The detection of antibodies against Shuni virus in cattle from Western Kenya

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Abstract

A serological survey was done to detect antibodies against Shuni virus (SHUV) from cattle in Western Kenya. In Kenya the disease status of SHUV in cattle has never been established. It is a zoonotic virus and even though studies have been carried out as early as the 1960s, little research has been published and SHUV is still not a well-recognised Orthobunyavirus.

One hundred serum samples were collected from healthy cattle in Kenya and tested for antibodies against SHUV by a serum neutralization assay. All antibody titre values were greater than 1:160, with most of the samples greater than 1:320. Of the samples tested, 87% had titres greater than 1:320, 12% had a titre of 1:320 and 2% had a titre of 1:160. Samples were classified as positive if the antibody titre was ≥ 1:10 and negative if < 1:10. This study suggests that cattle are exposed commonly to SHUV, which may be endemic in Kenya.
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## Abbreviations

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<thead>
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<tr>
<td>Agro-Ecological Zones (AEZ)</td>
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<td>Agricultural research Council-Ondersterpoort Veterinary Institute (ARC-OVI)</td>
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<td>Aino virus (AINV)</td>
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<td>Akabane virus (AKAV)</td>
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<td>Bunyamwera virus (BUNV)</td>
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<td>Heamagglutination-inhibition (HI)</td>
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<tr>
<td>Infectious Diseases of East Africa Livestock (IDEAL)</td>
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<td>International Livestock Research Institute (ILRI)</td>
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<td>Iquitos virus (IQT)</td>
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<td>Kasba virus (KASV)</td>
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<td>Lower Midlands (LM)</td>
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<td>Minimum Essential Medium (MEM)</td>
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<td>Oropouche virus (OROV)</td>
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<td>Phosphate buffered saline (PBS+ (Ca/Mg))</td>
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<td>Reverse transcriptase polymerase chain reaction (RT-PCR)</td>
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<td>Rift valley fever virus (RVFV)</td>
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<td>Sathuperi virus (SATV)</td>
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<td>Schmallenberg virus (SBV)</td>
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<td>Serum neutralisation test (SNT)</td>
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<td>Shamonna virus (SHA)</td>
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<td>Shuni virus (SHUV)</td>
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<td>Tissue Culture Infective Dose (TCID50)</td>
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<td>Upper Midlands (UM)</td>
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1. INTRODUCTION

This project forms part of the Infectious Diseases of East Africa Livestock (IDEAL) project, a collaborative effort between the University of Edinburgh (United Kingdom), the International Livestock Research Institute (ILRI) (Nairobi, Kenya) and the Department of Veterinary Tropical Diseases (DVTD) at the University of Pretoria, South Africa. The project was approved by the Animal Ethics Committee, in South Africa (Appendix 11.1). Results from this study will add knowledge which is of interest to epidemiologists and medical professional. It will also lay a foundation for further research on the disease in Kenya.

Shuni virus (SHUV) belongs to the Simbu serogroup of the genus *Orthobunyavirus*, family *Bunyaviridae*. The members of the *Orthobunyavirus* genus are arthropod-borne viruses (arboviruses) that are transmitted mainly by mosquitoes and *Culicoides* biting midges. SHUV was first isolated in 1966 from a cow in Sokoto, Nigeria during a surveillance programme for the study of viral infections by the University of Ibadan between 1964 and 1969 (Causey *et al.*, 1972).

In Kenya, little is known about the presence of SHUV in cattle. A recent study by Abutarbush *et al.*, (2015), confirmed that members of the Simbu serogroup group are present in Europe, Africa, Australia and the Middle East. SHUV has been confirmed in horses and wildlife in South Africa (van Eeden *et al.*, 2010; 2012) and recently in Israel (Golender *et al.*, 2015). Due to transboundary trade in animals, as well as the favourable climate in Kenya for arthropods, animals in Kenya are at risk for SHUV infections. Cases of foetal malformation and abortion have been reported in Kenya (Davies & Jessett, 1985), but there has been no laboratory confirmation of the presence of the SHUV in Kenya. There is thus a need to investigate whether SHUV occurs in Kenya.

Serum samples from cattle in Western Kenya were tested for the presence of antibodies to SHUV, using the serum neutralisation test (SNT), which is regarded as a very specific test.
2. LITERATURE REVIEW

2.1. Virus classification

Shuni virus (SHUV) belongs to the *Bunyaviridae* family which comprises a number of viruses of both veterinary and public health significance. Genera in this family are *Hantavirus*, *Nairovirus* (of significance is Dugbe virus as the cause of Nairobi sheep disease and Crimean-Congo haemorrhagic fever virus), *Orthobunyavirus*, *Phlebovirus* (Rift Valley fever virus) and *Tospovirus* (plant viruses). There are 48 species in the *Orthobunyavirus* genus, of which Bunyamwera virus is the type species (Soldan & González-Scarano, 2005).

SHUV belongs to the *Orthobunyavirus* genus. This group of viruses has a worldwide distribution and consists of important pathogens that may cause diseases in humans and animals (Table 2.1). Most of the viruses have been isolated from arthropods such as mosquitoes and *Culicoides spp.* midges, as well as from vertebrate hosts. Little is known of their veterinary significance. Some of the viruses in this group, such as SHUV, Akabane virus (AKAV), Oropouche virus (OROV), Sathuperi (SATV) and Shamonda virus (SHAV) viruses are potential pathogens. The most pathogenic of these viruses is AKAV, which has been studied the most extensively (St George & Kirkland, 2005).

