Thesis Ref. No._____

ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

MASTER OF VETERINARY SCIENCE THESIS

SCHMALLENBERG VIRUS: VIRAL DETECTION, MORPHOLOGICAL IDENTIFICATION AND A SPATIAL DISTRIBUTION MODELING OF ITS VECTORS

BY

GETACHEW TEKA DISASA

DEPARTMENT OF CLINICAL STUDIES MVSc PROGRAM IN VETERINARY EPIDEMIOLOGY

JUNE, 2020

BISHOFITU, ETHIOPIA

SCHMALLENBERG VIRUS: VIRAL DETECTION, MORPHOLOGICAL IDENTIFICATION AND A SPATIAL DISTRIBUTION MODELING OF ITS VECTORS

By

Getachew Teka Disasa

A Thesis submitted to Addis Ababa University College of Veterinary Medicine and Agriculture in Partial Fulfillment of the requirements for Master of Veterinary Science in Veterinary Epidemiology

JUNE, 2020

BISHOFITU, ETHIOPIA

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical Studies

As MVSc research advisors, we certify that we have read and evaluated this thesis prepared under our guidance by **Getachew Teka** titled**: Schmallenberg Virus: Viral Detection, Morphological Identification and A Spatial Distribution Modeling Of Its Vectors**

Dr. Haileleul Negussie	_____	_____
Major Advisor	**Signature**	**Date**
Dr. Samson Leta	_____	_____
Co-advisor	**Signature**	**Date**
Dr. Megarsa Bedasa	_____	_____
Co-advisor	**Signature**	**Date**

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical Studies

As the member of the Examining board of the final MSc virtual defense, we certify that we have evaluated the thesis prepared by Getachew Teka titled "**Schmallenberg Virus: Viral Detection, Morphological Identification and A Spatial Distribution Modeling Of Its Vectors"** We recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Epidemiology.

Dr. Teshale Sori	_____	_____
Internal Examiner	**Signature**	**Date**
Dr. Tesfaye Rufael	_____	_____
External Examiner	**Signature**	**Date**
Dr. Haileleul Negusei	_____	_____
Department Chairperson	**Signature**	**Date**

DEDICATION

This thesis manuscript is dedicated to my wife, Woynishet Asefa, my children Amerti and Latera Getachew and my mother, Akafete Abebe for having with affection and love and for their keen partnership in the success of my life.

DECLARATION SHEET

Firstly, I declare that this thesis is my bonafid *work* and that all sources of material used for this thesis have been properly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MVSc degree at Addis Ababa University College of Veterinary Medicine and Agriculture and is deposited at the College library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree or certificate.

Short quotations from this thesis are permissible without special permission provided accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of College when in my judgment; the proposed use of the material is in the interests of scholarship. In all other instances however permission must be obtained from the author.

Name: Getachew Teka Disasa

Signature:

Place: College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission:

TABLE OF CONTENTS **PAGES**

LIST OF ABBREVIATIONS ..... **IV**

LISTS OF ANNEXES ..... **VI**

LIST OF FIGURES ..... **VII**

LISTS OF TABLES ..... **VIII**

ABSTRACT ..... **IX**

1. INTRODUCTION **1**

2. LITERATURE REVIEW **3**

2.1. Schmallenberg virus **3**

2.2. Etiology **4**

2.2.1. *Virus Morphology* 4

2.2.2. Virus Sequence Analysis 5

2.2.3. Pathogenicity 6

2.3. Epidemiology **7**

2.3.1. Occurrence 7

2.3.2. Transmission 8

2.3.3. Risk factors 10

2.4. Signs of the disease **11**

2.5. Diagnosis **11**

2.6. Economic Importance **15**

2.7. Control and Prevention of the Disease **15**

3. MATERIAL AND METHODS **16**

3.1. Study Area **16**

3.2. *Culicoide* Collection and Identification **18**

3.3. *Culicoide* Processing **19**

3.4. Viral Extraction **19**

3.5. Real-time PCR and Visualization of RT-PCR Products **19**

3.6. *Culicoides* Distribution Modeling **20**

3.6.1. Potential Predictors of SBV Vector Culicoides Occurrence 20
3.6.2. GIS Operation 22
3.6.3. Modeling Approach 22
3.7. Clearance **24**
4. RESULTS **25**
4.1. *Culicoides* Survey **25**
4.2. Viral Detection **26**
4.3. SBV *Culicoides* Distribution Model **27**
4.3.1. Model Performance and Importance of Environmental Variables 27
4.3.2. Ensemble Models 35
5. DISCUSSIONS **38**
5.1. *Culicoides* Survey **38**
5.2. Viral Detection **39**
5.3. SBV Vector *Culicoides* Distribution Model **40**
6. CONCLUSIONS AND RECOMMENDATIONS **42**
7. REFERENCES **43**
8. LIST OF ANNEXES **51**

ACKNOWLEDGMENTS

Firstly, I would like to thank Almighty God for full protection, support and guidance me in all my works and daily life. Without his blessings, this reaching would not have been possible.

I sincerely want to thank my advisors Dr. Haileleul Negussie, Dr. Samson Leta and Dr. Megersa Bedasa for consistent help in all aspect of this study work from early proposal writing to field sample collections, laboratory work, Data processing, up to writing of the final of this paper. Without them this work might not accomplished to this stage.

I want to acknowledge the Oromia Livestock Agency for their sponsorship this master program. My special appreciations go to thematic research project of College of Veterinary Medicine and Agriculture of Addis Ababa University for the incredible support on the budget and logistics. My gratitude goes to Mr. Tesfaye Mulatu and his team staff of NAHDIC those helped me in many aspects of this research works particularly in material facilitating, field sample collections and *Culicoides* identifications. I also want to thank Tsedale Teshome and Dr. Eyerusalem Fetene who have been with me from collection to identification and processing of *Culicoides.*

I would like to thank National Animal Health Diagnostic and Investigation Center (NAHDIC, Sebata), Addis Abeba University College of Veterinary Medicine and Agriculture Parasitology laboratory, and Jimma University college of Agriculture and Veterinary medicine for provision of field materials and lab materials for morphological identification of *Culicoides.* National Veterinary Institute (NVI) for processing and detection of virus. My gratitude goes to the staff of these institutions those who supported me on work of this thesis research.

I am expanding my deepest thanks to my wife, Woynishet Asefa and my mother, Akafete Abebe who assist me in their day to day communicative and psychological support and aid. My lovely idiom goes to my beloved children Amerti and Latera Getachew for being with me in all my works.

Finally, I am grateful to all of those the people who have supported me to complete the research work.

LIST OF ABBREVIATIONS

AHS	African horse Sickness
AKAV	Akabane virus
ANN	Artificial neural networks
BTV	Bluetongue virus
CA	Committee averaging
CDC	Centers for Disease Control and Prevention
CTA	Classification tree analysis
CV	Coefficients of variation
DNA	Deoxyribonucleic acid
ESA	European Space Agency
FDA	Flexible discriminant analysis
GAM	General additive models
GBM	General boosted models or boosted regression trees
GIS	Geographic information systems
GLM	General linear models
IIKC	Interactive identification key for *Culicoides*
Kappa	Cohen's kappa statistics
MARS	Multiple adaptive regression splines
MAXENT	Maximum entropy
MODIS	Moderate Resolution Imaging Spectroradiometer
NADHIC	National Animal Health Diagnostic and Investigation Center
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
QGIS	Quantum Geographic Information System
RF	Random forests
RNA	Ribonucleic Acid

ROC	receiver operating characteristics
RT-PCR	Reverse transcriptase-polymerase chain reaction
rt RT-PCR	real-time Reverse transcriptase-polymerase chain reaction
SRE	Surface Range Envelope
SBV	Schmallenberg Virus
TSS	True Skills Statistics
VIF	Variance Inflation Factor
VNT	Virus Neutralization Test
WHO	World Health Organization

LISTS OF ANNEXES **PAGES**

ANNEX 1: - Protocol: Purification of Viral RNA 51
ANNEX 2: Ethical clearance 54
ANNEX 3 :-NVI Master-Mix preparation and Run PCR reaction 55
ANNEX 4:- INDIVIDUAL MODELS OUTPUTS 56

LIST OF FIGURES **PAGES**

Figure 1:- SBV morphological diagram with three segments. 5
Figure 2: Phylogenetic relationship between Schmallenberg virus and *Orthobunyaviruse*s. 6
Figure 3: Schmallenberg Virus diagnosis. 14
Figure 4: Map of the study areas 17
Figure 5:- real-time RT-PCR result of SBV genome detection for the *Culicoide's* pool 26
Figure 6: *C. imicola* Prediction value. 35
Figure 7: Coefficient of Variation 'clamping mask'. 36
Figure 8: Selected prediction areas of committee averaging. 37

LISTS OF TABLES **PAGES**

Table 1: Confirmed cases of Schmallenberg virus infections in Europe. 8
Table 2:- *Culicoides* Species identified in five districts of different parts of Ethiopia and their percentage out of the sorted species 25
Table 3:- Average Performance of individual Models 27
Table 4:- Variables Contribution in the models 28
Table 5:- Ensembel Variable Importance 34

ABSTRACT

Schmallenberg virus (SBV) is a novel *Orthobunyavirus* that causes drop milk production, diarrhea, fever, congenital malformation and abortion to the affected animal. First it spread rapidly throughout the European continent and then to other parts of the world. In Ethiopia, there was no previous study conducted on the detection of SBV in *Culicoides* and ensemble modeling of SBV vectors in Ethiopia. Thus, this study aimed to conduct entomological survey, SBV detection and ensemble modeling of the vectors. An entomological survey was conducted during the study from October, 2019 to May, 2020 in Adama, Bishoftu, Holeta, Jimma, and Sebeta. The result showed that from the total of 6356 adult *Culicoides* midges collected, *C. imicola* (32%), *C. kings* (30%), *C. deltus* (4%), *C.milnei* (13%), and not identified (20%) were recorded. Detection of SBV from a total of 29 pools of *Culicoides* midges containing 70-160 *Culicoides*/pool was performed using real-time RT-PCR. The results revealed that, none of the *Culicoides* midges were positive for SBV genome. Ensemble model were developed using predictor variables land cover data, livestock, mean annual maximum temperature, mean annual minimum temperature, precipitation, solar radiation, water vapour and wind. The overall contributions of the variables to the model were mean annual minimum temperature (49.5%) highest and land cover (3.9%) lowest. Of 30 individual models developed, 27 had ROC > 0.90 and 24 had TSS and Kappa > 0.80. The ensemble model of the 30 models showed most parts of Ethiopia are suitable for *C. imicola*. The suitability differs across zones. High probability of suitability occurs in Borena, Bale, East Hararge, East Shewa, North Shewa, West Shewa, Hadiya, North Wollo, Southern Tigray, and Eastern Tigray while low suitable areas were occurs in Benchimaji, Shaka, Kafa, Gambela, Zone 1 and 2 and Afar Regional State. Thus, our results suggested that more work is needed on viral detection of SBV, modeling of the spatial and temporal situations of both vector and the disease.

Keywords: *Culicoides, Ensemble modeling, Occurrence, Schmallenberg Virus, Viral detection*

1. INTRODUCTION

Schmallenberg virus (SBV) is a novel *Orthobunyavirus* identified in Germany during the summer of 2011 through the new technology of deep sequencing metagenomic analysis (Hoffmann *et al.*, 2012). SBV was verified to result in congenital deformities in calves and lambs when dams were infected in the first trimester following insemination and this has since been identified as SBV's primary impact on ruminant production (Davies *et al.*, 2012; Elbers *et al.*, 2012). The virus was found in malformed lambs, kids and calves in different European countries in different organs (Bilk *et al.*, 2012). Infection of animals during pregnancy causes arthrogryposis hydranencephaly syndrome, which results in congenital malformations, abortions, and stillbirths (Tarlinton *et al.*, 2012).

SBV spread rapidly throughout the European continent reaching the Scandinavian countries and the British Isles at the North, the Mediterranean region including Spain, Southern France, Italy, Greece and Turkey at the South and Eastern European countries such as Poland or Lithuania and Russia (Beer *et al.*, 2012; Claine *et al.*, 2013; Hoffmann *et al.*, 2012; Julia *et al.*, 2019; Zeynalova *et al.*, 2019). It also spreads to Turkey (Azkur *et al.*, 2013), China (Zhai *et al.*, 2018), and Lebanon (Abi-rizk *et al.*, 2017). In Africa, no genomic detection, but only serological screening of SBV reported in Mozambique (Blomstrom *et al.*, 2014), Tanzania (Mathew *et al.*, 2015), South Africa (Leask *et al.*, 2013), and Ethiopia (Sibhat *et al.*, 2018).

In the world *Culicoides* midges are relatively studied due to their ability to transmit African horse sickness, epizootic hemorrhagic disease, Bluetongue, and Schmallenberg viruses and parasites of domestic and wild animals (Carpenter *et al.*, 2013; McGregor *et al.*, 2019; Wanji *et al.*, 2019). Following detection, a range of *Culicoides* species was rapidly implicated in the transmission of SBV through a series of studies in the Netherlands (Elbers *et al.*, 2013) and Belgium (De Regge *et al.*, 2012). Some studies have confirmed SBV infection in *C. chiopterus, C. dewulfi, C. imicola, C. punctatus, C. pulicaris, C. obsoletus* and *C. scoticus.* This implicate that these species as possible competent vectors of the virus in Europe (De Regge *et al.*, 2012; Doceul *et al.*, 2013; Rasmussen *et al.*, 2012). The infection and transmission of SBV by *C. imicola* and *Obsoletus* complex were verified by experimental infection *Culicoides* (Pages *et al.*, 2017).

The detection of SBV is primarily based on RT-PCR systems either in the form of different commercially available real-time RT-PCR kits or various in-house real-time or conventional RT-PCR protocols (Bilk *et al.,* 2012; Fischer *et al.,* 2013; Hoffmann *et al.,* 2012). The viral genome was detected by RT-PCR in *Culicoides* (De Regge *et al.,* 2012). However, In Ethiopia, previous studies on morphologically identification of *Culicoides* were conducted and indicated the presence of different speciese of *Culicoides such as C. imicola, C. neavei, C. zuluensis, C. fulvithorax and C. isioloensish, C. king and C. schultezi* (Fetene, 2019; Mulatu and Hailu, 2019). However, detection of the SBV genome in *Culicoides* has not been done in Ethiopia in previous studies. Thus, assessing SBV genome in *Culicoides* is very crucial. Among *Culicoides* midges, *C. imicola* is one of the most widely spread vector in the world (Guichard *et al.*, 2014).

Health risk can be assessed and managed by using spatial modeling and temporal distribution of vector species (Hongoh *et al.,* 2011). In previous study of Leta *et al.*,(2019b) modeling the worldwide distribution of *C. imicola* has done by using an ensemble approach. Ensemble modelings join a variety of method to forecast the worldwide allocation of a species and contributions of each variable. To decrease variance, bias and improve forecasting ensemble model join various model in to single predictive model (Arau and New, 2006; Thuiller *et al.*, 2009a).

The ensemble modeling of *C. imicola* in this study comprises geo-positioned occurrence of *C. imicola* used by Leta *et al.,* (2019a) globally, Fetene, (2019) nationally, and the current collection in different parts of Ethiopia. However, ensemble modeling distribution of *C. imicola* focusing on SBV has not been done. This, ensemble modeling gives great emphasis to the national level with great distribution *C. imicola* as this could be a vector for SBV. Thus, this study aimed to conduct entomological survey, SBV detection and ensembel modeling of the vector.

Specific objectives:

- An entomological survey and morphological identification of *Culicoides*
- To detect the genome of SBV in *Culicoides*
- To conduct species distribution modeling of *C. imicola* vector of SBV.

2. LITERATURE REVIEW

2.1. Schmallenberg virus

Schmallenberg virus (SBV) is a new emerging virus identified in Germany during the summer of 2011 through new technology of deep sequencing metagenomic analysis (Hoffmann *et al.,* 2012). It was first detected in Europe in autumn 2011 and named after the place where the original isolate derived from, a town called Schmallenberg, located in the north-west of Germany. In autumn 2011, dairy cattle farmers from the Netherlands and Germany almost simultaneously reported disease outbreaks in their herds including mild fever, therapy-resistant diarrhoea and a reduction in milk yield (De Regge *et al.,* 2014; Hoffmann *et al.,* 2012). After ruling out several classical bovine endemic and emerging viruses as cause of these outbreaks, the Friedrich-Loeffler-Institute of Germany identified a new virus as causative agent utilizing a meta-genomic approach with next-generation sequencing (Hoffmann *et al.,* 2012). Similarities of nucleotide homology of the new virus made it to classify it in to the Simbu serogroup of the family *bunyaviridae*, genus *Orthobunyaviruses* and are closely related to other arthropod-borne viruses, which are known to primarily infect ruminants such as Akabane, Sathuperi, Aino and Shamonda virus. All these viruses are transmitted by vectors such as mosquitoes and biting midges and can induce congenital malformations in neonates if a susceptible dam is infected during a vulnerable period in early pregnancy (Beer *et al.,* 2012; Hoffmann *et al.,* 2012; Friedrich-Loeffler-Institut, 2015).

