Molecular and epidemiologic characterisation of selected tick-borne pathogens circulating in extensively reared cattle along the Ethiopian-Sudanese border region of Benishangul Gumuz, Western Ethiopia

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ABSTRACT: Ticks are widely distributed throughout Ethiopia, presenting a significant challenge to livestock and causing considerable economic losses that impact the livelihoods of resource-poor farming communities. This study aimed to assess the species composition and determine the prevalence of TBPs in extensively reared cattle populations at the border region of Ethiopia and Sudan. In a cross-sectional study conducted in the Benishangul-Gumuz region from July to November 2019, we collected 1,015 cattle blood samples. We identified TBPs in the samples by high-resolution melting analysis (HRM) analysis and representative sequencing of genus-specific PCR products. We detected TBPs in 78.1% of blood samples, including *Anaplasma marginale* (30%), *Anaplasma platys* (6.4%), *Candidatus Anaplasma cinensis* (7.4%), *Anaplasma* spp. (12.7%), *Theileria velifera* (29.4%), *Theileria orientalis* (20.9%), and *Theileria sergenti* (7.6%). Co-infection occurred in 36.6% of the samples, with *A. marginale* and *T. velifera* being the most frequent co-infection combination. Cattle in lowland regions faced a higher risk of TBP infections and the risk of *Anaplasma* spp. infection was greater in cattle from the Assosa District than those from the Bambasi District. *Anaplasma* was more prevalent in Assosa, while *Theileria* was more prevalent in Bambasi District. *Candidatus Anaplasma cinenis* was identified in the present study for the first time in Ethiopian livestock population. The findings of this study contribute to a better knowledge of the epidemiology of TBPs, enhancing the understanding of animal health practitioners and regional authorities in the context of transboundary tick-borne disease management in East Africa.

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1. Introduction

Ticks (*Acari: Ixodidae*) are obligate hematophagous arthropods of great veterinary and public health importance. The impact of ticks on health can be twofold; 1) as ectoparasites, ticks cause tissue damage, production loss, toxin-induced paralysis, and predisposition to secondary bacterial infection, and 2) ticks are reservoirs and vectors for a wide range of human and animal pathogens (Dantas-Torres *et al.*, 2012). Ticks are distributed worldwide. The distribution of individual tick species varies according to agroclimatic factors, including humidity, vegetation, and hosts. Changing climate and land use patterns have shifted the geographic ranges of some tick species. This, in turn, has led to an increase in the spectrum of tick-borne diseases (TBDs) affecting humans and animals (Dantas-Torres *et al.*, 2012; Nicholson *et al.*, 2010). Approximately 10% of tick species are estimated to carry human and animal pathogens (Jongejan and Uilenberg, 2004). Therefore, understanding the occurrence and distribution across the transmission pathway of tick-borne pathogens (TBPs) enables the identification of vulnerability where diseases pose significant health threats and where efforts can be focused to design control strategies and improve risk communication.

Similar to other parts of sub-Sahara Africa, ticks are common and widely distributed throughout Ethiopia, posing a significant challenge to livestock productivity (Mekonnen *et al.*, 2001). Over 60 species of ticks infesting domestic and wild animals have long been

recorded, and 33 are known to be the most common and important livestock parasites. The prevalence, species composition of ticks infesting livestock, and impact on the commercial values of skin and hides have previously been reported (Abera *et al.,* 2010; Kumsa *et al* 2012). Despite the significance of ticks, data on molecular detection, TBPs diversity and their interaction with specific tick and vertebrate host species remains an important research gap. To date, there are a handful of works that have used molecular methods to detect TBPs in Ethiopia (Tomassone et al., 2012; Kumsa *et al.,* 201; Hailemariam *et al.,* 2017).

A recent systemic review and meta-analysis reported sixteen molecularly confirmed TBPs from ticks and various animal species that belong to *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Theileria*, *Babesia*, and *Coxiela* species (Kaba, 2022). However, all studies included in the analyses were from only four administrative regions, no none from the border regions with neighbouring countries. This study aimed to fill part of the TBP surveillance gap in Ethiopia, by investigating TBPs in Benishangul-Gumuz, a border region bordering Sudan in the Northwest part of Ethiopia, which offers a different ecological zone that may influence the distribution of arthropod vectors distribution and the diseases they transmit.

Previous studies have shown the region has tick species that belong to four genera, including *Amblyomma*, *Rhipicephalus* (*Boophilus*) and *Hyalomma* (Said *et al.,* 2020). Due to trade and civil instability, livestock movement across the Ethiopian-Sudanese border is unrestricted, which may facilitate the transmission of transboundary animal diseases, including TBDs, into Ethiopia. The spread of diseases from livestock trade and migration is compounded by the asymptomatic presentation of some TBDs in cattle, hindering the ability of animal health practitioners to spot infected animals. The objectives of the study were to determine the molecular prevalence of *Anaplasma*, *Babesia*, *Coxiella*, *Ehrlichia*, and *Theilera* pathogens in cattle and identify the risk factors associated with the occurrence of TBDs.

2. Material and Methods

2.1 Study area

This study was conducted in Assosa and Bambasi districts of Benshangul Gumuz Region, Northwestern Ethiopia (Figure 1). Assosa district spans from 9.60° and 10.45° N latitude and 34.20° and 34.58° E longitude, with an altitude ranging from 580-1544 meters above sea level (m.a.s.l) and an annual rainfall varying between 850 and 1200 mm. The mean yearly temperature in this district ranges between 16.75°C and 37.9°C (NMSA, 2015).

Bambasi district is located at 9.45° - 9.75° N latitude and 34.35°- 34.88° E longitude, with the lowest and highest altitudes of 1350 and 1770 m above sea level. The annual rainfall in Bambasi varies from a minimum of 900 mm to a maximum of 1200 mm, while the average minimum and maximum tempratures are 23°C and 32°C, respectively (NMSA, 2015). Livestock species reared in the study areas include cattle, goats, sheep, donkeys and poultry (BGRBoA, 2022). The districts were selected as part of a larger vector control project because they are the bordering districts of the regions with high mobility of people and livestock.

