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FACULTY OF VETERINARY MEDICINE

SEROEPIDEMIOLOGICAL STUDY OF BLUETONGUE IN INDIGENOUS SHEEP IN SELECTED DISTRICTS OF AMHARA NATIONAL REGIONAL STATE, NORTHWESTERN ETHIOPIA

BY
DARSEMA GULIMA

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A thesis submitted to the Faculty of Veterinary Medicine, Addis Ababa University in partial fulfillment of the requirement for the Degree of Master of Science in Tropical Veterinary Epidemiology

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FMD	Foot and Mouth Disease
IFAT	Indirect Fluorescent Antibody Test
MAbs	Monoclonal Antibodies
mRNA	messenger Ribonucleic Acid
NS1-3	Non-structural protein 1 – 3
OARD	Office of Agriculture and Rural Development
OD	Optical Density
OIE	Office International des Epizooties
OoARD	Office of Agriculture and Rural Development
OPG	Oxalate Phenol Glycerin
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween-20
PCR	Polymerase Chain Reaction
PPR	Peste des Petits Ruminants
SPS WTO	Sanitary and Phytosanitary Measures of the World Trade Organization
USDA	United States Department of Agriculture
VMRD	Veterinary Medical Research and Development
VP1-7	Viral Protein 1 - 7

in Agam Wouha and Doshegn, respectively. The difference on village basis was statistically significant (χ^2= 144.9, df=8, P< 0.001).

Age specific seroprevalence increased from 24.2% (95% CI = 21.3 - 27.1), in yearlings (< 2 years) to 48.0% (95% CI = 44.7 - 51.4) in old sheep (> 5 years). The difference was statistically significant (χ^2= 31.5, df = 2, Pr < 0.001).

It was concluded that bluetongue virus was circulating in a wide area of the Amhara National Regional State, northwestern Ethiopia. This is an indication of endemicity of the virus in the region. Two risk factors, agroecology and age were found as major determinants for seropositivity of bluetongue virus antibodies.

Further study on the bluetongue virus on different animal species, entomological surveys on the biological vectors, awareness creation among both the livestock owners and animal health professionals, precautions during exotic breed importation are recommended.

Keywords: Agroecology, ANRS, Bluetongue, C-ELISA, Ethiopia, Seroprevalence, Sheep

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ABSTRACT

A seroepidemiological survey of bluetongue virus was conducted with the objectives of determining the distribution and seroprevalence of bluetongue and putative risk factors associated with seropositivity in indigenous Ethiopian sheep in Amhara National Regional State, northwestern Ethiopia. Competitive enzyme-linked immunosorbent assay and relevant information using a standard questionnaire format were used. In the study 860 serum samples were collected from indigenous sheep and 45 livestock owners were interviewed. The study was undertaken in three agroecological zones including lowland, middle altitude and highland.

The overall seroprevalence of bluetongue virus in the indigenous sheep in the study areas at individual animal and flock levels were 34.1% (95% CI = 30.9% - 37.2%) and 65.4% (95% CI = 62.2% - 68.6%), respectively.

Individual animal level bluetongue virus antibody seroprevalence was highest in lowland (53.4%, 95% CI = 50.1% – 56.7%) followed by middle altitude (37.2%, 95% CI = 34.0% - 40.5%) and highland (15.5%, 95% CI = 13.1% - 17.9%). The difference was statistically significant ($\chi^2 = 94.5$, df = 2, P< 0.001). Sheep in the lowland were 6.2 and 1.9 times more likely to be seropositive than sheep in the highland and middle altitude, respectively. Seropositivity of sheep at the middle altitude was 3.2 times more likely than sheep in the highland. On flock basis, the seroprevalence in their respective agroecologies were 97.1% (95% C.I = 96.0% - 98.2%), 82.8% (95% C.I = 80.2% - 85.3%) and 41.6% (95% C.I = 38.3% - 44.9%), which were much higher than individual animal seroprevalence.

At village level, the highest flock seroprevalence was observed in Agam Wouha and Metema Yohannes (100%, n=92 and 87) and the lowest was in Doshegn (30.0%, n=87). The altitudes of Metema Yohannes, Agam Wouha and Doshegn are 600, 700 and 2900 meters above sea level, respectively. The maximum and minimum individual animal level seroprevalences were 73.9% (95% CI = 71.0% -76.8%) and 11.49 (95% CI = 9.4%-13.6%)

1. INTRODUCTION

In Ethiopia, sheep are highly prized animals with significant socio-economic roles. Their smaller size gave the farmers many advantages. Sheep keeping needs low unit costs, which makes it easier to start or expand the holding. Sheep can be kept in the marginal pieces of land holdings. Sheep keeping minimizes hazards due to losses during droughts and epizootics. They are easy to market and this allows meeting unexpected quick expenditure (living bank). They are the means of earning foreign currency through export of them as live animal, and their products like mutton and skin, which is the second export commodity next to coffee (ESAP, 1997). Sheep farming also has a social role in the tradition of Ethiopian people, in religious context. In holydays and feasts, like the Ethiopian New Year and ceremonials, sheep is the most accepted animal for ritual sacrifices.

Livestock diseases inflict a heavy loss on the sub-sector. Apart from high mortality, they affect fertility, growth rate and produces. Several diseases have severely limited the livestock production and export potential of the country (APHIS, 2003).

Bluetongue (BT) is a non-contagious, vector-borne, list A, viral disease of ruminants (OIE, 2004). Sheep are the most clinically affected hosts. Bluetongue was first reported more than 125 years ago with the introduction of European breeds of sheep into southern Africa (Walton, 2004). Non-native sheep experienced a severe febrile disease with high morbidity and mortality (Seifert, 1996; CIDRAP, 2004). Bluetongue virus (BTV) strains have been identified in many tropical and temperate areas of the world since that time from latitude of approximately 50°N to 35°S (Lundervold *et al.*, 2003; Shringi and Shringi, 2005). However, bluetongue disease is a phenomenon of ruminants in the temperate zones. There is little, if any, clinical disease reported in the tropical and subtropical areas of the world, except when non-native ruminants were introduced into a virus-endemic area. At least 24 serotypes of bluetongue virus have been described internationally (OIE, 2004; Walton, 2004). While the viruses are classified antigenically and taxonomically as bluetongue virus, each serotype is unique and may or may not cause the disease (FAO, 2003; OIE, 2004). The

bluetongue viruses are transmitted among ruminants by competent vector species of the genus *Culicoides*, which are known as biting gnats or midges (Tweddle, 2002; CIDRAP, 2004; Walton, 2004).

Bluetongue can cause spectacular disease outbreaks and the Office International des Epizooties (OIE) categorized it as list A disease (OIE, 2004). The WTO SPS agreement and the OIE International Animal Health Code outline states that "if a country that lies between 40^0N and 35^0S and if that country did not confirm bluetongue virus clinical infection, it should establish a surveillance and monitoring program" (OIE, 2004).

Bluetongue virus is widely prevalent in Africa. It was reported including in Ethiopia's neighbors, Kenya and the Sudan in 1909 and 1980, respectively (Eisa *et al*., 1980; Sellers, 1981; Naresh *et al*., 1996). The seroprevalence results of BTV in sheep so far reported from different African countries (Table 2), range from 13.3% to 84.4% (Kanhai and De Silva, 1981; Ekue *et al*., 1985; Hyera and Lyaruu, 1995; Musuka and Kelly, 2000).

Bluetongue was not given attention in Ethiopia until a seroprevalence of 46.7% (n=90) was reported in sheep in three agroecological areas of central Ethiopia (Woldemeskel *et al*., 2000). That study, though on a limited number of samples collected non-systematically, has drawn a national interest to learn more about the spread as well as the distribution of the virus among domestic ruminants in different parts of the country. The current study was designed to determine the status of bluetongue virus antibodies in the Ethiopian indigenous sheep of the Amhara National Regional State, northwestern Ethiopia, where more than 39% of the nation's sheep population exists. The objectives of the study were as follows:

- To determine the distribution and seroprevalence of bluetongue virus group-reactive antibodies by agroecology in indigenous sheep in Amhara National Regional State and

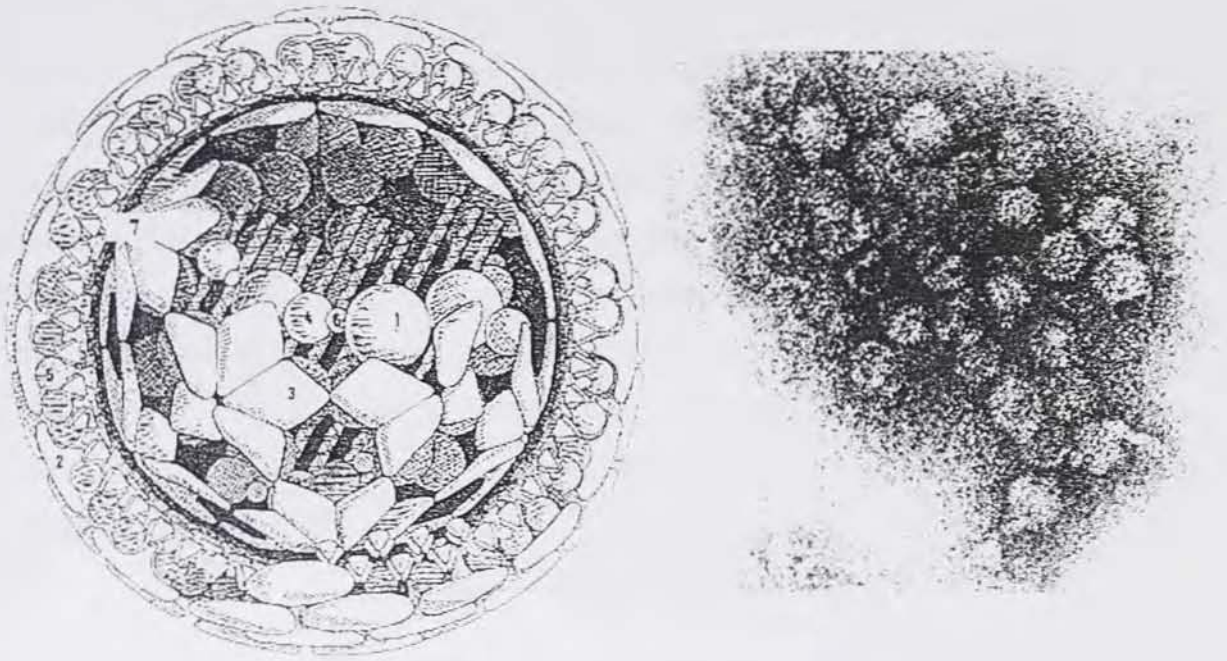
- To find out the possible associations between the presence of bluetongue virus antibodies and potential risk factors

2. LITERATURE REVIEW

2.1. Etiology

Bluetongue virus is the prototype species of the genus Orbivirus in the family Reoviridae. The members of the genus Orbivirus, unlike other members of the family (reoviruses and rotaviruses), are arthropod-borne (Murphy *et al.*, 1999; Roy, 2000). There is an outer capsid comprising two proteins (VP2 and VP5), which encapsulates an inner capsid, or core that is made up of 5 proteins and a genome of 10 segments of double-stranded RNA (Figure 1). While the two proteins of the outer capsid are responsible for virus entry and establishment of virus infection within the host cells, the core components are responsible for replication of the viral genome. Of the two outer capsid proteins, the VP2 contains the major virus neutralization antigen and has a variable sequence resulting in the 24 serotypes of the virus (Dadhich, 2004; OIE, 2004; Walton, 2004). The second outer capsid protein VP5, also varies considerably, although much less than the VP2. VP7 is highly conserved protein for identification of the BTV serogroup (Walton, 2004). Another characteristic of Orbivirus is accumulation of mutations and co-evolution of gene pools at different locations or regions, which is proposed as "topotype" (the Far East, Australian, African or North American). Genetic heterogeneity of field strains of BTV occurs as a consequence of both genetic drift and shift (CIDRAP, 2004; OIE, 2004).

Figure 1: An electron microscopic and a schematic diagram of the bluetongue virus (BTV) particle, showing the morphology, positions and structures of BTV components



Source: CIRAD (2003)

2.2. Epidemiology

2.2.1. Distribution

Currently, BTV is considered to be present in all continents, except for Antarctica (Caporale *et al.*, 2003; OIE, 2004). Canada is reported as bluetongue virus-free by national serologic surveys of sentinel cattle programs (CIDRAP, 2004).

The global distribution of BTV lies approximately between latitudes 35^0S and 50^0 N where *Culicoides* midges are present (Figure 2) (Caporale *et al.*, 2003; Lundervold *et al.*, 2003).