Signs of SBV disease do not be observed on Adult goats and sheep. Apparent clinical signs of SBV infection in adult cattle are reported to be short-lived. These include loss of appetite, loss of body condition, hyperthermia, diarrhoea, and reduction in milk production. In pregnant animals the growing fetus affected when the virus crosses the placenta. In cattle and sheep day 60-180 and 15-28 are stages of pregnancy most susceptible for fetal deformities respectively causing neonatal malformation affecting neuro-musculo-skeletal systems. Fetal nerve tissue damaged by the virus causing abnormalities of spinal cord and brain which result in to skeleton and muscles abnormalities (Beer *et al.,* 2012; Bilk *et al.,* 2012; Hoffmann *et al.,* 2012; Phil Scott, 2019). The syndrome is known as arthrogryposis hydraenchphally (AHS) and characterized by

arthrogryposis, severe torticollis, ankylosis, kyphosis, lordosis, and scoliosis, brachygnathia inferior and neurological disorders. Most of the anomalies were observed in cases of abortions and stillbirths, while some calves may be born alive with various pathologies and behavioral abnormalities (Friedrich-Loeffler-Institut, 2015; Helmer *et al.,* 2016; Tarlinton *et al.,* 2012). The economic impact of this disease high in meat and milk industry particularly milk loss was the most frequently reported clinical sign and was observed in 90.2% of clinically affected animals and abortion 11.9% (Lechner *et al.,* 2017).

2.2. Etiology

Schmallenberg virus is single-stranded, triple segmented, enveloped, negative-sense RNA virus of *Bunyaviridae* family, in *Orthobunyavirus* genus (Hoffmann *et al.,* 2012). It is grouped within a member of Simbu serogroup viruses that includes Aino, Akabane, and Shamonda viruses. Sathuperi and Douglas virus are those viruses that are most closely related to SBV (Hoffmann *et al.,* 2012; OIE, 2017; Tarlinton *et al.,* 2012).

2.2.1. *Virus Morphology*

Schmallenberg virus is an enveloped virus with a triple segmented genome composed of single-stranded, negative-sense RNA. The three segments indicated in Figure 1 below, designated large (L), medium (M), and small(S), code for at least 5 proteins. In general, the L segment codes for a polyprotein with replicase and transcriptase activity. A non-structural protein (NSm) and virion surface glycoproteins (G1 and G2) encoded by M segment. Non-structural protein (NSs) and nucleocapsid (N) protein coded by the S segment (Doceul *et al.,* 2013; Hoffmann *et al.,* 2012; Wernike *et al.,* 2015). Genomic studies have revealed significant sequence homology between the S segments of Shamonda virus and SBV resulting in the tentative characterization of SBV as a Shamonda-like virus (Hoffmann et al., 2012; Tarlinton et al., 2012). Type I integral membrane proteins embedded in the envelope are the surface glycoprotein Gc and Gn: N contact with the outer environment while C toward the intraviral space. These are essential for budding, fusion and entry into the cells (Strandin *et al.,* 2013).

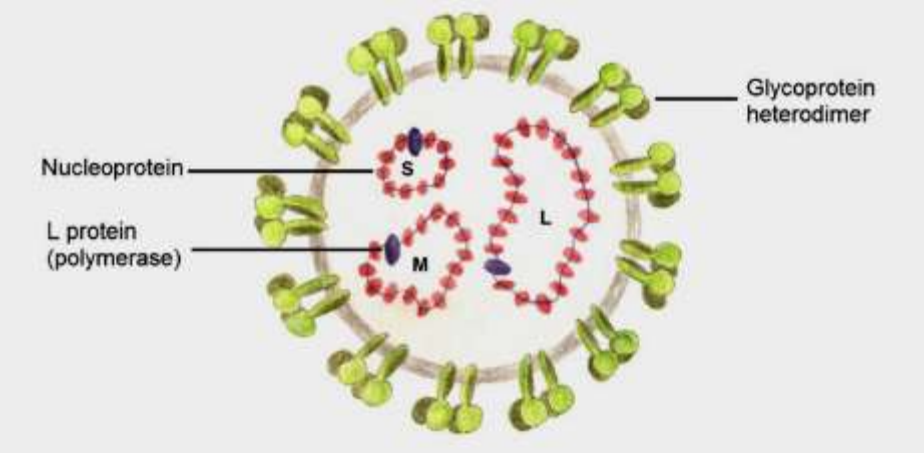


Figure 1:- SBV morphological diagram with three segments.

Adapted from Tarlinton *et al.* (2012).

2.2.2. Virus Sequence Analysis

Phylogenetic analysis of the SBV genomic segments indicated in Figure 2 below shows schmallenberg virus identity 71% the M segment with Aino virus, 97% the S segment with Shamonda virus and 69% the L segment with Akabane virus (Hoffmann *et al.*, 2012). Analysis of further sequence of SBV resulted higher similarity of the S and L segments of Shamonda virus; while the M segment of the Douglas and Sathuperi (Yanase *et al.*, 2012). Schmallenberg Virus segments could be resulted from reassortment of S and L segments of the Shamonda virus and M segments of the Sathuperi virus (Goller *et al.*, 2012). As described by Goller *et al.*, (2012) phylogenetic study of all of these sequences has revealed that SBV similar to the Sathuperi virus. As suggested by Doceul *et al.* (2013) SBV as an ancestor of Shamonda virus.

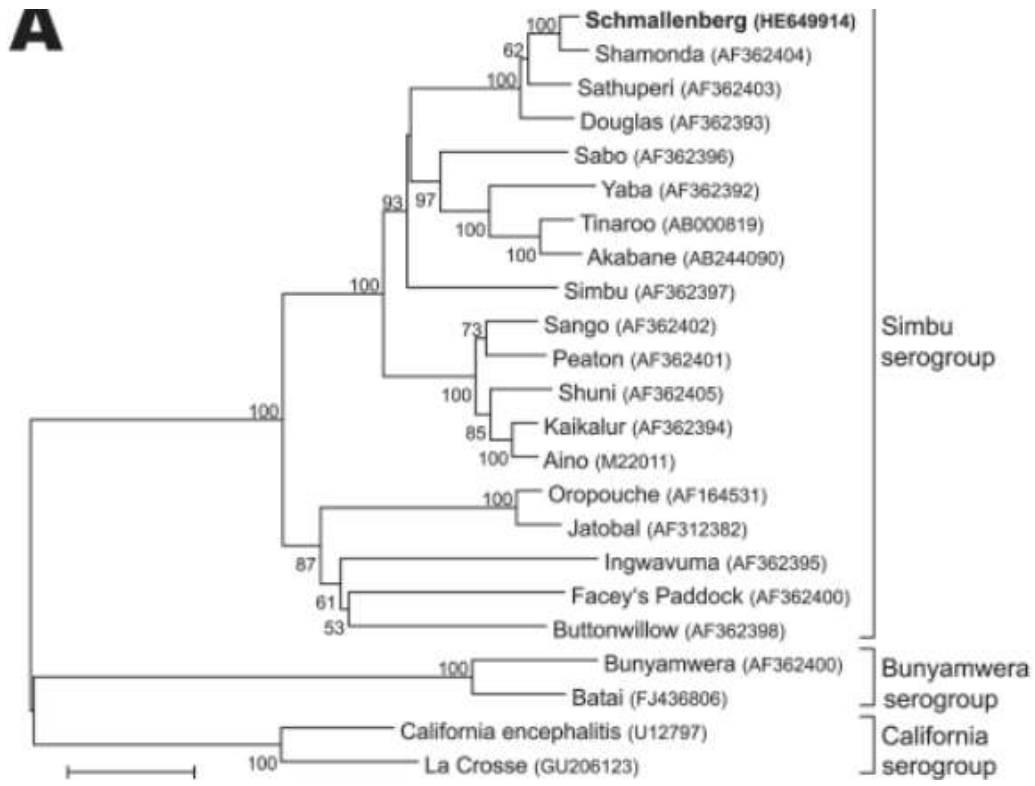


Figure 2: Phylogenetic relationship between Schmallenberg virus and *Orthobunyaviruse*s. Adapted from Hoffmann *et al.* (2012)**.**

2.2.3. Pathogenicity

SBV pathogenesis in ruminants is frequently similar to findings described for Akabane virus (AKV). Clinical studies have identified a pattern of malformations in Akabane outbreaks in cattle which suggest that the lesions in fetuses are dependent on the developmental stage of the fetus at the time of infection. Fetuses aborted at around 4–6 months of gestation are often the first indication of an outbreak, followed by dead, full-term fetuses with severe arthrogryposis (infected between 103 and 174 days of gestation), then live-born animals with less severe arthrogryposis but with neurological deficits due to hydranencephaly (infected between 79 and 104 days of gestation) (Kirkland, 2002). In comparison to Akabane virus, initial pathology reports indicated that SBV typically caused hydranencephaly, porencephaly, hydrocephalus, cerebellar hypoplasia and micromyelia (shortening of the spinal cord). Micromyelia particularly

affects the ventral horn of the spinal cord in calves. Histologically, affected animals displayed alymphohistiocytic meningoencephalomyelitis with glial nodules. Additional lesions included arthrogryposis, vertebral malformations and brachygnathia inferior, with myofibrillar hypoplasia of skeletal muscle in both calves and lambs (Herder *et al.,* 2012). As Varela *et al.* (2013) study indicated brain and spinal cord help for the Schmallenberg virus replication.

2.3. Epidemiology

2.3.1. Occurrence

It is unknown when and where SBV originated (Friedrich-Loeffler-Institut, 2015) and its emergence is the first detection of the Simbu virus serogroup in Europe (Hoffmann *et al.,* 2012). SBV was first detected in Germany in November 2011 and in the Netherlands in December 2011 (Hoffmann *et al.,* 2012; OIE, 2017). Spread of SBV from mainland Europe to Great Britain has been tentatively linked to natural movements of insects from infected areas, similar to the pattern of bluetongue virus in 2008(Tarlinton *et al.,* 2012). *Orthobunyaviruse*s in the Simbu serogroup are known in Africa, Asia, Australiawwa, and the Middle East (Tarlinton *et al.,* 2012).

As of March 2012, cases of Schmallenberg virus infection have been confirmed in Germany, the Netherlands, Belgium, France, the United Kingdom, Luxembourg, Italy and Spain. Reappearance of SBV happened in a sheep flock in Belgium (Claine *et al.,* 2013). Serum neutralization test of sample collected from October 2011 to June 2013 showed seroprevalence in the Republic of Ireland (IE) and United Kingdom (UK) (King *et al.,* 2015). In France the viral genome was detected by RT-qPCR of samples collected from malformed lamb's brain for the first time in north-eastern part of the country (Beer *et al.,* 2012; Doceul *et al.,* 2013).

In Austria Schmallenberg virus genome and antibodies were detected first in Autumn 2012, thus SBV persistently distributed in different European countries (Steinrigl *et al.,* 2014). Table 1 below showed serological and/or molecular evidence of the virus were available from several European countries including Belgium, France, Greece, UK, Italy, Spain, Luxembourg, Denmark, Poland, Sweden and Switzerland (Beer *et al.,* 2012); Turkey (Azkur *et al.,* 2013;

Tobank *et al.*, 2016) and China (Zhai *et al.*, 2018). In Lebanon the prevalence of SBV was reported for the first time in 2017 (Abi-rizk *et al.*, 2017). In Africa, the available research evidences on SBV are few; however, in Mozambique antibody detection has showed 43-97% in sheep, 72-100% in goats and 100% in cattle in September 2013 (Blomstrom *et al.*, 2014), in Tanzania seroprevalence has showed 61% (Mathew *et al.*, 2015) and in Ethiopia antibody detection of SBV has showed 56.6% (Sibhat *et al.*, 2018).

Table 1: Confirmed cases of Schmallenberg virus infections in Europe.

Holdings with confirmed cases						
Country	**Cattle**	**Sheep**	**Goats**	**Total**	**Date (2012)**	Adapted from
France	1544	1128	17	2689	31 July	ProMed-mail, 2012 France
Belgium	407	167	2	576	12 July	Claine *et al.*, 2013
The Netherlands	237	107	6	350	10 July	Beer *et al.* 2012
Luxembourg	6	6	-	12	2 April	ProMed-mail, 2012 Italy,Luxe
UK	53	220	3	276	13 August	King *et al.*, 2015
Italy	3	-	5	8	24 May	ProMed-mail, 2012 Italy,Luxe
Spain	-	1	-	1	13 March	ProMed-mail, 2012 Spain
Denmark	3	-	-	3	23 July	ProMed-mail, 2012 Denmark
Switzerland	21	-	-	21	14 August	Schorer *et al.*, 2012
Germany	877	866	19	1792	21 August	Beer *et al.* 2012
Total	3151	2495	82	5728		

The above report showed that SBV distributed within a short period in different European countries.

2.3.2. Transmission

Arthropod vectors particularly mosquitoes, phlebotoms, *Culicoides*, ticks and thrips can transmit the majority of *bunyaviruses*. The origin of Schmallenberg and its means of introduction to European domestic ruminants remain unknown. It is a vector-borne disease predominately

transmitted by *Culicoides* midges or mosquitoes (*Culicidae*). Biting midges in the genus *Culicoides* (Diptera: Ceratopogonidae) are like mosquitoes in that the females of nearly all species need a blood meal in order to develop eggs. These midges are smaller (1 – 3mm) than mosquitoes and can be much more abundant. More than a million blood-seeking females can be captured on a warm summer night with a single light trap near livestock. Although some European *Culicoides* species are notorious for their man biting habits most southern African species prefer to feed on animals and are night active and we can hardly notice them flying around (Venter, 2006).

Pools of midges, *C. obseletus* and *C. dewulfi,* tested positive for SBV via RT-PCR(De Regge *et al.,* 2012). *Culicoides* midges are widely distributed throughout the world in temperate and tropical climates. The spread of SBV through Europe has been rapid, similar to the 2006-2009 spread of bluetongue virus, which is also disseminated by a *Culicoides* vector. Midges have been trapped at several locations in Belgium as part of an ongoing blue tongue virus (BTV) surveillance programme. Returning to stored samples, SBV genetic material has been detected in three species of midge (*C. obsoletus, C. dewulfi and C. pulicaris*) trapped in September and October of 2011. That confirmed the transmission and spread of Schmallenberg virus was by two species of *Culicoides C. obsoletus* and *C. dewulfi* (Tarlinton *et al.,* 2012). There are similar reports of SBV genetic material in *C. obseletus* collected in September–November 2011 in Italy and in *Culicoides* spp. in Denmark in October 2011 (Doceul *et al.,* 2013): both countries have only reported single herds affected by SBV infection to date.

Schmallenberg virus do not transmit from animal to animal, it spreads by *Culicoides* midges. Its spread is intimately connected to the population of midges, which characteristically climax in late summer/early autumn and fall shrilly one time frosts start. The transmission of the virus can also occur during winter with reduced extent when low midge population. SBV could be excreted in semen but sexual transmission not confirmed yet (Lechner *et al.,* 2017). Vertical transmission, across the placenta, is also possible. Fetuses may be most vulnerable early in the pregnancy, as malformations to fetuses and newborns occur several months after an acute infection in the mother (Wernike *et al.,* 2015).

2.3.3. Risk factors

Insect Vectors (*Culicoides*)

Abundance of insect vectors (biting midges) specially Culicoids as Vectors of Schmallenberg Virus plays great role in transmission of disease of SBV (Rasmussen *et al.,* 2012). It is a vector-borne disease predominately transmitted by *Culicoides* midges or mosquitoes (*Culicidae*). Thus far, pools of midges, *C. chiopterus, C. dewulfi, C. imicola, C. obsoletus, C. pulicaris, C. punctatus,* and *C. scoticus,* tested positive for SBV via RT-PCR (De Regge *et al.*, 2012; Pages *et al.,* 2017; Rasmussen *et al.,* 2012). *Culicoides* midges are widely distributed throughout the world in temperate and tropical climates.

Climate Change

Climate change and the intensive growth of the global transportation systems which cause increase urbanization and conversion of land to agricultural use also make emerging and spreading of insect transmitted viruses (Kerstin and Beer, 2019).

Seasons

Seasonal breeding in sheep and differences in the vulnerable periods during which trans-placental infection lead to foetal malformations in cattle, goats and sheep (Beer *et al.,* 2012). The late summer/early autumn is the periods when the populations of midges naturally reach climax which is intimately connected to spread of the virus, but fall shrilly when frosts start. The transmission of the virus can also occur during winter with reduced extent when low midge population.

Physiological status and others

Studies by different authors have also showed that SBV might be affected by the type of farm, agroecology, herd size, breed, physiological status of the animals particularly pregnancy and age of animals (Claine *et al.,* 2013; De Regge *et al.*, 2013; Sibhat *et al.,* 2018; Steinrigl *et al.,* 2014).

If infection takes place between day 28 and 56 of gestation, it might result in birth of congenitally malformed or stillborn lambs (Helmer *et al.*, 2016).

2.4. Signs of the disease

The infected animals specially cattle shows high temperature, decreased milk production, in appetence, loss of body condition and diarrhoea (Hoffmann *et al.*, 2012). Clinical signs do not be observed on goats and sheep. During the Outbreak the disease present only for 2-3 weeks, thus if individual animals affected the disease persist only for 2-4 days(Friedrich-Loeffler-Institut, 2015; Tarlinton *et al.*, 2012). In pregnant animals the fetus affected by the virus after it crosses the placenta during a period of 60-180 days and 15-28 days in cattle and sheep respectively, thus result in malformation, deformities and abortion (Tarlinton *et al.*, 2012).

