Epidemiology of Epizootic Lymphangitis of cart horses in Northern Ethiopia Using Conventional diagnostic Methods and Nested Polymerase Chain Reaction

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Abstract

Background: Epizootic lymphangitis (EL), caused by *Histoplasma capsulatum variety farciminosum* (HCF) is a contagious chronic disease of equines characterized by development of nodular lesions in the lymph nodes, lymphatic vessels and skin. This disease is the most important diseases of equines in Ethiopia causing a significant economic loss, particularly cart pulling equines. Todate there is no sound diagnostic nor control measure implemented in the country. Furthermore, there is a shortage of data on the epidemiology of the disease in different regions of the country including northern Ethiopia. This study was conducted to investigate the epidemiology of EL in northern Ethiopia using the conventional methods and the nested polymerase chain reaction (PCR).

Methods: A total of 191 cart-horses were enrolled and used as sources of pus and blood samples. The blood was used for the extraction of the DNA of HCF from buffy coat for nested PCR while the pus samples were cultured on Sabourauds Dextrose Agar for isolation. Statistical Package for Social Sciences (SPSS) version 21 was used for data analysis by applying logistic regression, receiver operating characteristic (ROC) curve and Cohen's kappa coefficient test. In addition, the level of agreement between the clinical examination and the nested PCR was evaluated.

Results: Infection with HCF was confirmed in 44% (84/191) of the horses using nested PCR. Subclinical infection was observed in 18.18% (22/121) of the apparently healthy horses. Considering nested PCR as a gold standard, the sensitivity and specificity of the clinical examination were 74% and 95%, respectively while the area under the ROC curve (AUR) was 0.83 (95% confidence interval, 0.77, 0.896). Moreover, a moderate (k=0.675) agreement was observed between the nested PCR and clinical examination.

Conclusions: The findings of the present study showed the wide spread occurrence of EL in northern Ethiopia and the advantage of the nested PCR in detecting of the infection of HCF even before the clinical symptoms are apparent.

1. Background

Ethiopia is home for 9.6 million donkeys, 2.16 million horses, and 0.41 million mules. With 2.03 million head of horses, Ethiopia accounts for about 34.5% of total Africa's and 3.45% of global horse populations (1). In Ethiopia, equines are used for transportation of people and commodities and support crop production. On the other hand, Ethiopian equids are exposed to both husbandry and diseases of bacterial, viral, fungal and parasitic origins. Epizootic Lymphangitis (EL) also called Equine Histoplasmosis, has been a priority disease of high morbidity and mortality and hence its economics (2).

It is caused by Histoplasma (H.) capsulatum var. faciminosum (HCF); a dimorphic fungus that exists in soil as a mold and transforms into yeast forms once it parasitizes mammalian tissues. Most infections in humans are ascribed to Histoplasma (H.) capsulatum var. capsulatum (HCC), while HCF is an equine pathogen (3). EL is a contagious chronic disease of horses and other Equidae. Clinically it is characterized by a spreading, supprative, ulcerating pyogranulomatous dermatitis and lymphangitis (4).

The disease is endemic in sub Saharan Africa mainly in Ethiopia but previous reports indicated the presence of the disease within European, African and Asian countries including Iraq (5); Egypt (6); and Nigeria (7). However, knowledge on current global epidemiological situation of the disease is limited.

A gross examination study conducted in selected towns indicated that the disease is highly endemic in Ethiopia with a prevalence ranging from 0-39.1% (2;2;4;8;9). EL has a significant impact upon livelihoods within resource poor settings. (10) reported that losses to the owner due to morbidity of a horse with EL resulted in more than 50% reduction in daily earnings. A loss of US$ 1,683.00 per case of EL in cart-horses and an estimated total loss of 6,023,457 US$ was recorded by computing a loss for the total cases (3579) of cart-horses in Ethiopia (4). Successful EL control measures are non-existent in Ethiopia; therefore, it remains a number one priority disease of horses and affecting socio-economic advantage of communities' dependent of cart-horse as means of livelihood. In early cases of EL administration sodium iodide intravenously and potassium iodide orally, excision of lesions and topical treatments have all been attempted, either alone or in combination that showed promising results but usually with recurrence and signs of iodism. Moreover, iodides are expensive and not easily accessible (11;12).

Currently, diagnosis of EL in Ethiopia is based on clinical symptoms and microscopic examination for HCF yeast cells within pus. However, such classical diagnostic method, though useful for routine case management in endemic areas, has limited specificity and sensitivity to study full epidemiology of the diseases. There are asymptomatic early cases and potential carriers that cannot be detected. Thus, evaluation using the applications of PCR based protocols to rapidly identify HCF directly from equine clinical specimens is of paramount importance to understand the molecular epidemiology of the disease (13). The aim of this study was to investigate the epidemiology of EL in northern Ethiopia using the conventional methods and PCR and to evaluate the inherent diagnostic capacity of conventional tests (clinical sign and microscopic examination) for diagnosis of EL in horses taking PCR as a gold standard.

