. The suspension was placed in the microwave for 2 minutes until the agarose was completely melted. Then 35ml of melted agarose was cooled and poured in the gel tray loaded with a comb.

The agarose gel was submerged in the buffer basin of the electrophoresis apparatus (figure 2.2) filled with 1X TBE buffer containing 10 µl/500 ml ethidium bromide.

To prepare 10X TBE buffer, 108 gram of Tris- (hydromethyl)-aminomethane, 55 gram of Boric acid and 7.4 gram of EDTA were mixed and brought to 1 litre using double distilled water. The buffer was kept at room temperature and used at 1x concentration for gel preparation and electrophoresis.
Per each electrophoresis run, 5 µl of 100 bp ladder molecular weight marker (MW marker) stained with an indicator dye was placed in the first lane of the gel. 12 µl of each RT-PCR product were loaded in the gel after being stained with 3 µl of an indicator dye (Bromophenol blue).

Constant electric current of 90 mV then switched on for about 40 minutes to allow the migration of the amplified PCR products. The standard molecular weight marker (1KB) was incorporated in each reaction for determination of the size of the amplified product by comparing this size with the separate distinguishable bands of the MW marker which were seen when the gel was stained with ethidium bromide.

The gel was then visualized under UV light using gel documentation apparatus (figure 2.3).
Figure 2.1: Thermal cycler (TECHNE, TC-412, USA)
Figure 2.2: Electrophoresis apparatus
Figure 2.3: Gel documentation apparatus
CHAPTER THREE

Results

The BTV RT-PCR assay was a simple procedure that efficiently detected all BTV serotypes used in this study.

The described RT-PCR assay, with primers derived from genome segment 6 of BTV serotype 17, afforded sensitive and specific detection of all BTV serotypes used in this study. The specific 614 bp PCR product was visualized on ethidium bromide-stained agarose gel from 1ng RNA of North American BTV serotypes 2, 10, 11, and 13, and from 1ng RNA of Sudanese BTV serotype 2 (Figure 3.1).
Figure 3.1: Detection of BTV serotypes in infected cell cultures.

Visualization of the 614 bp PCR product on ethidium bromide-stained agarose gel from 1ng RNA of North American BTV serotypes 2, 10, 11, and 13; and Sudanese serotype 2. Lane MW: molecular weight marker; Lane 2: North American BTV-2; Lane 2: Sudanese BTV-2; Lane 3: North American BTV-10; Lane 4: North American BTV-11; Lane 5: North American BTV-13. Lane 6: Non-infected Vero cell (negative control).
Specificity of BTV RT-PCR

The specificity studies indicated that the specific 614 bp PCR product was not detected from 1 ng of RNA from epizootic hemorrhagic disease virus (EHDV) serotype 1 and 1 ng of RNA from Palyam virus (Figure 3.2).

**Figure 3.2:** Specificity of RT-PCR for BTV.

614 bp Amplification product was not detected from 1 ng RNA of EHDV-1 and Palyam virus. Lane MW: molecular weight marker; Lane 1: Sudanese BTV-2 (positive control); Lane 2: EHDV-1; Lane 3: Palyam virus.
Sensitivity of BTV RT-PCR

The sensitivity studies indicated that the specific 614 bp PCR product was obtained from the amounts of 1 ng, 500 pg, 250 pg and 125 pg RNA from Sudanese BTV serotype 2 (Figure 3.3).

Figure 3.3: Sensitivity of RT-PCR for BTV.

The specific 614 bp PCR product was obtained from the amounts of 1.0 ng, 500 pg, 250 pg and 125 pg RNA from Sudanese BTV serotype 2. Lane MW: molecular weight marker; Lanes 1-4: (Sudanese BTV-2) 1.0 ng, 500 pg, 250 pg and 125 pg, respectively.
Bluetongue virus is a serious veterinary problem (Shope et al., 1960), and the economic importance of the disease is mainly attributed to clinical disease in sheep (Holf and Trainer, 1974; Pini, 1976; Jessup, 1985). There is restriction on the international trade of livestock and animals products unless the animals are certified BTV-free by conventional virus isolation and serology (Osburn et al., 1994). Thus the disease is of interest to dairy producers and wildlife managers.

Rapid detection of BTV is important in disease outbreaks as well as for determining disease-free status of exporting animals. Many diagnostic tests have been developed for detection of BTV including antibody, antigen and nucleic acid detection techniques (Mecham and Wilson, 1994). Antibody detection indicates that an animal has been previously exposed to the virus but not necessarily an indicator of viraemia. An indirect enzyme-linked immunosorbent assay (ELISA) and a competitive (C) ELISA, using a group-specific monoclonal antibody against bluetongue virus (BTV), are described for the detection of antibodies to BTV in cattle and sheep sera (Afshar et al., 1987).
Antigen and nucleic acid detection assays are more indicative of viraemia, but can detect residual non-infectious molecules from a recent infection. None of the available antigen or nucleic acid detection assays have been validated for all the 25 serotypes of BTV. Although antigen detection assays are very sensitive, inexpensive and reliable, they take long time to perform. In addition, reagents are more difficult to develop than nucleic acid detection tests. Therefore, the development of a nucleic acid amplification-based assay for all serotypes of BTV was necessary (Aradaib et al., 1998; Dangler et al., 1990; Katz et al., 1993; Shad et al., 1997; Wilson and Chase, 1993).