The effect of viral infection during early pregnancy is not obvious, but after it crosses the placenta it damages the nerve tissue of brain and spinal cord which result in muscle and skeletal problems, so the infected fetus born with malformation or deformed and aborted at the end (Helmer *et al.*, 2016). SBV infection may result in malformation of new born fetus with persistent flexion or fusion of joints and limbs, deformed brain and spinal cord damages. The period of infection during pregnancy influence fetal deformities; there are also fetus born normally with some nervous problems like unable to see, unable to suck, unable to stand, ataxia and spasm (Helmer *et al.*, 2016). SBV infection during twin pregnancy also result in arthrogryposis for one and neural abnormalities for the other twin or one malformed and the other normal or with slow growth (Doceul *et al.*, 2013).

2.5. Diagnosis

Diagnostic procedures for the detection of SBV infections became available very soon after the discovery of the virus and were rapidly distributed to the infected areas. SBV diagnosis can be done by genomic and antibody detection of the virus as indicated in Figure 3. They include:-

Antibody detection

Neutralization tests

The Serum Neutralisation Test (SNT) is a serological test used to detect the presence of functional antibodies that prevent infectivity of a virus. The SNT is the best in detecting SBV antibodies and set it as a non-reference standard for further testing on the field samples (Loeffen *et al.,* 2012). There, it was demonstrated that the SNT could serve as the standard test and had outstanding diagnostic performance, sensitivity and specificity (Bréard *et al.,* 2013; Pejaković *et al.,* 2018). It was validated with the first virus isolate within a few weeks of SBV detection (Beer *et al.,* 2012).

Blood or colostrums samples were centrifuged at 1,100xg for 25 min at room temperature. Colostrum's lipid layer was removed to isolate lacto-serum. Serum samples were heat inactivated. Lacto serum samples were 12 times two-fold diluted in Minimum Essential Medium, starting at ½ in 96-well plates. Approximately100–200 TCID50 SBV (isolate SBV-BH80/11–4) was then added to each diluted serum before overnight incubation at 37°C. Results of VNT were expressed as the effective dilution neutralizing 50% of the challenge virus (ED50) and were considered positive if log2 ED50 was<3.49 (Claine *et al.,* 2018). There are two advantages of viral neutralization test, it carry out on sample from all animals and it gives quantitative results (Mansfield *et al.,* 2013). The weakness of viral neutralization test is that it takes much time about 6 days (Bréard *et al.,* 2013).

Indirect immunofluorescence

Indirect immunofluorescence assay is a laboratory test used to detect antibodies in serum or other body fluid. The specific antibodies are labeled with a compound that makes them glow an apple green color when observed microscopically under ultraviolet light. This test can be carried out on virus infected BHK-21 cells and is the least sensitive for antibody detection of schmallenberg virus (Bréard *et al.,* 2013).

Enzyme linked immunosorbent assay (**ELISA**)

Enzyme linked immunosorbent assay (ELISA): commonly used analytical biochemistry assay that uses a solid-phase enzyme immunoassay (EIA) to detect the presence of a protein in a liquid

sample using antibodies directed against the protein to be measured. ELISA has been used as a diagnostic tool in medicine, plant pathology, and biotechnology, as well as a quality control check in various industries. In the simplest form of an ELISA, antigens from the sample are attached to a surface. Then, a matching antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change. It allowing mass screening available within about 5 months of first virus isolate; a first commercial SBV antibody ELISA has been in use in several countries since May 2012 (Beer *et al.,* 2012). These techniques allowed the unambiguous diagnosis of SBV infections in malformed neonates by PCR or demonstration of precolostral antibodies with high sensitivity and specificity.

The short viraemia limits the use of RT–PCR for the detection of SBV infections in adult animals to the acute phase of the infection. The sensitivity is highest in animals presenting with fever. The ELISA tests were able to detect diseased population in higher seroprevalence, from 20–99%, using a higher number of animals tested compared to SNT. However, for low theoretical seroprevalence, from 1–10%, the ELISA tests performance was inadequate to calculate a number of sheep needed to test positive to determine infected flock, which would be of importance in SBV re-emerging situations (Pejaković *et al.,* 2018). As showed by Pejaković *et al.* (2018) ID.Vet ELISA test, with almost perfect agreement with SNT, could be used as an appropriate substitution for Serum Neutralization Test.

Detection of viral genome

Real-time reverse transcriptase (RT)–qPCR

Presently viral genome can be detected by Real-time qPCR by means of specialized PCR machines with high sensitivity and specificity providing quantitative analysis with low contamination risk (Aebischer *et al.,* 2019). For SBV genome detection it was implemented and preliminary validated within days; validated commercial kits available after about 3 months(Bilk *et al.,* 2012), RT-qPCR used was invented for the first time at the Friedrich Loeffler Institute (Hoffmann *et al.,* 2012) and carried out on RNA genome extracted from serum. The result of

RT-qPCR articulated by cycle threshold that positive at Ct < 40 as indicated by Claine *et al.*, (2018).

It is generally accepted that the detection of viral RNA using rt-RT PCR presents the most reliable option in confirming acute SBV infection. However, due to the destruction of viral RNA (depending on the time of the sampling or the transport and storage conditions), short time of viremia or missing clinical signs in male animals, the clinical case number could be underestimated (Claine *et al.*, 2018). Results obtained in Belgium showed the presence of antibody while there is no viral genome of SBV (De Regge *et al.*, 2013). In Denmark genome of SBV was detected by RT-qPCR from pools of *Culicoides* midges collected in October 2011 (Rasmussen *et al.*, 2012).

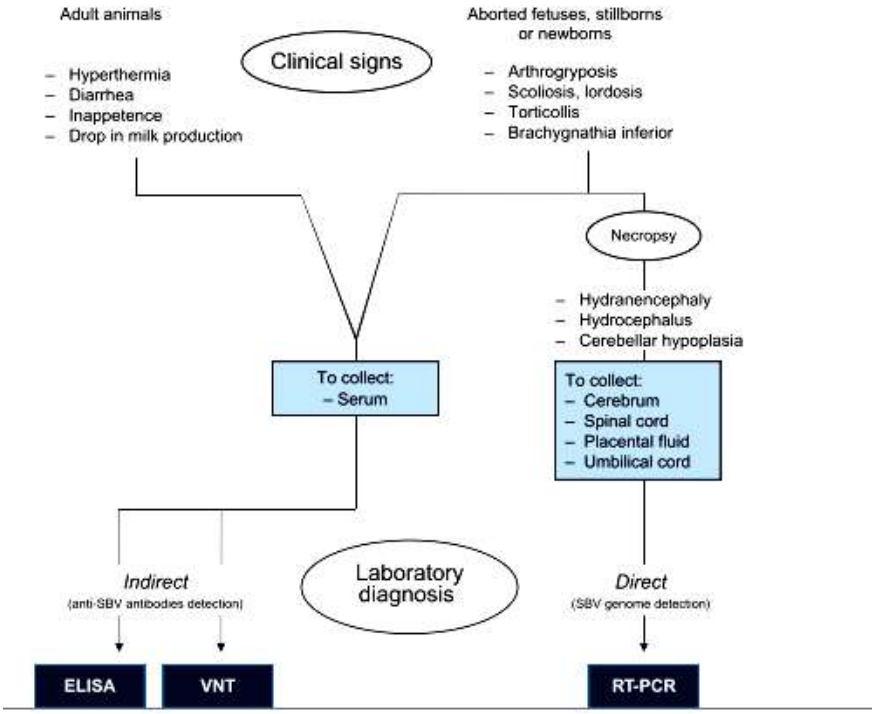


Figure 3: Schmallenberg Virus diagnosis.

Adapted from Claine *et al.* (2015).

2.6. Economic Importance

As many studies showed the prevalence of congenital malformations associated with SBV infection in naïve herds appears to be much lower than that of AKAV and has a low or limited impact on animal health (Collins *et al.,* 2018). From Poskin *et al.* (2017) report up to 4% of calves and 19% of lambs were aborted, stillborn or deformed in Belgium due to SBV infection during the 2011–2012 SBV epizootic. In contrast, the incidence of AKAV lesions in affected herds can be very high with up to 50% of calves and 80% of lambs malformed, respectively (Kirkland, 2002).

Schmallenberg virus causes low impact of health and production at herd level, but it differs on overall meat and milk industries due to restriction of import of these products from SBV declared countries which are affected by important fiscal impact (Claine *et al.*, 2015). Milk loss was the most frequently reported clinical sign and was observed in 90.2% of clinically affected animals, followed by diarrhoea 83.2%, fever 58.2% and abortion 11.9% (Lechner *et al.*, 2017). If new born animals and fetal infected with SBV during pregnancy, it result in congenital abnormality and malformation (Claine *et al.*, 2018; Phil Scott, 2019).

2.7. Control and Prevention of the Disease

SBV disease does not have specific treatments. Deformed fetus should be removed by caesarean operation, if deformed fetus born alive should be euthanized. As indicated by agri food and bioscience institute, (2018) commercial vaccines against the Schmallenberg virus are also available for use in cattle and sheep. Control of midge populations during the vector time even though it is difficult with high population. Hygienic calving to prevent contamination and it is important to breed animals during low midge population (OIE, 2017). Shifting or restricting the timing of mating in cattle and sheep to outside of the vector active season (Phil Scott, 2019). Clearly transporting naïve animals to endemic areas for mating or during pregnancy is to be avoided.

3. MATERIAL AND METHODS

3.1. Study Area

The current study was conducted from October, 2019 to May, 2020 in different parts of Ethiopia. These study areas were located mostly in central parts of the country, in administrative zones of Oromia Regional State, and were selected based on the presence of livestock, a large number of *Culicoides,* and the central milk sheds in which prior study showed a seropositive to SBV. *Culicoides* were collected from Adama, Bishoftu, Holeta, Jimma and Sebeta in some milk shed areas of the country as shown in Figure 4. Seroprevalence of Schmallenberg virus has been studied from these sites on sample collected for Bluetongue Virus outbreak in dairy farm (Sibhat *et al.,* 2018).

Adama town is one of the districts in the East Shoa zone of Oromia regional state, Ethiopia. The total livestock population of Adama districts based on the report of district's agency is estimated to constitute 40,150 cattle, 24,700 sheep, 26,844 goats, and 9,092 equines and 552,980 poultry. There are also many livestock sector activities going in area like fattening, poultry farming, and dairy farming. *Culicoides* were collected from the dairy farms pen which is located at 1718 elevation, 8.568708N, and 39.276272E.

Bishoftu town is one of the districts in East Shoa zone of Oromia regional state. Its topography is undulating and characterized by flat land on the North and East parts of the city, locked by several lakes, while the South is dominated by hills. The total livestock population of Bishoftu districts based on the report of district's agency is estimated to constitute 172,256 Cattle, 54,162 sheep, 42,725 goats, and 52,594 equine and 303,958 poultry. It is a place where poultry production and dairy farming is highly practiced. Data were collected from the dairy farms located at an elevation of 1885-1905, 8.762258- 8.780658N, and 38.988732- 38.996563E.

Holeta is located in the Oromia Special Zone Surrounding Addis Ababa. The area is special source of milk for Holeta and Addis Ababa as it has a high potential dairy farm. The total livestock population of Holeta districts based on the report of district's agency is estimated to constitute 156,780 Cattle, 48,924 sheep, 41,262 goats, and 38,640 equines and 254,760 poultry.

Data for this study were collected from the dairy farms at an elevation of 2341-2436, 9.0511-9.0735N, and 38.487-38.499 E.

Jimma is the largest city in south-western Oromia. It is a special zone of the Oromia Regional state and is surrounded by Jimma Zone. The total livestock population of Jimma, based on the report of district's agency is estimated to constitute 53,250 Cattle, 25,230 sheep, 12,570 goats, and 10,030 equines and 101,057 poultry. Data for this study were collected from the dairy farms at an elevation of 1690-1726, 7.662564- 7.694192N, and 36.819065- 36.834049E.

Sebeta is a town located in the Oromia Special Zone surrounding Addis Abeba. The town is the most active as it is located on the road between Addis Abeba and Jimma and Buttajira. In addition to agriculture the area is known for dairy farm production. The total livestock population of Sebeta districts based on the report of district's agency is estimated to constitute 54,280 Cattle, 29,482 sheep, 20,456goats, and 9,456 equines and 92735 poultry. The data for this study were also collected from the dairy farm located at an elevation of 2057- 2144, 8.802922- 8.898135N, and 38.504238- 38.890276E.

Study area

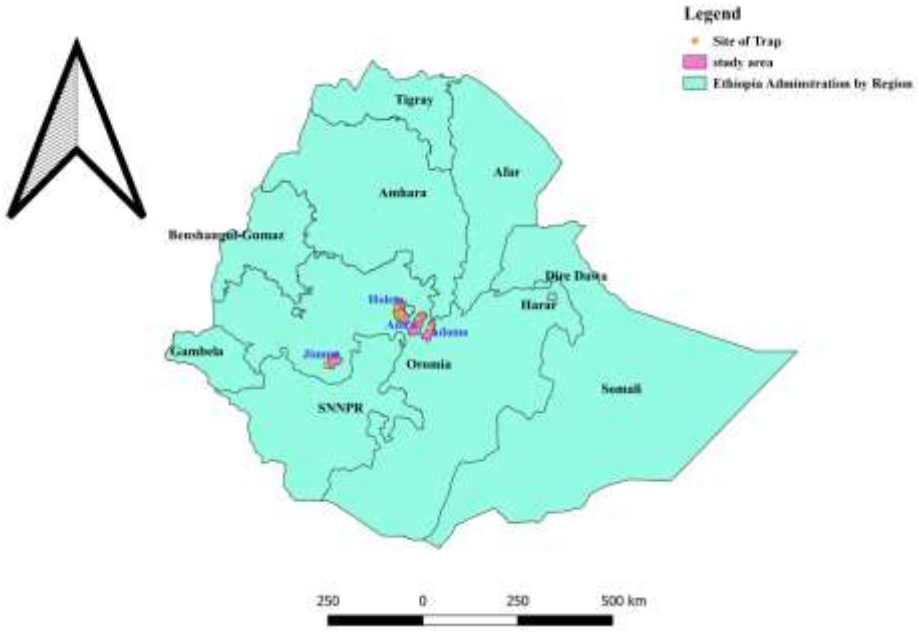


Figure 4: Map of the study areas

3.2. *Culicoide* Collection and Identification

Culicoides trapping was performed from October, 2019 to May, 2020 time period. *Culicoides* were collected at each collection site which was conducted in every zone. On each sampling day, ultraviolet light onderstepoort type (OVI) 220 V down draught suction light traps each fitted with 8 W black fluorescent (Ultraviolet) light tubes and the power for light was fed by 12volt batteries. The traps were suspended at a height of 1.4 to 2 m above the ground and set in close proximity to animals where there was suspected as a potential for breeding and feeding sites for *Culicoides.* Each UV light trap (OVI trap) was run overnight in the vicinity of livestock set up from approximately 1 h before sunset to 1 h after sunrise. Collected *Culicoides* were transported to Jimma University, College of Agriculture and Veterinary Medicine, NAHDIC, and Addis Ababa University, College of Veterinary Medicine and Agriculture, parasitology laboratory. Then, *Culicoides* were collected using plastic jar suspended below the trap's fan and put in deepfreeze (-20°C) for 15 minutes to kill *Culicoides.* Then, sorting and identification into genus and species levels were made by using Interactive Identification Key for Palaearctic Biting Midge *Culicoides* (Diptera: Ceratopogonidae) under the aid of a stereo light microscope and with the assistance of entomology expert from NAHDIC. The *Culicoides* were pooled in to cryovial tube from 50-160 *Culicoides* per pool. It was preserved in liquid nitrogen (-196°C) and -80°C deep-freezer till processed.

Culicoides identification

Species identification and counting were carried out by observing morphological features under a stereomicroscope. Most *Culicoides* midges have a wing pigmentation pattern and the distribution of wing macrotrichia that is composed of grey and white spots; these patterns are unique to each species and can be easily observed under a dissecting microscope. Then, we observed the antennal XI/X ratio (length of segment XI divided by length of segment X), and the shape and size of the 3rd palpal segment. Finally, we compared all observed traits with IIKC (interactive identification key for *Culicoides*) database pictures (Mathieu *et al.,* 2012). Slide mounting was performed in the cases of morphologically similar species, atypical variations of the wing pattern, identification confirmation, or if the specimen was damaged. Slide mounted specimens those

observed under a light microscope were morphological features like shape, size, and the number of female spermathecae and eye separation distance (Mathieu *et al.*, 2012).

3.3. *Culicoide* Processing

Culicoides from each catch collection site were processed at National Veterinary Institute of Ethiopia (NVI) laboratory for viral detection. The *Culicoides* samples (50-160) pools were grounded and homogenized in bio-safety level II laboratory at NVI. *Culicoides* were grounded using sterile mortar and pestle by adding 0.5ml of sterile phosphate buffered saline containing antibiotic. The supernatant was harvested after centrifuged at 12,000 rpm for 10 minutes and stored in a 2 ml cryovial at -20°C for further testing.