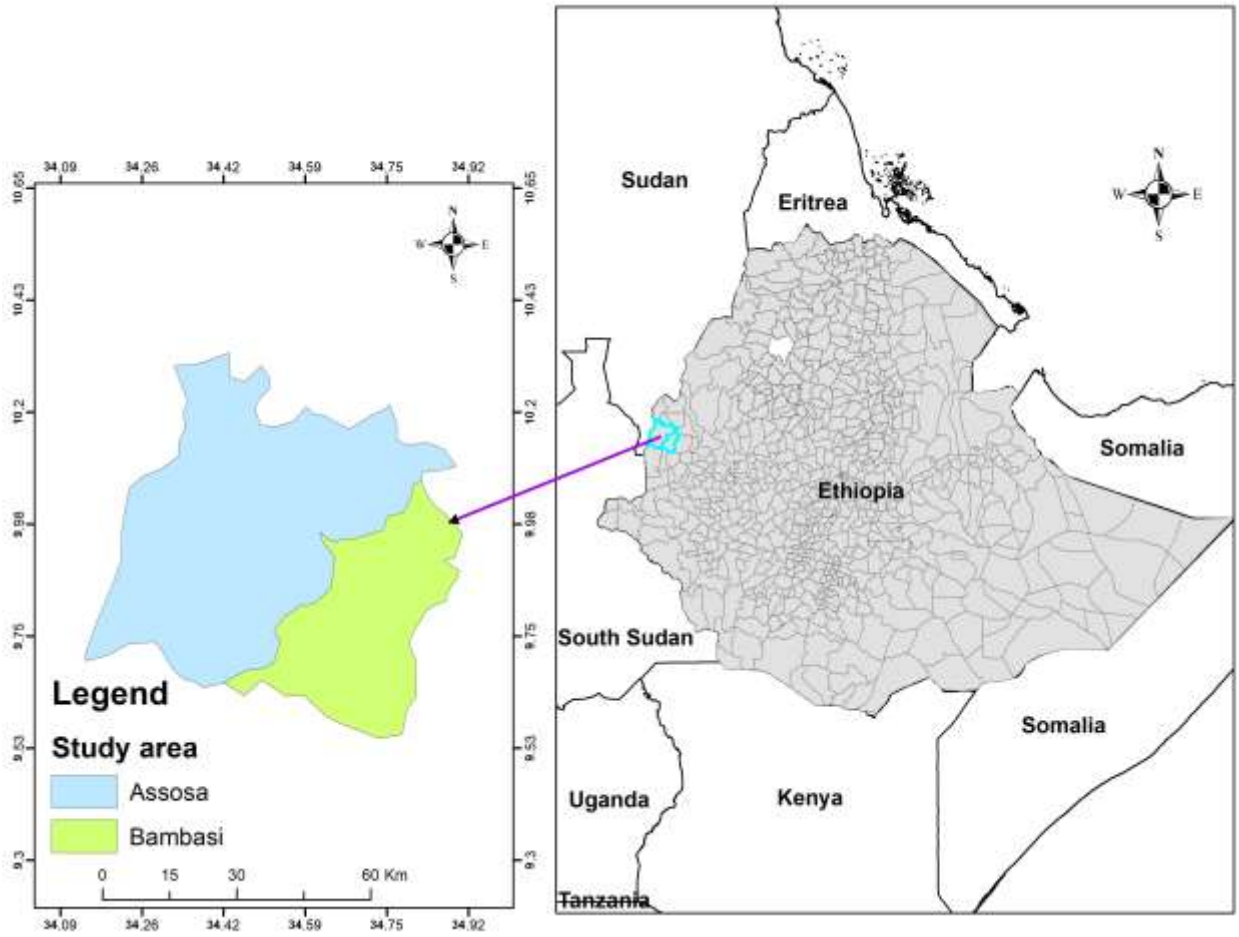

Figure 1: Map of the study area

2.2 Study setting, design and sample size determination

Using cross-sectional study design, we screened cattle blood samples for the presence of TBPs from June 2019 to November 2019. Extensively reared cattle owned by smallholder farmers were included in the study. The true representatives of the study population were determined by a combination of simple random and multistage cluster sampling methods. The study cattle populations were first stratified by district (first stage), and then by peasant associations (PA, second stage). Prior to the sampling, animal inventory, site assessment, and livestock extension system were gathered. Selection of PA and herds (third stage) was done in collaboration with district animal health experts. Herds grazing within the same grazing area (sharing a similar ecology and thus tick exposure) were defined as the primary sampling units. While, individual animals were considered as secondary samplining unit.

The sample size was estimated using the method described by Dohoo et al., (2009) for estimating a single proportion in a two-sided test ($n = \frac{Z^2pq}{L^2}$), with adjustment for clustering $n' = n(1 + p(m - 1)$. Due to the lack of TBP prevalence data from Benishangul Gumuz region, we used a priori prevalence of 50%. With a confidence level of 95%, desired precision of 5%, an average herd size of five cattle, and an intra-cluster correlation coefficient of 0.29 for *Anaplasma marginale* Oundo *et al.*, (2022), a minimum sample size of 830 cattle was estimated. We accounted for at least a 20% non-response rate, and the final dataset consisted of 1015 cattle; the number of samples was allocated to districts proportionally.

$n = \frac{Z^2pq}{L^2}$……(Equation 1)

where,

n = required sample size
Z= percentile of a standard normal distribution of 95% confidence level (Z= 1.96)
p = a priori estimate of TBP prevalence
q= 1-p
L = the precision of the estimate

$n' = n(1 + \rho(m - 1))$……(Equation 2)

Where n' is the adjusted sample size, n is the original size estimate, ρ is the intra-cluster corelation coefficient, and m is the herd size (Dohoo et al., 2009).

To increase the precision by a factor of k, we used to increase sample size by a factor of k^2 (Cornish, 2006). Therefore, to increases precision by 1.3% the sample size was increased by 2.6 folds so that total of 1015cattle blood sample collected from Assosa and Bambasi districts of Assosa zone Benishangul gumuz regional state. Hence, based on the two districts population size 463 blood sample from Bambasi and 552 blood sample from Assosa districts was collected from individual animal.

2.3. Ethical approval

This study strictly adhered to the experimental guidelines and procedures approved by the Institutional Animal Care and Use Committee at the International Centre of Insect Physiology and Ecology (*icipe*) and Haramaya University's ethical review committee. Ethical approval was also obtained from Benishangul Gumuz Regional Bureau of Agriculture before the commencement of the study (P3/1-10/631 Date 23/8/2019). Blood samples were collected after receiving informed verbal consent from cattle owners, with blood collection collected by experienced veterinarians. The Ethiopian National Biodiversity Institute authorised the transfer of genomic DNA from Ethiopia to *icipe's* Martin Lüscher-Emerging Infectious Diseases Laboratory in Kenya (EBI17/2565/2022 Date 27/01/2022).