<table>
<thead>
<tr>
<th>1. Acara virus (ACAV)</th>
<th>17. Caraparu virus (CARV)</th>
<th>33. Nyando virus (NDV)</th>
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<tr>
<td>2. Akabane virus (AKAV)</td>
<td>18. Catu virus (CATUV)</td>
<td>34. Olifantsvlei virus (OLIV)</td>
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<td>3. Alajuela virus (ALJV)</td>
<td>19. Estero Real virus (ERV)</td>
<td>35. Oriboca virus (ORIV)</td>
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<td>6. Bakau virus (BAKV)</td>
<td>22. Guama virus (GMAV)</td>
<td>38. Sathuperi virus (SATV)</td>
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<td>8. Benevides virus (BVSV)</td>
<td>24. Kaeng Khoi virus (KKV)</td>
<td>40. Shuni virus (SHUV)</td>
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<td>11. Botambi virus (BOTV)</td>
<td>27. Madrid virus (MADV)</td>
<td>43. Tete virus (TETEV)</td>
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<td>12. Bunyamwera virus* (BUNV)</td>
<td>28. Main Drain virus (MDV)</td>
<td>44. Thimiri virus (THIV)</td>
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<td>13. Bushbush virus (BSBV)</td>
<td>29. Manzanilla virus (MANV)</td>
<td>45. Timboteua virus (TBTV)</td>
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Table 2.1: Species of the genus *Orthobunyavirus*, family Bunyaviridae
14. Bwamba virus (B WAV)  30. Marituba virus (MTBV)  46. Turlock virus (TURV)
15. California encephalitis virus (LACV)  31. Minatitlan virus (MNTV)  47. Wyeomiyia virus (WYO V)

* - type species. Schmallenberg virus is an unclassified Orthobunyavirus.

There are 18 serogroups in the Orthobunyavirus genus. The largest serogroup is the Simbu serogroup. There are over twenty five virus species in the Simbu serogroup (Saeed et al., 2001). Cross neutralization (NT) test and cross-heamagglutination-inhibition (HI) tests are used to distinguish these virus species in this serogroup (Plyusnin et al., 2012).

2.2. Structure of Orthobunyavirus

The genome of the genus Orthobunyavirus consists of three single-stranded, negative-sense RNA segments designated as small (S), medium (M) and large (L) RNA segments based on their size. The S RNA segment encodes the nucleocapsid protein (N) and non-structural protein (NSs); the M RNA segment encodes the two surface glycoproteins (Gn and Gc) and the other non-structural protein (NSm); and the L RNA segment encodes the RNA dependent RNA polymerase (L). The Gc protein, which is the larger glycoprotein, is present on the surface of viral particles and is thus recognized by neutralizing antibodies (L). Of the three segments, the M RNA segment is the most variable among Orthobunyaviruses, suggesting that host immunity has imposed evolutionary pressure on its sequence. As is known for other segmented viruses, genetic reassortment of Orthobunyaviruses plays an important role in their evolution and potentially changes their antigenicity, virulence and host rage (Elliott & Blakqori, 2011).

2.3. Epidemiology of Shuni and related viruses

2.3.1. Transmission

The majority of Bunyaviruses are transmitted by arthropod vectors, in particular mosquitoes, phlebotoms, Culicoides, ticks and thrips, with the exception of hantaviruses, which are transmitted by rodents. Studies have shown that viruses within the Simbu serogroup are transmitted by arthropods, mainly Culicoides midges, but also by mosquitoes from the Aedes
and *Culex* genus and by several species of ticks (Oya *et al*., 1961; St Georges *et al*., 1980; Coetzer *et al*., 2004). Environmental factors, especially seasonal high rainfall and warm temperatures, promote the proliferation of vectors that transmit SHUV (William *et al*., 2010). SHUV is a zoonotic pathogen and it can also be spread by exposure to infected tissues (van Eeden *et al*., 2010).

### 2.3.2. Host range and geographic distribution

SHUV was first isolated from the blood of sheep from Ibadan, cattle from Northern Nigeria and from pools of *Culicoides* *spp.* midges. Neutralizing antibodies were found in dairy cattle, in 25 out of 28 trade cattle and in 23 out of 72 sheep (Causey *et al*., 1969, 1972; Kemp *et al*., 1973; Lee, 1979). SHUV was recovered twice from pools of *Culex theileri* mosquitoes caught near Johannesburg, from seven apparently healthy cattle and a goat in Natal between 1970 and 1979 (McIntosh 1972; 1980). SHUV was also isolated twice from *Culicoides* *spp.* during field surveillance in Nigeria (Lee, 1979).

In December 2014, the Israeli Veterinary Field Services were notified of the appearance of arthrogryposis-hydranencephaly syndrome (Inaba *et al*., 1975) in two herds of sheep in the villages of Yokneam and Sde Ya’akov, respectively. Both villages were located in the Izre’el Valley, in Israel’s northern valleys. SHUV was isolated from the foetal brain of a malformed lamb and the presence of SHUV in sheep in Israel was confirmed during the winter of 2014 – 15, suggesting a northward expansion of SHUV from sub-Saharan Africa (Golender *et al*., 2015).

SHUV, SHAV and Simbu virus (SIMV) have been isolated from apparently healthy cattle, sheep and goats, horses, a rhinoceros and a crocodile with neurological disease in South Africa (van Eeden *et al*., 2010).

In 1977, SHUV was isolated from the brains of two horses with nervous disease submitted for rabies virus examination; one from South Africa (Coetzer & Howell, 1998) and one from Zimbabwe (Coetzer & Erasmus, 1994). The identification of SHUV in seven horses with neurological symptoms between June 2009 and December 2010 (van Eeden *et al*., 2012)
highlighted the role of SHUV as a cause of neurological syndromes of horses in South Africa.