2. Methods

2.1. Study Area:

The study was conducted in the selected cities of Northern Ethiopia: Mekelle, Kombolcha, Bati, and Kamisse towns where huge numbers of cart-horses are used for the transportation of man and goods. Mekelle is located 783 kms North of Addis Ababa at latitude and longitude of 13^{o} 29N 39^{o} 28E and it is located at 2000-2200 meter above sea level. The weather condition is hot and dry. The mean annual rainfall of the area is 628.8 mm. The annual minimum and maximum temperature is 17°C and 24°C, respectively (1). Respectively Kamisse and Bati are located in North-Eastern and North-central parts of Ethiopia in Amhara National Regional State. Kombolcha, Kamisse, and Bati are located 367, 315 and 406 kms north of Addis Ababa, at latitude and longitude of 11°5'N 39°44'E, 11°11'N 40°1'E, and 10°43'N 39°52'E respectively. The altitudes of Kombolcha, Bati, and Kamisse are 1842 masl, 1502 masl, and 1424 masl, respectively. The topography of the districts generally is marked by the presence of numerous mountains, plateaus, hilly and sloppy area. The average annual rainfall of Kombolcha, Bati,

and Kamisse are 1248, 926 and 972.8 mm, respectively. The average annual temperature of Kombolcha, Bati, and Kamisse are 15.9 °C, 20.4 °C and 20.2°C, respectively.

2.2. Study Design

A cross-sectional study was conducted on cart-horses with signs of lymphangitis or lymphadenitis or both (EL typical lesions) and apparently healthy ones sharing a similar husbandry system in the area. Owners were briefed about the purpose and relevance of the study and carthorses from willing owners were recruited in the study. Sampling was purposive to recruit both to include both sick and apparently heathy ones.

2.3 Study Animals and sampling

Working cart-horses in the selected towns with symptoms of EL lesion as described by (14), and apparently healthy cart horses were sampled. EL horse cases were defined as a naturally infected horse with symptoms of lymphangitis or lymphadenitis or both upon clinical examination. Apparently healthy horse cases, in this study, were horses that did not demonstrate any sign of EL lesions upon clinical examination. From each study animal, body condition, age, and clinical condition were recorded. Dentation and owner history were used to classify the age as young (≤ six years old) and adult (> six years old).

2.4. Sample Collection and Transport

From each study animal, blood samples from jugular vein were collected in 10 ml Vacutainer tubes containing EDTA anticoagulant. The blood was centrifuged at 3000 rpm for 5 minutes thereafter buffy coat was extracted for later use in molecular tests. From horses with un-ruptured nodules or lesion of EL, pus samples were aseptically collected with sterile syringe and needle and striped into two universal bottle containing Sabarouds Dextrose Agar (SDA) enriched with horse serum (each for isolation of yeast and mycelial forms of HCF at varying temperatures). Part of the pus was smeared on glass slide for gram staining. Specimens were transported at +4 ℃ cool transport box to the College of Veterinary Sciences, Mekelle University. Buffy coat samples were kept in -20 ℃ until use. The SDA culture media inoculated with pus was incubated at room temperature and 37 ℃ and 5% $Co_{2,}$ respectively to isolate mycelial and yeast form. A total of 191 cart-horses were sampled. Out of the 70 samples taken from cart-horses with visible EL lesion, only 32 pus samples of closed lesions were collected for gram stain and culture (Table 1).

2.5. Smear Preparation and Gram Staining of Pus Sample Collected from Closed Lesion of EL

Pus smears were prepared in field from 32 cart-horses. The smears were fixed with methanol for 3 minute, and stained with Gram's stain for the identification of the yeast form of HCF. Examination was made using oil immersion at 1000x magnification (100 objective*10 ocular). Search was conducted for typical yeast form of the organism, which should appear as Gram-positive, pleomorphic, ovoid to globose structures, approximately 2–5 μm in diameter. They may occur singly or in groups, and either extracellularly or within macrophages. A halo around the organisms (unstained capsule) is frequently observed (5).

2.6. Fungal Culture and Isolation

Collected pus samples were inoculated aseptically onto slants of SDA containing chloramphenicol (0.5g/liter) and enriched with 2.5% glycerol. For the isolation of the mycelia form, incubation was made at 26 $^{\circ}$C while isolation of the yeast form was made at 37 $^{\circ}$C with 5% CO_2 tension and high humidity (14;15). The culture was checked periodically and Gram-stained preparations were made from suspicious growth (2).