2.4. Sample collection and processing

Approximately 4 ml of whole blood was drawn from the jugular vein, using sterile vacutainer tubes containing anticoagulant ethylene diamine tetra acetic acid (EDTA). About 1.5 ml of whole blood samples were filled into cryovial tubes with a capacity of 1.8 ml at the sample collection site and stored in a portable -80°C freezer liquid nitrogen container daily and transported to the Assosa Regional Animal Health Diagnostic and Disease Investigation Laboratory. The remaining whole blood sample from vacutainer tubes was used to draw ¾th of a capillary tube for PCV measurements. Then, the whole blood for each sample was centrifuged using a centrifuge (HERMLE LABORTECHNIK, GERMANY) at 13500 rpm for 5 minutes, and PCV measuring for each sample was carried out at field site. Then, samples in liquid nitrogen were transported to Animal Health Institute (AHI) (Sebeta, Ethiopia) and stored there at -80°C until DNA extraction.

2.5. Genomic DNA extraction

Total genomic DNA was extracted using the non-enzymatic salting out method from EDTA-treated bovine blood samples (Suguna *et al.,* 2014). Briefly, RBC lysis was performed briefly by adding 300 µl of whole blood to 900 µl low salt buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA) followed by 50 µl of 1% Triton X-100. Then the samples were incubated at 56°C for 10 minutes and centrifuged to obtain white pellets. To ensure complete lysis and to obtain a white pellet, the centrifugation step was repeated with decreasing amounts of triton X. The step was followed by addition of 300 µl high salt buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA, 400 mM NaCl) and 40 µl of 10% sodium dodecyl sulphate (SDS) to the white pellet, thoroughly mixed and incubated at 56°C for 10 minutes. Maximum protein precipitation was carried out using 100 µl of NaCl. Following centrifugation at 8000 rpm for 5 minutes, 300 µl of isopropanol was added to the supernatant and inverted the eppendorf slowly for optimum DNA precipitation. The contents were centrifuged at 10000 rpm for 20 minutes to pellet down the DNA. The pellets were washed with 70% isopropanol, centrifuged again, the supernatant discarded and the microcentrifuge with pellets inverted to air dry. The pellets (DNA) were resuspended in 50 µl of Tris-EDTA buffer and transported to the Martin Lüscher Emerging Infectious Disease (ML-EID) laboratory at the International center of Insect Physiology and Ecology (ICIPE) in Nairobi, where they were stored at −80 °C before molecular identification of pathogens.

2.6. Molecular characterisation

Molecular identification of microorganisms was performed using PCR amplification coupled with high-resolution melt analysis (HRM), which targeted the 16S rDNA region (for *Anaplasma*, *Erhlichia* spps) and 18S rRNA (for *Babesia* and *Theileria* spps*).* For each of these species, the reaction constituted 5.0 µl of PCR grade water, 2.0 µl of 5x HOT FIRE Pol Eva Green HRM mix-no ROX (Solis Bio Dyne, Estonia), 0.5 µl of 10 pmol of each working primer for the respective genus-specific reactions and 2.0 µl of the extracted genomic DNA making up the final volume to 10 ul. For each of the pathogens, the amplifications were performed in Quant Studio 3(Applied Bio Systems) with the PCR cycle parameters set as; enzyme activation step at 95°C for 15 min, followed by 40 cycles of denaturation at

94°C for 20 sec, annealing for 30 sec at temperatures in Table 4, and extension at 72°C for 30 sec. A final extension step was performed at 72°C for 7 minutes. Immediately post PCR, HRM was carried out with an increasing temperature from 72°C to 95°C at a rate of 0.1°C/sec. Positive controls and negative controls were run concurrently with the samples. Melt curves were visualised with reference to changes in fluorescence with changes in temperature. The positive samples for *Anaplasma* and *Theileria* spps were identified mainly by comparing the melting profiles alongside the positive controls (Kassaza *et al.*, 2018).

Table 1. Standard PCR for confirmation of *Anaplasma, Babesia, Ehrlichia* and *Theileria* spps

Target Gene	**Primer name**	**Primer sequences 5'-3'**	**Annealing (°C)**	**Product size (bp)**	**Genus**
16S rDNA	AnaJVF	CGGTGGAGCATGTGGTTTAATTC	55	300	*Anaplasma*
	AnaJVR	CGRCGTTGCAACCTATTGTAGTC			
	EHRSD	GGTACCYACAGAAGAAGTCC		1030	
	PH1492	GGTTACCTTGTTACGACTT			
16S rDNA	16S8FE	GGAATTCAGAGTTGGATCMTGGYT CAG	60.5	448	*Ehrlichia/ Anaplasma*
	B-GA1B	CGGGATCCCGAGTTTGCCGGGACT TCTTCT			
	PER-1	TTTATCGCTATTAGATGAGCCTATG	58	451	
	PER-2	CTCTACACTAGGAATTCCGCTAT			
18S rRNA	RLB-F2	GAGGTAGTGACAAGAAATAACAAT A	60.5	460–520	*Babesia/ Theileria*
	RLB-R2	TCTTCGATCCCCTAACTTTC			
18S rRNA	BJ1	GTCTTGTAATTGGAATGATGG	55	500	
	BN2	TAGTTTATGGTTAGGACTACG			
16S rDNA	Rick-F1	GAACGCTATCGGTATGCTTAACAC A	55	350–400	*Rickettsia/ Coxiella*
	Rick-F2	CATCACTCACTCGGTATTGCTGGA			
rOmpB	120-2788	AAACAATAATCAAGGTACTGT	53		*Rickettsia strains*
	120-3599	TACTTCCGGTTACAGCAAAGT			
	Trans-1	TATGTATCCACCGTA GCCAGTC	61		*Coxiella*
	Trans-2	CCCAACAACACCTCCTTATTC			

2.7. Data management and analysis

Sample metadata on the district, PAs, altitude, age, sex, body weight, PCV and skin color were entered into Microsoft Excel 2016 spreadsheets. Altitude and latitude data captured during data collection with GPS and HRM-PCR output were also recorded in Microsoft Excel 2016 spreadsheets. Data validation were assessed by applying several checks to ensure the accuracy and quality of data in Microsoft Excel, and 3 samples were dropped during data evaluation and analysis because of missing values. Variable coding, and analysis were performed using STATA version 14. The prevalence of *Anaplasma* and *Theileria* spp. and their corresponding mixed infection was assessed using a frequency table. Binary logistic regression was applied to estimate the relationship between dichotomous outcome variables i.e. presence or absence of *Anaplasma* and *Theileria* spps and dichotomous and ordinal predictor variables i.e. district, Keble, altitude, age and sex independent variables according to (Thrusfield, 2018). The risk factors; age and sex assessed has no statistically significance difference even if all has an association with the existence of pathogen with the odd ratio between (0 and 1) and their confidence interval don't include zero. The average normal body weight of Zebu cattle age categories for male and female used according to (Kashoma *et al.*, 2011). While, only altitude was statistically sifnificant. The model fit for predictor variables were checked using wald test statistic and the removal of any of these variable affects the model so; all predictor variables were kept. The effect of risk factors on outcome variable i.e. positive to TBPs was determined by binary logistic regression and the test was considered statistically significant at $p \leq 0.05$.