First bluetongue has been detected in South Africa, in 1876 and subsequently spread to all parts of sub-Saharan Africa where exotic breeds of sheep were introduced (OIE, 2004). It

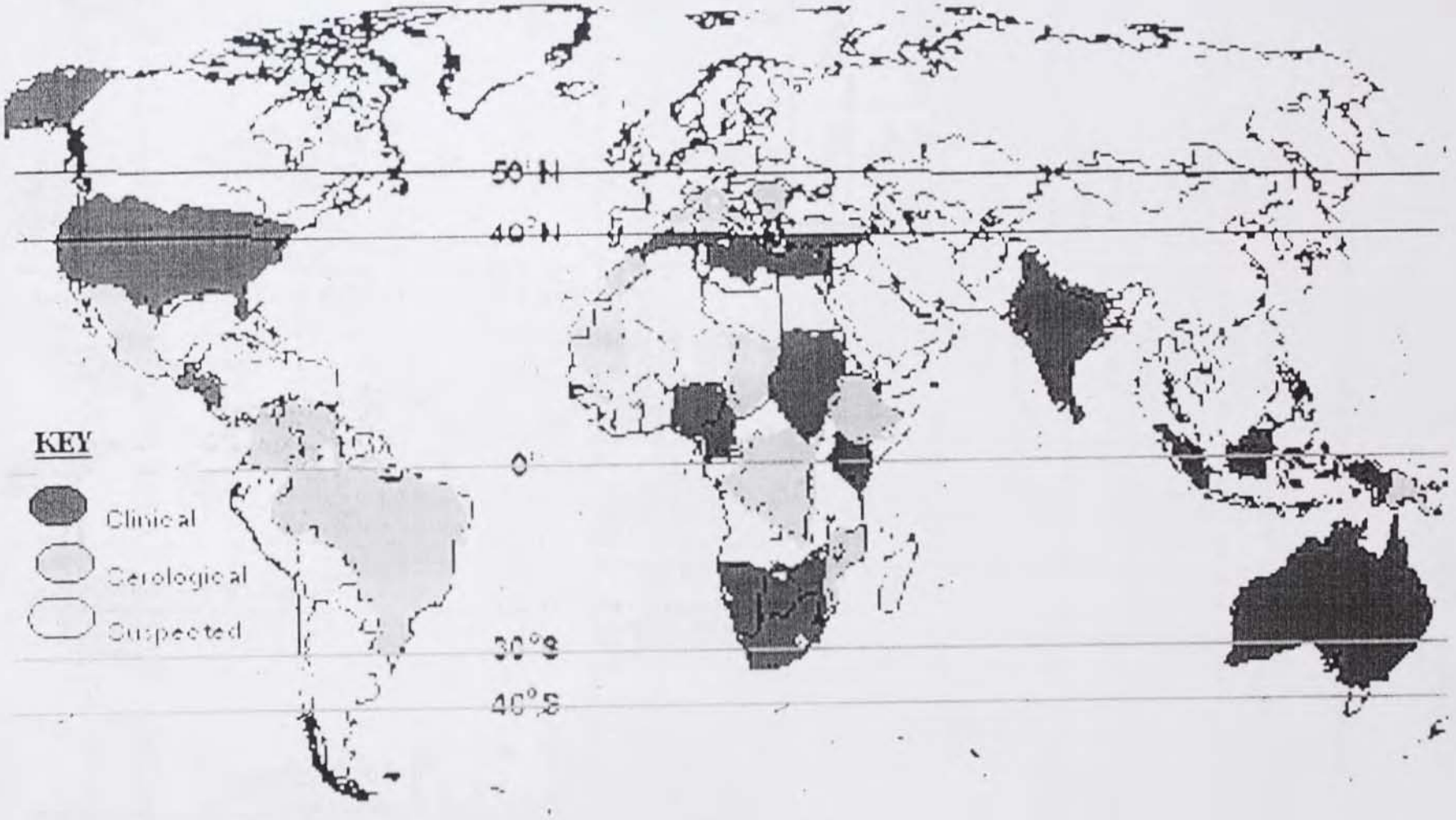
was reported in Kenya in 1909 (Sellers, 1981). In 1943, bluetongue was reported outside Africa (Roy, 2000; OIE, 2004). Since the year 1998, BTV has emerged in Mediterranean European countries where it had never been reported. Six strains have spread across 12 countries and 800km further north in Europe than has previously been reported. It was suggested that spread has been driven by climate changes (Purse *et al.*, 2005). The recently affected areas are not in the classical latitudes for BTV (Roger *et al.*, 2003). The latest outbreak reports were in Spain (Feb. 2005), Portugal (Jan. 2005), China (Dec.2004), Croatia (Dec. 2004), Morocco (Dec. 2004), France (Corsica, Sep. 2004), Namibia (Jul. 2004), Cyprus (May 2004), Lesotho (May. 2004), Israel (Nov. 2003) & Italy (Sep. 2003) (OIE, 2005). Global distribution of bluetongue virus serotypes and their primary *culicoides* vectors, some of the seroprevalence results of BTV in different countries of Africa and other continents are listed in Tables 1, 2 and 3.

Table 1: Global distribution of bluetongue virus (BTV) serotypes and primary *Culicoides* spp. vector, by geographical region.

Region	Bluetongue virus serotypes	Vector species
Africa	1-16, 18, 24	*C. imicola, C. bolitinos*
Asia	1-4, 7, 9, 12,16, 17, 20, 21, 23	*C. imicola, C. fulvus, C. orientalis*
Australia	1, 3, 9, 15, 20, 21, 23	*C. brevitarsis, C. fulvus, C. wadai.*
Europe	2, 4, 9,10	*C. imicola, C. obsoletus.*
North America	2, 10, 11, 17	*C. sonorensis; C. insignis*
South and Central America, Caribbean	1, 3, 4, 6, 12, 17	*C. insignis*

Source: Baldet *et al.* (2003) and Walton (2004).

Figure 2: The current global distribution of bluetongue virus



Source: CIRAD (2003) and OIE (2005)

Table 2: Reports of BTV antibody seroprevalence from different African countries

Author(s)	Origin/ Country	Animal species	Sample size	Prevalence (%)	Discussed risk factors
Musuka and Kelly (2000)	Zimbabwe	Sheep Cattle Goats	Ng Ng Ng	13.3 6.7 13.3	-
Woldemeskel *et al.* (2000)	Central Ethiopia	Sheep	90	46.7	Altitude
Hyera and Lyaruu (1995)	Tanzania	Sheep Goats	292 1173	73.9 77.4	Age, location
Formenty *et al.* (1994)	Ivory Coast	Sheep Cattle	623 215	52.0 95.0	Age
Mushi *et al.* (1992)	Botswana	Sheep Cattle Goats	369 491 452	25.5 90.2 48.0	-
Ekue *et al.* (1985)	Cameroon	Sheep	37	16.2	-
Exeifeka *et al.* (1983)	Nigeria	Sheep Cattle Goats Camels	210 425 407 64	27.6 21.6 21.6 17.2	Temperature, Rainfall, Humidity, Sex
Kanhai and de Silva (1981)	Mozambique	Sheep/Goats Cattle	250 230	82.4 87.4	-

Ng = Not given

Table 3: Reports of BTV antibody seroprevalence results from countries out of Africa

Authors(s)	Origin/ Country	Animal species	Sample size	Prevalence (%)	Discussed risk factors
Shringi and shringi (2005)	India (Rajasthan)	Sheep	178	30.3	-
Listes *et al.* (2003)	Croatia	Sheep Cattle Goats	1268 357 1693	13.7 49.9 16.0	-
Lundervold *et al.* (2003)	Kazakhstan	Sheep Cattle Goats	542 279 137	21.4 25.4 25.5	Age
Bhalodiya and Jhala (2002)	India (Gujarat)	Sheep Cattle	90 437	55.55 67.04	Age
Govindarajan *et al.* (2002)	India (Tamil Nadu)	Sheep Goats	4089 874	15.85 7.78	-
Hendrikx *et al.* (2001)	France (Corsica)	Sheep Cattle	4704 2237	28.2% 31.3%	-
Martinez *et al* (1999)	Mexico	Deer (wt)	350	81.0	-
Dunbar *et al.* (1998)	Florida, USA	Bears	61	5.0	
Uhaa *et al* (1990)	California, USA	Cattle	3774	51.6	Age
Stanley (1990)	Yemen	Sheep	Ng	24.0	-
Homan *et al.* (1985)	Costa Rica Colombia	Cattle Cattle	1435 635	48.1 51.8	Altitude
Vankamman and Cybinski (1981)	New Guinea	Sheep Cattle	62 1854	10 59.6	-

2.2.2. Host range

Bluetongue, so named because it can cause a loss of oxygen and a blue tinge to the tongue. Bluetongue virus can cause infections and diseases of economic importance in domestic and wild ruminants, which includes sheep, goats, cattle, camels, deer, African antelope and various other herbivores such as elephants (Formenty *et al.*, 1994). However, sheep and white-tailed deer are severely affected (FAO, 2003; OIE, 2004). Among sheep temperate breeds are severely affected (FAO, 2003; CIDRAP, 2004).

2.2.3. Modes of transmission

2.2.3.1. Vector transmission

Bluetongue virus is an Orbivirus that has evolved a lifecycle where alternate cycles of virus replication in vertebrate and invertebrate hosts are essential for virus persistence; and infections are transmitted between their vertebrate hosts by hematophagous arthropods (Tweddle, 2002; FAO, 2003; Roger *et al.*, 2003; CIDRAP, 2004). The cyclical vectors are the midges *Culicoides* species (Figure 3). The genus *Culicoides* (Diptera: Ceratopogonidae) encompasses more than 1247 species of biting midges with about 20 species considered as vectors (Baldet *et al.*, 2003).

Each broad geographic region of the world has a different *Culicoides* species as the primary BTV vector: *C. imicola* in Africa, Middle East, Asia and Southern Europe (Baldet *et al.*, 2003; Roger *et al.*, 2003). *C.variipennis* in North America, *C.insignis* in Central and South America, *C. bravitarsis* in Japan and Australia (Table 1) (Walton, 2004).

For transmission to occur, vectors must feed on animals while they are viraemic and climatic conditions must be suitable for the virus to complete development within the vector (CIDRAP, 2004). The extrinsic incubation period in insects is 1-2 weeks (FAO, 2003). The duration of viraemia in the infected vertebrate is an important factor in the transmission to

the biting, competent midges. The maximum viraemic time reported in cattle and sheep are 65 and 54 days respectively (FAO, 2003; CIDRAP, 2004). MacLachlan (2003) stated that viraemia is associated with erythrocytes. Infected midges remain infective for the rest of their lives (Roger *et al.*, 2003; CIDRAP, 2004).

Vector competence of *Culicoides* for Orbiviruses is partly influenced by climate. Orbivirus development in *Culicoides* vectors is unable to occur at temperatures below about 10°C to 15°C depending on the Orbivirus species and serotype. The BTV is assumed to survive above a temperature threshold of 12.5^{0}C, which is below the activity threshold for flight of *C. imicola* (minimum 17-18^{0}C). The generation interval is reported to be shorter at higher temperature (Braverman *et al.*, 2001). Paweska *et al.* (2002) reported that there was no individual vector with BTV transmission potential (TP) detected at 15°C during their work on oral susceptibility of competent BTV vectors. Increasing environmental temperature (climate change) was reported to extend the vector season (Tweddle, 2002). Mullens *et al.* (2003) clearly stated in his investigation report that environment governed dynamics and intensity of vertebrate-vector contact in both time and space. They also discussed that temperature had dramatic and consistent influence, altering vector developmental rates and life history parameters since temperature was best known to control rates of virogenesis time to transmission for bluetongue viruses in *Culicoides*. The feeding and breeding activities of the vectors are highest in areas with high rainfall and relative humidity, low wind force, high population of definitive and reservoir hosts and night temperature of 22^{0}C. The optimum temperature lies between 13^{0}C and 35^{0}C (Exeifeka *et al.*, 1983; Braverman *et al.*, 2001; Wittman *et al.*, 2002).

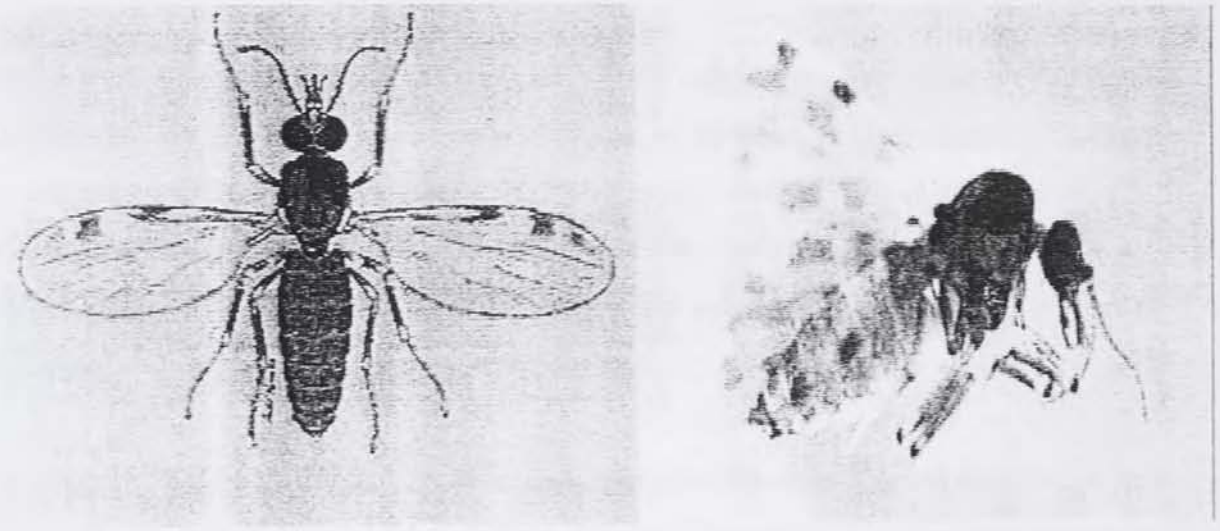
Culicoides mosquitoes breed in moist conditions in variety of habitats, like wet manure, organic soil, rotten plants (Braverman *et al.*, 2001). They have nocturnal feeding habits. Females take a blood meal prior to egg laying, feed at roughly 4-days interval and live for about 2-3 weeks. Midges were reported from altitudes of 170 to 4000m. However, up to 1000 m. is the most appropriate altitude (Braverman *et al.*, 2001).

Studies conducted by White and colleagues (2005) suggested that bluetongue viruses could overwinter in the insect vectors (experimentally, 3 out of 1500 adult *Culicoides* vectors were found to live for more than 90 days, even though the average life span was 10-20 days).

2.2.3.2. Non-vector transmission

Aside from biting insects, bluetongue virus is suggested to be vectored by means of instruments (blood transfer) or experimental inoculation of semen containing red or white blood cells from viraemic bulls; in utero depending on the stage of development, viral strain, and immune status of the dam (Hoar *et al.*, 2004).

Figure 3: *Culicoides imicola*, the principal vector of bluetongue in Africa, Middle East and Europe, at different positions



Source: CIRAD (2003)

2.3. Pathogenesis

All of the pathology of bluetongue can be assigned to vascular endothelial damage resulting in changes to capillary permeability and fragility, with subsequent disseminated intravascular coagulation and necrosis of tissues supplied by damaged capillaries. These changes result in edema, congestion, hemorrhage, inflammation and necrosis. Mechanical stress, exposure to sunlight and abrasion exacerbate the severity of the disease (Seifert, 1996; MacLachlan, 2003).

2.4. Clinical manifestations

All breeds, sexes, and ages of sheep are reported to be susceptible, but breeds native to endemic areas probably are more resistant than breeds from BT-free regions (Seifert, 1996). The average incubation period of BT following natural infection in sheep is 7 to 10 days (Radostitis *et al*, 1994; Merck, 1998).

The average duration of the febrile (41°C) reaction may range from 2 to 11 days (Tweddle, 2002). Other common clinical signs include edema of lips, nose, face, submandibulum, eyelids and ears; congestion of mouth, nose, nasal cavity, conjunctiva, skin and coronary bands; lameness and depression (Radostitis *et al*, 1994; Merck, 1998; CIDRAP, 2004). There is frequently a nasal discharge, at first watery, but later muco-purulent or even blood stained, appears and eventually dries out to form crusts.

In very severe cases, the tongue becomes markedly swollen and edematous and even protrudes from the mouth. It may be dark-blue in color ("bluetongue"). Complete anorexia and marked depression are generally present at this stage (Seifert, 1996; FAO, 2003; CIDRAP, 2004).

2.5. Pathology

Most common lesions include mucosal edema, pulmonary edema, hydrothorax, hydropericardium, serosal hemorrhages, and other hemorrhagic and thrombotic phenomena. A curious feature of BTV infections in sheep is hemorrhage at the base of the pulmonary artery (Seifert, 1996; Merck, 1998; FAO, 2003).