SHUV is a zoonotic pathogen, as was reported from a single human isolation of the virus from a one and a half year old child in Nigeria in August 1966 (Moore, 1975). Antibodies were detected in the sera of veterinarians that had regular exposure to horses, livestock, or wildlife in South Africa in 2011 and 2012 (van Eeden et al., 2014). The presence of SHUV in Kenya has not been documented, although the related AKAV has been reported in Kenya (Davies & Jessett, 1985). Other closely related Simbu serogroup viruses have been found recently in Tanzania, which borders Kenya (Matthew et al., 2015).

SHAV was first recovered from cattle blood and pools of Culicoides imicola collected at Ibadan in Northern Nigeria. Neutralizing antibodies were found in a sentinel group of dairy cows (Causey et al., 1969; Kemp et al., 1971, 1972; Kemp et al., 1973; Lee, 1974). In the Kagoshima and Miyazaki prefectures, southern Japan, in 2002, six virus isolations were made from Culicoides midges from samples of resident cattle (Yanase et al., 2005).

SATV was isolated originally in India from a pool of Culex vishnui mosquitoes, from dairy cattle and pools of Culicoides spp. (Dandawate et al., 1969; Causey et al., 1972; Lee, 1979). A serological survey conducted on four to nine year old Nigerian cattle showed that 17 out of 24 had neutralizing antibodies.

AKAV is distributed widely throughout Australia, south/east Asia, east Asia, the Middle East and Africa (Ogawa et al., 2007). AKAV was first isolated in Japan in 1959. Serum neutralizing antibodies to AKAV were found in a high proportion of cattle sampled in Kenya (50 - 95 %), and in a lower proportion of sampled sheep and goats (13 - 33 %). Camel and horse sera also contained antibodies to the virus (70 % and 50 % respectively), as well as the sera of a wide range of Kenyan wild ruminants and zebras (Davies & Jessett, 1985). Antibodies were present in animals from the high altitude temperate grasslands, drier bushed and wooded grasslands and semi-desert. AKAV infection in adult animals is usually subclinical, but encephalomyelitis has been associated recently with AKAV infection in adult cattle (Lee Park et al., 2002). In pregnant cows, the virus can cause abortions and congenital malformation of
calves, characterized by arthrogryposis and hydranencephaly, which was first seen in Israel in 1969. In February 2002, the first cases of 'blind newborn calves' were observed on farms located in the northern valleys of Israel (Brenner et al., 2004). Economic losses were due to decreased fertility and reduction in milk yield in cows (Horikita et al., 2005).

SBV is a provisional name for a newly discovered virus in the Orthobunyavirus genus and is a member of the Simbu serogroup viruses. SBV affects ruminants and transmission occurs by insect vectors, mainly Culicoides spp. and vertically in utero. SBV was first detected in November 2011 in Germany from samples collected in summer/autumn 2011 from sick dairy cattle (fever, reduced milk yield) (Conraths et al., 2013; Gibbens, 2012; Hoffmann et al., 2012). It was also detected in dairy cows in the Netherlands. From early December 2011, congenital malformations were reported in newborn lambs in the Netherlands and SBV was detected in and isolated from the brain tissue. Up to May 2012, The Netherlands, Belgium, Germany, United Kingdom, France, Luxembourg, Spain and Italy reported stillbirth and congenital malformations with PCR positive results.

BUNV is a teratogenic virus from North America that affects mainly sheep. A recent survey of cattle indicated that up to 28 % were positive for specific antibodies to BUNV (Sahu et al., 2002). BUNV was first isolated from a mosquito pool in Utah, USA in 1956 (Holden & Hess, 1959) but was only linked to disease during an epizootic of neonatal loss and malformed lambs in a sheep flock in Texas in 1987 (Crandell et al., 1989). The virus has also been isolated from a horse and a clinically healthy cow. BUNV is similar to Akabane disease, except that it affects only sheep.

2.1. Clinical signs

Most Simbu serogroup viruses cause sub-clinical infections in non-pregnant animals. In pregnant animals, these viruses may cross the placenta causing foetal infections that are associated with abortion, premature birth, still birth and congenital abnormalities in calves, lamb, and kids. The abnormalities include arthrogryposis, orencephaly, hydrocephalus, cerebella hypoplasia and congenital hydranencephaly (St George & Kirkland 1994). From a recent study of SHUV in Israel by Golender et al., (2015), clinical evidence showed that
malformations appeared up to six months after infection with SHUV and after the virus had been eliminated from the host by the immune system. Isolation of SHUV from malformed brains may indicate strong neurotropism of this putative pathogen. The possibility of its replication in the foetal nervous system should also be considered because an affected fetus that is born alive is likely a reservoir (Golender et al., 2015).

If clinical signs caused by SHUV are present, they may be difficult to distinguish clinically from other Orthobunyaviruses, such as SHAV, SATV, AKAV, OROV, SBV and BUNV, as they all cause abortions and malformations.

Wildlife can also be affected by SHUV. In South Africa, SHUV was found in rhinos and infected animals presented with a sudden onset of symptoms, paralysis and sudden death. Crocodiles also exhibited signs of paralysis and died (Venter et al., 2010). In horses neurological signs are usually seen (van Eeden et al., 2012). In humans febrile signs were observed in a child at a hospital in Nigeria (Moore, 1975). In experimental baby mice inoculated intracerebrally with SHUV, characteristic neurologic signs of nervousness were exhibited (Golender et al., 2015).

2.2. Pathology

The gross pathology findings include lung oedema and congestion, with variable presence of hydropericardium or other cavity fluids, subcutaneous and other regional oedema, and mild surface or cardiac haemorrhages (Williams et al., 2010).