3.Results

3.1. Prevalence

Based on RT-PCR analysis followed by DNA sequence *TBPs i.e. A. marginale, A. playts, Ca. A. cinenis*, Anaplasma* spp, *T. velifera, T. orientalis and T. sergenti* were identified from the study area. While, no positive result observed form the total DNA sample for *Babesia*, *Ehrlichia* and *Coxiella* species. Among the 1015 cattle examined for TBP status 78.1%, (793) (95% CI, 75.5-80.6) were infected by at least one TBP. Sergeant, ESG, 2(018) Epitool was used to assess the prevalence and confidence interval. Overall, *Anaplasma* and *Theileria* spps were identified from 578 and 589 cattle, respectively (Table 2).

Table 2. Prevalence of *Anaplasma* and *Theileria* spps in cattle in Assosa and Bambasi districts, Benishangul gumuz, Ethiopia.

Pathogenies	No. Positive	Prevalence %	[95% Conf. Interval]	
Overall *Anaplasma* and *Theileria*	793	78.1	75.5	80.5
Overall *Anaplasma*	578	56.9	53.8	59.9
A. marginale	306	30.1	27.4	33.0
A. playts	67	6.6	5.2	8.3
*Ca. A. cinenis**	75	7.4	5.9	9.2
Anaplasma spp	130	12.8	10.9	15.0
Overall Theileria	589	58	54.9	61.0
T. velifera	301	29.6	29.6	32.5
T. orientalis	210	20.7	18.4	23.4
T. sergenti	78	7.7	6.2	9.5

Note: Ca. A. cinenis = Candidatus Anaplasma cinenis**

Co-infection with two pathogens from both genera of pathogens was identified with the prevalence of 36.6%. Among the 793 positive animals, 374 were found mixed positive by two pathogens in various combinations. Amoung mixed infection the prevalence of *A. marginale and T. velifer* was found 9.9% and the most mixed infection observed (Table 3).

Table 3. Prevalence of mixed-infection with *Anaplasma* and *Theileria* spps.

Pathogenies	No. Positive	Prevalence %	[95% CI]	
A. marginale and T. velifera	102	10.0	8.4	12.0
A. marginale and T. orientalis	69	6.8	5.4	8.5
Anaplasma spp and T. velifera	46	4.5	3.4	5.9
A. playts and T. velifera	28	2.8	1.9	3.9
Ca. A. cinensis and T. velifera	20	1.9	1.3	3.0
A. marginale and T. sergenti	31	3	2.2	4.3
A. playts and *T. orentalis*	16	1.6	0.9	2.5
A. platy and *T. Sergenti*	5	0.5	0.2	1.2
Ca. A. cinensis and *T. orentalis*	16	1.6	0.9	2.5
Ca. A. cinensis and *T. sergenti*	4	0.4	0.2	1.0
Anaplasma spp and *T. orentalis*	23	2.3	1.5	3.4
Anaplasma spp and *T. sergenti*	15	1.5	0.9	2.4
Total mixed positive	374	36.8	33.9	39.8

All identified *Anaplasma* and *Theileria* species from the present study were recorded from both Bambasi and Assosa districts and all PAs with varied prevalence except *Ca. A. cinensis* and *T.sergenti* was not recorded from two and one PAs of Bambasi district respectively (Supplementary materials; table 7).

3.2. Risk Factors

Based on logistic regression analysis using full and reduced model, district and altitude was statistically significant with P= 0.001< 0.05 for *Anaplasma* spps. The odd ratio 0.56 shows samples from midland has 0.56% less likely positive result than lowland for *Anaplasma* spps. *Anaplasma* spps was seen more prevalent from underweight female cattle 70.58% (408) than healthy cattle weight of female cattle 5.36% (31)(Table 4).

Table 4. Summary of Frequency, *Odds Ratio*, P- value and CI of risk factors of *Anaplasma* spps

Risk factors	*Risk factors Category*	*Anaplasma spps*		*Odds Ratio*	*P value*	*CI*
District		*Freq.*	*%*			
	Bambasi	288	49.82	*.8316847*	*0.001*	*.53 - 1.31*
	Assosa	290	50.17			
Age	*≤2 year*	87	15.05	*1.167326*	*0.062*	*.99 - 1.37*
	2-4 years	127	21.97			
	≥ 4 years	364	62.97			
Sex	*Male*	155	26.82	*1.053441*	*0.899*	*.66 - 1.67*
	Female	423	73.18			
Altitude	*Lowland*	337	58.30	*.6524892*	*0.001*	*.42 - 1.02*
	Midland	241	41.70			
B.Wt. (KG)	*Male normal wt.*	71	12.30	*.8881476*	*0.056*	*.67 - 1.18*
	Male Under wt.	68	11.76			
	Female normal wt.	31	5.36			
	Female Under wt.	408	70.58			

District and altitude also shows statistically significance difference to *Theileria* spps with P= 0.001 < 0.05. And the odd ratio of altitude 0.46 indicates samples from midland has 0.46% less likely positive result than lowland for *Theileria* spps. *Theileria* spps was more prevalent from underweight female cattle 72.32% (426) than healthy female cattle with normal weight 3.23% (19). Based on age groups TBPs were more prevalent from older cattle than younger. Also, among sex category, the prevalence of *Anaplasma* and *Theileria* spps were higher from female than male cattle (Table 5).