2.6. Immunity

Sheep indigenous to tropical countries in Africa, the Middle East and Asia can be infected with BTV, but may not usually exhibit disease. Group-specific and type-specific antibodies are formed within 7-10 days of infection with BTV. The group-specific antibodies persist for about 6-18 months; whereas type-specific neutralizing antibodies can often still be demonstrated after 3 years. Protective immunity is associated with neutralizing antibodies. Thus recovery from natural infection results in a type-specific humoral immunity, which usually lasts a year but can be life long (Verwoerd and Erasmus, 1994; Merck, 1998). The lambs of immune ewes will be partially protected by colostral immunoglobulins that persist for a maximum period of 6 months and is serotype specific (Verwoerd and Erasmus, 1994; Merck, 1998).

In addition to neutralizing antibodies, cell-mediated immunity (CMI) also plays an important role in recovery from infection and protection against reinfection (Deshmukh and Gujar, 2000). The average duration of immunity is estimated to be 33 months ranging from 1 to 60 months (Hoar *et al.*, 2004).

2.7. Diagnosis

It is not possible to confirm a BTV infection solely on clinical grounds. Diagnosis is, therefore, dependent upon the laboratory based isolation and identification of the virus, of viral antigens or genomic RNA sequences, or a demonstration of BTV-specific antibodies (Murphy *et al.*, 1999; Roy, 2000; CIDRAP, 2004; OIE, 2004).

2.7.1. Virus isolation

A number of virus isolation systems are in common use, but two of the most efficient are embryonated chicken eggs (ECE) and sheep (Merck, 1998; Murphy *et al.*, 1999). Direct inoculation of viraemic blood into cell culture is an inefficient way to isolate BTV. A high-titer virus preparation is most readily generated by one or two passages in ECE (OIE, 2004).

2.7.2. Serological tests

Bluetongue virus antibody generated in infected animals can be detected in a variety of ways that depend on the sensitivity and type of test used. The recommended tests for the detection of bluetongue serogroup-specific antibodies are Agar Gel Immunodiffusion (AGID) and competitive Enzyme-Linked Immunosorbent Assay (cELISA), with the later becoming more popular because of its greater accuracy and adaptation to conventional rapid laboratory testing (FAO, 2003; OIE, 2004).

Competitive ELISA is currently recommended by OIE for international animal movement due to its high sensitivity and specificity (OIE, 2004). The test can detect BTV specific antibodies in animal sera. It is based on the competition between the antibodies in serum to be tested and a monoclonal antibody, which is coupled to the peroxidase. This monoclonal antibody is directed to the N-terminal part of the VP7 protein, a major core protein of the BTV (specific for the BT serogroup). This method is easy to implement, rapid and reliable and is particularly suited to the analysis of a large number of samples (Ward *et al.*, 1996; FAO, 2003; OIE, 2004).

Competitive Enzyme-Linked Immunosorbent Assays (cELISA) basically involve methods that measure the inhibition of a reactant for a pretitrated system. The degree of inhibition reflects the activity of the unknown. We can, therefore, measure antibodies or antigens so that, antigens' subtyping may be performed by comparing the relative avidity (OIE, 2004). The BT competitive or blocking ELISA measures BTV-specific antibody without detecting cross-reacting antibody to other Orbiviruses (Ward *et al.*, 1996; OIE, 2004). The specificity is the result of using one of a number of BT serogroup-reactive MAbs. The antibodies all appear to bind to the amino-terminal region of the major core protein VP7. Here, the antibodies in test sera compete with the MAbs for binding to antigen (Abdella *et al.*, 2003).

In cELISA the kit uses a competitive enzyme linked immunosorbent assay for detection of specific BT antibody in the serum. The test depends on inhibition of the binding of a mouse/ guinea-pig monoclonal antibody to a BTV specific epitope in the presence of positive serum. Inhibition is detected as a reduction in optical density (OD) reading obtained with the MAb alone following the addition of peroxidase labelled anti-mouse conjugate and substrate/chromogen mixture. A threshold value of 50% inhibition is adopted for routine screening (Tweddle, 2002; OIE, 2004).

2.7.3 Polymerase chain reaction

Primer-directed amplification of viral nucleic acid has revolutionized BT diagnosis. Results to date indicate that polymerase chain reaction (PCR) techniques may be used, not only to detect the presence of viral nucleic acid, but also to 'serogroup' orbiviruses and provide information on the serotype and possible geographical source (topotype or genotype) of BTV isolates within a few days of receipt of a clinical sample such as infected sheep blood (Hawkes *et al.*, 2000; Johnson *et al.*, 2000). Traditional approaches, which rely on virus isolation followed by virus identification, may require at least 3-4 weeks to generate information on serogroup and serotype and yield no data on the possible geographical origin of the isolated virus (Aradaib *et al.*, 1998, 2003; Abdella *et al.*, 2003).

2.7.4. Differential diagnosis

Conditions that should be considered in differential diagnosis of BT in sheep are: Foot and Mouth Disease (FMD), Peste des Petits Ruminants (PPR), contagious pustular dermatitis (Orf), sheep pox, *Oestrus ovis*, pneumonia, polyarthritis, mange mites and foot-rot (CIDRAP, 2004). Differentiations based on clinical grounds are difficult.

2.8. Prevention and control

There is no specific drug recommended. Control is difficult because of persistent viral infection and efficient transmission by *Culicoides* species, which are abundant throughout the world. The wide spread movement of domestic and wild ruminants in their natural habitats as well as for purposes of exhibition and sale provides a hazard of introduction into areas (Merck, 1998; Dungu *et al.*, 2004). Since a wide variety of game can be a reservoir of infection in endemic areas, the feasible alternative to control the disease is vaccination (Seifert, 1996; FAO, 2003; OIE, 2004).

In endemic areas, prophylactic immunizations of sheep remain the most practical and effective control measure (Deshmukh and Gujar, 2000; Dungu *et al.*, 2004). The present Onderstepoort BT vaccine (Reg. No. G 358 Act No. 36/1947) comprises 3 bottles (vaccines A, B, and C). The three pentavalent vaccines are administered separately at 3-week intervals. After 2 to 3 annual immunizations, most sheep were protected with long lasting immunity to all serotypes in the vaccine (Dungu *et al.*, 2004). Alternatives to live attenuated vaccines may be inactivated whole virus, recombinant virus-like particles, recombinant core-like particles or of constructing recombinant which would combine the safety of inactivated product and the advantages of live virus. VP2 (inducing neutralizing antibodies) has been studied as a candidate for recombinant vaccine (CIDRAP, 2004). Combined vaccine of VP2 and VP5 proteins was reported to protect all the vaccinated sheep and also to elicit a higher neutralizing antibody response (Dungu *et al.*, 2004).

3. MATERIALS AND METHODS

3.1. Study design

A cross-sectional seroepidemiological survey of bluetongue virus antibodies in Ethiopian indigenous sheep was conducted between October and December 2004 to estimate the overall, district and village seroprevalence at different levels of geographic areas and assess risk factors. A total of 860 indigenous sheep in 182 flocks (ranging from 10 to 42 flocks per village), with an average flock size of 9.3 (ranging from 2 to 35), were sampled. All animals under individual ownership were recognized as one flock, irrespective of their small size.

3.1.1. Study area description

This study was conducted in three selected districts within the Amhara National Regional State, nortwestern Ethiopia, namely *Metema*, *Guangua* and *Kutaber*. The districts were selected purposively based on agroecological zones. Agroecologies for sampling were defined traditionally as lowland (<1000 m.a.s.l), middle altitude (1000-2000 m.a.s.l) and highland (>2000 m.a.s.l). The region was stratified into three agroecological zones and one district was selected as a representative to that stratum. The laboratory analysis was conducted at the National Veterinary Institute, Debre Zeit, Ethiopia.

The Amhara National Regional State lies in the northwestern part of Ethiopia bordering Tigray to the North, Afar to the East, Oromyia to the South, Benshangul gumiz to the West and the Sudan to the Northwest (Figures 4 and 5). The region covers 25% of the total area of the country, and is estimated to be 150,173.66 sq.km. with a population of 17.205 million people (CACC, 2003). It lies between $9^{0}20'$N - $14^{0}20'$N, and $36^{0}20'$E - $40^{0}20'$E. The altitude ranges from less than 500 meters above sea level at Matebia to 4620 meters above sea level at Ras Dashen. Moisture availability varies from humid to arid with average annual rainfall ranging from 700mm in the arid zones to 2500mm in the humid zones. The rain pattern is bimodal. The main rainy season extends from the month of June to September and the shorter rainy season is from March to May. The mean annual

temperatures range from less than 11^0C in the mountainous areas to more than 40^0C in the lowlands (BOA, 2000). Due to the presence of many running rivers and lakes, there is an abundance of water in those bodies, which are favourable for insect vectors. Some water bodies that are stagnant form boggy and marshy land. Vector-borne diseases such as malaria and kala-azar disease in humans, babesia and African horse sickness in animals are widely distributed.

Agroecological zones were defined based on altitude, climatic conditions, and geographic characteristics that affect the habitat of insect vectors (*Culicoides* species) and the bluetongue virus (BTV). These are traditionally termed as '*Kolla*' (lowlands), '*Woinadega*' (middle altitudes) and '*Dega*' (highlands), covering 31%, 44% and 25% of the total area of the ANRS, respectively.

Smallholder mixed farming dominates (80%) and livestock is an integral part of the farming system in the region. Out of the total area about 36% is under cultivation, 23% under grazing and browsing, 12% covered by bush and shrubs, 2% covered with forest, 16% not utilized, 7% settlement and construction site, and 4% belong to water bodies (BOA, 2000).

In the region free grazing is mostly practiced and the grazing system is communal. The general feeding practices are estimated as: grazing 48.6%; crop residues 35.3%; Hay 13.1%; by-products 1.1%; improved feed 0.2%; and others 3.5% (CACC, 2003). In high and mid altitudes, stall-feeding (dry season) is practiced. For dry seasons (when feed is least available), farmers used to save their crop residues, like straw and hay to practice stall-feeding. Better feed is available during the late rainy season (September to November), when the pasture is green and the feed biomass, relatively high. Water source may be from rivers, streams, ponds, wells and lakes. The distance of watering point varies from less than 1 to 10 kms away from the house. Animals are kept indoor (installed) in the highlands and in some parts in the middle altitude. In the lowland areas, herders use loose kernel or fences.

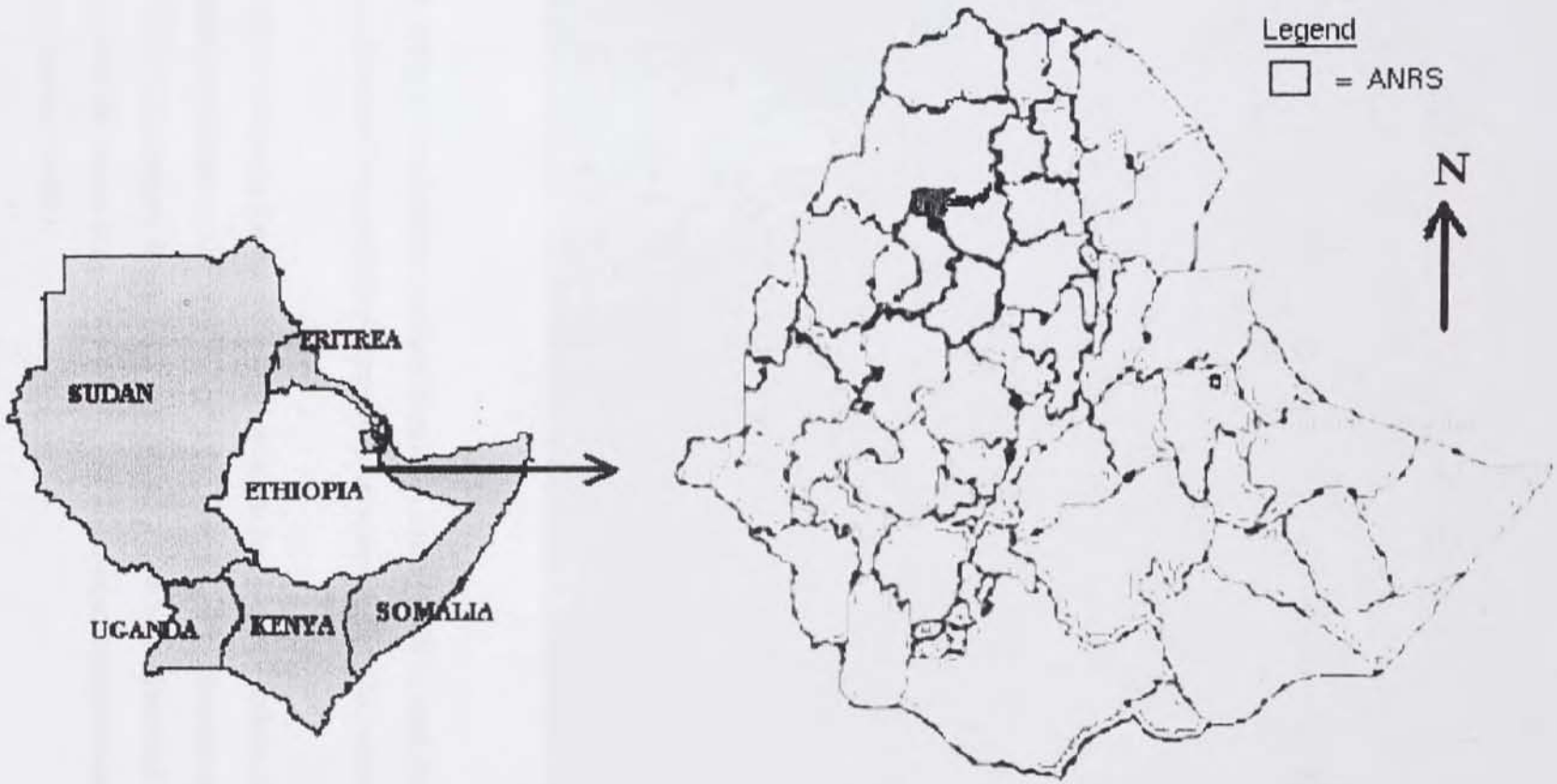


Figure 4: Map of Ethiopia with neighboring countries and zonal boundaries, to show the Amhara National Regional State