3.4. Viral Extraction

RNA was extracted using the QIAamp Viral RNA Mini Kit for nucleic acid extraction (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions (Annex-I). Briefly, 140 µl *Culicoide* samples were homogenized with 560 µl of AVL buffer by centrifuging for 3 min at maximum speed. Then add 560 µl of ethanol to the sample mix and centrifuge. From these up to 630 µl of the sample transferred to a QIAamp Mini column placed in a 2 ml collection tube, and centrifuge for 1min at ≥8000 rpm; then flow-through discarded. About 500 µl AW1 Buffer added to the QIAamp Mini column and each centrifuged for 1min at ≥8000 rpm, the flow-through was discarded. Another 500 µl AW2 buffer was added and centrifuged for 3min at ≥14,000 rpm. Finally, QIAamp Mini column placed in a new 1.5 ml collection tube, and then 60 µl of buffer AVE equilibrium was added directly to the spin column membrane, and centrifuged for 1 min at ≥8000 rpm to elute the RNA. Eventually, viral RNA extracted was immediately stored at -20ºC until amplification.

3.5. Real-time PCR and Visualization of RT-PCR Products

SBV genome detection in *Culicoides* pools was conducted using real-time RT-PCR (RT-PCR) that primers and probe sequences were from Germany. RT-PCR amplification was performed

using specific primers (forward primers SBV-L-F: 5'-TTG CCG TTT GAT TTT GAA GTT GTG-3' and reverse SBV-L-R: 5'-TCA GGG ATC GCA AAT TAA AGA ACC-3') and specific probe (SBV-L-FAM: 5'-TCA TCC GTG CTG ACC CTC TGC GAG-3') targeting the region of L-segment of the viral genome according to Fischer *et al.,* (2013). Reactions were performed on a CFX96^{TM} C1000 Touch Thermal Cycler Real-Time PCR Detection System (Bio Rad, Hercules, CA, USA). Briefly, for one reaction 10 µl 5x RT-PCR buffer, 1.0 µl RT-PCR enzyme mix, 3 µl RNase-free water, 1 µl 10mM dNTPs mix, 5 µl RNA template or RNase free water for the no template control (NTC) was added and 2 µl SBV-specific primers each, 1 µM SBV-specific probes) solution was prepared as a master mix. The region of interest was amplified by 10 min at 45°C, 10 min 95°C, followed by 45 cycles of 15s at 95°C and 45s at 60°C (De Regge *et al.*, 2012; Fischer *et al.,* 2013) .

3.6. *Culicoides* Distribution Modeling

3.6.1. Potential Predictors of SBV Vector Culicoides Occurrence

Factors which may influence the number of *Culicoides* specimens include the presence of breeding sites and other light sources near the light trap, the height of the trap above ground level, wind-speed, the phase of the moon, and even the tides. Climatic conditions such as temperature and wind velocity, rainfall, relative humidity, and the age of the population during the trapping night may also influence the numbers of *Culicoides* midges collected (Venter, 2006).

The distribution, abundance, and seasonal occurrence of *Culicoides* are determined by the availability of moisture-rich habitats that are essential for the developments of immature stages egg, larval, and pupal forms. All *Culicoides* species only breed in moist low-lying areas. The basic requirements are moisture and a medium containing organic matter. As described by Mellor *et al.,* (2000) *Culicoides* species may breed in situations varying from those which are almost aquatic, e.g. pond margins; to those where no free water is present but the humidity is close to 100%, e.g. interior of dung pads of large animals, decomposing fruit, tree-holes, plants and rock activities. By the current study four species of *Culicoides* had identified, but only *C. imicola*

could be a vector of Schmallenberg Virus as stated by Pages *et al.,* (2017) that transmit the virus experimentally.

For this study ensemble modeling method identified suitable area for *C. imicola* by using R package biomod2 (Thuiller *et al.,* 2009). The models join factors like livestock distribution, land cover and climatic to forecast the national allocation of *C. imicola* describing the particular involvement of different factors. Geo-positioned presence of *C. imicola,* environmental and livestock data were used to build the exact forecast distribution of *C. imicola.*

Climatic Factors: The survival of a given vector/species and thereby their geographical distribution is influenced by climatic and other environmental features. Temperature, rainfall, Solar radiation (Cianci *et al.,* 2015), wind speed (Dom *et al.,* 2013; MØLLER, 2013), and water vapor pressure (Lucio *et al.,* 2013) influence the existence of various insect's lifecycle. The WorldClim database obtained from (http://worldclim.org/) were used to explain climatic impact on distribution of *C. imicola.*

Data of Land cover: - Land cover data used in this study were obtained from the European Space Agency's GlobCover Portal (http://due.esrin.esa.int/page_globcover.php) which is the most up to date GlobCover v2009 that denote 22 classes (Defourny *et al.,* 2011), at a high resolution (300 m).

Livestock data allocations were obtained from website of (http://www.fao.org/livestock-systems/) to describe happening of vectors. Gilbert *et al.,* (2018) reviewed livestock dataset of 2010 of the world at global extent of 5 minutes of arc (~10 km2) resolution.

Occurrence of *Culicoides* :-Worldwide point of existence of *C. imicola* indicated by Leta *et al.,* (2019a), the national occurrence from the southern part of Ethiopia by Fetene, (2019) and the central part of the country by the current study, were incorporated in this research. About 1140 points of existence of *C. imicola* were identified across the world of which 101 geo-positioned occurrences of *C. imicola* were identified in Ethiopia. Therefore, 1140 thinned existence of *C.*

imicola accounts were used for ensemble modeling in this paper. Finally, the spatial modeling of *C. imicola* of Ethiopia was clipped by mask from the global model.

3.6.2. GIS Operation

In the current study various explanatory variables had different spatial resolutions incorporated in modeling. However, for modeling in R using 'biomod2' all GIS layers have to share the same projection system, extent and resolutions. Hence, by using Quantum geographic information system (QGIS Desktop 3.4.5 software) tools include clip and raster calculation all raster layers were rescaled to have the same extent and resolution. Finally, all layers of global have clipped by mask to Ethiopia context (3.0°, 15.0°N: 32.0°, 48.0°E) with the resolution of 5arcminutes and the same coordinate system (EPSG: 4326 - WGS 84 - Geographic) and GeoTIFF (*.*tif*) raster format.

Analysis of Variance of inflation factor were used to test multicollinearity between descriptive variables by means of "*vifstep"* in "*usdm"* of R package (Naimi, 2015; Naimi *et al.*, 2014). Based on multicollinearity test result only those with less than or equal to 10 VIF value were included for analysis (Craney and Surles, 2002) by stepwise selection practice. The larger the value of VIF, the more collinear the variables indicate multicollinearity. As a rule of thumb, if the VIF of a variable exceeds 10, which will happen if multiple correlation coefficients for a variable R^2 exceed 0.90, that variable is said to be highly collinear. Calculates variance inflation factor (VIF) for a set of variables and exclude the highly correlated variables from the set through a stepwise procedure. The final ensemble model out was also plotted and mapped with using QGIS software.

3.6.3. Modeling Approach

The model developed using sampling points (geo-referenced data) and different climatic and environmental data. In this study the '*biomod2'* package of R software were used to estimate distributions of *C. imicola* by ensemble species distribution modeling. To forecast allocation of

Culicoides and to describe involvement of different factors the ensemble model joins livestock distribution, land cover and climatic factors.

Ten diverse algorithms models used in developing 'biomod2' package were artificial neural networks (ANN), classification tree analysis (CTA), flexible discriminant analysis (FDA), general additive models (GAM), general boosted models (GBM), general linear models (GLM), maximum entropy (MAXENT), multiple adaptive regression splines (MARS), random forests (RF), and surface range envelope (SRE) (Thuiller *et al.,* 2009). All of these ten methods require the presence and absence results to decide the range of suitability for *C. imicola.* In our case there was no absence report, but Surface Range Envelope (SRE) model were used to generate pseudo-absence data (Thuiller *et al.,* 2009).

The data was evaluated by dividing in to two portions; calibrated the model as training data and another portion was validated the predictions as testing data. The model predictive score was evaluated by using a calibration (training) subset of 80% of the input data set and an evaluation (testing) subset of 20%. The models' performance were assessed by means of true skill statistic (TSS), receiver operating characteristics (ROC), and Cohen's kappa statistics (Kappa) (Allouche *et al.,* 2006; Shabani *et al.,* 2018). A total of 30 models were performed from 3 runs (10 modeling methods × 3 folds), from which the average values of TSS, Kappa and ROC were taken. The three evaluation metrics were run giving 30 models (10 models x 3 folds), thus the average value of ROC, Kappa, and TSS of the cross-validation was made. TSS valued from −1 to 1, +1 indicate perfect agreement between predictions and observations and values of 0 or less shows agreement no better than random classification (Allouche *et al.,* 2006).

The model performance evaluation was performed in each run for species distribution; as a result averages of the runs were taken as report. Only modeling algorithms with an average TSS score above 0.8 were included in the subsequent ensemble modeling procedure (Allouche *et al.,* 2006; Shabani *et al.,* 2018). This ensures that the individual models used to build the ensemble models were the best suited and most accurately predicted occurrences. The model techniques passing the TSS test were used in the final modeling steps using the full data set. The resulting models are referred to as "full models" as they use all the presence and absence points and not just the

80% as the evaluation models. TSS was only calculated for the evaluation models, as the full models do not contain a data split and, therefore, cannot be evaluated by TSS. After running all steps in the model development, the model projected and ensemble for the potential distribution of the species. An ensemble model of *C. imicola* distribution was combining all excellent models or mixed algorithms which help to overcome uncertainty in model selection. The models' mean, coefficient of variation (CV) and committee averaging (CA) evaluated with high predictive distribution model were combined to build ensemble model for species distribution.

3.7. Clearance

The ethical review committee of College of Veterinary Medicine and Agriculture of Addis Ababa University has approved and given Ethical clearance (Annexes II) for collecting *Culicoides* samples from central parts of the country. Permission from the district and respective village authorities for the study was obtained prior to study.

4. RESULTS

4.1. *Culicoides* Survey

A total of 6356 adult *Culicoides* midges (752; 12% in Ada'a, 152; 2% in Adama, 1460; 23% in Holeta, 3482; 55% in Jimma and 504; 8% in Sebeta) were captured during study period between October, 2019 and May, 2020 from the selected twenty-one trap sites. From the captured *Culicoides* midges 4424 (70% of total caught) were pooled for RNA extraction and 1932 (30% of total midges caught) were sorted *C. imicola* (32%), *C. kings* (30%), *C. deltus* (4%), *C. milnei* (13%), and not identified (20%). Their distribution however was very different from site to site. All traps indicated that *C. imicola* is the most abundant *Culicoides* species and it was the only common species that were collected in all collection sites. All species of *Culicoides* were collected from the Holeta district. The identified *Culicoides* species and their percentage out of the sorted species are shown in Table 2 below.

Table 2:- *Culicoides* Species identified in five districts of different parts of Ethiopia and their percentage out of the sorted species

	Districts						
Spp	Ada'a	Adama	Holeta	Jimma	Sebeta	Tota	%
C. imicola	354(17.3%)	158(7.7%)	548(26.7%)	843(41.1%)	148(7.1%)	**2051**	32
C. kings	171(8.9%)		332(17.2%)	1270(65.8%)	156(1.5%)	**1929**	30
C. deltus			75(27.3%)		200(72.7%)	**275**	4
C. milnei			289(35.2%)	533(64.8%)		**822**	13
unidentified	227(17.7%)		216(16.9%)	836(65.4%)		**1279**	20
Total Culicoides	**752**	**158**	**1460**	**3482**	**504**	**6356**	
%	12	2	23	55	8		

4.2. Viral Detection

SBV genome detection was conducted, a total of 29 pools of *Culicoides* midges containing 50-160 *Culicoides*/pool were examined using real-time RT-PCR, of which none of the *Culicoides* midges were positive for SBV genome as shown in Figure 5 below. The ct-value showed by the light cycler was negative since there is no signal or product amplified in real time PCR.

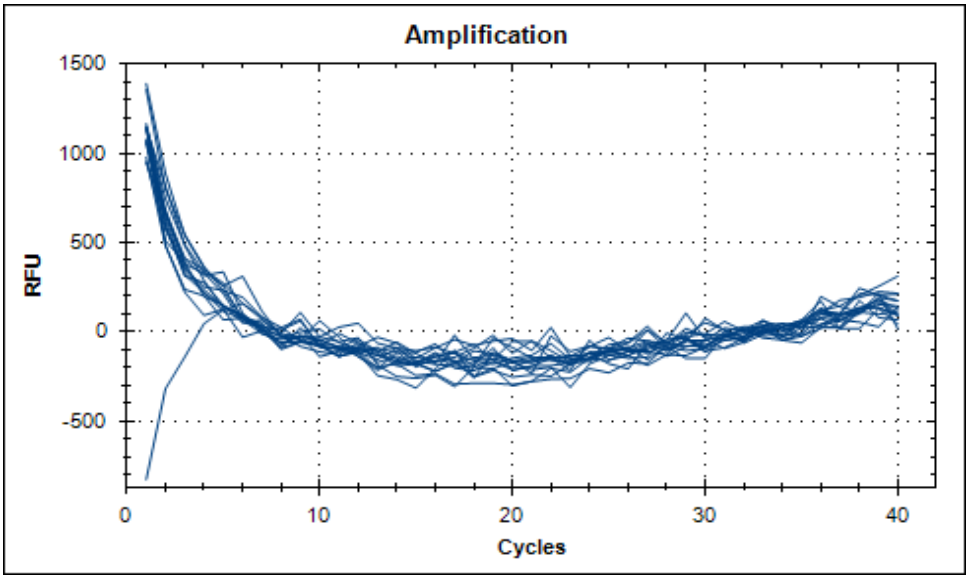


Figure 5:- real-time RT-PCR result of SBV genome detection for the *Culicoide's* pool

4.3. SBV *Culicoides* Distribution Model

4.3.1. Model Performance and Importance of Environmental Variables

Thirty individual models were developed initially thus individual model performance evaluation of 27 models had ROC > 0.90 ($ROC_{average}$= 0.955), according to Swets, (1998) classification this could be measured as good accuracy. Of all ROC performance only SRE model accounts the smallest accurate, while RF account for the most accurate on average.

Of 30 models, 24 had TSS > 0.80 ($TSS_{average}$ = 0.837) and KAPPA>0.80($KAPPA_{average}$=0.838), according to LBOUY *et al.*, (2010) categorization this measured as excellent accuracy. The models with (TSS > 0.80 and KAPPA>.80) were combined to form ensemble forecasting of *C. imicola*. The model developed performed very well (ROC =0.999, TSS=0.898 and KAPPA=0.898).

Table 3:- Average Performance of individual Models

performance evaluator	RF	GAM	GLM	GBM	CTA	ANN	SRE	FDA	MARS	MAXENT. Phillips
KAPPA	0.89	0.87	0.83	0.88	0.85	0.77	0.77	0.84	0.85	0.82
ROC	0.99	0.98	0.97	0.98	0.95	0.93	0.88	0.97	0.98	0.91
TSS	0.89	0.87	0.83	0.88	0.85	0.77	0.77	0.84	0.85	0.82

The contribution of each variable in all ten models was stable for variables that were accepted as good predictors. The overall reviews of variable contributions were described in each model. The distribution of *C. imicola* were extensively determined 49.5% by mean annual minimum temperature, 23.6% by mean annual maximum temperature and 20.1% by solar radiation for individual and ensemble models. Wind (5.1%) and land cover (3.9%) with fewer determinants to models. The table 4 below shows contribution of variables in the models.

Table 4:- Variables Contribution in the models

Variables	RF	GAM	GLM	GBM	CTA	ANN	SRE	FDA	MARS	MAXENT. Phillips	Overall contribution
Tmin	22	70	65	24	43	76	28	74	69	23	49.5%
Tmax	3	35	41	3	1	42	31	34	28	18	23.6%
Srad	14	24	31	10	15	21	30	18	18	19	20.1%
Vap	13	8	7	8	28	44	31	15	3	9	16.6%
Livestock 5km	25	1	0	29	32	29	30	0	9	10	16.4%
Prec	7	19	13	8	15	12	17	17	13	18	14.0%
Wind	2	16	3	1	2	1	15	1	0	11	5.1%
LandCover.Nodata_Value	1	21	0	0	0	7	7	0	0	3	3.9%

Key: ANN: Artificial Neural Network; CTA: Classification Tree Analysis; FDA: Flexible Discriminant Analysis; GAM: Generalized Additive Model; GBM: Generalized Boosting Model or usually called Boosted Regression Trees; GLM: Generalized Linear Model; MAXENT.Phillips: Maximum Entropy; MARS: Multiple Adaptive Regression Splines; RF: Random Forest; SRE: Surface Range Envelop or usually called BIOCLIM.

Those models which met the inclusion criteria for TSS > 0.8 were integrated to develop ensemble model (these models were CTA, FDA, GAM, GBM, GLM, MARS, MAXENT.Phillips and RF models). Based on importance of variables in the ensemble model 39.9% were accounted by mean annual minimum temperature, 12.4% by mean annual maximum temperature, 12.4% by radiation, 9.7% by precipitation, 6% by livestock distribution, 4.3% by pressure, 0.7% by speed, and 0.2% by land cover.