Table 5. Summary of Frequency, *Odds Ratio*, P- value and CI of risk factors of *Theileria* spps

Risk factors	*Risk factors Category*	*Theileria spp*		*Odds Ratio*	*P value*	*CI*
District		*Freq.*	*%*	*.85449*	*0.001*	*.53 - 1.37*
	Bambasi	307	*52.23*			
	Assosa	282	*47.77*			
Age	*≤2 year*	*89*	15.11	*1.113015*	*0.198*	*.95 - 1.31*
	2-4 years	*136*	23.1			
	> 4 years	*364*	61.79			
Sex	*Male*	*155*	26.32	*1.76751*	*0.899*	*1.09 - 2.85*
	Female	*434*	73.68			
Altitude	*Lowland*	*357*	*61.29*	*.4647959*	*0.001*	*.29 - .74*
	Midland	*225*	*38.71*			
B.Wt. (KG)	*Male normal wt.*	*79*	13.41	*.6584835*	*0.056*	*.49 - .88*
	Male Under wt.	*65*	11.04			
	Female normal wt.	*19*	3.23			
	Female Under wt.	*426*	72.32			

The effect of *Anaplasma* on PCV was assessed on pastive sample and 7/578 (1.21%) Severely anemic, 162/578 (28.03%) moderately anemic while 409/578 (70.76%) has normal PCV value. *Anaplasma* has no statistically

significance difference on PCV with P= 0.394 and Odd ratio 0.89 shows *Anaplasma* positive samples were 0.89% less likely has anemic sataus. Also, the outcome of *Theileria* on PCV was measured and only 7/589 (1.19%) Severely anemic, 166/589 (28.18%) moderately anemic while 416/589 (70.63%) has normal PCV value. *Theileria* has no statistically significanne difference on PCV with P= 0.556 and Odd ratio 0.95 shows *Theileria* positive samples were 0.95% less likely has anemic sataus.

Logistic regression analysis of mixed infection with *Anaplasma* and *Theileria* spps indicates statistically significance difference with P= 0.001< 0.05 for district and altitude using full and reduced model. The odd ratio of altitude 0.44 shows samples from midland has 0.44% lesslikly negative result than lowland for mixed infection. All other possible risk factors age, sex and body weight assessed with binary logistic regression were not found statistically significant both under the full and reduced model (Table 6*)*.

Table 6. Summary of Frequency, *Odds Ratio*, P- value and CI of risk factors of mixed infection.

Risk factors	*Risk factors Category*	Mixed infection		*Odds Ratio*	*P value*	*CI*
District		*Freq.*	*%*	*.4780509*	*0.000*	*.37 - .62*
	Bambasi	209	55.88			
	Assosa	165	44.12			
Age	*<2 year*	41	10.96	*.9942656*	*0.926*	*.88 - .12*
	2-3 years	105	28.07			
	>=4 years	228	60.97			
Sex	*Male*	106	28.34	*.8511327*	*0.275*	*.64 - 1.14*
	Female	268	71.66			
Altitude	*Lowland*	133	35.56	*.4431093*	*0.000*	*.34 - .57*
	Midland	241	64.44			
B.Wt. (KG)	*Male normal wt.*	46	12.30	*.8560918*	*0.084*	*.72 - 1.02*
	Male Under wt.	52	13.90			
	Female normal wt.	260	69.52			
	Female Under wt.	16	4.28			

The correlation analysis between predictor variables: district, altitude, age, sex, body weight and outcome variable *Anaplasma* spps has weak correlation coefficient from the least 0.014 to age category and the highest 0.143 to altitude. Also, the correlation analysis between all predictor variables and outcome variable for *Theileria* spps has weak correlation coefficient from the least 0.0009 to skin color and the highest 0.143 to altitude.