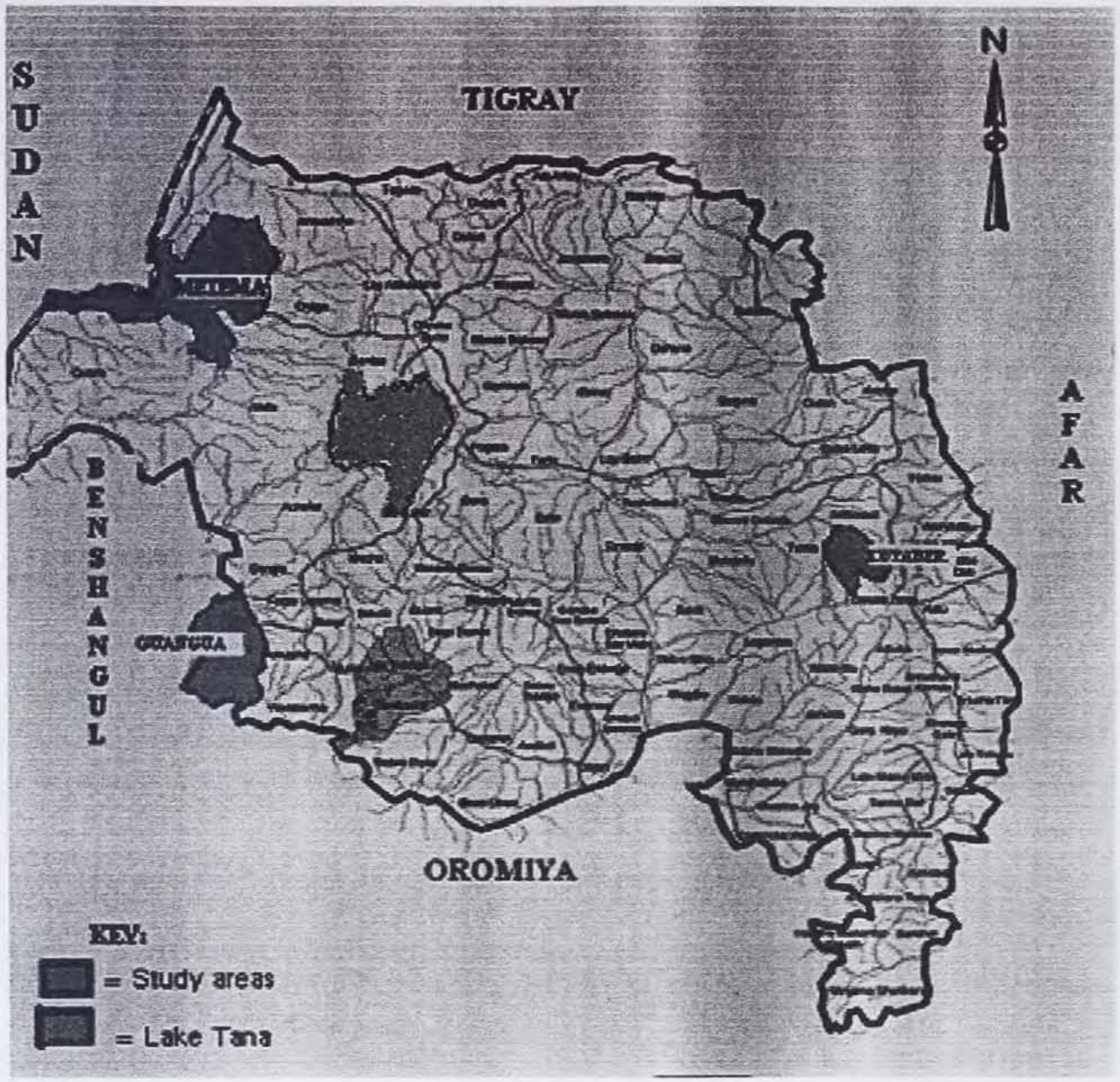


Figure 5: Map of the Amhara National Regional State (ANRS) with district boundaries showing the locations where sheep sera were taken (red shaded)

Metema district: lies in the extreme northwestern part of the Amhara Region as well as the nation bordering Sudan to the west (Figure 5). The area is estimated to be 4057 km^2 (BOA, 2000). The altitude ranges from 500 to 700 m.a.s.l. The annual rainfall ranges from 700mm to 900mm and the mean annual minimum and maximum temperatures are 28^0C and 43^0C (Data from district OARD).

Most part of this district is covered by forest, bush and savannah grass. Extensive grazing predominates and animals are not housed. Currently, the regional state is using this area for settlement of people from the over populated highlands. Its location gives it the proximity to neighboring country (Sudan, which is a bluetongue endemic).

The ecosystem (ecology), representing '*Kolla*' (lowland), is favourable for insect breeding. In the district there are 4,956 sheep, 103,756 cattle, 29,863 goats, 7114 equines and 446 camels (CCAC, 2003).

Guangua district: lies in the extreme southwestern part of the Amhara National Regional State bordering Benshangul gumiz national region to the southwest (Figure 5). The area is estimated to be 1784 km^2 (BOA, 2000). The altitude ranges from 1500 to1700 m.a.s.l. The annual rainfall ranges from 1300mm to 1800mm and the mean annual minimum and maximum temperatures are 20^0C and 25^0C (Data from district OARD). Small-scale mixed farming is the dominating practice. Stall-feeding is partially practiced. This district is densely populated with livestock as well as people and most of the land is intensively cultivated in the rainy season. Irrigation is also highly practiced.

The ecosystem, representing '*Woinadega*', is very ideal for insect breeding. In the district there are 248,566 sheep, 638, 869 cattle, 91,959 goats and 104,972 equines (CCAC, 2003).

Kutaber: district is located in the eastern part of the region (Figure 5). The area is estimated to be 936 km^2 (BOA, 2000). This district is included in the study for the representativeness of the third agroecology (cold highlands). The altitude ranges from 1759 to 3455 m.a.s.l. The annual rainfall ranges from 500mm to 995mm and the mean annual minimum and maximum temperatures are 10^0C and 20^0C. The mean relative humidity is 68%. Frost usually occurs between months of October and January (annual report of the Kutaber district Office of Agriculture, 2003/2004). This district is densely populated and most of the land is intensively cultivated in the rainy season. Grazing land is scarce and livestock owners used to practice stall-feeding using hay and crop residues. In the district there are

72,077 sheep, 67, 996 cattle, 43,247 goats and 19,505 equines (CCAC, 2003). A summary of the description of the study areas is presented in Table 4.

Table 4: A summary description of the study sites surveyed for bluetongue seroprevalence in nortwestern Ethiopia (Oct.– Dec. 2004)

		Annual Average	
	Altitude	Temperature	Rainfall
Study area/ Village	(m.a.s.l)	(Min-Max; °C)	(Min-Max; mm)
Metema district			
Metema Yohannes	600	28-43	700-900
Agam Wouha	700	28-43	700-900
Kokit	600	28-43	700-900
Guangua district			
Addis Alem	1700	20-25	1300-1800
Dangulla	1650	20-25	1300-1800
Bezrakani	1600	20-25	1300-1800
Kutaber didtrict			
Alansha	2700	10-20	500-995
Kelemderba	2850	10-20	500-995
Doshegn	2900	10-20	500-995

3.1.2. Target population

The target population comprises the total population of indigenous sheep in the Amhara National Regional State that is estimated as 5,320,330 (CCAC, 2003). This amount accounts to 39% of the Ethiopian sheep population.

A combination of probability and non-probability sampling methods were applied in this study. The study population was stratified into three strata based on the agroecology (traditional), lowland, middle altitude and highland. The stratification was required because the study population is kept under different agroecological zones, which could influence the existence and prevalence of the biological vector of bluetongue virus which in turn influences the seroprevalence of BTV to be estimated. Then, three study districts namely, *Metema*, *Guangua* and *Kutaber* were selected purposively from the Amhara National Regional State, each to represent one of the three agroecological zones. The considerations for selection were agroecology, livestock holdings, proximity to endemic areas and accessibility. Selected districts were considered as strata. Then a list of villages within each district was compiled from a data obtained from the respective district's agricultural office with the help of animal health professionals, and study villages were sampled randomly from the list (sample frame). The sampling frame of the households in each village was prepared with the help of administrative bodies and agricultural development agents in the locality.

The required number of livestock owners (households) were selected based on lottery system from a list of farmers in that village (sample frame) with replacement, and sera were collected from all sheep owned by the selected farmers until the required number was attained.

The criteria for inclusion of animals for sampling were all indigenous sheep older than 6 months of age. This was to avoid discrepancy due to maternal antibodies. A total of 860 serum samples of sheep from 182 flocks were collected using plain 10 ml vacutainer tubes.

3.1.3. Sample size

A total of 860 sheep sera were collected to examine for the presence of antibodies against bluetongue virus. This sample size was determined using standard procedures as described by Thrusfield (1995) for an infinite population. The following points were considered for the calculation: 50% estimated prevalence, 95% confidence and 5% allowable error for the estimate. A two-stage cluster sampling technique has been used to calculate the minimum sample size. The formula used for sample size determination was:

$$Ts = \frac{1.96^2 * g * P * (1-P)}{g * d^2 - 1.96^2 Vc}, \text{ where,}$$

Ts= Total sample size

g= Number of clusters to be sampled (9X20= 180)

P= estimated prevalence (0.5)

d= desired absolute precision (acceptable error)

Vc= between cluster variance = $s.d^2$=0.0625 (s.d= standard deviation= $P^2 = 0.5^2 = 0.25$)

1.96= z value for the 95% confidence interval

Therefore, $Ts = \frac{1.96^2 * 180 * 0.5 * 0.5}{180 * 0.05^2 - 1.96^2 * 0.0625}$

$Ts = 824$

Based on that the minimum sample size calculated was 824 to be divided equally among the 9 villages.

3.1.4. Study type

This study was a cross-sectional study to determine the seroprevalence of bluetongue virus group-specific antibodies using the competitive Enzyme-Linked Immunosorbent Assay (cELISA) configuration on sera samples collected from sheep in three agroecological zones namely, warm lowland (*Metema*), middle altitude (*Guangua*) and cool highland (*Kutaber*) in the Amhara National Regional State (Figure 5).

The risk factors associated with bluetongue virus occurrences were collected using a standard questionnaire format. The map indicating the study districts is produced and indicated (Figure 5).

3.1.5. Methodology

3.1.5.1. Sera collection and storage

The sheep (no = 860) were securely restrained at standing position. During sampling the ages of sheep were estimated using dentition or by asking their owners.

About 10ml blood samples were collected from the jugular vein of each animal by venepuncture using sterile, plain vacutainer tubes. The blood containing tubes were provided identification number. Data including the owner's name, flock number, flock size, species, age, sex, housing, body condition, symptoms for disease, vial number, and other relevant information were recorded at the time of bleeding. The blood was allowed to clot for at least four hours under shade. After 4 to 12 hours, centrifuged and the separated sera were collected in separated sterile vials and transported in ice. Then, the sera were stored at -20^0C until subjected to cELISA test.

3.1.5.2. Serological testing

Serum samples were submitted to the National Veterinary Institute, Debre Zeit, for the laboratory analysis. A total of 860 sheep sera samples were tested using cELISA for the

detection of group-specific antibodies to bluetongue virus. Competitive ELISA is reported to be the best sensitive and specific test from any other serological methods with ability to distinguish between antibodies to viruses in the bluetongue and epizootic hemorrhagic disease serogroup (Hoar *et al.*, 2004; Shringi and Shringi, 2005), and also the OIE handbook recommends use of the cELISA for detection of bluetongue virus antibodies (OIE, 2004). The reported sensitivity and specificity from the supplier was 99%. A commercial competitive enzyme-linked immunosorbent assay (cELISA) was used following the procedure described by the supplier of the kit (VMRD, Pullman, USA) as follows:

1. Controls and serum samples (25 µl each) were transferred to the Antigen-Coated Plates (Annexes V and VI) according to the setup record. The plate contents were mixed gently by tapping the sides several times to coat the bottom of the wells. The plates were allowed for 15 minutes at room temperature (24°C), uncovered.

2. Antibody-peroxidase conjugate (25 µl) (Annex V) was added to each well. The plate contents were mixed gently and allowed for an additional 15 minutes at room temperature, uncovered.

3. The plates were washed three times:

4. Substrate solution (50µl) was added to each well. The side of the loaded assay plate tapped several times to coat the bottom of the wells and allowed for 10 minutes at room temperature, uncovered.

5.S top solution (50µl) (Annex V) was added to each well, and mixed gently.

6. Immediately after adding the stop solution, the plates were read on a plate reader at optical density (O.D.) reading wavelength of 620 nm.

Result interpretation

•Test sera were positive when they produced an optical density less than 50% of the mean of the negative controls (% inhibition greater than 50%).

•Test sera that produced an optical density greater than or equal to 50% of the mean of the negative controls were considered negative.

3.1.5.3. Questionnaire survey

A pretested standard questionnaire format was developed for relevant data collection in relation to bluetongue disease (Annex II). The questionnaire was administered to 45 selected stockowners (five from each of the 9 villages) in order to test their level of awareness on bluetongue disease and other directly or indirectly related evidences, such as livestock husbandry and management practices, common animal diseases, abundance and possible harm of insect vectors.

3.1.5.4. Data collection

A standard format was developed and used to collect information related to bluetongue and relevant data for analysis. Risk factors at flock and individual levels were included. The data comprised area location, altitude, climatic conditions, identification number, owner's name, flock number, flock size, housing, species, breed, sex, age, body condition, disease symptoms, and other relevancies (Annex I).

The results of cELISA were compiled for each individual animal tested.

3.1.5.5. Statistical Analyses

Data were stored in Microsoft excel software (Microsoft corporation). The apparent seroprevalence was calculated as the number of test positive samples divided by the total

number of samples tested. Flock level seroprevalence was computed as the number of flocks with at least one sero-reactor sheep divided by the total number of flocks tested.

Ninty-five percent (95 %) confidence interval (CI) estimation with upper and lower limits, was calculated using excel spreadsheet and stata software for all rates obtained in the study. The formula used for the seroprevalence at different levels was:

CI = Seroprevalence $\pm$ 1.96 * Standard Error

True seroprevalence, positive and negative predictive values were calculated from the apparent seroprevalence employing sensitivity and specificity of the test, which was 99% as reported by the kit supplier (VMRD), using WIN EPISCOPE 2.0 software. The general formulae for the true prevalence, positive and negative predictive values as described by Pfeiffer (2002) are:

- True prevalence (P) = $\frac{AP + (Sp-1)}{Se + (Sp-1)}$; where,

AP = Apparent Prevalence

Sp = Specificity

Se = Sensitivity

- Positive predictive value (PV+) = $\frac{P*Se}{(P*Se) + (1-P)*(1-Sp)}$; where,

P = True prevalence; Se and SP sensitivity and specificity, respectively

and

- Negative predictive value (PV-) = $\frac{Sp*(1-AP)}{Sp*(1-P) + (1-Se)*P}$, where

Sp = specificity, Se = Sensitivity and P= True prevalence

Descriptive statistics were used to analyze the distribution of antibluetongue antibodies in different agroecological zones, villages, flock size, ages and sexes. The analyses were performed using STATA 7.0 statistical software (Stata corporation). Based on the type of

data, the association between risk factors and bluetongue seroprevalence were analyzed in a univariate and multivariate statistical analyses using the chi-square test for bivariate associations and multiple logistic regressions for multiple factors, respectively. The strengths of the associations were determined using estimates of odds ratio. The putative risk factors were agroecology, flock size, age and sex. Univariate analysis using logistic regression was conducted for assumed risk factors separately to determine their epidemiological importance. A backward elimination procedure was employed to construct a final model. The p-value for statistically analyzed data was considered significantly different at 5% probability level (Thrusfield. 1995).