Table 5:- Ensembel Variable Importance

Variables	EMmean	EMca
Tmin	0.399	0.403
Tmax	0.124	0.148
Srad	0.124	0.153
Prec	0.097	0.111
Livestock 5km	0.06	0.06
Water vapour pressure	0.043	0.56
Wind speed	0.007	0.011
Land Cover Nodata value	0.002	0.003

Key: Land cover = Land cover type, Livestock = Livestock population (livestock population/5 arc minute), Tmax = Mean annual maximum temperature (°C), Tmin = Mean annual minimum temperature (°C), Prec = Mean annual precipitation (mm/year), Srad = Solar radiation (kJ m^{-2} day^{-1}), Vapr = water vapor pressure (kPa), Wind = wind speed (m s−1).

4.3.2. Ensemble Models

The above ensemble model incorporated to develop a suitability map of *C. imicola.* The suitability map (Figure 6) estimated the mean probability of *C. imicola* in Ethiopia. The vector estimated to occur in different parts of Ethiopia and the predicted occurrence of *C. imicola* differs from place to place. The ensemble model of clipped Ethiopia from the global ensemble model showed most parts of Ethiopia is suitable for *C. imicola* which could be a vector for SBV. The range of suitability differs from zone to zone in that high probability of suitability occurrence in Borena, Bale, East Hararge, East Shewa, North Shewa, West Shewa, Hadiya, North Wollo, Southern Tigray, Mekele, and Eastern Tigray. The intermediate probability of predictive occurrence shown in South Omo, Gamo gofa, Wolayita, East wollega, Awi, West Gojam, Afar zone 3, 4 &5, and Dege Habur. The low suitability areas Benchimaji, Shaka, Kafa, Ilu Ababor, Southern Somali, Welwel and Wader, Shinille, West Tigray, North Gondor, Awi, Metekel, Gambela zone 1&2.

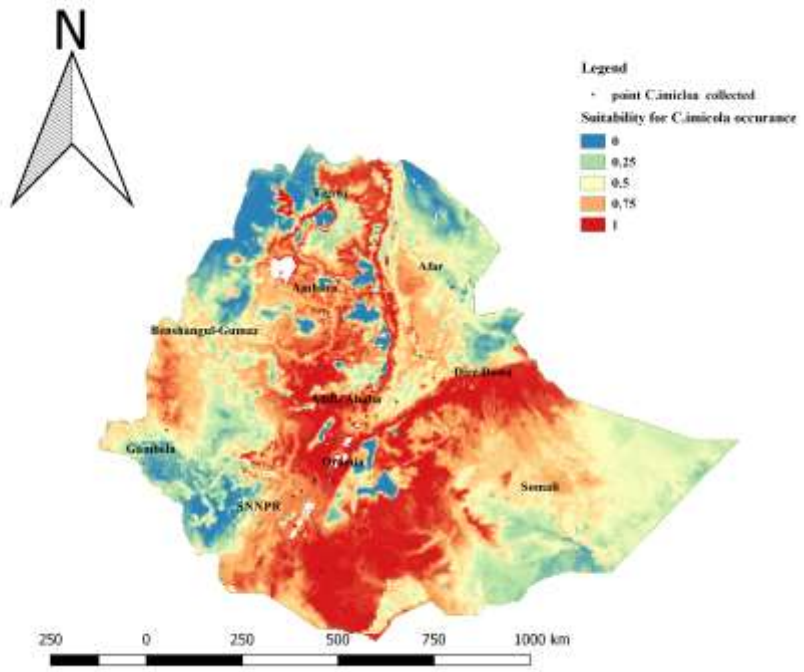


Figure 6: *C. imicola* Prediction value.

The warmer shows most fitting, while the cooler shows less suitable environment.

Figure 7 shows the 'clamping mask' value which showed how predictive occurrence deviates from the mean probability of occurrence. This map was the complement of the mean probability of *C. imicola* presence in the area. The 'clamping mask' indicated in the following Figure 7 below showed uncertainty within various regions.

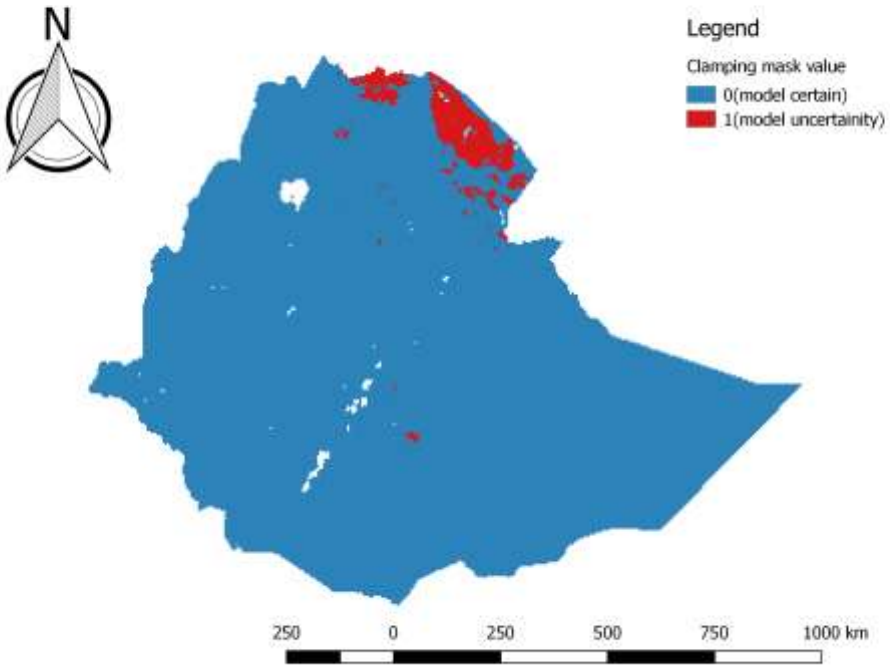


Figure 7: Coefficient of Variation 'clamping mask'.

Uncertain indicated by red

The committee averaging model described in Fig. 8 below indicates measure of uncertainty and a prediction. In this model the presence and absence areas for *C. imicola* were selected from each model. It was developed by measuring both prediction of *C. imicola* occurrence and uncertainty on *C. imicola* observation. Afar Zone 1&2, and North East Tigray are areas were high

uncertainty observed from Ethiopia. This is because the values of the variables are outside the range used for calibration.

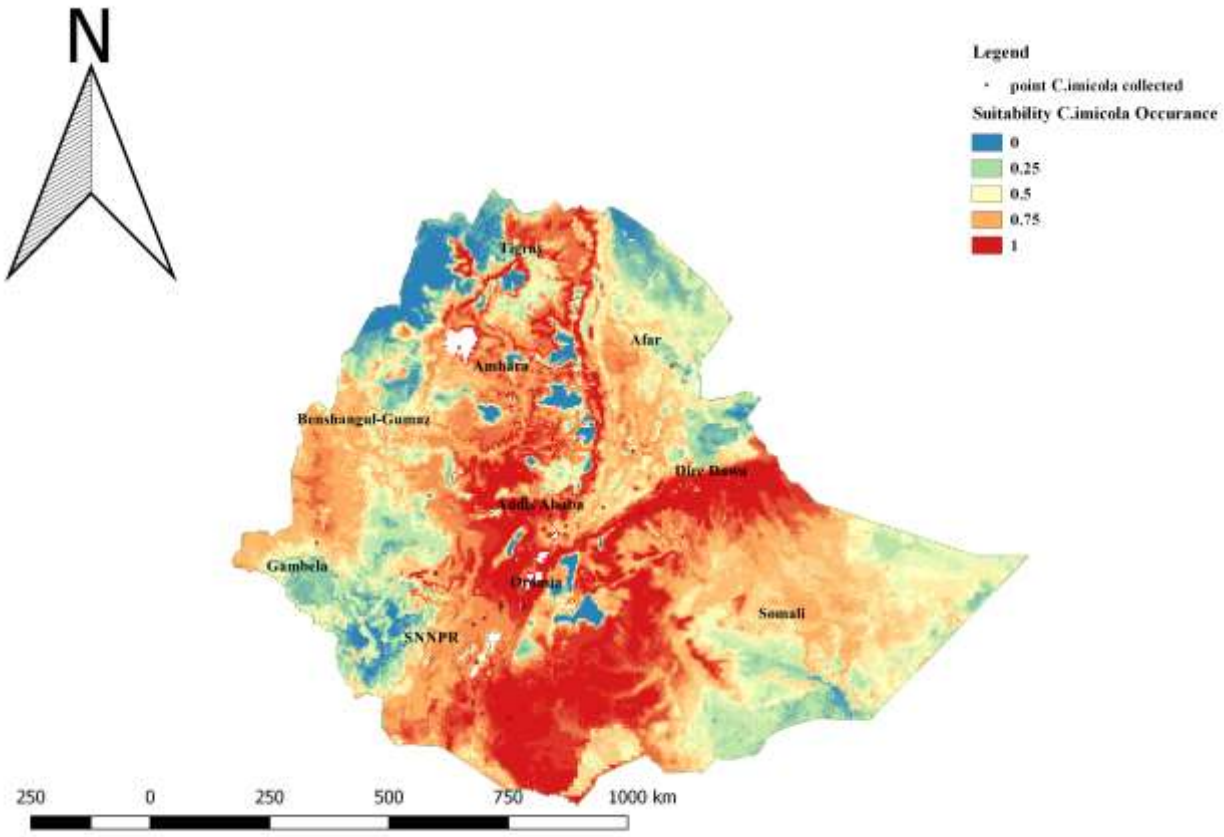


Figure 8: Selected prediction areas of committee averaging.

The warmer colors indicate most suitable, light colors indicates less suitable with uncertain prediction, while cooler colors indicates unsuitable environment with certain prediction

5. DISCUSSIONS

5.1. *Culicoides* Survey

Culicoides are biting midges that can be involved in transmission of different viral diseases affecting both animals e.g. bluetongue (BTV), Schmallenberg (SBV), Akabane, African horse sickness, and epizootic hemorrhagic disease viruses and humans (e.g. Oropouche fever virus). In this study, morphological identification confirmed the presence of four species and a group of unidentified *Culicoides* midges of which *C. imicola* was the highest count followed by *C. kings*, unidentified group, *C. milnei* and *C. deltus*, respectively. The previous study done by Mulatu and Hailu, (2019) showed the existence of *C. imicola* and *C. milnei* in Western parts of Ethiopia especially the extreme abundance of *C. milne*. A study done by Fetene, (2019) showed high abundance of *C. imicola* followed by *C. king* in the Southern part of the country. Our study revealed that *C. imicola* is the most abundant *Culicoides* species and it was the only common species that was collected in all studied sites.

The presence of *C. imicola* in all districts and trap shown in this study coincide with the study conducted in Morocco (Lhor *et al.*, 2015), Portugal(Wilson *et al.*, 2012), and Spain(ORTEGA *et al.*, 1997). Generally, this supports that *C. imicola* is the most abundant species of *Culicoides* in the world (Guichard *et al.*, 2014). *C. kings* which had the second-highest count have been distributed in Ada'a, Holeta, Jimma and Sebeta districts but absent in Adama. *C. deltus* present in Holeta and Sebeta, but not present in Ada'a, Adama and Jimma districts; *C. milne* was identified only in Holeta and Jimma but absent in Ada'a, Adama and Sebeta districts and unidentified group of midges present in Ada'a, Holeta and Jimma, but absent in Adama and Sebeta.

The lowest counts of *Culicoides* species found in Adama might be associated with the climatic and geophysical conditions which could not favor optimal vector breeding and larval development. The basic requirements are moisture and a medium containing organic matter as indicated by Venter, (2006).

5.2. Viral Detection

The current study demonstrated that SBV genome in all pools of *Culicoides* was not detected using real-time RT-PCR. The absence of significant quantities of SBV RNA genome in *Culicoides* may not suggest that the sites are not a risk for the disease. It may have been due to the time of sampling that *Culicoides* collected from November to February. In difference, August, September, and October were the time at which *Culicoides* detected positive for SBV as indicated by (De Regge *et al.*, 2012; Rasmussen *et al.*, 2012). However, studies showed that SBV has been detected by real-time RT-PCR and reverse transcription-quantitative PCRs (RT-qPCRs) in *C. chiopterus, C. dewulfi, C. obsoletus, C. pulicaris, C. punctatus,* and *C. scoticus* (De Regge *et al.*, 2012; Elbers *et al.*, 2013; Rasmussen *et al.*, 2012) experimentally in laboratory *C. sonorensis*(De Regge *et al.*, 2014) and *C. imicola* (Pages *et al.*, 2017).

No study has shown the detection of SBV genome in *C. King, C. milnie* and *C. Deltus* which were collected by the current study. In contrast to this SBV genome has detected in *C. imicola* experimentally in Italy and Spain (Balenghien *et al.*, 2014; Pages *et al.*, 2017). Regardless of Sibhat *et al.*, (2018) report which showed high sero-prevalance of SBV in Ethiopia, no genomic detection in *Culicoides* in the current study. Despite, high *C. imicola* and some clinical signs like abortion and reduced milk production in the study areas, SBV genome has not been reported. Similarly, there were no reports of genomic detection of SBV from the neighboring countries during the study periods.

The primer and probe we used in the current study detects the L-segment of the virus. Weak positive pools of *Culicoides* were not suitable to detect L segment by rRT-PCR(De Regge *et al.*, 2012). So this could be the reason why these pools were not confirmed for the L segment.

5.3. SBV Vector *Culicoides* Distribution Model

In this study, four species of *Culicoides* has collected, identified and pooled for detection of SBV. None of the pool detected positive for SBV genome. Even though our result being negative; among the four species identified by this study only *C. imicola* can be a vector for SBV as it has detected experimentally by Pages *et al.,* (2017). *C. imicola* was the most abundant and collected in all traps in this study, so that we used this species in developing predictive distribution maps by using an ensemble modeling technique. We used additional sets of data compiled globally by Leta *et al.,* (2019a) and some parts of Ethiopia by Fetene, (2019).

The current *C. imicola* distribution models provide valuable information on the spatial suitability habitat for *C. imicola* occurrences, thus greatly assist informed risk-based surveillance, prevention, and control activities. Our study focused on modeling the distribution of *C. imicola* broadly in Ethiopia. This vector was considered as a potential vector for SBV (Pages *et al.*, 2017).

The TSS and ROC were selected as robust measures of model performance (Allouche *et al.*, 2006). Biotic and abiotic factors are required to model *C. imicola* distribution. The national distribution of *C. imicola is* determined highly with mean annual minimum temperature and mean annual maximum temperature. Wittmann *et al.,* (2001) showed the minimum of the monthly minimum temperatures was high significant determinant of the distribution of *C. imicola* resulted in a high degree of accuracy in predicting the occurrence of *C. imicola* in Iberia. Global ensemble modeling of *C. imicola* by Leta *et al.,* (2019b) showed temperature contribute 64% to the model. Our ensemble model influenced highly by mean annual minimum temperature and by mean annual maximum temperature. Fertility, hatching and survival rate of *C. imicola* were influenced by higher temperature which supported by Veronesi *et al.,* (2009). When temperatures start increasing, adults emerge and populations grow progressively to reach a peak of abundance in spring or summer depending on locations, as a function of spring temperatures and summer dryness. Temperature decreases the larval development time, the time between two blood meals, and therefore increases the laying frequency, which leads to a positive effect on the population dynamics (and its growth), and therefore we expected the temperature to have a positive effect on abundance (Villard *et al.,* 2019)

Our investigation showed that solar radiation and precipitation were influential to the distribution of *C. imicola* that each contributes to the overall model 20.1% and 16.6% respectively this result coincide with Leta *et al.,* (2019b). The occurrence of *C. imicola* was higher in the warmest parts of Spain, where the highest seasonal amount of precipitation coincided with the lowest seasonal temperatures (Calvete *et al.,* 2008).

C. imicola uses livestock as a source of blood meals was established as confirmation by Martinez DE LA Puente *et al.,* (2017). In this paper the distribution of *C. imicola* was influenced about 16.4% by livestock distribution. The risk of transmission and amplification of a pathogen is influenced by frequencies of contact between host and *C. imicola* as it is blood sucking insects (Martinez DE LA Puente *et al.,* 2017; Purse *et al.,* 2012).

Land covers account 3.9% to overall contribution to the model which was the least of all variables. According to Purse *et al.,* (2012) land cover characteristics of *C. imicola* includes rain fed about 50-70% mosaic cropland and Vegetation based on land cover classification.

According to this paper, Ethiopia has a wide suitable area for *C. imicola* distribution. The model described all regions have small to wide range of suitable area where Oromia region has wide and Gambela, and some Amahara and Tigray region has few patchy suitable area. The areas with wider distribution of *C. imicola* were Borena, Bale, East Hararge, Jijjiga, Hundene, Arsi, East Shewa, North Shewa, West Shewa, Hadiya, North Wollo, Southern Tigray, Mekele, and Eastern Tigray. While those areas with intermediate suitable distribution were South Omo, Gamo gofa, Wolayita, East Wollega, West Gojam, Afar zone 3, 4 &5, and Dege Habur. The suitability map indicated that Benchimaji, Shaka, Kafa, Ilu Ababor, Southern Somali, Wader, Shinille, Afar Zone 1&2, West Tigray, North Gondor, Awi, Metekel, Gambela zone 1&2 are with few patches suitable for *C. imicola* distribution.