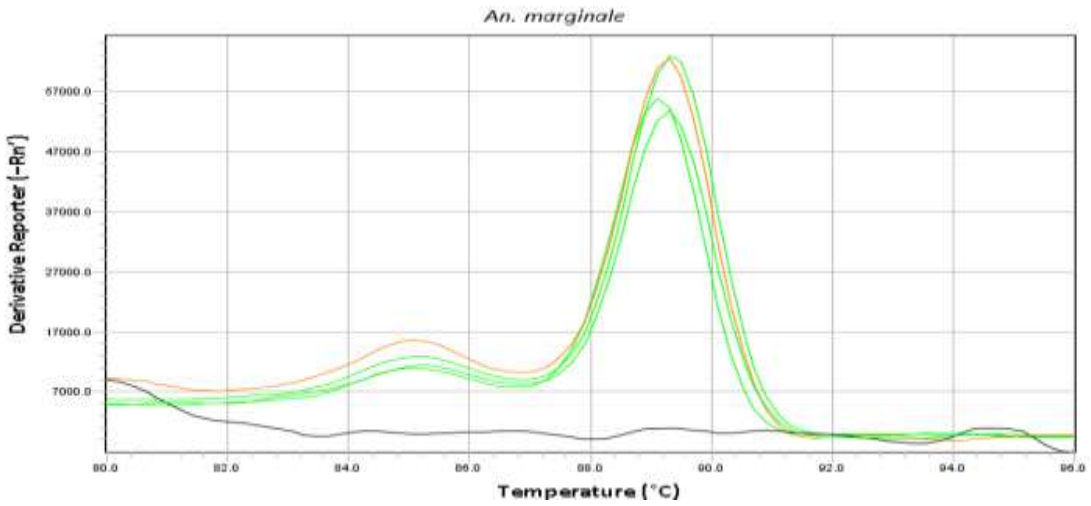

Figure *2:* HRM characterisation of *Anaplasma marginale*

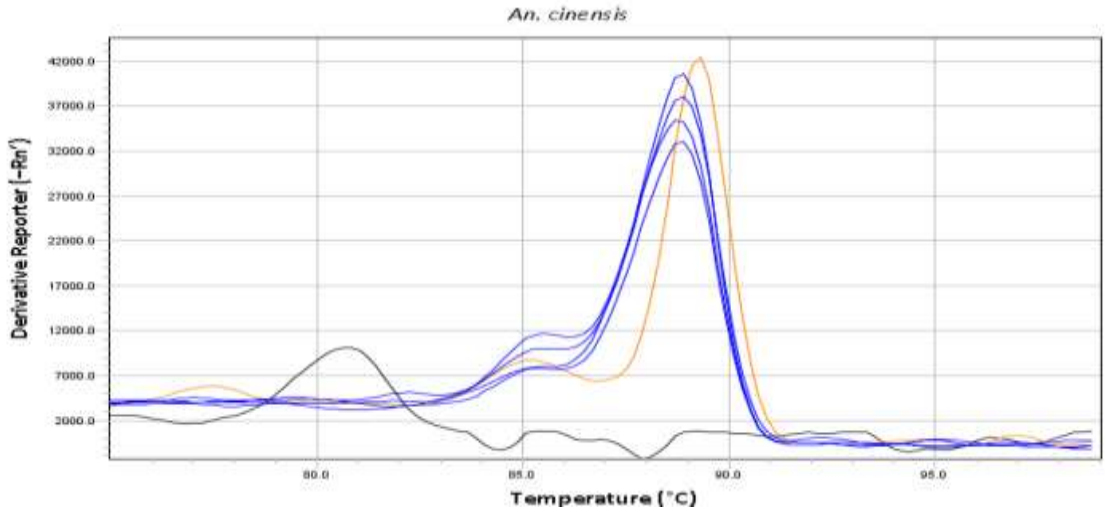

Figure 3: HRM characterisation of *Anaplasma cinensis*

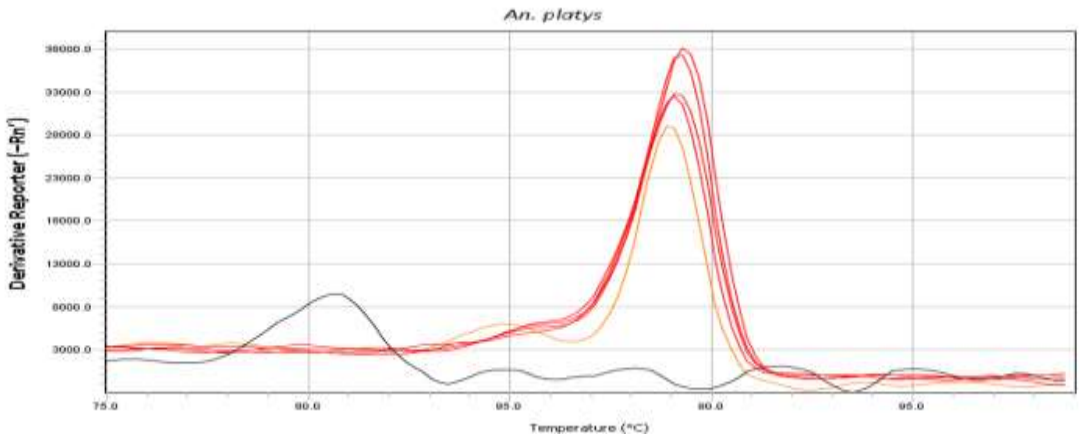

Figure 4: HRM characterisation of *Anaplasma platys*

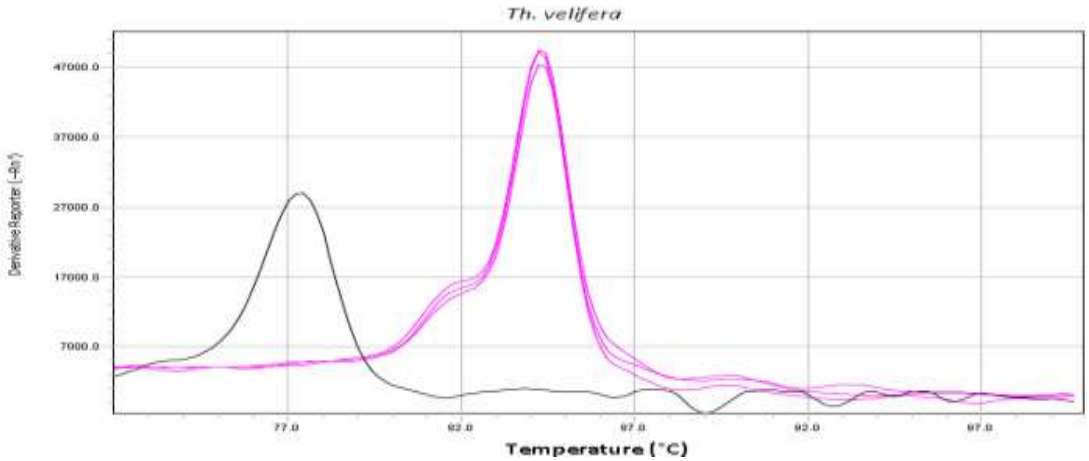

Figure 5: HRM characterisation of *Theileria velifera*

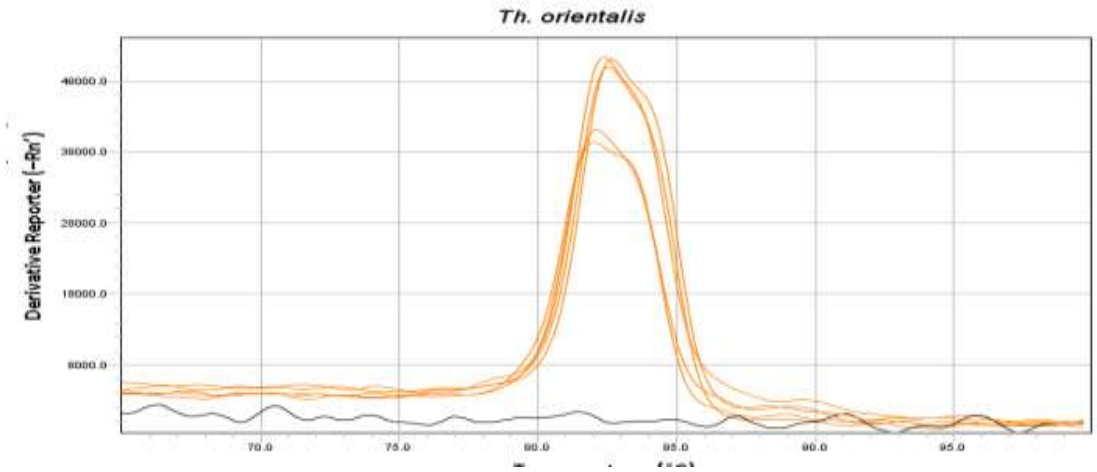

Figure 6: HRM characterisation of Theileria orientalis

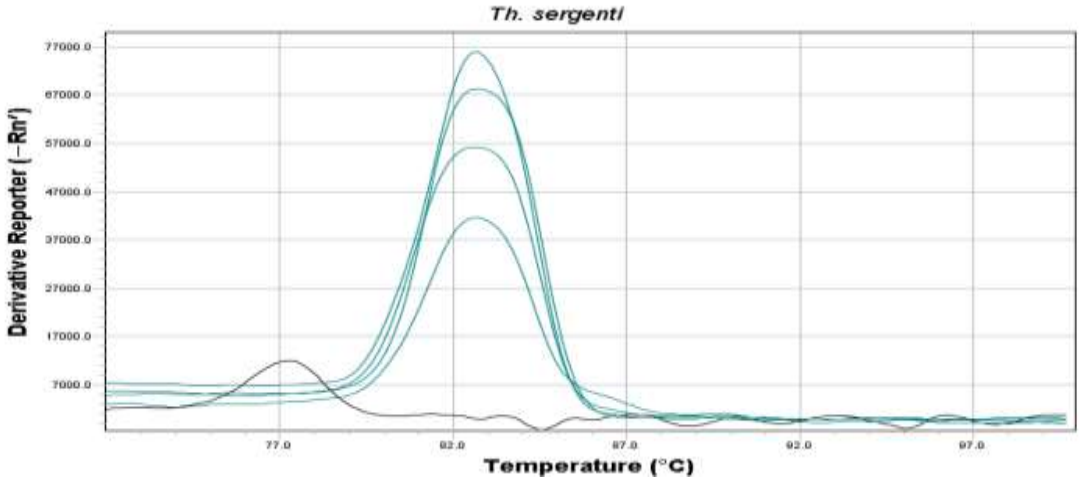

Figure 7: HRM characterisation of Theileria sergenti

4. Discussion

The occurrence of tick-borne pathogens has not been well documented with highly sensitive molecular test from domestic animals of Beneshangul gumuz region Ethiopia. The present study, identified *Anaplasma and Theileria* spps from whole blood sampled from cattle population. Blood sample collected from apparently healthy cattle of Bambasi and Assosa districts of Benishangul Gumuz region was subjected to molecular identification of microorganisms performed PCR amplification coupled with HRM and sequence for phylogenetic analyses. It shows the overall prevalence of tick borne pathogen 78.1%, (793/1015) positive for both *Anaplasma* spps and *Theileria* spps or at least one of *Anaplasma* spps and *Theileria* spps. This finding was very higher than the study by (Yang *et al.,* 2015) that shows the mean prevalence of single infection with each species was 17.6, 4.8 and 40.5% for *A. phagocytophilum*, *A. bovis* and *A. ovis* respectively based on phylogenetic analysis of the 16S rRNA gene). That could be due to ecological difference between the study areas and vector occurrence. *Ca. A. cinenis* was identified from the present study for the first time in Ethiopian livestock population.

The present finding agreed with the report by Agina *et al.,* (2021). *Theileria* species was the blood pathogen with the highest molecular detection rate 72.13%. But, it was lower than the study conducted by (Hailemariam *et al.,* 2017). molecular detection of tick-borne pathogens in cattle from South western Ethiopia 96.9% positive for at least one hemoparasite with PCR/RLB. This relatively lower prevalence from previous study from Ethiopia, could be due to coldest season of present sampling time and acaricide spray and treatment given to sick animals.

Among identified TBPs in the present study the most frequently occurring were *A. marginale* 30.1% from total of 56.9% prevalence of *Anaplasma* spps which was similar to the finding of Abanda *et al.,* (2019) *A. marginale* 30.7%, and also similar to the finding of Peter *et al.,* (2020) based on BLASTn analysis of the sequences against non-redundant GenBank nucleotide database report *A. marginale* 31% from Kenya. Also, from present finding *T. velifera* 29.6% was the highest prevalence from total of 58% *Theileria* spps. which is higher than the finding of Hailemariam *et al.,* (2017) *T. velifera* 13.0% this relatively higher prevalence of present finding could be due to higher tick vectors of *A. marginale* and *T. velifera* occurrence in the present study area. Furthermore, the presence of species of hematophagous Diptera could be potential mechanical vectors of *Anaplasma* spps (Fuente *et al.,* 2005).

Mixed infection was detected between *Theileria* and *Anaplasm* spps from present study. Accordingly, the highest co-infection of two pathogens was recorded from *A. marginale and T. velifera* 10%, *A. marginale* and *T. orientalis* 6.8%, which indicates