4. RESULTS

A seroepidemiological survey of bluetongue virus was conducted with the objectives of determining the distribution and seroprevalence of bluetongue virus antibodies and putative risk factors associated with seropositivity in indigenous Ethiopian sheep in the ANRS, northwestern Ethiopia. In this study, a serological investigation of bluetongue virus antibodies using competitive enzyme-linked immunosorbent assay (cELISA) test was employed on 860 sheep sera. Relevant information for data analysis were collected from 45 interviewed livestock owners using a standard questionnaire format.

4.1. Serological survey

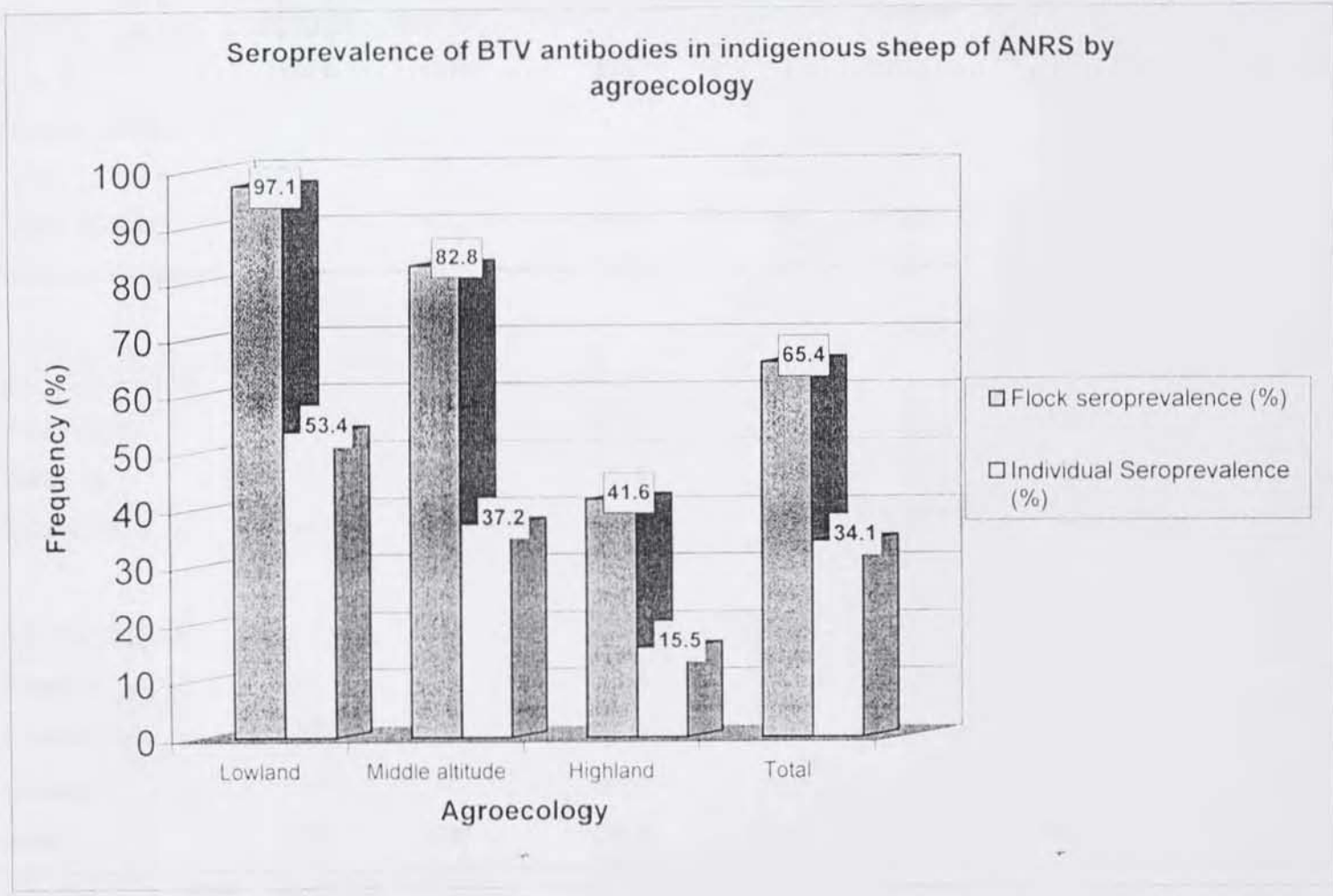
During this study a total of 182 flocks (households) of sheep within 9 villages and 3 districts were bled between October and December 2004 in three agroecological zones of the ANRS, northwestern Ethiopia. Sheep sera were stratified according to agroecological zone, flock size, age and gender for statistical analyses for significant test as risk factors for bluetongue virus seropositivity. There was an overall flock level seroprevalence of 65.4% in 182 flocks with at least one infected sheep per flock and 34.1% at individual level (Tables 5 and 6). The flock level seroprevalence ranged 41.7% in the highlands to 97% in the lowland areas.

Figure 6 presents the seroprevalence, flock and individual sheep level of bluetongue virus antibodies in indigenous sheep sampled from different agroecological zones of the ANRS, northern Ethiopia.

All the three sampled districts and the 9 villages were seropositive (with at least one reactor) for bluetongue virus antibodies (Figure 6 & Table 5). The individual animal level bluetongue virus antibody seroprevalences were highest in lowland (53.4%, 95% CI = 50.1% – 56.7%) followed by middle altitude (37.2%, 95% CI = 34.0% – 40.5%) and highland (15.5%, 95% CI = 13.1% - 17.9%).

On flock basis, the seroprevalence in their respective agroecologies were 97.1% (95% C.I = 96.0% - 98.2%), 82.8% (95% C.I = 80.2% - 85.3%) and 41.6% (95% C.I = 38.3% - 44.9%), which were much higher than individual animal seroprevalence. Of the total (293) positive animals, 141 (48.1%) were from lowland, 102 (34.8%) from middle altitude and 50 (17.1%) from highland. The difference of seroprevalence between the three agroecological zones was significant ($\chi^2 = 94.5$, df = 2, $p < 0.001$). Sheep in the lowland were 6.2 (95% CI = 4.2 – 9.2) and 1.9 (95% CI = 1.4 – 2.7) times more likely to be seropositive than sheep in highland and middle altitude, respectively. The sheep at the middle altitude to be seropositive for bluetongue virus was 3.2 (95% CI = 2.2 – 4.8) times more likely than sheep in the highland.

Figure 6: A summary of seroprevalence of BTV antibodies using competitive ELISA in sheep of different agroecological zones in the Amhara National Regional State (Oct. to Dec. 2004)



($\chi^2 = 94.5$, df = 2, $p < 0.001$

Table 5: A summary of seroprevalence, flock and animal level of bluetongue virus antibody in sheep sera of different villages in the ANRS

Village	Flock			Individual			
	Number tested	Number positive	Seroprevalence (%)	Number tested	Number positive	Seroprevalence (%)	(95 % confidence interval)
Metema district							
Kokit	13	12	92.3	85	32	37.6	(34.4–40.9)
Agam Wouha	13	13	100.0	92	68	73.9	(71.0-76.8)
Metema Yohannes	9	9	100.0	87	41	47.1	(43.8-50.5)
Guangua district							
Addis Alem	24	20	83.3	110	47	42.7	(39.4-46.0)
Dangulla	24	17	70.8	111	25	22.5	(19.7-25.3)
Bezrakani	11	10	90.9	53	30	56.6	(53.3-59.9)
Kutaber district							
Alansha	16	10	62.5	101	15	14.8	(12.5-17.2)
Kelemdereba	42	19	42.9	134	25	18.7	(16.1-21.3)
Doshegn	30	9	30.0	87	10	11.5	(9.1-13.6)
Total	182	119	65.4	860	293	34.1	(30.9-37.2)

χ^2= 144.9, df=8, p = 0.000

The percent seropositivity among sheep from different villages, as shown in Table 5, varied from 11.5% (n = 87) in Doshegn (Altitude 2900 m.a.s.l) to 73.9% (n = 92) in Agam Wouha (Altitude 700m.a.s.l). The highest flock seroprevalence was observed in Agam Wouha and Metema Yohannes (100%) followed by Kokit and Bezrakani (92.3%, 95% CI = 90.5% - 94.1%, and 90.9%, 95 % CI = 89.0% - 92.8%,) villages, respectively and the lowest was in Doshegn (30.0%, 95% CI = 26.9% - 33.1%). The difference in seroprevalence on village basis was statistically significant (χ^2= 144.9, df=8, P< 0.001).

The average number of sheep sampled per flock was 9.3 (ranging from 2 to 35). Considering at least one seropositive sheep in a flock, the overall seropositivity recorded in this study for bluetongue virus antibodies was 65.4% (Table 6). The seroprevalence was found to increase with flock size from small to large, ranging from 25.8% (95% CI = 22.9% - 28.7%) to 38.4% (95% CI = 35.2% - 41.7%). However, the difference among the different flock sizes was not significant (p> 0.05).

Table 6: A summary of seroprevalence of bluetongue virus antibodies in sheep of the Amhara National Regional State based on flock size category (Oct. to Dec. 2004)

Flock size category	Number of sheep tested	Number of positives	Seroprevalence (%)	(95% Confidence interval)
< 7 sheep	248	64	25.8	(22.9 – 28.7)
7-15 sheep	336	123	36.6	(33.4 – 39.8)
>15 sheep	278	106	38.4	(35.2 – 41.7)
Total	860	293	34.1	(30.9 – 37.2)

Table 7 shows the seroprevalence of bluetongue virus antibodies in relation to sex. The total numbers of male and female sheep tested were 226 and 634, respectively. A statistically significant difference (χ^2 = 7.7, p< 0.01, OR = 1.6) in seroprevalence of bluetongue virus was observed between female and male sheep, which seems higher in females 36.7% (95% CI = 33.5% – 39.8%) as compared to 26.5% (95% CI = 23.6% – 29.5%) in males.

Table 7: A summary of seroprevalence of bluetongue virus antibodies in sheep of the Amhara National Regional State based on sex category (Oct. to Dec. 2004).

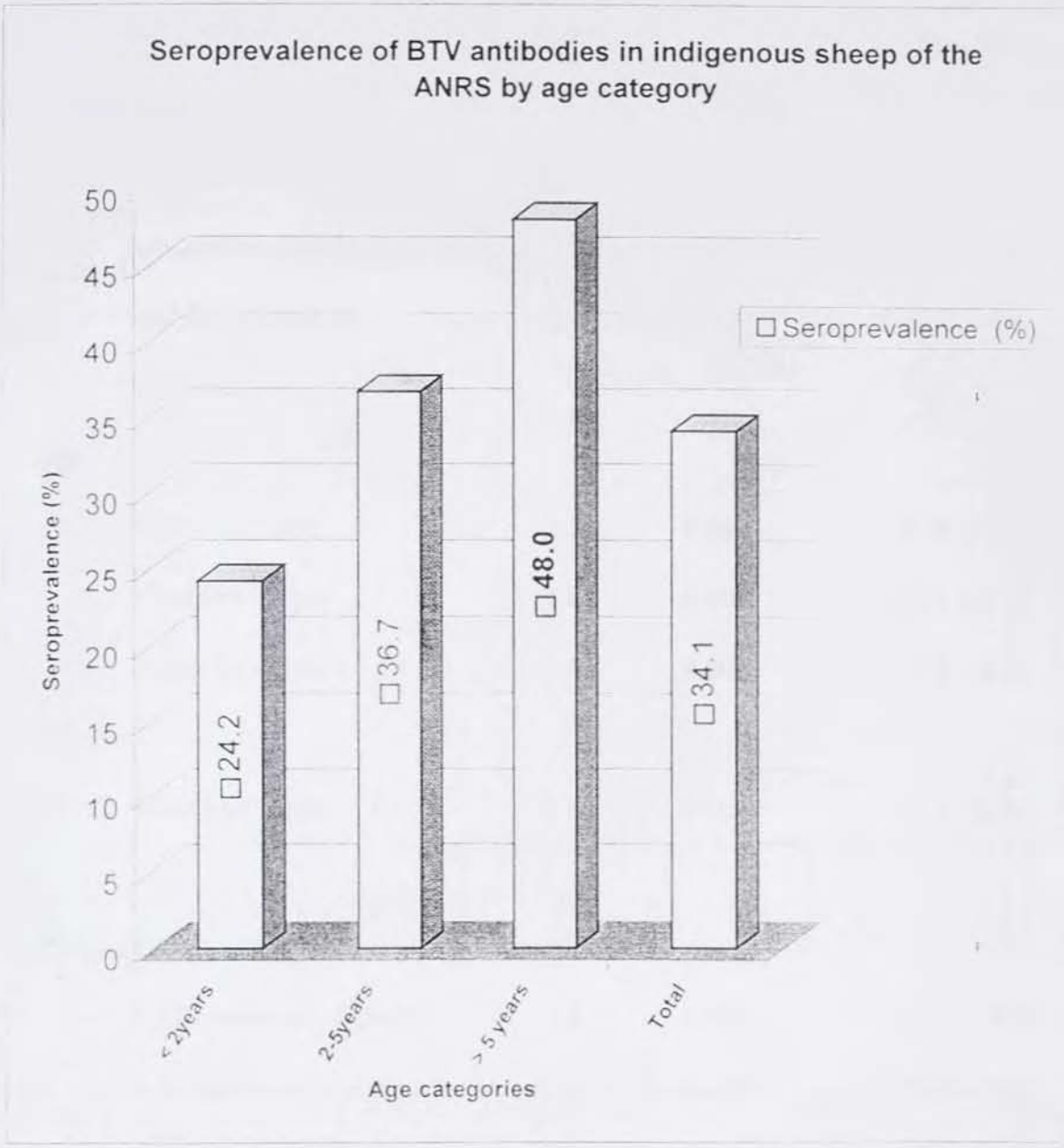
Sex category	Number of sheep tested	Number of sheep positives	Seroprevalence (%)	(95% Confidence interval)
Male	226	60	26.5	(23.6 – 29.5)
Female	634	233	36.7	(33.5 – 39.8)
Total	860	293	34.1	(30.9 – 37.2)

The minimum and maximum ages of sampled sheep were 0.5 and 13 years with an average of 2.82 years. Ages were categorized into three groups as sheep less than 2 years old, 2 to 5 years and greater than 5 years of age. The respective numbers of sheep in each group were 343 (39.9%), 338 (39.3%) and 179 (20.8%).