Conventional virus isolation and serology are time consuming and laborious (Pearson et al., 1992). The traditional approaches that rely on virus isolation followed by virus identification may require at least three to four weeks to generate information on BTV serogroup and serotypes. Also, conventional virus isolation may not provide data on the possible geographic origin of the isolates (Zientara et al., 2004). So, the development of molecular diagnostic techniques for detection of BTV would be advantageous in a variety of circumstances including clinical and sub clinical disease investigation, vaccination programs and epidemiological studies (Pearson et al., 1992; Aradaib et al., 1994, 1995).

RT-PCR based detection assays have been described for detection of bluetongue virus infection in susceptible ruminants (McColl and
Gould, 1991; Wade-Evans et al., 1991; Wilson and Chase, 1993). BTV RT-PCR can provide rapid, sensitive and specific viral identification for BTV infections (Zientara et al., 2004). The primary gene target for group-specific amplification was genome segment 6, which codes for non-structural protein 1 (NS1) as it is highly conserved among cognate genes of BTV serogroup (Jensen and Wilson, 1995).

The BTV RT-PCR assay was a simple procedure that efficiently detected all BTV isolates used in this study. The described RT-PCR assay specifically detected BTV RNA in infected vero cell cultures. Selection of the primers was based on the observation that NS1 gene of BTV is the most conserved among cognate genes of BTV serogroup (Aradaib et al., 1998).

The specific 614 bp PCR products, visualized on ethidium bromide-stained agarose gel, were obtained from all BTV RNA samples tested (1.0 ng each). The specificity studies indicated that the specific 614 bp PCR product was not amplified from 1.0 ng of RNA from epizootic hemorrhagic disease virus (EHDV)-1 and RNA from Palyam virus under the same stringency condition described in this study. This confirmed that BTV NS1 genome is highly conserved among cognate genes of BTV serogroup.

A nested PCR assay using primers derived from the non structural protein 1 (NS1) gene of North American BTV serotype 11 was
developed to detect the United States BTV serotypes 2, 10, 11, 13 and 17 and the Sudanese BTV serotypes 1, 2, 4 and 16 and BTV serotype 4 from South Africa and BTV serotype 2 from Senegal. The primary specific 790 bp PCR products and the nested 520 bp amplification products were not detected from closely related Orbiviruses including, EHDV serotypes 1, 2, 4; Sudanese isolate of Palyam virus and total nucleic acid extracts from uninfected Vero cells (Aradaib et al., 2005).

A duplex, one-step RT-PCR assay was developed and evaluated to detect genome segment 7 from any of the BTV serotypes. Assay sensitivity was evaluated using tissue culture derived virus, infected vector insects and clinical samples (blood and other tissues). No cross-reactions were detected with members of closely related Orbivirus species (African horse sickness virus, Epizootic hemorrhagic disease virus and Palyam virus) (Anthony et al., 2007).

Two new Real Time qPCRs were developed and validated to detect and amplify BTV segments 1 and 5 from all of the BTV serotypes. These two methods are complementary and could be used in parallel to confirm the diagnosis of a possible incursion of BTV (Toussaint et al., 2006).

A new rapid single step RT-PCR with infra red (IR)-dye-labeled primers was reported as a sensitive and specific assay for detecting BTV RNA in Culicoides biting midges. All serotypes of BTV and none of the
eight serotypes of the closely related EHDV were detected (Kato and Mayer, 2006).

The sensitivity studies indicated that the specific 614 bp PCR product was obtained from the amounts of 1 ng, 500 pg, 250 pg and 125 pg RNA from Sudanese BTV serotype 2. Thus, in the present study, the described BTV RT-PCR protocol indicated that the PCR assay was capable of detecting the amount of 125 pg of BTV genomic dsRNA.

However, the interpretation of positive BTV PCR results must be analyzed carefully, particularly in BTV-free areas before officially reporting BTV cases. In the absence of epidemiological data, virus isolation is strongly recommended to confirm molecular diagnosis (Zientara et al., 2004).

The BTV RT-PCR assay provides supportive diagnostic technique to the lengthy cumbersome conventional virus isolation procedures. While the nested PCR assay required 7 hours for submission of the final results (Aradaib et al., 2005), the BTV RT-PCR assay described in this study, required 4 hours to obtain the final results.

The rapidity, sensitivity and specificity of the RT-PCR assay would greatly facilitate detection of BTV infection among susceptible ruminants.
In conclusion, the described BTV RT-PCR assay, using primers derived from genome segment 6 of BTV-17, provides a simple, rapid, specific and sensitive diagnostic method for detection of BTV.

In addition, the PCR assay could be used for detection of the virus in areas of endemicity or incursion of the virus in BTV-free zones.
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