existence of the burden due to mixed infection of pathogen on the cattle population of the present study area, that could be due to co-infection of vectors. From present study, the least mixed infection of pathogens was seen from *Ca. A. cinensis* and *T. sergenti* 0.4%. Unlike, report from Hailemariam *et al.,* (2017) who described level of co-infection ranged from double to sextuple, the present study confirms only two pathogens mixed existence and there was no interspecies mixed infection of *Theileria* and *Anaplasma* from present study.

The present study indicates only district and altitude were statistically significant for *Anaplasma* and *Theileria* spps occurrence. The remaining risk factors; age, sex and body weight assessed has no statistically significance difference even if all has an association with the existence of pathogen with the odd ratio between (0 and 1) and their confidence interval don't include zero.

Risk factor assessment shows that *Anaplasma* spps, has statistically significant difference to altitude with more prevalence on lowland 58.30% and midland 41.70%. Also, *Theileria* spps has statistically significant difference to altitude with more prevalence on lowland *61.29*% than midland 38.71%. The present study was agreed with the finding of Adugna and Tamrat, (2022) prevalence of tick infestation from midland 47.7% and lowland 57.8%. It was not inconsistent with Walker and Blanton, (2014) dry environmental conditions are a danger to ticks, particularly to the questing larvae, which are very susceptible to drying out fatally. This could be due to the lowland of present study area was at the most upper level of lowland altitude (>1300 -1500) and the season of sampling was also during wet and the coldest season. The range of altitude midland (1500–2300) lowland (500–1500) (Etana *et al.,* 2020).

Concerning the two districts relatively higher *Anaplasma* spps were recorded from Assosa and higher *Theileria* spps were recorded from Bambasi. This could be due to more prevalence of vectors of *Anaplasma* spps in Assosa and more vectors of *Theileria* spps found in Bambasi than Assosa district. All *Anaplasma* and *Theileria* spps recorded from present study area were found from both Bambasi and Assosa districts and all PAs with varied prevalence except *Ca. A. cinensis* and *T. sergenti* which was not recorded from two and one PAs of Bambasi district respectively. This distribution of TBPs in all study site could be due to existence and evenly distribution of vectors.

The present study results reviled that younger animals were relatively free of tick borne pathogens in all cases and more prevalent in older cattle even if they were kept in the same grazing area. The lowest occurrence of pathogen among less than one year old may be due to innate and acquired immunity. Adults are a slightly free of pathogens than weaners (12–36 months) Haji *et al.,* (2022) which could be due to used up passive immunity and unexperienced to active immunity. The higher prevalence of TBPs in older cattle present study is in line with (Radostits *et al.,* 2007). endemic stability developed in older cattle between host, agent, vector and environment for vector borne diseases is such that clinical disease occurs rarely or not at all, and it could be due to immunosuppression. Strong immunity occurs after natural infection with TBPs (Radostits *et al.,* 2007). Additionally, the present result was in line with the idea of the larger size of older animals, provide more habitat for ectoparasites including TBD vectors (Anderson *et al.,* 2013). This may contribute to the increase in prevalence of hemoparasites as age increases. The herd structure of the present cattle population was dominated with more older cattle and higher number of old cattle were sampled.