The age was categorized in three groups as indicated in Figure 7. Age specific seroprevalence increased from 24.2% (95% CI = 21.3% - 27.1%), in <2 years old group to 48.1% (95% CI = 44.7% - 51.4%) in >5 years group. The seroprevalence difference was significant (χ^2= 31.5, df = 2, p< 0.001) among age categories. Logistic regression analysis for age and seroposetivity provided an odds ratio (OR) value of 2.9 (95%CI = 2.0 – 4.2) for age group 3 (>5 years) compared to 1 (<2 years). And 1.8 (95% CI= 1.3 – 2.5) for age

group 2 (2-5 years) compared to age group 1 (<2 years). Odds ratio value for age group 3 compared to age group 2 was 1.6 (95%CI = 1.1– 2.3).

Figure 7: A summary of seroprevalence of bluetongue virus antibodies in sheep of the Amhara National Regional State based on age categories (Oct. to Dec. 2004)



$\chi^2 = 31.5$. df = 2 p = 0.000

Table 8: A summary of univariate logistic regression estimates for different putative risk factors of bluetongue virus seropositivity in the ANRS (Oct to Dec. 2004)

Putative risk factor	Odds ratio (OR)	P-value	(95% confidence interval for OR)
Agroecology			
- lowland vs highland	6.2	0.000	(4.2 –9.2)
- lowland vs middle altitude	1.9	0.000	(1.4 – 2.7)
- middle altitude vs highland	3.2	0.000	(2.2 – 4.8
Age			
- >5yrs vs < 2yrs	2.9	0.000	(2.0 – 4.2)
- > 5yrs vs 2-5yrs	1.6	0.000	(1.1 – 2.3)
- 2-5yrs vs < 2yrs	1.8	0.000	(1.3 – 2.5)
Sex			
- female vs male	1.6	0.006	(1.1 - 2.2)
Flock size			
- > 15 sheep vs < 7 sheep	1.8	0.002	(1.2 - 2.61)
- > 15 sheep vs 7- 15 sheep	1.1	0.460	(0.8 - 1.5)
- 7-15 sheep vs < 7 sheep	1.7	0.006	(1.2 – 2.4)

Multivariate analysis of logistic regression for the different putative risk factors indicated that two of the four putative risk factors (agroecology and age) were significant ($p<0.05$) and thus included with the final model (Annexes VII, VIII and XI). Flock size and sex, which were significant in the univariate analyses, did not show any significant association ($p>0.05$) in the final model.

Upon clinical evaluation during sampling and information obtained from owners, a proportion of sheep were found to have symptoms assumed to be signs of bluetongue in sheep (nasal discharge, coughing, history of abortion and diarrhoea). Of the 293 bluetongue virus seropositive sheep 42.0% had clinical signs but there was no statistical association ($\chi^2 = 2.99$, df. = 1, $P> 0.05$) between related clinical signs and bluetongue virus seroposetivity. Moreover, there was no significant correlation between any of clinical sign and seropositivity in a correlation analysis by stata. It was also observed that only nasal discharge and coughing were correlated among the four bluetongue related clinical signs considered.

4.2. True prevalence and Predictive values of the test

The calculated true seroprevalence, positive and negative predictive values of the test on sheep sera in the Amhara National Regional State (overall, by agroecology, by age and sex) were presented in table 9. The overall true seroprevalence, positive and negative predictive values were 33.7%, 98.2% and 99.5%, respectively.

Character	Sample tested	Number of positive	Apparent seroprevalence (%)	True (Estimated) seroprevalence (%)	Positive predictive value (%)	Negative predictive value (%)
Total	860	293	34.1	33.7	98.2	99.5
Agroecology						
Lowland	264	141	53.4	53.5	99.1	99.8
Middle altitude	274	102	37.2	37.0	98.3	99.4
Highland	322	50	15.5	14.6	94.4	99.8
Age						
<2 years	343	83	24.2	23.7	96.9	99.7
2 to 5 years	338	124	36.7	36.4	98.3	99.4
>5 years	179	86	48.0	48.0	98.9	99.1
Sex						
Male	226	60	26.5	26.0	97.2	99.7
Female	634	233	36.7	36.4	98.3	99.4

Table 9: A summary of true seroprevalence, positive and negative predictive values of the test on sera of indigenous sheep in the Amhara National Regional State.

4.3. Questionnaire survey

Forty-five selected livestock owners were questioned using a pretested standard questionnaire format developed for relevant data in relation to bluetongue disease.

All the 45 interviewees questioned have responded, as they did not see a specific disease showing the common known clinical signs of bluetongue, rather there was a tendency to incline to other endemic diseases such as PPR, ORF, FMD, up on trying to give them clues based on common clinical signs of bluetongue disease (pictures of sheep with common symptoms of bluetongue were shown). Moreover, none of the livestock owners did sense the negative impact of small biting insects such as mosquitoes. They complained of the larger biting flies.

This study also showed that there was a wide animal movement around *Metèma* district. *Metema* is a main route for animal trading to Sudan (300 to 800 cattle were driven daily at the time of sera collection as estimated by the district's agricultural office). The Sudanese livestock also enter the Ethiopian territory for grazing land and water.

The existing husbandry system is free grazing with mixed crop and livestock farming. Stall-feeding, during the dry seasons for all livestock and regularly for working oxen, is practiced in the middle and higher altitudes. Water sources used were rivers, streams, ponds and wells. The distance of watering point varies from less than 1 to 15 kms away from the house. Stalling of animals during night is practiced in the highlands and in some parts in the middle altitude. In the lowland areas, herders use loose kernels or fences.

Figure 8 shows the frequency of sheep diseases mentioned by livestock owners in lowland/midland and highland. The major diseases of sheep complained in lowlands were Peste des Petits Ruminants (PPR) (24/30 respondents), contagious pustular dermatitis (ORF) (23/30), pneumonia (17/30), sheep pox (9/30), foot-rot (8/30) and diarrhea (8/30). While those mentioned in highlands were pasteurellosis (14/15), PPR (9/15), foot-rot (6/15) and pneumonia (5/15) (Figure 8).

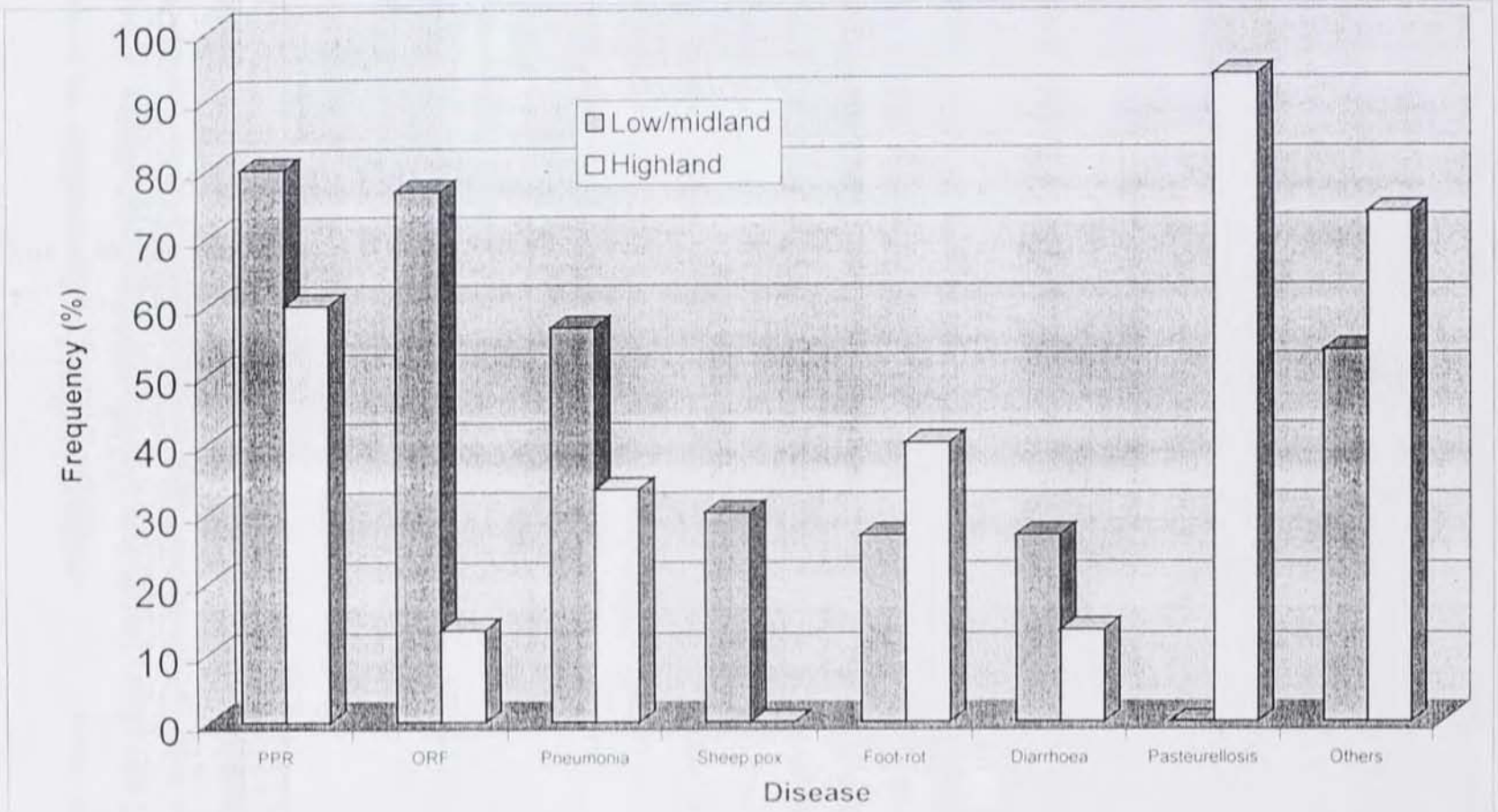


Figure 8: A summary of sheep diseases categorized by owners according to their economic importance in lowland and highland areas of Northwest Ethiopia

5. DISCUSSION

After the first detection of bluetongue antibodies in sheep in central Ethiopia (Woldemeskel *et al.*, 2000), there was a national interest to learn more about the prevalence as well as the distribution of the virus among domestic ruminants in different parts of the country. A seroprevalence study of bluetongue in indigenous sheep was employed in three agroecological zones of the Amhara National Regional State (ANRS), northwestern Ethiopia from October to December 2004. One study site was bordering Sudan, which is endemic to bluetongue virus.

Based on this study, bluetongue virus antibody was found widely distributed in indigenous sheep in the Amhara National Regional State, northwestern Ethiopia, with a seroprevalence of 34.1%. This result confirms the earlier work of Woldemeskel *et al.* (2000) who reported the presence of seropositive sheep with a seroprevalence of 46.7% in central Ethiopia by detecting antibodies to bluetongue virus, using the same ELISA configuration, though he employed a non-random sampling design to test 90 sheep (about 30 from each of the tree agroecological zones). However, since the disease is not reported clinically and not known by the livestock owners while the ecosystem of most part of Ethiopia is ideally favourable to the biological vector (*Culicoides* species), the potential danger of spreading the infection throughout the country should be considered.

The moderately higher seroprevalence result obtained in the current study could be explained through the differences between agroecologies, which may create a favorable condition for the activities of the vector, and the existing management practices employed for agricultural activities favouring the transmission of bluetongue virus.

Generally the fact that this moderately higher seroprevalence of bluetongue virus antibodies in sheep sera is in agreement with investigation reports of other workers in different countries. Among these were those reported by Exeifeka *et al.* (1983) 27.6% in Nigeria; Mushi *et al.* (1992) 25.5% in Botswana; Formenty *et al.* (1994) 52.0% in Ivory Coast;

Woldemeskel *et al.* (2000) 46.7% in central Ethiopia; Hendrikx *et al.* (2001) 28.2% in France (Corsica); Bhalodiya and Jhala (2002) in India (55.5%) and Shringi and shringi (2005) 30.3% in India, too.

However, lower seroprevalences were recorded from indigenous sheep in New Guinea (10%); Cameroon (16.2%); Zimbabwe (13.3%); India (15.8%); Croatia (13.7%); Kazakhstan (21.4%), (Vankamman and Cybinski, 1981; Ekue *et al.*, 1985; Musuka and Kelly, 2000; Govindarajan *et al.*, 2002; Listes *et al.*, 2003; Lundervold *et al.*, 2003; respectively).

Extremely high seroprevalences in sheep also were reported in Mozambique (82.4%) by Kanhai and De Silva (1981) and Tanzania (73.9%) by Hyera and Lyaruu (1995).

The seroprevalence in different areas may vary depending upon the breed involved and the seasonal concentration of the vector. Several species of domestic and wild animals may also be involved in maintaining the infection in a given locality than the other (Martinez *et al.*, 1999; Govindarajan *et al.*, 2002; OIE, 2004).

Moreover, bluetongue has been endemic in Sudan and Kenya as confirmed about 25 and 96 years ago, respectively (Eisa *et al.*, 1980; Sellers, 1981; Naresh *et al.*, 1996). These countries have long borders with Ethiopia. There is a free animal movement across national boundaries between Sudan and Ethiopia (Sudanese livestock enter to Ethiopian territory for grazing and water during the dry season). Therefore, the occurrence of bluetongue virus infection in Ethiopian indigenous sheep at higher seroprevalence may not be surprising.

The seroprevalence of bluetongue virus antibodies in relation to the three agroecological zones clearly indicated that there is appreciably higher seropositivity in the lowland and middle altitude than in the highland sheep. There was a statistically significant ($p<0.001$) difference in seroprevalence to bluetongue virus antibodies. Sheep in the lowland were 6.2 and 1.9 times more likely to be seropositive than sheep in highland and middle altitude, respectively. The odds ratio of sheep at the middle altitude was 3.2 times more likely to be

seropositive than sheep in the highland. The higher seroprevalence of bluetongue virus antibodies in the lowland and middle altitude and very low in the highland sheep suggests that lower altitudes are more favourable for the activities of the *Culicoides* vectors. This result showed similar pattern with the previous reports by different workers (Exeifeka *et al.*, 1983; Homan *et al.*, 1985; Woldemeskel, *et al.*, 2000). Mullens *et al.* (2003) clearly described in their investigation report that environment governed dynamics and intensity of vertebrate-vector contact in both time and space. They also discussed that temperature had dramatic and consistent influence, altering vector developmental rates and life history parameters since temperature was best known to control rates of virogenesis time to transmission for bluetongue viruses in *Culicoides*.