The global ensemble model of Leta *et al.,* (2019b) overlap in many aspects with this current model. This paper gives emphasize to the national level elaborating the suitability distribution of *C. imicola* in different regions and zones within the country. Even though the virus detection of pooled *Culicoides* was negative for SBV there is presence of suitable habitats and high distribution of *C. imicola* which could be a vector for SBV and other diseases such as BTV and AHS.

Ensemble species distribution modeling was constructed by using of climatic data source from worldclim. Even though wide uses of worldclim data for ensemble species modeling distribution, the data has some limitations (Deblauwe *et al.,* 2016). The current reality may deviate from displayed result due to the change of climate in the last decades as world version 2 contains precipitation of 1970-2000.

6. CONCLUSIONS AND RECOMMENDATIONS

In this study, the entomological study showed the abundance of *C. imicola,* followed by *C. king, C. deltus and C. milnie* species of *Culicoides* in different parts of Ethiopia. This study also demonstrated that SBV genome in all pools of *Culicoides* was not detected using real-time RT-PCR. Species distribution modeling for *C. imicola* indicates vector mean minimum temperature, mean maximum temperature and solar radiation are the most dominant predictors as major environmental preferences. The species distribution model shows the intensity of *C. imicola* varied from zone to zone and high occurrence is found in Borena zone, Bale zone Jijjiga, Hundene, West Shewa, North Shewa, Hadiya and East Shewa.

The occurrence of these *C. imicola* species might have the ability to create SBV, BTV and AHS disease emergence in different parts of Ethiopia. This predicted distribution model helps in understanding the risk of introduction and distribution of SBV, BTV and AHS disease vector *C. imicola* and for application of nationwide vector and disease control programs.

Based on the above conclusion the following recommendations are forwarded:

- Further investigation is essential on ecological modeling targeting seasonal dynamics of vector and disease
- Detail studies are needed for the molecular detection and characterization of the viral genomes in vectors
- Further research is required to model more SBV vectors in other areas with records of disease epidemics

7. REFERENCES

Abi-rizk, A., Kanaan, T., Hage, J. El. (2017). Seroprevalence of Schmallenberg virus and other Simbu group viruses among the Lebanese sheep. *Open Vet. J.* **7**, 290–293.

Aebischer, A., Wernike, K., Hoffmann, B., Beer, M. (2019). Rapid Genome Detection of Schmallenberg Virus and Bovine Viral Diarrhea Virus by Use of Isothermal Amplification Methods and High-Speed Real-Time Reverse Transcriptase PCR Downloaded from. *J. Clin. Microbiol*. **52**, 1883–1892.

Agri food and bioscience institute, (2018). Schmallenberg Virus Spreading West | *Agri-Food and Biosciences Institute* [WWW Document]. agri food Biosci. Inst. Home pages. URL https://www.afbini.gov.uk/.

Allouche, O., Tsoar, A., Kadmon, R. (2006). Assessing the accuracy of species distribution models : prevalence , kappa and the true skill statistic (TSS). *J. Appl. Ecol.* **43,** 1223–1232.

Arau, M.B., New, M. (2006). Ensemble forecasting of species distributions. TRENDS *Ecol. Evol*. **22,** 42–47.

Azkur, A.K., Albayrak, H., Risvanli, A., Pestil, Z., Ozan, E., Yılmaz, O., Tonbak, S., Cavunt, A., Kadı, H., Macun, H.C. (2013). Antibodies to Schmallenberg virus in domestic livestock in Turkey. *Trop. Anim. Health Prod.* **45,** 1825–1828.

Balenghien, T., Pagès, N., Goffredo, M., Carpenter, S., Augot, D., Jacquier, E., Talavera, S., Monaco, F., Depaquit, J., Grillet, C. (2014). The emergence of Schmallenberg virus across Culicoides communities and ecosystems in Europe. *Prev. Vet. Med.* **116**, 360–369.

Beer, M., Hs, N.A.T., De R P O, A.N. (2012). REVIEW ARTICLE 'Schmallenberg virus '-a novel orthobunyavirus emerging in Europe. *Epidemiol. Infect.* **141**, 1–8.

Bilk, S., Schulze, C., Fischer, M., Beer, M., Hlinak, A., Hoffmann, B., (2012). Organ distribution of Schmallenberg virus RNA in malformed newborns. *Vet. Microbiol.* **159**, 236–238.

Blomstrom, A.-L., Stenberg, H., Scharin, I., Figueiredo, J., Nhambirre, O., Abilio, A.P., Fafetine, J., Berg, M. (2014). Rapid Communication Serological Screening Suggests Presence of Schmallenberg Virus in Cattle, Sheep and Goat in the Zambezia Province, Mozambique. *Transbound. Emerg. Dis*. **61,** 289–292.

Bréard, E., Lara, E., Comtet, L., Viarouge, C., Doceul, V., Desprat, A., Vitour, D., Pozzi, N., Cay, A.B., De Regge, N. (2013). Validation of a commercially available indirect ELISA using a nucleocapside recombinant protein for detection of Schmallenberg virus antibodies. *PLoS One* **8,** 534–546.

Briese, T., Calisher, C.H., Higgs, S. (2013). Viruses of the family Bunyaviridae: are all available isolates reassortants? *Virology* **446,** 207–216.

Calvete, C.., ESTRADA, R.., MIRANDA, M.A.., BORRÁS, D.., CALVO, J.H.., LUCIENTES, J.. (2008). Modelling the distributions and spatial coincidence of bluetongue vectors Culicoides imicola and the Culicoides obsoletus group throughout the Iberian peninsula. Med. *Vet. Entomol.* **22,** 124–134.

Carpenter, S., Groschup, M.H., Garros, C., Felippe-bauer, M.L., Purse, B. V. (2013). Culicoides biting midges , arboviruses and public health in Europe. *Antiviral Res.* **100**, 102–113.

Cianci, D., Hartemink, N., Zeimes, C.B., Vanwambeke, S.O., Ienco, A., Caputo, B. (2015). High resolution spatial analysis of habitat preference of Aedes albopictus (Diptera: Culicidae) in an urban environment. *J. Med. Entomol.* **52,** 329–335.

Claine, F., Coupeau, D., Wiggers, L., Muylkens, B., Kirschvink, N. (2018). Modelling the evolution of Schmallenberg virus seroprevalence in a sheep fl ock after natural infection. *Prev. Vet. Med*. **154**, 132–138.

Claine, F., Coupeau, D., Wiggers, L., Muylkens, B., Kirschvink, N. (2015). Schmallenberg virus infection of ruminants : challenges and opportunities for veterinarians. *Vet. Med. Res. Reports* **6,** 261–272.

Claine, F., Coupeau, D., Wiggers, L., Muylkens, B., Kirschvink, N. (2013). Schmallenberg Virus among Female Lambs, Belgium, 2012. *Emerg. Infect. Dis*. **19**, 1115–1117.

Collins, Á.B., Mee, J.F., Kirkland, P.D. (2018). Pathogenicity and teratogenicity of Schmallenberg virus and Akabane virus in experimentally infected chicken embryos. *Vet. Microbiol.* **216**, 31–37.

Craney, T.A., Surles, J.G. (2002). Model-Dependent Variance Inflation Factor Cutoff Values. *Qual. Eng.* **14,** 391–403.

Davies, I., Vellema, P., Roger, P. (2012). Schmallenberg virus–an emerging novel pathogen. *In Pract.* **34**, 598–604.

De Regge, N., Deblauwe, I., De Deken, R., Vantieghem, P., Madder, M., Geysen, D., Smeets, F., Losson, B., van den Berg, T., Cay, A.B. (2012). Detection of Schmallenberg virus in different Culicoides spp. by real-time RT-PCR. *Transbound. Emerg. Dis*. **59**, 471–475.

De Regge, N., Madder, M., Deblauwe, I., Losson, B., Fassotte, C., Tomme, M., Cay, A.B., Demeulemeester, J., (2014). Schmallenberg Virus Circulation in Culicoides in Belgium in 2012 : Field Validation of a Real Time RT-PCR Approach to Assess Virus Replication and Dissemination in Midges. *PLoS One* **9**. e.0087005

De Regge, N., van den Berg, T., Georges, L., Cay, B. (2013). Diagnosis of Schmallenberg virus infection in malformed lambs and calves and first indications for virus clearance in the fetus. *Vet. Microbiol.* **162**, 595–600.

Deblauwe, V., Droissart, V., Bose, R., Sonké, B., A., B.-O., Svenning, J.-C., Wieringa, J.J., Ramesh, B.R., Stévart, T., Couvreur, T.L.P. (2016). Remotely sensed temperature and

precipitation data improve species distribution modelling in the tropics Remotely sensed temperature and precipitation data improve species. *Glob. Ecol. Biogeogr*. **25**, 443–454.

Defourny, P., Bogaert, E. Van, Kalogirou, V., Perez, J.R. (2011). GLOBCOVER 2009 Products Description and Validation Report, in: *Product Discription Manual*. pp. 1–53.

Doceul, V., Lara, E., Sailleau, C., Belbis, G., Richardson, J., Bréard, E., Viarouge, C., Dominguez, M., Hendrikx, P., Calavas, D., Desprat, A., Languille, J., Comtet, L., Pourquier, P., Eléouët, J.-F., Delmas, B., Marianneau, P., Vitour, D., Zientara, S.(2013). Epidemiology, molecular virology and diagnostics of Schmallenberg virus, an emerging orthobunyavirus in Europe, *Veterinary Research*. https://doi.org/10.1186/1297-9716-44-31

Dom, N.C., Ahmad, A.H., Ismail, R., 2013. Habitat Characterization of Aedes Sp . Breeding in Urban Hotspot Area. *Procedia - Soc. Behav. Sci*. **85**, 100–109.

Eifan, S., Schnettler, E., Dietrich, I., Kohl, A., Blomström, A.-L. (2013). Non-structural proteins of arthropod-borne bunyaviruses: roles and functions. *Viruses* **5,** 2447–2468.

Elbers, A.R.W., Loeffen, W.L.A., Quak, S., Boer-luijtze, E. De, Spek, A.N. Van Der, Bouwstra, R., Maas, R., Spierenburg, M.A.H., Kluijver, E.P. De, Schaik, G. Van, Poel, W.H.M. Van Der, (2012). Seroprevalence of Schmallenberg Virus Antibodies among Dairy. *Emerg. Infect. Dis.* **18,** 1065–1071.

Elbers, A.R.W., Meiswinkel, R., Weezep, E. Van, M.Sloet, M., Oldruitenborgh-Oosterbaan, V., A.kooi, E. (2013). Schmallenberg Virus in Culicoides spp . Biting Midges , the Netherlands ,. *Emerg. Infect. Dis*. **19**, 106–109.

Fetene, E. (2019). Survey Of Culicoides (Diptera: Ceratopogonidae) And Modeling The Spatial Distiribution Of Culicoides Imicola In Ethiopia.*DVM Thesis unpublished*

Fick, S.E., Hijmans, R.J. (2017). WorldClim 2 : new 1-km spatial resolution climate surfaces for global land areas. *Int. J. Clim.* https://doi.org/10.1002/joc.5086

Fischer, M., Schirrmeier, H., Wernike, K., Wegelt, A., Beer, M., Hoffmann, B. (2013). Development of a pan-Simbu real-time reverse transcriptase PCR for the detection of Simbu serogroup viruses and comparison with SBV diagnostic PCR systems. *Virol. J.* **10**, 1–9.

Friedrich-Loeffler-Institut, (2015). Profile Schmallenberg Virus , 2/Profile/FLI/ *Federal Research Institute for Animal Health Südufer* **10**, D-17493.

Gilbert, M., Nicolas, G., Cinardi, G., Boeckel, T.P. Van, Vanwambeke, S.O., Wint, G.R.W., Robinson, T.P. (2018). Data Descriptor : Global distribution data for cattle , buffaloes , horses , sheep , goats , pigs , chickens and ducks in 2010. *SCIENTIFIC DATA*. **5**, 1–11.

Goller, K. V, Höper, D., Schirrmeier, H., Mettenleiter, T.C., Beer, M. (2012). Schmallenberg virus as possible ancestor of Shamonda virus. *Emerg. Infect. Dis.* **18**, 1644.

Graham, M.H. (2003). Confronting Multicollinearity in Ecological Multiple Regression. *Ecology*

84, 2809–2815.

Guichard, S., Tran, A., Garros, C., Balenghien, T., Darren, J., 2014. Worldwide niche and future potential distribution of Culicoides imicola , a major vector of bluetongue and African horse sickness viruses. *PLoS One* **9,** e112491.

Helmer, C., Eibach, R., Humann-ziehank, E., Tegtmeyer, P.C. (2016). Original Article Seroprevalence of Schmallenberg virus infection in sheep and goats flocks in Germany , 2012 – 2013. *Vet. Med. Sci*. **2,** 10–22.

Herder, V., Wohlsein, P., Peters, M., Hansmann, F., Baumgärtner, W. (2012). Salient lesions in domestic ruminants infected with the emerging so-called Schmallenberg virus in Germany. *Vet. Pathol*. **49,** 588–591.

Hoffmann, B., Scheuch, M., Höper, D., Jungblut, R., Holsteg, M., Schirrmeier, H., Eschbaumer, M., Goller, K. V, Wernike, K., Fischer, M., Breithaupt, A., Mettenleiter, T.C., Beer, M. (2012). Orthobunyavirus in Cattle ,. *Emerg. Infect. Dis.* **18,** 469–472.

Hongoh, V., Hoen, A.G., Aenishaenslin, C., Waaub, J., Bélanger, D., Michel, P., Consortium, T.L. (2011). Spatially explicit multi-criteria decision analysis for managing vector-borne diseases. *Int. J. Health Geogr*. **10,** 1–9.

Julia, K., Larska, M., Collins, Á.B., Rola, J. (2019). Post-Epidemic Distribution of Schmallenberg Virus in Culicoides Arbovirus Vectors in Poland. Viruses **11,** 1–18.

Kerstin, W., Beer, M. (2019). International proficiency trial demonstrates reliable Schmallenberg virus infection diagnosis in endemic and non-affected countries. *PLOSONE* **14**, 1–11.

King, B., Brown, T.O.S., Tarlinton, R., Daly, J.M. (2015). Seroprevalence of Schmallenberg virus in the United Kingdom and the Republic of Ireland : 2011 – 2013. *Vet. Microbiol*. **180**, 36–40.

Kirkland, P.D. (2002). Akabane and bovine ephemeral fever virus infections. Vet. Clin. North Am. *Food Anim. Pract.* **18,** 501–514.

LBOUY, C., LASRAM, F.B.R., GUILHAUMON, F., SOMOTz, S., THUILLER, W., MOUILLOT, D. (2010). The Mediterranean Sea as a ‘ cul-de-sac ’ for endemic fishes facing climate change. *Glob. Chang. Biol.* **16**, 3233–3245.

Leask, R., Botha, A.M., Bath, G.F. (2013). Schmallenberg virus-Is it present in South Africa? *J. S. Afr. Vet. Assoc.* **84**(1), 1-4.

Lechner, I., Wüthrich, M., Meylan, M., Borne, B.H.P. Van Den, (2017). Association of clinical signs after acute Schmallenberg virus infection with milk production and fertility in Swiss dairy cows. *Prev. Vet. Med*. **146,** 121–129.

Leta, S., Fetene, E., Mulatu, T., Amenu, K., Jaleta, M.B., Beyene, T.J., Negussie, H., Kriticos, D., Revie, C.W. (2019a). Updating the global occurrence of Culicoides imicola , a vector for

emerging viral diseases. *Sci. Data* **6,** 1–8.

Leta, S., Fetene, E., Mulatu, T., Amenu, K., Jaleta, M.B., Beyene, T.J., Negussie, H., Revie, C.W. (2019b). Modeling the global distribution of Culicoides imicola : an Ensemble approach. *Sci. Rep.* **9,** 1–9.

Lhor, Y., Khayli, M., Bouslikhane, M., Harrak, M. El, Fihri, O.F. (2015). Spatial and Seasonal Distribution of Culicoides Species in Morocco in Relation to the Transmission of Bluetongue Viruses. *Br. J. Virol*, **2,** 88–95.

Loeffen, W., Quak, S., De Boer-Luijtze, E., Hulst, M., Van Der Poel, W., Bouwstra, R., Maas, R. (2012). Development of a virus neutralisation test to detect antibodies against Schmallenberg virus and serological results in suspect and infected herds. *Acta Vet. Scand*, **54**, 1–8.

Lucio, P.S., Degallier, N., Servain, J., Hannart, A., Durand, B., de Souza, R.N., Ribeiro, Z.M. (2013). A case study of the influence of local weather on Aedes aegypti (L.) aging and mortality. *J. Vector Ecol*, **38**, 20–37.

Mansfield, K.L., La Rocca, S.A., Khatri, M., Johnson, N., Steinbach, F., Fooks, A.R. (2013). Detection of Schmallenberg virus serum neutralising antibodies. *J. Virol. Methods*, **188,** 139–144.

Martinez DE LA Puente, J., NAVARRO, J., FERRAGUTI, M., SORIGUER, R., FIGUEROLA, J. (2017). First molecular identification of the vertebrate hosts of Culicoides imicola in Europe and a review of its bloodâ feeding patterns worldwide: implications for the transmission of bluetongue disease and African horse sickness. *Med. Vet. Entomol*, **31,** 333–339

Mathew, C., Klevar, S., Elbers, A.R.W., Van der Poel, W.H.M., Kirkland, P.D., Godfroid, J., Mdegela, R.H., Mwamengele, G., Stokstad, M. (2015). Detection of serum neutralizing antibodies to Simbu sero-group viruses in cattle in Tanzania. *BMC Vet. Res*, **11**(1)**,** 208.