The present finding of PCV value shows no statistically significant difference for presence and absence of TBPs. PCV values were considered as ≥25% normal, 16 to 24% as moderate anemic, and ≤ 15% as severely anemic (Hofmann *et al.,* 2004). Our finding shows surprisingly higher prevalence of TBPs from samples with normal PCV than moderately anemic and severely anemic samples which was not agreed with the finding of, Kumar *et al.,* (2015) who reported the most marked and common clinical signs in all the cases of Theileriosis, and Anaplasmosis, were severe anemia (hemoglobin=3-6 g/dl) (Kessell, 2015). *Theileria* spps can cause regenerative anemia and non-regenerative anemia. The variation of present result from previous findings could be due to infected and sick animals might be treated before sampling and appear free of TBPs at a time of sampling. Or the cause of lowered PCV could be because of other pathogens than tick borne pathogens.

The present finding shows frequency of TBPs on the sex basis with higher prevalence in female than males. It was agreed with (Bariso and Worku, 2018). reported tick borne haemo-parasite infectivity with sex of animals with higher prevalence in female than male. But our finding from present study was not consistent with the finding of Okal *et al.,* (2020) who stated risk of infection was not associated with sex. This could be due to relatively more stress on female because of lactation and hormonal disturbance and local farmers

used female cattle for farm plowing for crop cultivation.

The present study also revealed the non-existence of *Babesia, Ehrlicha* and *Coxiella* in the study area based on HRM-PCR coupled with DNA sequencing result. Therefore, it was not consistence with the finding of Hailemariam *et al.,* (2017) who reported *B. bigemina* (14.0%), *E. ruminantium* (0.5%) and *E. minasensis* (0.26%) from Ethiopia. This difference could be due to ecological difference between present and previous study area and possibly due to vector distribution deference.

5. Summary and Conclusion

In Summary, the aim of this study was molecular characterisation of *Anaplasma, Babesia, Coxcella, Eherlicha and Theilera* pathogens in cattle population. The representatives of the study population were selected by simple random sampling methods and 1013 cattle blood samples were collected from Assosa and Bambasi districts. *Anaplasma, and Theilera* pathogens were confirmed by PCR amplification coupled with HRM and finally DNA sequencing. Binary logistic regression was employed using STATA version 14 statistical software. And overall prevalence of TBPs from present study were 78.1%, shows the existence of highly prevalent *Anaplasma* and *Theileria* spps from cattle of Bambasi and Assosa districts. *Anaplasma marginale* and *T. veliferia* were relatively more prevalent species identified from present study. The prevalence of *Anaplasma* and *Theileria* spps was higher in lowland than midland and shows statistically significant difference with $P < 0.05$. The prevalence of *Anaplasma* and *Theileria* spps shows statistically significant difference between Assosa and Bambasi District with $P < 0.05$. *Anaplasma* spps was more prevalent from Assosa District while, *Theileria* spps was more prevalent from Bambasi District. Co-existence between different two species of *Anaplasma* and *Theileria* were recorded on individual animal. But, there were no co-existence with in species of either *Anaplasma* or *Theileria* spps.

In general, the present study shows higher prevalence and species diversification of *Anaplasma* and *Theileria* spps in cattle population of the study area. The distribution of pathogens also confirms the presence of tick vectors for *Anaplasma* and *Theileria* spps in target population. The implication of highly significant difference between lowland and midland with more prevalence in lowland indicates lowland as more risky area. The co-existence of pathogen on individual animal reviled burden on cattle. The present study also confirms the non-existence of *Babesia, Ehrlicha* and *Coxiella* in the study area based on HRM-PCR and DNA sequencing.

Based on the above conclusion the following recommendations are forwarded:

- Since *Anaplasma* and *Theileria* spps were highly prevalent in the present study area strategic prevention and controlling of tick and TBPs should be implemented keeping enzootic stability.
- Special attention should be given to lowland areas where TBPs were more prevalent.
- Animals brought to this area from other area expected to have no previous exposure to tick and tick-borne pathogens need Chemoprophylaxis administration.
- Farther study is essential to investigate the economic impact of thus TBPs in the present study area.

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8. Supplementary Materials

Table 7. Prevalence of different *Anaplasma* and *Theileria* spps from study site PAs

		Anaplasma spps				*Theileria spps*		
	PA code	*A.marginale*	*A.platys*	*Ca. A.cine*	*Anaplasma spp*	*T.velifer*	*T.orienta*	*T.sergent*
Bambasi	M.43	46.9	6.5	1.5	9.1	28.8	27.3	9.1
	M.47	46.3	5.9	5.9	7.5	40.3	22.4	7.5
	Sonka	30.7	7.7	0	38.5	38.5	15.4	0
	Shobora	24.3	18.9	0	13.5	43.2	24.3	8.1
	Qeshemando	47.1	8.6	1.4	12.8	44.3	14.3	15.7
	Qeshemando 4	49.3	4.1	4.1	9.6	28.7	26.0	8.2
	Garabichea w.	47.4	7.0	3.5	1.7	40.3	21.0	5.2
	M.55	37.1	4.8	4.8	11.3	33.8	25.8	11.3
Assosa	Amba 5	18.7	8.3	14.5	12.5	22.9	16.6	8.3
	Selga 23	13.8	8.3	15.2	22.2	23.6	16.6	11.1
	Megle 37	17.0	8.5	4.2	25.5	23.4	14.8	12.7
	Megle 32	39.5	11.1	4.9	4.9	37.0	23.4	3.7
	Amba 13	11.2	3.2	11.2	17.7	20.9	25.8	6.4
	Amba 2	18.0	2.7	19.4	12.	18.0	16.6	2.7
	N.Komishga	15.3	1.9	8.6	14.4	17.3	18.2	4.8
	Hoha 18	17.7	5.0	8.8	15.1	26.5	17.7	5.0
	Total	30	6.4	7.4	12.8	29.7	20.5	7.6

9. Author Contributions

Bayisa Kenaw Data curation, Investigation formal analysis, writing original. Dr Shewit Kalayu: formulate research design, Supervision, edition and conceptualisation. Dr Shihun Shimelis, Dr Berhanu Sibhat, Proff. Teshale Sori: Validation, review and editing. Dr Getachew Abichu involved on DNA analysis. Dr Dan Masinga: Funding acquisition, project administration, resources mobilisation. Mr Odhiambo Peter Otieno and Jandouwe Villinger: perform Molecular characterisation and Sequencing and Phylogenetic analysis.

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11. Conflicts of Interest

The authors declare no conflict of interest

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