Histopathological lesions found most commonly in spinal cord sections were assymetrical, not always bilateral and included haemorrhages; vascular congestion especially of grey matter but also multifocal distended white matter vessels; perivascular cuffing with mononuclear cells and occasional neutrophils most prominent in ventral horn grey matter but sometimes occurring in dorsal and lateral horns and elsewhere; variable mononuclear meningitis and occasional vascular wall necrosis; gliosis; glial nodules which at times contained neutrophils; occasional poliomalacia; and occasional neuronal chromatolysis or necrosis. Lesions similar to those found in spinal cord occurred in the brain and cerebellum,
but especially affecting lower white matter regions. Meningitis with severe lesions in the cerebellum may be observed (Williams et al., 2010).

2.3. Diagnosis

Simbu sero-group viruses are endemic in Africa. There were reports of detection of antibodies to viruses like SHAV, SHUV, MANV, SATV and AKAV in the 1970s (Causey et al., 1972; Lee, 1979; Metselaar et al., 1976; Theodoridis et al., 1979). Due to the extensive cross reactivity that can occur between these viruses that are both genetically and antigenically closely related, it can be difficult, if not impossible to determine which virus(es) an animal has been infected with (Lee, 1979; Kinney et al., 1981; Saeed et al., 2001).

Diagnosis of infections caused by Simbu sero-group viruses has been accomplished traditionally by detection of specific antibodies using virus neutralization assays, but more recently by enzyme-linked immunosorbent assays (ELISAs), which may be available commercially (Matthew et al., 2015). In general, commercially available ELISAs are sensitive, specific and robust, but as these viruses were originally clustered on the basis of serological assays, extensive cross reactivity is often observed (Kinney et al., 1981; McPhee et al., 1988; Bréard et al., 2013; Matthew et al., 2015). The initial validation of the ELISA assay was undertaken in Europe where other Simbu sero-group viruses were not detected in cattle or sheep. As there is a high degree of similarity between the N protein antigens of members of the Simbu sero-group (the basis upon which these viruses were initially grouped), it was not surprising that cross reactivity was observed in the ELISA when animals were infected with one or more other Simbu sero-group viruses (Matthew et al., 2015).

Although a number of reports have described the serological relationships between some of the Simbu serogroup viruses (Takahashi et al., 1968; Calisher et al., 1969; Reeves et al., 1970; David-West, 1972; Causey et al., 1972), the most comprehensive analysis was performed by Kinney & Calisher (1981), who studied the serological relationships between all the recognized members of the serogroup. These authors showed that although most Simbu serogroup viruses were readily distinguishable in neutralization assays, they exhibited complex relationships by complement fixation tests.
Based on this review, the SNT is regarded as the gold standard for differentiating viral species and was the reason this assay was chosen for this study. It can be used as a confirmation for commercially available ELISA kits. The SNT was used to detect SHUV antibodies from human sera in a study by van Eeden et al., (2014).

A SNT was developed for SBV, optimized and evaluated (Loeffen et al., 2012). This SNT had a specificity of > 99% and a sensitivity very close to 100%. The assay was highly repeatable and reproducible. The assay can be used at a relative high throughput for testing of animals for export, surveillance, screening and research purposes, and can also be used as a confirmation test for commercially available ELISAs, or for (relative) quantification of antibodies. It allows semiquantitative detection of antibodies against the virus. SNT remains a possible alternative as a screening test, but is also useful as a confirmation test or a semiquantitative test to be used in experimental infections, when amounts of antibodies need to be determined.

Virus isolation can be achieved in cell cultures and direct virus detection and identification is possible using RT-PCR and other molecular detection methods. Subsequent sequencing of the genome can give more detailed information of the type of the virus infecting animals. Electron microscopy can also be used to identify SHUV. It was used to identify Bunyavirus-like particles in one virus isolate from a horse with severe and fatal neurological disease. Confirmation of the presence of SHUV was achieved through screening of specimens with Bunyavirus family primers and identification of the virus using Bunyavirus specific primers and phylogenetic analysis (van Eeden et al., 2012).

2.4. Prevention and treatment

There is currently no specific treatment or vaccine for SHUV. Future prospects in research in vaccine production are necessary, considering the value of species affected. Control of potential vectors during the vector-active season may decrease the transmission of virus although it may be impossible practically. Possible intervention may include rescheduling of breeding outside the vector season, which should decrease the number of foetal
malformations.

3. JUSTIFICATION

SHUV can result in reproductive losses as well as ill health of animals. The overall effect of such outbreaks can result in a reduced income through cattle losses and potentially pose a threat to trade. The disease is also of public health concern and knowledge from this study will be of interest to medical authorities and will prompt further research on the disease in humans. The disease is a cause of concern, as it is re-emerging and neglected zoonotic disease, considering that historical isolation dates go back to 1966. Rapid and warning alert system will need to be developed and further research undertaken.

The confirmation of SHUV in South Africa poses a great threat to Kenya, Zimbabwe and other SADC countries. Although the serological study was performed on samples from Western Kenya, the Zimbabwean Masters student will establish the test at the Central Veterinary Laboratory, Zimbabwe. This will help Zimbabwe and other SADC countries to carry out further research studies.

4. AIM

To establish the extent of infection by SHUV in cattle in Kenya as a contribution to studies on effects of infection to animal health and production.

5. OBJECTIVES

- To be trained on selected serological methods used in diagnosis and survey investigations for SHUV.
- To use selected serological method to screen for SHUV antibodies in sera obtained from one hundred cattle in Western Kenya
- To analyse test results and publish research outputs to disseminate research findings to stakeholders.
6. MATERIALS AND METHODS

6.1. Experimental design

6.1.1. Study site

The study site was an area of western Kenya, which is considered representative of smallholder livestock farmers in east Africa. The area was approximately 4590 km² and covered some or all of Busia (95.9 %), Teso (96.3 %), Siaya (55.5 %), Butere/Mumias (26.9 %) and Bungoma (20.4 %) sub locations. A sub location is the smallest administrative unit and was typically about 10 km across and contained households where approximately 60 % of households own cattle (Figure 6.1).