Braverman *et al.* (2001) clearly suggested that climatic variables influenced bluetongue virus spatial and seasonal distributions through modifications of vector abundance and capacity which incorporates biting rates, vector survival, extrinsic incubation and vector competence. Same workers also stated that both temperature and rainfall, which are functions of agroecology, were significant factors, as temperature had a profound influence on the survival of these vector capacity components and since the immature stages of *Culicoides imicola* are found in areas of damp or wet organic matter, rainfall has a major positive influence on vector population densities. Caporale *et al.* (2003) described that bluetongue vector distribution in the world was based on climatic and environmental conditions instead of political boundaries.

Braverman and Chechik (1993) suggested that altitude of up-to one km is the most appropriate height level, as regards temperature and relative humidity, for survival of *Culicoides* species in long time transportation. Moreover, Dungu and colleagues (2004) indicated that bluetongue virus stops replicating in the *Culicoides* vectors when the temperature was below 15°C and the activity of *C. imicola* the main insect vector of bluetongue, was also temperature dependent.

The current study showed the presence of bluetongue virus antibodies in areas with a wide range of altitudes. Seropositive sheep were obtained in all heights included in the study

(600-2900m.a.s.l) although seroprevalence declined as the altitude advanced. This work also showed significant clustering of bluetongue virus antibodies at the village level, which may be a function of local variations in the distribution and activity of the vector *Culicoides*.

Interestingly, there was a statistically significant ($p<0.001$) difference in seroprevalence to bluetongue among different age groups. Old sheep were 2.9 and 1.6 times more likely to be seropositive than yearlings and young sheep (2-5 years), respectively as indicated by the logistic analysis. Similarly, young sheep were 1.8 times more likely to be seropositive than yearlings. This difference is attributed to the fact that older animals are usually more exposed to bluetongue virus infection than younger ones due to their longer life. Infection of sheep with bluetongue virus leads to production of long-lasting protective humoral immunity (precipitating antibodies). The risk of sero-conversion exhibited as age advances agrees with reports by various workers (Uhaa *et al.*, 1990; Formenty *et al.*, 1994; Hyera and Lyaruu, 1995; Bhalodiya and Jhala, 2002; Lundervold *et al.*, 2003).

Howard (1999) suggested that group-specific antibodies persist for about six to eighteen months, whereas type specific neutralizing antibodies were still been demonstrated after three years. Shringi and Shringi (2005) reported a significantly different seroprevalence of bluetongue virus antibodies in different age groups of Indian sheep. That was 6.0% in less than 2 years old and 10.0% in more than 2 years old. The higher seroprevalence in older sheep, therefore, was not unexpected. The higher seropositivity among the older indigenous sheep indicates that these adult animals may be a potential source for spread of bluetongue virus in the region in particular and in the nation in general.

Upon univariate analysis, a significant difference ($\chi^2 = 7.7$; $P < 0.01$; OR = 1.6, 95% CI = 1.1 - 2.2) in seroprevalence of bluetongue virus antibodies was observed between female and male sheep. It was higher in females 36.7% as compared to 26.5% in males. However, the final model rejected sex as being risk factor. Sex may be confounded by age. The average age of female sheep sampled in this study was 3.38 years and that of males was

1.26 years. Shringi and Shringi (2005) reported similar seroprevalence of bluetongue virus antibodies (9.8% in male and 9.5% in female) in Indian sheep.

In the multivariate logistic regression analysis the most parsimonious model of bluetongue virus seropositivity included agroecology and age as major determinant factors.

This study showed that the livestock owners had no knowledge about bluetongue disease and the negative effect of potential biological vectors (*Culicoides* mosquitoes). This might be due to similarities of clinical symptoms of BT with other endemic diseases like foot and mouth disease, contagious pustular dermatitis and peste des petits ruminants. Due to its mild nature and great variation of clinical signs even animal health professionals may miss it to consider in differential diagnosis.

The high seroprevalence of bluetongue virus in the indigenous sheep without any clinical report and lack of awareness among the livestock owners might not be surprising as bluetongue is reported to be mild in African breeds while being highly fatal disease in European breeds (FAO, 2003; CIDRAP, 2004; OIE, 2005). Mushi *et al.* (1992) reported a 25.5% (n =369) seroprevalence of bluetongue virus antibodies without any clinical report in Botswana. They also described the subclinical or inapparent nature of bluetongue in African indigenous sheep. The lack of correlation between seropositivity and any of bluetongue resembled clinical signs observed during sampling also can justify the inapparent nature of bluetongue in the local sheep.

Walton (2004) stated that bluetongue viruses have been identified in many tropical and temperate areas of the world but the disease occurs in ruminants in the temperate zones. The same author also remarked the presence of little or none clinical disease in the tropical areas of the world, except when non-native ruminants were introduced into virus endemic areas. The variation of clinical signs in sheep both between individuals and particularly between breeds was described by Lundervold *et al.* (2003). MacLachlan (2003) also discussed the occurrence of clinical bluetongue in incursion regions of the world where infection was not endemic.

In general, the present study revealed that there was a wide spread occurrence of seropositivity to bluetongue virus antibodies among the indigenous breeds of Ethiopian sheep encompassing a large area of the Amhara National Regional State of Ethiopia, with an overall seroprevalence of 34.1%. All sampled districts and villages were found with seropositive animals for bluetongue virus antibodies. This may be an indication for endemicity of bluetongue virus in the Amhara National Regional State of Ethiopia. All the sampled sheep were indigenous type with no exotic blood.

6. CONCLUSIONS AND RECOMMENDATIONS

The results indicated that bluetongue virus was circulating in a wide area of the Amhara National Regional Stare. Clinical disease is not reported so far in Ethiopia. No vaccination programme against bluetongue either in sheep or other species is in practice in Ethiopia. Hence, the occurrence of bluetongue virus antibodies in the sera of the local sheep at higher seroprevalence in such a wide area should have been arisen as a consequence of either natural infection with the virus or of passive immunization with maternal antibodies. However, the chance of detecting colostral antibody in this study was minimized by excluding sheep less than 6 months of age during sampling. The higher seroprevalence and lack of clinical report indicated the possibility of bluetongue virus to be endemic in the region. This condition may lead to infection of susceptible exotic breeds of sheep imported in to Ethiopia, and may act as a deterrent to breed improvement programs.

The most parsimonious model selected included agroecology and age as major determinant factors. Lack of awareness about the bluetongue disease among the community together with the existing livestock management system (free animal movement) may serve as favourable means of spreading of bluetongue virus

Much remained to be learned about the ecological, climatic and environmental factors that lead to expansion of bluetongue virus ecosystems, interims both of insect vectors and specific serotypes of bluetongue virus suggesting the need for further works in the area.

Based on the results of this study, the following recommendations are forwarded:

- Further studies on the bluetongue virus including, isolation and identification of the virus and the serotypes on different animal species (all ruminants) as well as entomological surveys on the biological vectors are of paramount importance.
- Awareness creation among both the livestock owners and animal health professionals to consider bluetongue in differential diagnosis seems very important.

- Special attention should be given to those specific areas with higher seroconversion while attempting any corrective measures relating to bluetongue virus.
- Exotic sheep should be vaccinated against bluetongue virus prior to importation.

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8. ANNEXES

Annex I: Serum sample collection format

Region_____ Zone_____ District_____ Village_____

Date _____ Altitude_____ Average RF_____Average T°_____ CODE_____

Ser No.	Owner's name	Flock no.	Flock size	Sex	Age	Housing	Body condition	Clinical symptoms (H=Healthy, S= Sick,	Tube no.

Annex II: Bluetongue questionnaire format (Livestock owners ' observation)

1. REFERENCE

Region_____ Zone_____

District_____ PA/Kebele_____

Village_____ Date _____

Name (Livestock owner)_____-Age_____

2. NUMBER OF LIVESTOCK OWNED (by species)

Cattle_____ heep_____ Goats_____ Camels_____ Equine_____

3. HUSBANDRY SYSTEM

Sedentary: ☐ Transhumant: ☐ Nomadic ☐

4. ANIMAL MOVEMENT FACTORS

Grazing and watering ☐ Trade ☐ Both ☐ theft ☐

Cultural/ceremonial purposes ☐ Massive human movement ☐

GRAZING AND WATERING AREAS

Locality	Wereda	Zone	Season Wet/Dry/ Drought	Distance from the homestead in	
				hrs	Km

MAJOR CATTLE MOVEMENT ROOTS

FROM	VIA	TO	Purpose

5. DISEASE PROBLEMS

5. 1. List major diseases of cattle in the area (in order of importance).

	LOCAL NAME		SCIENTIFIC NAME
1			
2			
3			
4			
5			
6			

5. 2. List major diseases of sheep & Goats in the area (in order of importance).

	LOCAL NAME		SCIENTIFIC NAME
1			
2			
3			
4			
5			
6			
7			

5.3 Have you ever observed a disease with mouth and tongue lesions in animals? Y☐ N☐

5.4. What is the traditional name of that disease? .....

5.5 Which species of animals are affected? .....

5.6 How does the disease transmit among domestic animals?.....

5.7 When did you observe the disease for the first time?

Before 5 years ☐ Before 1 year ☐

Before 2 years ☐ Less than a year ☐

5.8 What are the suspected source(s) of the disease?

Seasonal herd movement for grazing and watering ☐

Newly introduced animal (trade, gift, Change, etc) ☐

Unknown ☐

5.9 Is the disease present currently? Yes ☐ No ☐

5.10 Is your herd affected by the disease now? Yes ☐ No ☐

5.11 How many animals are affected?

Cattle.....Sheep.....Goats.....Camels.....

5.12 How frequently you have observed the disease (outbreaks)?

each year ☐ every two years ☐

twice a year ☐ every five years ☐

5.13 Is there any treatment to cure the disease? Yes ☐ No ☐

5.14 If yes, what? Traditional .....

Conventional.....

5.15 Is there any control method? Yes ☐ No ☐

5.16 If yes, what?.....

5.18 Do (animal) biting insects exist in your locality? Yes ☐ No ☐

5.19 If yes, list their names and describe their size and appearance? .....

5.20 In which season (s) they do appear?.....

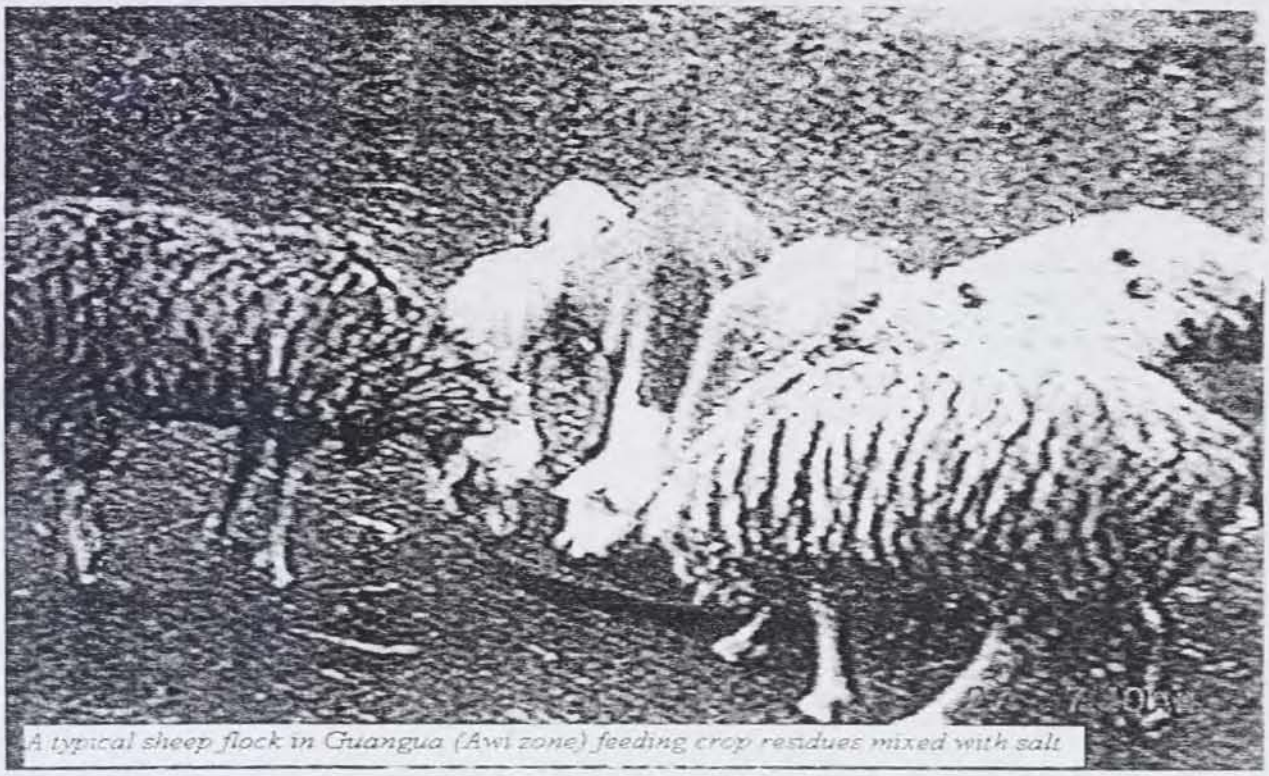
5.21 Do you think that they are harmful? What do they do? Yes ☐ No ☐

5.22 Can they transmit disease (s)? Yes ☐ No ☐

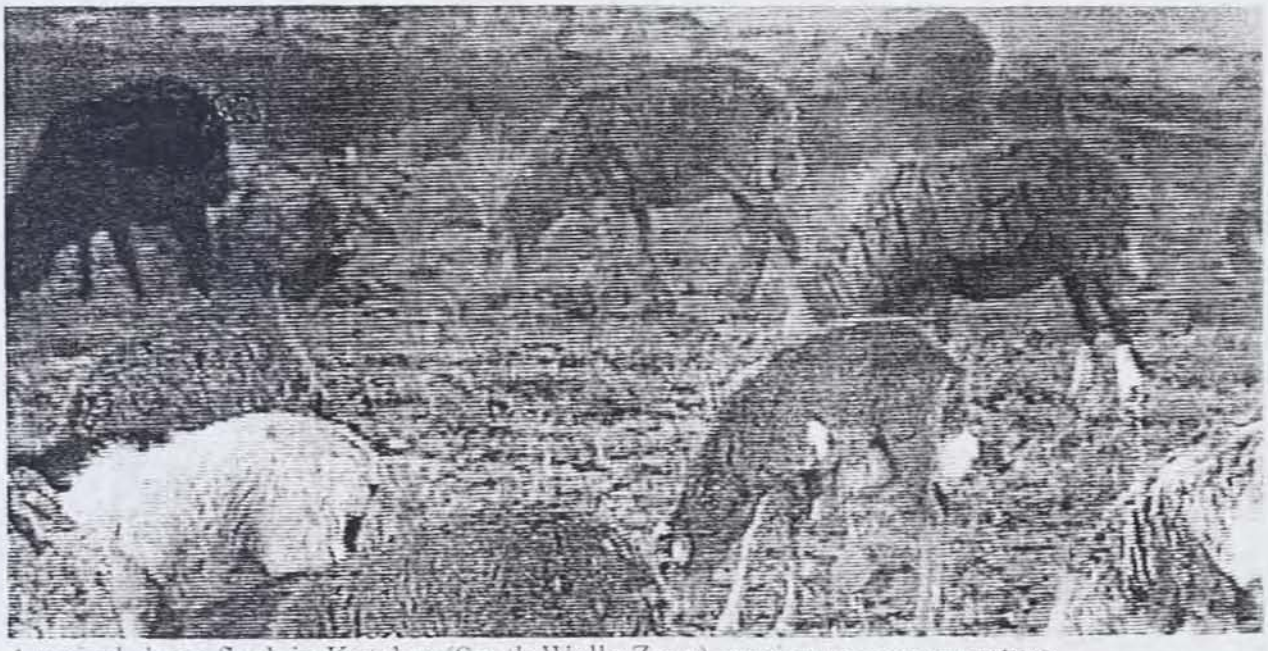
5.23 If yes, which disease(s)? .....