Mathieu, B., Cêtre-sossah, C., Garros, C., Chavernac, D., Balenghien, T., Carpenter, S., Vigneslebbe, R., Ung, V., Candolfi, E., Delécolle, J. (2012). Development and validation of IIKC : an interactive identification key for Culicoides (Diptera : Ceratopogonidae) females from the Western Palaearctic region. *Parasit. Vectors* **5,** 137.

McGregor, B.L., Sloyer, K.E., Sayler, K.A., Goodfriend, O., Krauer, J.M.C., Acevedo, C., Zhang, X., Mathias, D., Wisely, S.M., Burkett-Cadena, N.D. (2019). Field data implicating Culicoides stellifer and Culicoides venustus (Diptera: Ceratopogonidae) as vectors of epizootic hemorrhagic disease virus. *Parasites and Vectors* 12, 1–13.

Mellor, P.S., Boorman, J., Baylis, M., 2000. C ULICOIDES B ITING M IDGES : Their Role as Arbovirus Vectors. *Annu. Rev. Entomol*. **45**, 307–340.

MØLLER, A.P., 2013. Long-term trends in wind speed , insect abundance and ecology of an insectivorous bird Study area. *Ecosphere* **4,** 1–11.

Mulatu, T., Hailu, A. (2019). The Occurrence and Identification of Culicoides Species in the Western Ethiopia. *Acad. J. Entomol.* **12**, 40–43.

Naimi, B. (2015). On uncertainty in species distribution modelling. https://doi.org/10.3990/1.9789036538404

Naimi, B., Hamm, N.A.S., Groen, T.A., Skidmore, A.K., Toxopeus, A.G. (2014). Where is positional uncertainty a problem for species distribution modelling? *Ecography (Cop.)*. https://doi.org/10.1111/j.1600-0587.2013.00205.

OIE, (2017). SCHMALLENBERG VIRUS:- OIE TECHNICAL FACTSHEET. *Journal of veterinary Microbiology*, **4,** https://doi.org/10.1016/

ORTEGA, M.D., LLOYD, J.E., HOLBROOK, F.R. (1997). Seasonal And Geographical Distribution Of Culicoides Imicoi , A , Kieffer Oiptera : Ceratopogonidae) In Southwestern Spain. *J. Am. Mosq. Control Assoc.* **13,** 227–232.

Pages, N., Talavera, S., Verdun, M., Valle, M., Bensaid, A., Pujols, N. (2017). Schmallenberg virus detection in Culicoides biting midges in Spain : First laboratory evidence for highly efficient infection of Culicoides of the Obsoletus complex and Culicoides imicola s. *Transbound Emerg. Dis*. **00**, 1–6.

Pejaković, S., Wiggers, L., Coupeau, D., Kirschvink, N., Mason, J., Muylkens, B. (2018). Test selection for antibody detection according to the seroprevalence level of Schmallenberg virus in sheep. *PLoS One* **13**, 1–16.

Phil Scott, (2019). Schmallenberg Virus (SBV), in: National Animal Disease Information Service. *National Animal Disease Information Service*, www.nadis.org.uk

Poskin, A., Méroc, E., Behaeghel, I., Riocreux, F., Couche, M., Van Loo, H., Bertels, G., Delooz, L., Quinet, C., Dispas, M. (2017). Schmallenberg virus in Belgium: estimation of impact in cattle and sheep herds. Transbound. *Emerg. Dis.* **64,** 264–274.

Purse, B. V, Falconer, D., Sullivan, M.J., Carpenter, S., Mellor, P.S., Piertney, S.B., Mordue, A.J., Albon, S., Gunn, G.J., Blackwell, A. (2012). Impacts of climate, host and landscape factors on Culicoides species in Scotland. *Med. Vet. Entomol*. **26,** 168–177.

Rasmussen, L.D., Kristensen, B., Kirkeby, C., Rasmussen, T.B., Belsham, G.J., Bødker, R., Bøtner, A. (2012). Culicoids as Vectors of Schmallenberg Virus. *Emerg. Infect. Dis*. **18,** 1204–1206.

Shabani, F., Kumar, L., Ahmadi, M. (2018). Assessing Accuracy Methods of Species Distribution Models: AUC, Specificity, Sensitivity and the True Skill Statistic. *Glob. J. HUMAN-SOCIAL Sci.* **18**, 1–18.

Sibhat, B., Ayelet, G., Gebremedhin, E.Z., Skjerve, E. (2018). Acta Tropica Seroprevalence of Schmallenberg virus in dairy cattle in Ethiopia. *Acta Trop.* **178**, 61–67.

Steinrigl, A., Schiefer, P., Schleicher, C., Peinhopf, W., Wodak, E., Bagó, Z., Schmoll, F. (2014). Rapid spread and association of Schmallenberg virus with ruminant abortions and foetal death in Austria in 2012 / 2013. *Prev. Vet. Med.* **116**, 350–359.

Strandin, T., Hepojoki, J., Vaheri, A. (2013). Cytoplasmic tails of bunyavirus Gn glycoproteins—could they act as matrix protein surrogates? *Virology* **437**, 73–80.

Swets, J.A. (1998). Measuring the Accuracy of Diagnostic Systems. *Science (80-.).* **240,** 1285–1293.

Tarlinton, R., Daly, J., Dunham, S., Kydd, J. (2012). The challenge of Schmallenberg virus emergence in Europe. *Vet. J.* **194,** 10–18.

Thuiller, W., Lafourcade, B., Engler, R., Arau, M.B. (2009). BIOMOD-a platform for ensemble forecasting of species distributions. *Ecography (Cop.)*. **32,** 369–373.

Tobank, Ş., AZKUR, A.K., Pestil, Z., Biyikli, E., Abayli, H., Baydar, E., BULUT, H. (2016). Circulation of Schmallenberg virus in Turkey, 2013. *Turkish J. Vet. Anim. Sci.* **40,** 175–180.

Varela, M., Schnettler, E., Caporale, M., Murgia, C., Barry, G., Mcfarlane, M., Mcgregor, E., Piras, I.M., Shaw, A., Lamm, C., Janowicz, A., Kohl, A., Beer, M., Glass, M., Herder, V., Hahn, K., Baumga, W., Palmarini, M. (2013). Schmallenberg Virus Pathogenesis , Tropism and Interaction with the Innate Immune System of the Host. *PLoS Pathog* **9**(1), 1-13.

Venter, G. (2006). Arthropod vectors---->Culicoides. *Creative Commons Attribution license* , 1–26, http://www.culicoides.net.

Verhoef, F.A., Venter, G.J., Weldon, C.W. (2014). Thermal limits of two biting midges , Culicoides imicola Kieffer and C . bolitinos Meiswinkel (Diptera : Ceratopogonidae). *Parasit. Vectors* **7**, 1–9.

Veronesi, E., Venter, G.J., Labuschagne, K., Mellor, P.S., Carpenter, S. (2009). Life-history parameters of Culicoides (Avaritia) imicola Kieffer in the laboratory at different rearing temperatures. *Vet. Parasitol.* **163**, 370–373.

Villard, P., Muñoz, F., Balenghien, T., Baldet, T., Lancelot, R. (2019). Modeling Culicoides abundance in mainland France : implications for surveillance. *Parasit. Vectors* **12,** 1–10.

Wanji, S., Tayong, D.B., Ebai, R., Opoku, V., Kien, C.A., Patrick, W., Ndongmo, C., Njouendou, A.J., Ghani, R.N., Ritter, M., Debrah, Y.A., Layland, L.E., Enyong, P.A., Hoerauf, A., 2019. Update on the biology and ecology of Culicoides species in the South - West region of Cameroon with implications on the transmission of Mansonella perstans. *Parasit. Vectors* **166,** 1–12.

Wernike, K., Hoffmann, B., Conraths, F.J., Beer, M. (2015). Schmallenberg Virus Recurrence, Germany, 2014. *Emerg. Infect. Dis*. **21**, 2014–2016.

Wilson, A., Ramilo, D.W., Diaz, S., Pereira, I., Dele, J., Lucientes, J., Ribeiro, R., Boinas, F.,

(2012). First Report of 13 Species of Culicoides (Diptera : Ceratopogonidae) in Mainland Portugal and Azores by Morphological and Molecular Characterization. *PLoS One* **7**(4): e34896

Wittmann, E.J., Mellor, P.S., Baylis, M. (2001). Using climate data to map the potential distribution of Culicoides imicola (Diptera : Ceratopogonidae) in Europe. *Rev. sci. tech. Off. int. Epiz* **20,** 731–740.

Yanase, T., Kato, T., Aizawa, M., Shuto, Y., Shirafuji, H., Yamakawa, M., Tsuda, T. (2012). Genetic reassortment between Sathuperi and Shamonda viruses of the genus Orthobunyavirus in nature: implications for their genetic relationship to Schmallenberg virus. *Arch. Virol.* **157,** 1611–1616.

Zeynalova, S., Vatani, M., Asarova, A., Lange, C.E., (2019). Schmallenberg virus in Azerbaijan 2012–2018. *Arch. Virol*. https://doi.org/10.1007/s00705-019-04278-x

Zhai, S.-L., Lv, D.-H., Wen, X.-H., Zhu, X.-L., Yang, Y.-Q., Chen, Q.-L., Wei, W.-K. (2018). Preliminary serological evidence for Schmallenberg virus infection in China. *Trop. Anim. Health Prod*. **50,** 449–453.

8. LIST OF ANNEXES

ANNEX 1: - Protocol: Purification of Viral RNA

Kit Contents

QIAamp Viral RNA Mini Kit	(50)
Catalog no.	52904
No. of preps	50
QIAamp Mini Spin Columns	50
Collection Tubes (2 ml)	150
Buffer AVL*	31ml
Buffer AW1* (concentrate)	19ml
Buffer AW2† (concentrate)	13ml
Buffer AVE†	3x2ml

Protocol: Purification of Viral RNA

Things to do before starting

Equilibrate samples to room temperature (15–25°C).

Equilibrate Buffer AVE to room temperature for elution in step 11.

Check that Buffer AW1 (19ml concentration+25ml Ethanol) and Buffer AW2 (13ml concentration+30ml Ethanol) have been prepared.

Procedure

1. Pipet 560 µl prepared Buffer AVL containing carrier RNA into a 1.5 ml micro-centrifuge tube.

Note: If the sample volume is larger than 140 µl, increase the amount of Buffer AVL–carrier RNA proportionally (e.g., a 280 µl sample will require 1120 µl Buffer AVL–carrier RNA) and use a larger tube.

2. Add 140 µl plasma, serum, urine, cell-culture supernatant or cell-free body fluid to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.

Note: To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.

3. Incubate at room temperature (15–25°C) for 10 min.

Note: Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA.

4. Briefly centrifuge the tube to remove drops from the inside of the lid.

5. Add 560 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

Note: Use only ethanol, since other alcohols may result in reduced RNA yield and purity.

Do not use denatured alcohol, which contains other substances, such as methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl ethanol). To ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.

Note: Close each spin column to avoid cross-contamination during centrifugation.

Note: Centrifugation is performed at 6000 x *g* (8000 rpm) to limit micro-centrifuge noise.

Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

7. Carefully open the QIAamp Mini column, and repeat step 6. If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

8. Carefully open the QIAamp Mini column, and add 500 µl Buffer AW1. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Note: It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.

9. Carefully open the QIAamp Mini column, and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Continue directly with step 11, or to eliminate possible Buffer AW2 carryover, perform step 10 and then continue with step 11.

Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flowthrough, containing Buffer AW2, contacting the QIAamp Mini column.

Removing the QIAamp Mini column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp Mini column. In these cases, the optional step 10 should be performed.

Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

10. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided).

Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.

11. Centrifuge at 6000 x *g* (8000 rpm) for 1 min.

A single elution with 60 µl Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini column. Performing a double elution using 2 x 40 µl Buffer AVE will increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate.

Viral RNA is stable for up to one year when stored at –30°C to –15°C or at –90°C to –65°C.

ANNEX 2: Ethical clearance

አዲስ አበባ ዩኒቨርሲቲ
የእንስሳት ሕክምናና
ግብርና ኮሌጅ
ቢሾፍቱ/ደብረ ዘይት

ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/04/01/12/2020

Name of Applicant: Getachew Teka (DVM, MVSc fellow)

Address: College of Veterinary Medicine and Agriculture (Addis Ababa University)

Title of the project: *Entomological survey, molecular detection of virus and modeling spatial distribution of Schmallenberg virus vector Culicoides in Ethiopia*

Date of application: **10/11/2019**

Nature of the project: **non-invasive**
Target animal species: **no animals used**
Number of animals involved: **No**
Study area: **Different parts of Ethiopia**

Minutes No. and date of review: **VM/ERC/01/12/020, 03/01/2020**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia

መልሱን በሚጽፉልን ጊዜ እባክዎን የሮን ደብዳቤ ቁጥር ይጥቀሱልን
Please quote Our Ref. No. When replying

ፋክስ} Fax 251-11-4339933
ስልክ } Tel. +251 114338450
ፖ.ሣ.ቁ P.o.x. Box}34
ቢሾፍቱ/ደብረዘይት፣ ኢትዮጵያ Bishoftu/Debre Zeit, Ethiopia

ANNEX 3 :-NVI Master-Mix preparation and Run PCR reaction

	NATIONAL VETERINARY INSTITUTE	Document No. NVI -QMS - QF - 41		
Title:- Master mix preparation and PCR work sheet		Effective Date 20/11/2016	Issue No. 1	Page No. 1of 1

Date:-16/05/2020

Reference no MB 56/20

Real time RT- PCR for Schmallenberg virus Isolation procedure

1-Master mix preparation

Ser.no	Type of reagent	For one reaction	Total 18 reaction	Remark
1	RNase free water	3 µl	54 µl	
2	Primer- SBV-F-10pm/ µl 5'TTGCCGTTTGATTTTGAAGTTGTG-3'	2 µl	36 µl	
3	Primer-SBV-R -R-10pm/ µl 5'TCAGGGATCGCAAATTAAAGAACC-3'	2 µl	36 µl	
4	SBV-L-FAM 5'TCATCCGTGCTGACCCTCTGCGAG 3'BHQ1	1	18 µl	
5	5X RT- PCR buffer	10 µl	180µl	
6	10mM dNTPs mix	1 µl	18 µl	
7	RT- PCR enzyme mix	1 µl	18 µl	
8	Add Template (DN A)	5 µl		
	Total volume	25 µl		

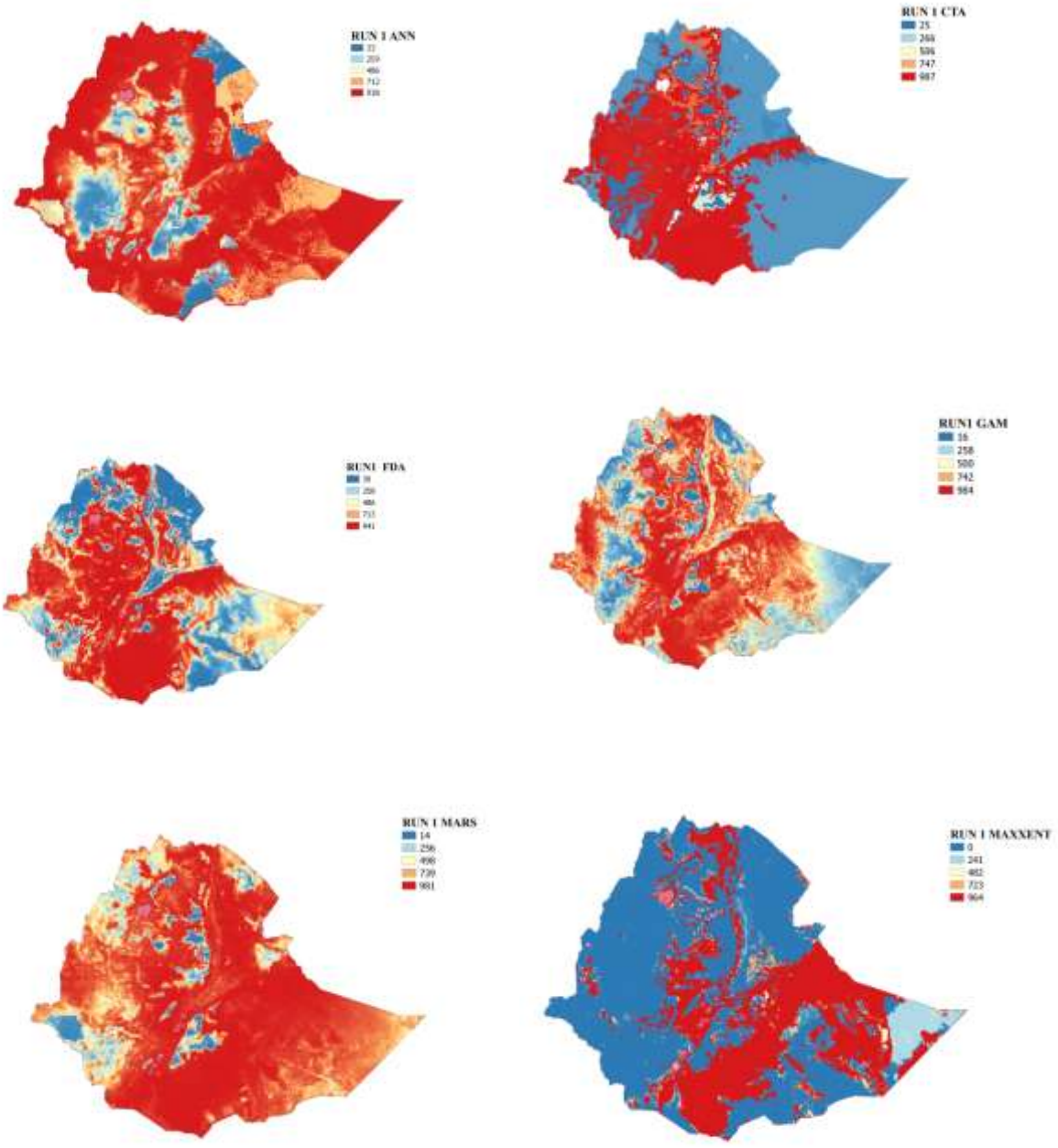
2-Run PCR Reaction

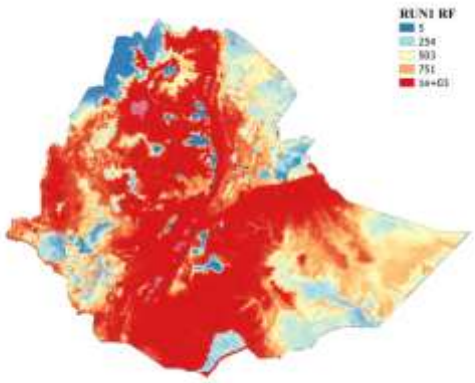
	Temperature	Time	Cycle	reference
cDNA synthsis	45 °c	10 mints	1-Cycle	(Nick De Regge *et al., 2012*
Initial Denaturation	95°c	10 mints	1-Cycle	
Denaturation	95 °c	15 Sec	40 Cycles	
Annealing & Elongation	60°c	45 Sec		
Put at	10 °c	Until machine off		