Figure 6.1: Map of Western Kenya showing the study area, agro-ecological zones and sublocations (selected sub-locations highlighted) (Bronsvoort et al., 2013)
6.1.2. Study design

The survey design was a “stratified 2-stage cluster sample” (Bronsvoort et al., 2013). Stratification was by agro-ecological zones (AEZ) that was described first by Pratt, Green & Guynne (1966). It is based upon climate and vegetation characteristics which are relevant to vector distribution and agricultural potential. AEZs that were represented in the study area were Lower Midlands (LM) 1, LM2 (with a northern middle and southern section), LM3, LM4 and Upper Midlands (UM) 3. It was anticipated that the random cluster sampling would ensure that there was reasonable representation of farmers with varying herd sizes and management systems. The first stage cluster (sub location selection) was selected using stratified random sampling with replacement. The second stage (calf selection) was selected using ordinary random sampling without replacement, with a total sample size of 100. If the stratifying by AEZ was justified (i.e. differences are observed) then the effect of AEZ would be taken into account in analyses.

6.1.3. Sampling of cattle

One hundred blood samples were collected from cows. Sampling was restricted to cows whose calves survived up to 51 weeks of age (the period of monitoring). The dam samples were collected during recruitment of calves into the study. Calves that survived up to 51 weeks were not evenly distributed along the sublocations, resulting in selection of unequal numbers from each sublocation. The sample was weighted so that the number of calves selected randomly in a given sublocation was proportional to the number of calves that completed the 51 week visit in that sublocation.

Blood was collected in 10 ml vacutainer tubes without anticoagulant, the serum separated and stored in cryo tubes at -20 °C in the ILRI Laboratory, Busia. Aliquots of the samples were transported to the Exotic Diseases Division, Agricultural Research Council, Onderstepoort Veterinary Institute (ARC-OVI), South Africa. Samples were inactivated before being transported to DVTD for further testing.
6.2. Identification of the test virus

The reference virus used in this study was SHUV Dr Vasco Mendes Reference strain #2V, which had been adapted in baby hamster kidney (BHK) cells and passaged twice in Vero cells (21/12/10). The Karber method (Karber, 1931) was used to determine the initial concentration of the virus at a TCID\textsubscript{50} of $10^{4.48}$ per ml.

The identity of the reference virus was tested using a pan–Simbu real time RT-PCR assay (Fischer \textit{et al.}, 2013), a broad screening tool for identification of Simbu serogroup viruses. The reference virus was tested together with the following viruses: AKAV, SIMV, MANV, SATV and SHAV virus.

In addition, nucleic acid samples extracted from the reference virus were sent to the Zoonosis Research Unit, Department Medical Virology, Faculty of Health Sciences, University of Pretoria for testing by a nested real-time RT-PCR assay confirmation of the identity of the virus (Van Eeden \textit{et al.}, 2014).

6.2.1.1 RNA extraction

Total nucleic acid extractions from 50 µl of the reference viruses were made using a MagMAX\textsuperscript{TM} Total Nucleic Acid Isolation kit (Lifetech), according to the manufacturer’s instructions. The samples were mixed with 10 µl RNA Binding Beads, 10 µl Lysis/Binding Enhancer, 65 µl isopropanol and 65 µl Lysis/Binding Solution. The samples were placed in a MagMaxTM Express Particle Processor (Lifetech) and a custom protocol run (Appendix 2). RNA Binding Beads were washed twice with Wash Solution 1 and twice with Solution 2 before elution of the nucleic acid in 50 µl Elution Buffer.

6.2.1.2 RT-PCR assay

Complementary DNA was synthesised using 8 µl of extracted nucleic acid and a PrimeScript\textsuperscript{TM} RT Reagent Kit (Takara) according to the manufacturer’s instructions. The reactions were incubated in a thermal cycler (Applied Biosystems\textsuperscript{®} 2720) at 37 °C for 15 min, followed by 85 °C for 5 sec.
Amplification and detection of the L segment of Orthobunyaviruses was achieved with the use of a a Power SYBR® Green Master Mix (Thermo Fisher Scientific), forward primer panOBV-L-2959F (5’-TTGGAGARTATGARGCTAARATGTG) and reverse primer panOBV-L-3274R (5’-TGAGCACTCCATTTNGACATRTC) according to Table 6.1.

Table 6.1. Simbu RT-PCR reagent quantities.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power SYBR green mastermix (2x)</td>
<td>10 µl</td>
</tr>
<tr>
<td>panOBV-L-2959F (10 µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>panOBV-L-3274R (10 µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>7 µl</td>
</tr>
<tr>
<td>Template</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>TOTAL VOLUME</strong></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Samples were run in a StepOnePlus™ System, according to the run profile in Table 6.2.

Table 6.2. Simbu RT-PCR cycle conditions.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Sec</th>
<th>Optics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 °C</td>
<td>120</td>
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</tr>
<tr>
<td>2</td>
<td>95 °C</td>
<td>120</td>
<td>OFF</td>
</tr>
<tr>
<td>3</td>
<td>95 °C (45 cycles)</td>
<td>30</td>
<td>OFF</td>
</tr>
<tr>
<td>3</td>
<td>55 °C (45 cycles)</td>
<td>30</td>
<td>ON</td>
</tr>
<tr>
<td>4</td>
<td>95 °C</td>
<td>15</td>
<td>OFF</td>
</tr>
<tr>
<td>4</td>
<td>55 °C</td>
<td>60</td>
<td>OFF</td>
</tr>
<tr>
<td>4</td>
<td>95 °C (ramp 0.3 °C)</td>
<td>15</td>
<td>Continuous</td>
</tr>
</tbody>
</table>

6.2.2. Serum neutralisation test (SNT)

The tests were conducted in duplicate.