5.24 What are the traditional methods used to control insects (their effect)? .....

Annex III: Pictures of indigenous sheep flocks in middle altitude and highland, to show the different visible phenotypic characteristics in different agroecological zones



A typical sheep flock in Guangua (Awi zone) feeding crop residues mixed with salt



A typical sheep flock in Kutaber (South Wollo Zone) grazing on a poor pasture

Annex IV: Bluetongue data code book used in the study

District (agroecology):	1= Metema (lowland)	2= Guangua (mid altitude)	
	3= Kutaber (highland)		
Villages:	1= Kokit	2= Agam Wuha	3= Metema Yohannes
	4= Addis Alem	5= Dangulla	6= Bezrakani
	7= Alansha	8= Kelemderba	9= Doshegn
Flock size category:	1= < 7 sheep	2= 7 - 15 sheep	3= > 15 sheep
Sex	1= Male	2= Female	
Age category	1= < 2years	2= 2 - 5years	3= >5years
Housing (night)	0= Outdoor	1= Indoor	
Body condition	1= Poor	2= Medium	3= Good
Clinical signs (related to BT)	0= None	1= Nasal discharge	2= Coughing
	3= Diarrhea	4= Abortion	
Test Result	0= Negative	1= Positive	

Annex V. Bluetongue Antibody Test Kit, cELISA (VMRD, Inc., Pullman, USA)

General Description

This competitive enzyme-linked immunosorbent assay (cELISA) detects bluetongue virus antibodies in ruminant sera. Sample serum bluetongue virus anti-body inhibits binding of horseradish peroxidase (HRP)-labeled bluetongue virus-specific monoclonal antibody to bluetongue viral antigen coated on the plastic wells. Binding of the HRP-labeled monoclonal antibody conjugate is detected by the addition of enzyme substrate and quantified by subsequent color product development.

Strong color development indicates little or no blockage of HRP-labeled monoclonal antibody binding and therefore the absence of bluetongue virus antibody in sample sera. Weak color development due to inhibition of the monoclonal antibody binding to the antigen on the solid phase indicates the presence of bluetongue virus antibodies in sample sera.

Kit Contents

	Component	287-5
A	Antigen-Coated Plates	5 plates
B	Positive Control	4 ml
C	Negative Control	4 ml
D	Antibody-Peroxidase Conjugate	16 ml
E	50X Wash Solution Concentrate	60 ml
F	Substrate Solution	30 ml
G	Stop Solution	30 ml

Materials Required

- Single and multichannel adjustable volume pipettors and disposable plastic tips.

- Test tubes or non-antigen-coated transfer plates.
- ELISA microplate reader or spectrophotometer with 620 nm filter.
- Deionized or distilled water.
- Paper towels.
- Multichannel pipettor reservoirs.
- Graduated cylinder.
- Wash bottle, manual, multichannel washing device.
- Timer.

Storage and Stability

All reagents were stored at 4°C until preparation for test procedure was started.

Preparation

- The serum samples, reagents and plates were brought to room temperature prior to starting the test.
- Positive and Negative Controls were run in duplicate, regardless of the number of serum samples to be tested. Controls were run on every plate. The control and serum sample IDs were entered on a photocopy of the attached Setup Record.
- The plates removed from the foil pouches (A). Any unused strips were returned to the pouch and securely sealed. Call VMRD for extra pouches and sealer. Place strips to be used in the frame and number the top of each strip to maintain orientation with the Setup Record. Always mark the strips in case they fall out of the frame during washing.
- Antibody-Peroxidase Conjugate (D) was READY-TO-USE. No dilution was necessary.
- Wash Solution (1X) was prepared by diluting one part of the 50X Wash Solution Concentrate (E) with 49 parts of distilled water. Approximately 1 ml was allocated for each well.
- Serum samples were tested UNDILUTED.

- Test tubes or non-antigen-coated transfer plates.
- ELISA microplate reader or spectrophotometer with 620 nm filter.
- Deionized or distilled water.
- Paper towels.
- Multichannel pipettor reservoirs.
- Graduated cylinder.
- Wash bottle, manual, multichannel washing device.
- Timer.

Storage and Stability

All reagents were stored at 4°C until preparation for test procedure was started.

Preparation

- The serum samples, reagents and plates were brought to room temperature prior to starting the test.
- Positive and Negative Controls were run in duplicate, regardless of the number of serum samples to be tested. Controls were run on every plate. The control and serum sample IDs were entered on a photocopy of the attached Setup Record.
- The plates removed from the foil pouches (A). Any unused strips were returned to the pouch and securely sealed. Call VMRD for extra pouches and sealer. Place strips to be used in the frame and number the top of each strip to maintain orientation with the Setup Record. Always mark the strips in case they fall out of the frame during washing.
- Antibody-Peroxidase Conjugate (D) was READY-TO-USE. No dilution was necessary.
- Wash Solution (1X) was prepared by diluting one part of the 50X Wash Solution Concentrate (E) with 49 parts of distilled water. Approximately 1 ml was allocated for each well.
- Serum samples were tested UNDILUTED.

Annex VI: Competitive ELISA Plate Layout

Controls Test serum samples (in duplicate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	C-	C-	1	2	3	4	5	6	7	8	9	10
B	C-	C-	1	2	3	4	5	6	7	8	9	10
C	C+	C+	11									
D	C+	C+	11									
E	Cc	Cc										
F	Cc	Cc										
G												40
H												40

Key: Cc Conjugate control

C+ Positive serum control

C- Negative serum control

Note: The plane plan can be modified to suit specific software packages. The number of controls should be kept the same.

Annex VII: Multistage logistic regression output of the four putative risk factors, agroecology, age, flock size and sex, for BTV seropositivity

Result	Coef.	Std. Err.	z	P>|z|	[95% Conf. Interval]
_Iaez_2	-.2148347	.0431464	-4.98	0.000	(-.2994 -.1302694)
_Iaez_3	-.4327073	.0427926	-10.11	0.000	(-.5165793 -.3488353)
_Iagecate_2	.1676152	.037347	4.49	0.000	(.0944165 .2408139)
_Iagecate_3	.2926204	.0451671	6.48	0.000	(.2040944 .3811464)
_Isex_2	-.0978845	.0404179	-2.42	0.015	(-.1771022 -.0186668)
_Iflscateg_2	.02100	.0386993	0.55	0.585	(-0.0800 .12000)
_Iflscateg_3	.05500	.044765	1.22	0.223	(-0.1102 0.2111)
_cons	.525777	0586533	8.96	0.000	(.4108186 .6407353)

aez = agroecological zone

agecate = age category

sex = sex

flscateg = flock size category

Annex VIII: Multistage logistic regression output of the three putative risk factors, agroecology, age and sex, after the model rejected flock size

Result	Coef.	Std. Err.	z	P>|z|	[95% Conf. Interval]
_laez_2	-.177681	.0380918	-4.66	0.000	(-.2523397 -.1030224)
_laez_3	-.404464	.0372554	-10.86	0.000	(-.4774833 -.3314447)
_lagecate_2	.1687197	.0372731	4.53	0.000	(.0956658 .2417735)
_lagecate_3	.2954651	.0451853	6.54	0.000	(.2069034 .3840267)
_lsex_2	-.0909105	.0399292	-2.28	**0.053**	(-.1691703 -.0126507)
_cons	.4879579	.0399715	12.21	0.000	(.4096152 .5663006)

Annex XI: Multistage logistic regression output of the two putative risk factors, agroecology and age, after the model rejected sex

Result	Coef.	Std. Err.	z	P>|z|	[95% Conf. Interval]
_laez_2	-.1689127	.0379893	-4.45	0.000	(-.2433703 -.0944551)
_laez_3	-.385527	.0364039	-10.59	0.000	(-.4568773 -.3141768)
_lagecate_2	.1319953	.0336844	3.92	0.000	(.0659752 .1980155)
_lagecate_3	.2492345	.0404647	6.16	0.000	(.1699252 .3285438)
_cons	.4351098	.0326209	13.34	0.000	(.371174 .4990455)

9. CURRICULUM VITAE

Personal Details:

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Time of birth: June, 1968

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Education:

Name of School	Year	Award
Faculty of Veterinary Medicine (Addis Ababa University)	2003-5	MSc degree
Undergraduate associate at the Dire Dawa Regi. Vet. Laboratory in collaboration with Addis Ababa University	1990-91	Research paper (DVM thesis)
Faculty of Veterinary Medicine (Addis Ababa University)	1985-91	DVM degree
Faculty of Science (Addis Ababa University)	1985	Pre-veterinary transcript
Kolfe Comprehensive Secondary School	1985	National Certificate
Kolfe Comprehensive Secondary School	1981-85	School Certificate
Yekitit Junior Secondary School	1981	National Certificate
Hailu Tesfaye Elementary School	1975-81	School Certificate

Professional experience:

June 2000 to date: Research Officer at Bahir Dar Regional Veterinary Laboratory, Amhara National Regional State, Ethiopia. Here with in the department of Epidemiology and Economics, the major responsibilities are undertaking researches on economically important livestock diseases, investigation of disease outbreaks, preparation of training materials and giving trainings to field staff and participate in related laboratory activities.

May 1999 to July 1999: Trainer at Kombolcha Agricultural Training Centre. A training (refresher course) was given to more than 250 Animal Health Assistants working in public animal health services in the Amhara National Regional State. I was taking part to teach the courses Clinical Diagnosis, Public Health and Apiculture.

April 1998 to June 2000: Veterinarian at the Agricultural Department of South Gondar Zone, Amhara National Regional State, Debre Tabor, Ethiopia. Responsibilities include solving and managing animal health problems of districts, handle different disease outbreaks, coordinated nine veterinary clinics. Compiling and processing disease information and epidemiological data submitted from district veterinary offices, abattoirs and animal breeding stations and delivering the available and summarized data to the Federal Epidemiology and Economics Unit, Regional Bureau of Agriculture and Regional Veterinary Laboratories. Focal person to GTZ and FINIDA (NGO projects) to participate in the activities in collaboration with zonal and regional administrations.

January 1994 to April 1998: Veterinarian at the Office of Agriculture for the Fogera district, South Gondar Zone, Amhara National Regional State, Woreta, Ethiopia. Responsibilities include solving and managing animal health problems in the district. Handle different disease outbreaks, regularly supervised small scale dairy farms, and coordinated six veterinary clinics).

January 1993 to January 1994: Head, Department of Animal and Fisheries development, Office of Agriculture for the Fogera district, South Gondar, Amhara National Regional

State, Woreta, Ethiopia. Responsibilities include Coordination of all activities concerning livestock development (especially forage development), animal health services, Lake Fisheries Development Project (LFDP) and Animal and Animal products marketing activities. Actively participate in the district's subject mater specialist team (SMS).

November, 1991 to January 1993: Veterinarian at the Office of Agriculture for the Dera district, South Gondar Zone, Amhara National Regional State, Dera, Ethiopia. Responsibilities include solving and managing animal health problems in the district. Coordinated six veterinary clinics).

Certificates awarded :

1. A certificate from Kombolch Agricultural Training Centre for giving training at the center (1999).
2. A certificate from the International Centre for Insect Physiology and Ecology (ICIPE) for the participation in the course on Tsetse and Trypanosomosis Control (August, 1997, Debre Zeit, Ethiopia).
3. A certificate from an officially recognized Computer Training Center for completing the courses on MsDose, Windows, Word and Excel computer programs (1999)

Languages :

Amharic- Written (excellent) and Spoken (fluent)
English-Written (excellent) and Spoken (fluent)
Guraghigna (Local)- Spoken

Research Activities and Publications :

1. Seroepidemilogical study of bluetongue in indigeneous sheep in the Amhara National Regional State (MSc thesis, Faculty of Veterinary Medicine, Addis Ababa University, June, 2005)

2. Epidemiological study of helminthosis in draught horses in the Awi zone of Amhara National Regional State, Ethiopia (2005).

3. Prevalence of Nosemosis, pests and enemies of honeybees in two selected zones in northwestern Ethiopia (2003).

4. Main causes of calf mortality in cattle ranches and a dairy enterprise in northwestern Ethiopia (2003).

5. Prevalence of Bovine Mastitis in Dire Dawa Autonomous and Eastern Harerge Administrative Zone (DVM thesis, Faculty of Veterinary Medicine, Addis Ababa University, July, 1991).

Seminar Presentations:

1. Bluetongue in Domestic Ruminants (presented at the Faculty of Veterinary Medicine, Addis Ababa University, April, 2003).

2. The problem of Colibacillosis in Animals (Seminar on Livestock Problems, Faculty of Veterinary Medicine, Addis Ababa University, 1990).

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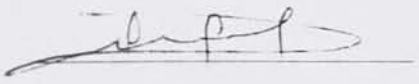
10. SIGNED DECLARATION SHEET

I, the undersigned, declare that the information presented here in my thesis is my original work, has not been presented for a degree in any other University and that all sources of valuable materials used in it have bee acknowledged.

Name: Darsema Gulima

Signature: 

Date of submission: 24/06/05

This thesis has been submitted for examination with my approval as university advisor:

1. Dr. Ademe Zerihun

Signature: _____

Seroepidemiological Study Of Bluetio
ngue In Indienous Sheep In Selected
Districts Of Amhara National Regiona
State North Western Ethiopia

Darsema Gulima