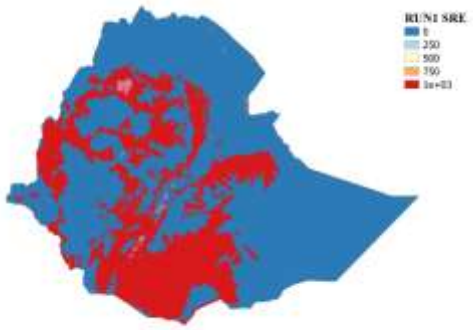
Result: The sample is negative since there is no signal or product amplified in real time PCR.

ANNEX 4:- INDIVIDUAL MODELS OUTPUTS

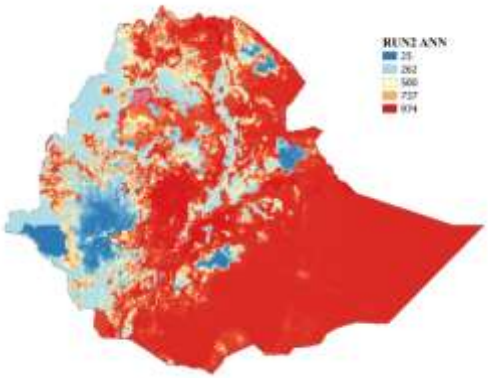
RUN1

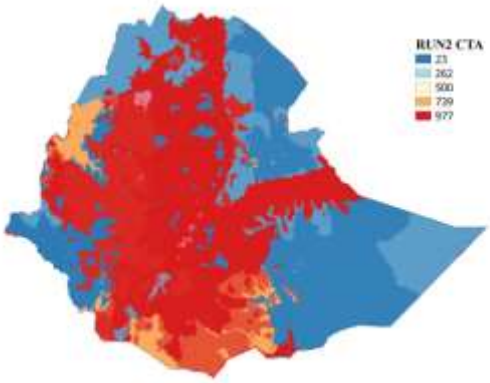


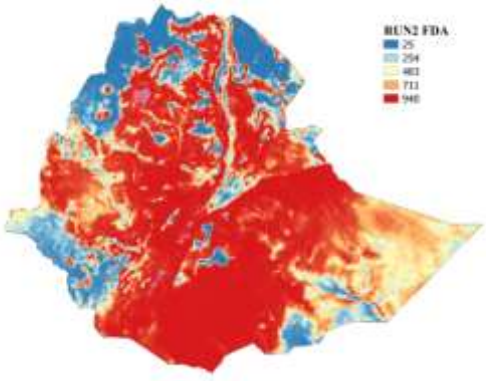

RUN1 RF
5
254
503
751
1e+03

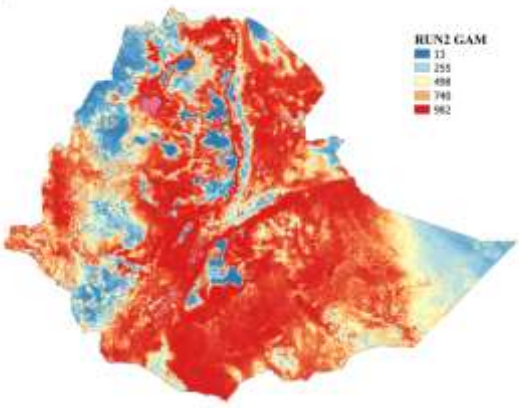

RUN1 SRE
0
250
500
750
1e+03

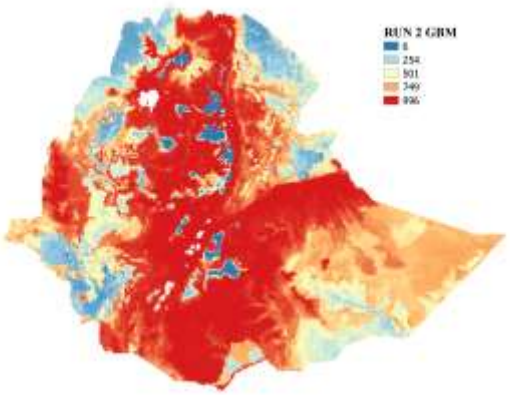
RUN2

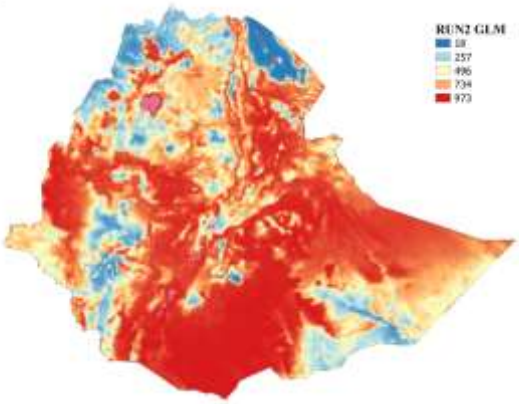

RUN2 ANN
25
262
500
737
974

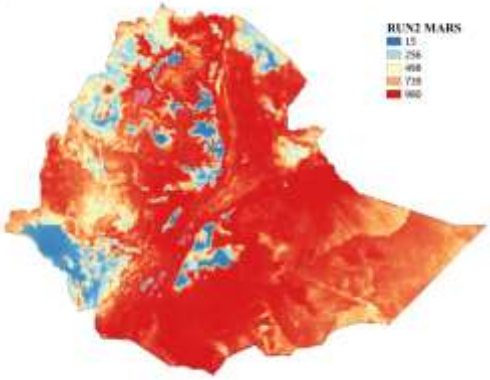

RUN2 CTA
23
262
500
739
977

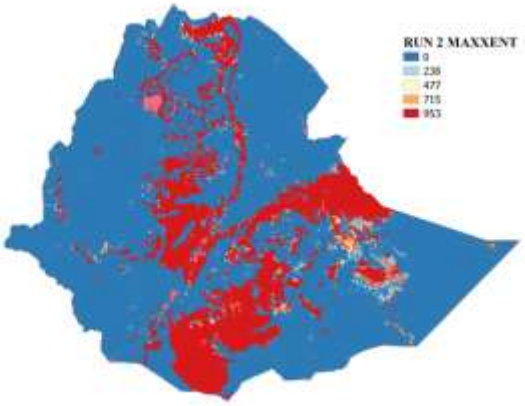

RUN2 FDA
25
254
483
711
940

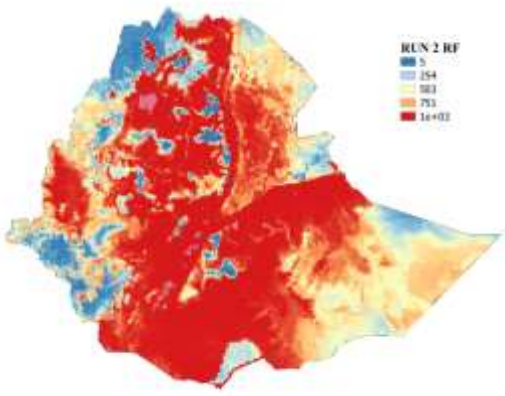

RUN2 GAM
13
255
498
740
982

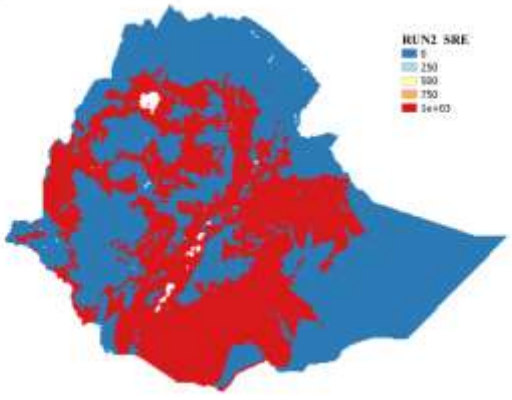

RUN 2 GBM
0
254
501
749
996


RUN2 GLM
18
257
496
734
973

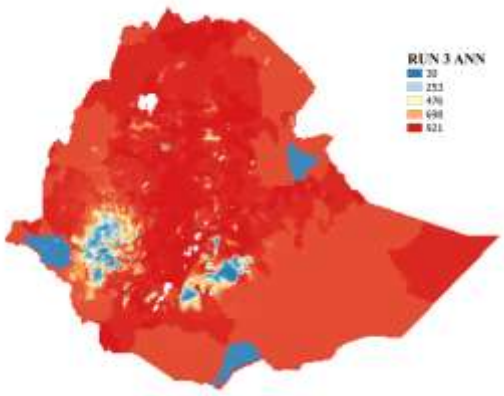

RUN2 MARS
15
256
498
738
980

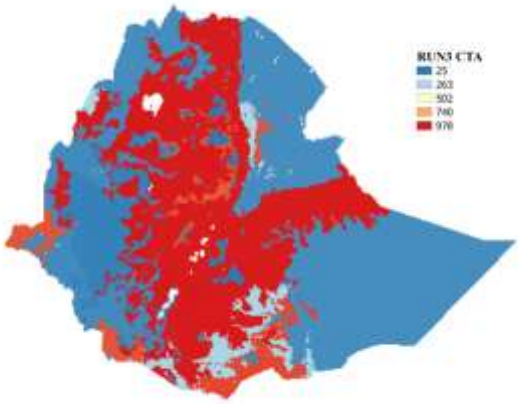

RUN 2 MAXXENT
0
238
477
715
953

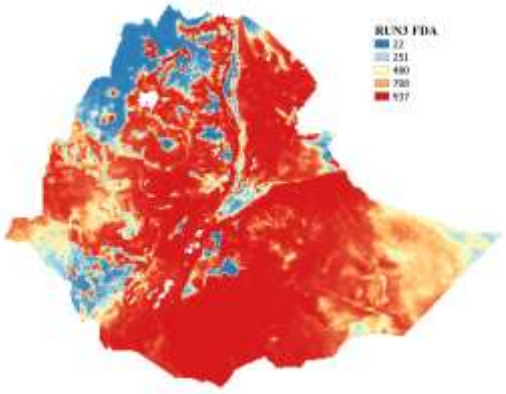

RUN 2 RF
5
254
503
751
1e+03

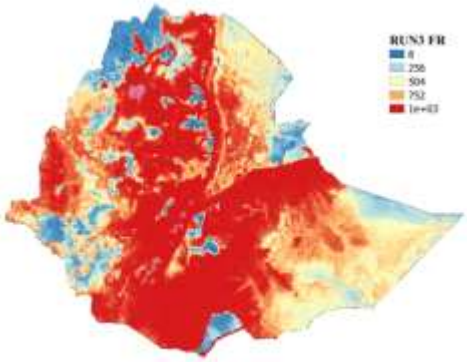

RUN2 SRE
0
250
500
750
1e+03

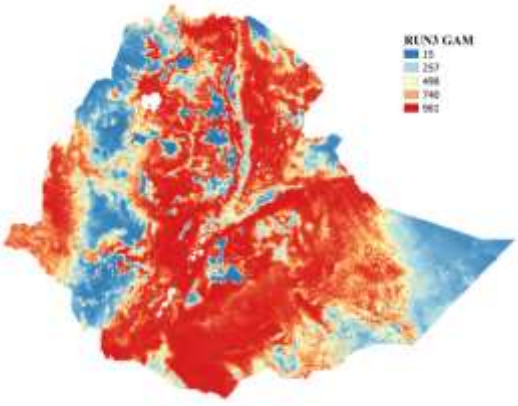
RUN3

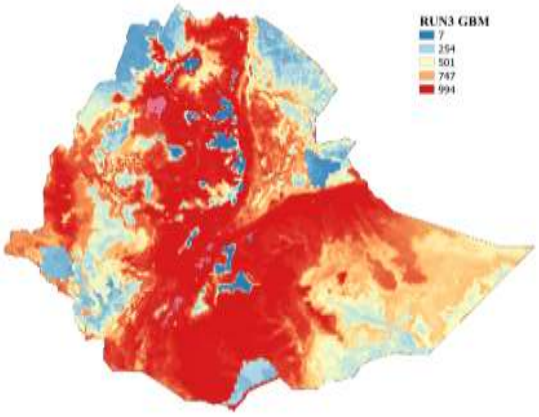

RUN 3 ANN
30
253
476
698
921

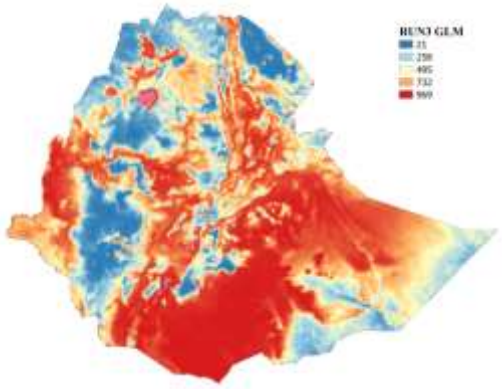

RUN3 CTA
25
263
502
740
978

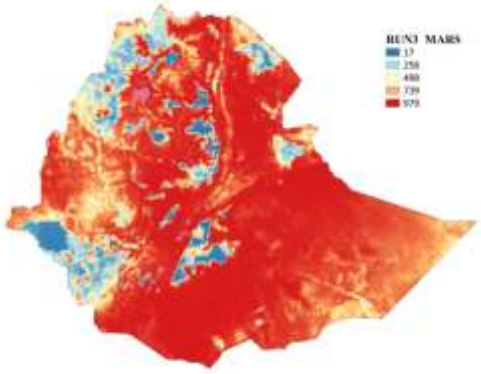

RUN3 FDA
12
251
490
730
737

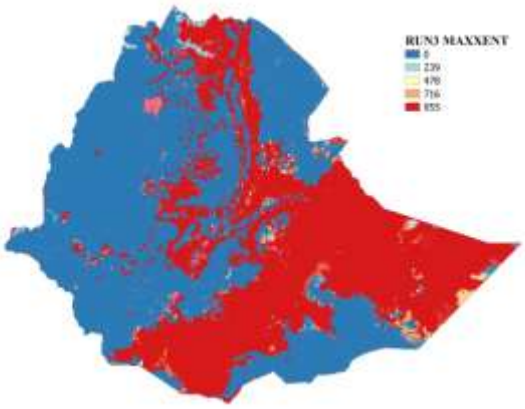

RUN3 FR
0
256
504
752
1e+03

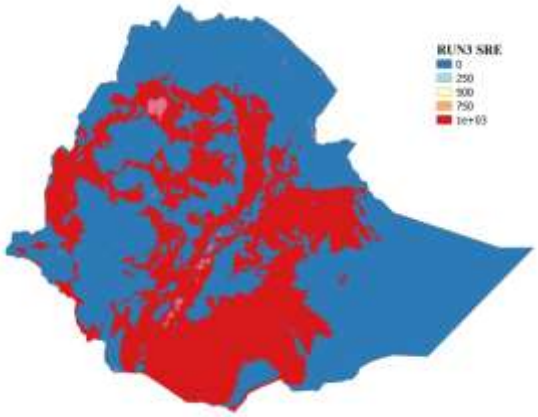

RUN3 GAM
15
257
498
740
981


RUN3 GBM
7
254
501
747
994


RUN3 GLM
15
258
495
732
969


RUN3 MARS
17
258
498
739
979


RUN3 MAXXENT
0
239
478
716
955


RUN3 SRE
0
250
500
750
1e+03