6.2.2.1 Cell culture and media

Vero cells were used in the SNT as the indicator cells. Vero cells are kidney cells derived from the African Green Monkey (Cercopithecus aethiops). Cells were grown in 75 mm² flasks in Minimum Essentials Medium (MEM) with 5 % foetal calf serum and 1 % gentamycin.
Dulbecco’s phosphate buffered saline (PBS) with calcium & magnesium was used to dilute the serum.

6.2.2.2 Positive and negative controls

Known positive and negative sera were titrated as part of the controls for the SNT assay. The negative control that was used was foetal calf serum from Highveld Biological and the positive a field sample provided by the ARC-OVI.

6.2.2.3 Test methodology

The protocol used was from the University of Pretoria standard operating procedure for SNT that was adapted for the SHUV SNT assay. The protocol was validated using a positive control. Sera were diluted 1:5 in PBS+ (Mg/Ca) and inactivated in a water bath for 30 min at 56 °C. A series of six, two fold dilutions in Minimum Essential Medium containing 5 % foetal calf serum was made in flat bottomed 96 well micro titre plates from 1:10 dilution to 1:320. Volumes of 100 µl were used.

The stock virus was diluted 1:300 in MEM containing 5 % foetal calf serum to obtain a concentration of 100 TCID₅₀. One hundred µl of the 100 TCID₅₀ antigen was added to all wells containing the diluted test sera.

A back titration was prepared by making a series of four ten-fold dilutions (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) from the 100 TCID₅₀ antigen and was used for the virus control. The virus control was set up over three rows and six columns as follows: 100 µl MEM containing 5 % foetal calf serum was added to all the wells, 100 µl of the 100 TCID₅₀ virus was added to the first two columns. One hundred µl of the four dilutions was added accordingly to the remaining four columns, starting with the highest virus dilution (10⁻⁴ to up the stock virus 100 TCID₅₀).

The plates were incubated for one hour at 37 °C (± 2 °C) in a humid atmosphere of 5 % CO₂ in air to allow interaction of the virus with the possible antibodies in serum to react. Eighty µl of Vero cells (480 000 cells/ml) were added to all the wells.
The cell control was set up in duplicate rows by adding 200 µl MEM containing 5 % foetal calf serum and 80 µl of the Vero cells suspension. The plates were incubated up to six days at 37 °C (± 2 °C) in a humid atmosphere of 5 % CO₂ in air, until the back titration indicated that the virus control show a 50 % CPE at the 10⁻² dilution of the 100 TCID₅₀ virus (stock virus). The presence of specific antibodies in the test serum inhibits the production of CPE. The end point dilution was taken as the dilution at which 50 % of the cells were infected.
7. RESULTS

7.1. Identification of the test virus

A composite of the results from the pan–Simbu real time RT-PCR assay (Fischer et al., 2013) is shown in Figure 7.1. Melt curve temperatures between 75 and 85 °C were considered positive for a Simbu-group virus. Curves below 75 °C were considered negative. SHUV had a melt curve temperature of 77.97 °C (Figure 7.2) and was positively identified as a Simbu-group virus, but could not be distinguished on the melt temperatures from AKAV, SIMV and MANV (melt curve temperature range between 77.07 - 77.97 °C). SHUV could be distinguished on the melt temperature from SATV (79.01 °C) and SHAV (79.31 °C) (Table 7.1).

![Melt Curve](image)

Figure 7.1. Composite of melt curves from Simbu-group viruses and controls.
Figure 7.2. Melt curve for SHUV.

Table 7.1: Melt curve temperatures for Simbu-group viruses and control.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Δ T&lt;sub&gt;m&lt;/sub&gt; peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHUNI</td>
<td>77.97 °C</td>
</tr>
<tr>
<td>AKAV</td>
<td>77.07 °C</td>
</tr>
<tr>
<td>SIMV</td>
<td>77.22 °C</td>
</tr>
<tr>
<td>MANV</td>
<td>77.67 °C</td>
</tr>
<tr>
<td>SATV</td>
<td>79.01 °C</td>
</tr>
<tr>
<td>SHAV</td>
<td>79.31 °C</td>
</tr>
<tr>
<td>Neg control H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>67.98 °C</td>
</tr>
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</table>

The test virus was confirmed as positive for SHUV by the Zoonosis Research Unit of the University of Pretorius (Appendix 11.3).

7.2. Serum neutralisation test

The positive control showed no cytopathic effect (CPE), while the negative control showed 100 % CPE. The cell control did not show CPE, which meant that no cytotoxicity was present in the wells. The virus control had 100 % CPE in all test wells. There was 75 % CPE in the 10⁻¹
dilution and 50 % CPE in the $10^{-2}$ dilution which meant that the test was valid. The titre was the reciprocal of the end point dilution where 50 % of cells were infected. A titre $\geq 1:10$ was considered positive. Titres of $< 1:10$ were considered negative.

In a sample size $n = 100$, all cattle tested positive for SHUV antibodies (Table 7.2). All titre values were above 1:160 with most of animals $> 1:320$. Out of the animals sampled, 87 % had titres above 1:320, 12 % had a titre of 1:320 and 2 % had a titre of 1:160 (Figure 7.3)

Table 7.2: Sample identities and titre values for antibodies against Shuni virus using the serum neutralisation test.

<table>
<thead>
<tr>
<th>Sample Identity</th>
<th>Titre value</th>
<th>Sample Identity</th>
<th>Titre value</th>
<th>Sample Identity</th>
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Figure 7.3: Distribution of antibody titres to SHUV of cattle.
8. DISCUSSION

The aim of the study was to test for the presence of SHUV antibodies in cattle from Kenya. This is the first report on the study of this disease in this country. There was previously no suspicion of the presence of the disease in Kenya, as no clinical cases have been documented there. However a closely related virus in the same serogroup, AKAV, was detected in Kenya by Davies & Jessett (1985). Recently a serological study by Matthew et al. (2015) in Tanzania which shares a border with Kenya, showed the presence of Simbu serogroup antibodies in cattle.

The pan-Simbu real time RT-PCR assay was able to detect SHUV with a melt temperature of 77.97, which confirmed it as a Simbu-group virus. Positive identification of the test virus as SHUV was obtained from the nested real-time RT-PCR assay (van Eeden et al., 2014), as performed by the Zoonosis Research Unit of the University of Pretoria.

In this study the SNT was chosen, as it is sensitive, reliable, less prone to variation and subjectivity in interpretation. There was cross reactivity of SHUV and Aino virus (AINOV), which resulted in merging of the two to form SHUV. Cross-reactivity between SHUV and other Simbu serogroup viruses is unlikely, as the SNT is a specific test used to characterize strains. However there is a challenge as reference virus strains are always required and may be difficult to source. Positive control serum was also used in the test to validate the results and the virus strain has been confirmed using pan Simbu real time RT-PCR by Fischer et al. (2013). Cross reactions have been seen using commercial ELISA kits. The SNT method has been used previously to detect antibodies in humans, in particular veterinarians in South Africa (van Eeden et al., 2014). Kinney & Calisher (1981) showed that although most Simbu serogroup viruses were readily distinguishable in neutralization assays, they exhibited complex relationships with complement fixation tests.

The results obtained in this study support the hypothesis that SHUV is endemic in Kenya. All cattle tested positive for SHUV antibodies, which means that all animals were exposed to the virus. The possible reason for this is that the sampled animals were adults and could have been exposed over a number of seasons to the Culicoides spp. vector, which are widely
distributed in all agro-ecological zones in Kenya. Cattle are a preferred feeding host for *Culicoides* spp. in Kenya (Davies & Walker, 1974a, Walker & Boreham, 1976).

SHUV has been isolated previously in *Culicoides* spp. (Lee, 1979 & McIntosh 1972; 1980). *Culicoides* spp. occur in a region between 40 °N and 35 °S, which coincides with the region the samples were collected from. Since all the samples tested positive and were collected from all AEZ, it means that the vector is distributed in all AEZ.

The results of this study provide support to previous studies that SHUV can be detected in apparently healthy cattle, sheep and goats (Da Costa Mendes, 1984). This study managed to establish that animals in LM1, LM2 (with a northern middle and southern section), LM3, LM4 and UM3 of the AEZs have been exposed to SHUV. However there were limitations, as there is no exact identification of samples tested in relation to the exact place of collection. More accurate mapping of the SHUV seropositive distribution in the different AEZ is needed.

The majority of animals tested had very high titres above 1:320, which suggests that the cattle had a recent or continuous exposure to the virus. Since the animals tested were adults, the high titres may have been due to the continuous exposure to the vector and hence SHUV. There was a smaller percentage of animals with relatively low titres and there is a possibility of the link with an AEZ with lower vector density. There is a limitation in the study as we could not map the titre level of seropositivity to the exact geographical point where samples could have originated.
9. CONCLUSION AND RECOMMENDATIONS

It can be concluded that most animals in western Kenya have been exposed to SHUV. Further studies still need to be carried out in order to estimate prevalence, as this project managed to establish exposure of cattle in western Kenya to the virus. There is still a need to assess if clinical cases of the disease exist in humans, cattle and other species. Studies to link presence of antibodies and reproductive orders are necessary to determine the impact of SHUV on the economy. More confirmatory tests such as virus isolations and molecular studies should be employed.

Previous studies have indicated that a subspecies of SHUV is associated with malformations through experimental infections of pregnant ewes (Kurogi et al., 1977; Parsonson, 1982; Tuda et al., 2004) and this needs to be explored further although recently a study in Israel by Golender et al., (2015) confirmed malformations in foetuses due to SHUV. Furthermore, studies to see if a link exists between abortion cases and co-infection with other arboviruses needs to be carried out. Molecular studies have only focused previously on horses in South Africa (van Eeden et al., 2012; Venter et al., 2010; Williams et al., 2010). However, a study by van Eeden et al. (2014) in humans was done, but more information is still missing on clinical cases in humans.

The first isolation of SHUV involved a human case, a child in Nigeria (Moore, 1975), confirmed the zoonotic nature of the disease. Subsequently veterinarians in South Africa were screened for SHUV antibodies and were positive (van Eeden et al., 2014). In light of this more studies need to be done in order to determine the extent of the disease in human beings.
10. REFERENCES


KEMP, G.E., CAUSEY, O.R. & CALISTA, E., 1971. Virus isolations from trade cattle, sheep, goats and


OYA, A., OKUNO, T., OGATA, T., KOYASHII, J & MATSUYAMA, T., 1961, Akabane, a new arbor virus


11. APPENDIX

11.1. Appendix 1 - Ethical clearance

Animal Ethics Committee

<table>
<thead>
<tr>
<th>PROJECT TITLE</th>
<th>The detection of antibodies against Akabane and Polyam Viruses in cattle from western Kenya</th>
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<tbody>
<tr>
<td>PROJECT NUMBER</td>
<td>V050-12</td>
</tr>
<tr>
<td>RESEARCHER/PRINCIPAL INVESTIGATOR</td>
<td>Ms. B Bshebe</td>
</tr>
<tr>
<td>STUDENT NUMBER (where applicable)</td>
<td>113 450 48</td>
</tr>
<tr>
<td>DISSERTATION/THESIS SUBMITTED FOR</td>
<td>Degree</td>
</tr>
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<td>ANIMAL SPECIES</td>
<td>Cattle</td>
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<tr>
<td>NUMBER OF ANIMALS</td>
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<tr>
<td>Approval period to use animals for research/testing purposes</td>
<td>January 2011 - January 2013</td>
</tr>
<tr>
<td>SUPERVISOR</td>
<td>Dr. M Quan</td>
</tr>
</tbody>
</table>

**KINDLY NOTE:**

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment.

<table>
<thead>
<tr>
<th>APPROVED</th>
<th>Date</th>
<th>28 January 2013</th>
</tr>
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<tbody>
<tr>
<td>CHAIRMAN: UP Animal Ethics Committee</td>
<td>Signature</td>
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11.2. Appendix 2 - Customized protocol for the MagMaxTM Express Particle Processor (Lifetech) for nucleic acid extraction.

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Protocol template version = 2.6.0
Instrument type = KingFisher
Creator = mquan
Created = 30/9/2009 9:31:36
Description = RNA isolation from whole blood.
Kit = MagMAX-96 viral RNA Isolation Kit, AM1836
Plate layouts = Default

[ PLATE LAYOUTS ]
Default
Plate type = KingFisher plate 200 ul
Plate change message = Change Default

A:
- volume = 20, name = Bead Mix (10 ul RNA binding beads, 10 ul Lysis/Binding Enhancer)
- volume = 50, name = Whole blood
- volume = 130, name = 65 ul Lysis/Binding Solution, 1 ul Carrier RNA, 2 ul Xeno RNA, 65 ul Isopropanol
B:
- volume = 150, name = Wash Solution 1
C:
- volume = 150, name = Wash Solution 1
D:
- volume = 150, name = Wash Solution 2
E:
- volume = 150, name = Wash Solution 2
F:
- volume = 50, name = Elution Buffer
G:
- EMPTY
H:
- EMPTY

[ STEPS ]
BIND
Step parameters
• Name = Lysis Binding 5 min
• Well = A, Default

Beginning of step:
• No Action = Yes

**Bind parameters:**
• Bind time = 5min 0s, speed = Fast dual mix

**End of step:**
• Collect beads = Yes, count = 5

---

**WASH**

**Step parameters**
• Name = 1st Wash I 1 min
• Well = B, Default

**Beginning of step:**
• Release = Yes, time = 0s, speed = Fast

**Wash parameters:**
• Wash time = 1min 0s, speed = Fast

**End of step:**
• Collect beads = Yes, count = 3

---

**WASH**

**Step parameters**
• Name = 2nd Wash I 1 min
• Well = C, Default

**Beginning of step:**
• Release = Yes, time = 0s, speed = Fast

**Wash parameters:**
• Wash time = 1min 0s, speed = Fast

**End of step:**
• Collect beads = Yes, count = 3

---

**WASH**

**Step parameters**
• Name = 1st Wash II 1 min
• Well = D, Default

**Beginning of step:**
• Release = Yes, time = 0s, speed = Fast

**Wash parameters:**
• Wash time = 1min 0s, speed = Fast

**End of step:**
• Collect beads = Yes, count = 2

---

**WASH**

**Step parameters**
• Name = 2nd Wash II 1 min
• Well = E, Default

**Beginning of step:**
• Release = Yes, time = 0s, speed = Fast

**Wash parameters:**
• Wash time = 1min 0s, speed = Fast

**End of step:**
• Collect beads = Yes, count = 2

---

**DRY**

**Step parameters**
• Name = Dry 1 min
• Well = E, Default
• Dry time = 1min 0s
• Tip position = Outside well

---

**ELUTION**

**Step parameters**
• Name = Elution 3 min
• Well = F, Default

**Beginning of step:**
• Release = Yes, time = 0s, speed = Fast

**Elution parameters:**
• Elution time = 3min 0s, speed = Bottom medium

**Pause parameters:**
• Pause for manual handling = No

**Remove beads:**
• Remove beads = Yes, collect count = 5, disposal well = B
11.3. SHUV RT-PCR assay report

User Developed or Modified Test Method
JS test SHUV for Melvin

Experiment

<table>
<thead>
<tr>
<th>Field</th>
<th>Value</th>
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<td>Last Modified Date</td>
<td>Feb-1-2016 15:19:03</td>
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Programs

Program Name: Denature

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<td>Hold (hh:mm:ss)</td>
<td>Slope (°C/s)</td>
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Program Name: Melt curve

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<th>Melting Curves</th>
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<td>Hold (hh:mm:ss)</td>
<td>Slope (°C/s)</td>
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Program Name: Cooling

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# User Developed or Modified Test Method

## Samples

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## Tm Calling

### Results

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<th>High</th>
<th>Peak 2 Tm</th>
<th>Area</th>
<th>Wide</th>
<th>High</th>
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</table>

## Melting Curves

![Melting Curves Graph](image)

JS test SHUV for Melvyn

Feb-1-2016

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User Developed or Modified Test Method

Melting Peaks

- 1: M ShUv 1
- 2: M ShUv 2
- 3: Ntc 1
- 4: Ntc 2
- 5: Pbs ShUv

Temperature (°C)

1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
40 45 50 55 60 65 70 75