2.7. PCR Based Detection of HCF

2.7.1. DNA Extraction from Buffy Coat and Isolated Yeast Form

DNA extraction from buffy coat samples and isolated yeast form were made with little modifications in the recommended procedures indicated in Qiagen DNeasy blood and Tissue Kit (Lot 157043215, QIAGEN, Hilden, Germany). Buffy coat sample (100 μl) or pieces of cultured cells were transferred into a 1.5 ml micro centrifuge tube. Because we noted, compact nature of the stored buffy coat sample or solid nature of yeast cell culture, tissue lysis buffer (ATL, 100 μl) was applied and incubated at 56^{o}C for 2 hours by frequent vortexing every 15 minutes. Thereafter, 20 μl proteinase K and 200 μl buffer AL (lysis buffer) were added, mixed thoroughly by vortexing, incubated at 56^{o}C for 10 minutes. A 200 μl of 99% ethanol was added and mixed thoroughly by vortexing. To avoid occlusion of columns, the mix was centrifuged at 1000 rpm for 5 minutes. The supernatant was transferred into a DNeasy Mini spin column placed in 2 ml collection tube and centrifuged at 10000 rpm for 5 minute. After discarding the flow through and collection tube, the spin column was placed in a new 2 ml collection tube; 500 μl buffer AW1 (wash buffer) was added and centrifuged at 10000 rpm for 3 minute. The flow through and collection tube were discarded and the spin column was placed in a new 2 ml collection tube then 500 μl buffer AW2 (wash buffer) was added and centrifuged at 13000 rpm for 4 minute. The flow through and collection tube was discarded while the spin column was transferred into a new 1.5 ml micro centrifuge tube and to elute the DNA, 100 μl buffer AE was added into the spin column and incubate for 2 minute at room temperature and centrifuged 10000 rpm for 2 minute. DNA yield was assessed using agarose gel electrophoresis method. 10μl sample of the isolated DNA and 2μl loading dye was loaded into a well of the 2% agarose

gel pre-stained in Ethidium bromide, electrophoresed for 45 minutes with voltage of 92 and visualized in UV light. Extracted DNA was stored in -20^{o}C until use.

2.7.2. PCR Protocol Please reduce the size of this section by citing references

Except little modification in the cycling conditions, primers and PCR protocols were adopted from (13). Primers were sequenced in genetic facility of Iowa State University, Ames, Iowa, USA. PCR amplifications were carried out in 200 µL thin wall PCR tubes (KASVI RCR tube) in a thermocycler (Tianlong PCR thermal cycler, China). The first-round PCR was performed using P3/2R8 primers (primer P3 5'-3', CGGAAGGATCATTACCACGCCG and primer 2R8 5'-3', CAGCGGGTATCCCTACCTGATC) and cycling conditions of 95°C for 10 min (activation) and then 39 cycles of 94°C for 1 min (denaturation), 49°C for 1 min (annealing), and 72°C for 1 min (elongation) and followed by a final extension cycle of 72°C for 10 min. The product from the first round was expected to be 587 bp. A 1-in-10 (vol/vol) dilution of the product from this first reaction was added to fresh master mix including primers of F5 and 2R5 (primer F5 5'-3', CTACCCGGCCACCCTTGTCTAC and primer 2R5 5'-3', CCTACCTGATCCAGTCAACC). The thermocycler program for the second round was the same as that for the first round, except that the annealing temperature was raised to 55°C for 1 min. The ladder used in this protocol was 100 base-pair DNA Ladder (HIMEDIA™, MBTO049). The expected product was 514 bp and was visualized via electrophoresis at 92 V for 45 min on a 2% (wt/vol) agarose gel stained with Ethidium bromide (13).

2.8. Data Processing and Analysis

After data collection, the corresponding code number was written carefully at each margin. The data generated was entered in to the Microsoft excel. The data was imported to and analyzed using Statistical Package for Social Sciences (SPSS) software version 21.0. Descriptive statistics was computed and categorical result was presented using Tables. The association between exposure and outcome variables was assessed using binary logistic regression analysis. Binomial logistic regression through crude odds ratio (COR) was used to asses strength of association of EL with body condition score, age and study area. For those variables that showed significant difference through COR, to avoid confounding factors, multiple logistic regression through adjusted odds ratio (AOR) was computed. Test agreement between two diagnostic tests was computed by Kappa test statistics. The level of agreement between the diagnostic tests was determined using Cohen's kappa coefficient (16;17). Probability (p) values < 0.05 were considered as significant.

3. Results

3.1 Description of study animals and clinical observations

Distribution of study subjects by study area was 61 (31.9%) from Mekelle, 36 (18.8%) from Bati, 42 (22 %) from Kamisse and 52 (27.2%) from Kombolcha. The age category of the study animals was young (20.4%, n= 39) and adult (79.6%, n= 152). The body condition of the cart-horses was poor (9, 4.7%), moderate (30, 15.7%), and good (152, 79.6%).

Out of 191 cart-horses observed, 70 of them showed signs of EL clinically. Generally, the observed clinical signs were loss of body condition or emaciation, swollen lymph nodes, conjunctivitis, restlessness, lameness, purulent nasal discharge, coughing, dyspnea and one or more nodular lesions on different body parts. The nodules were varied in size and in most of the case aligned following lymphatic vessels. Aged and un-ruptured nodules were usually soft with no hair, which discharge white to yellow pus and in some cause pus with blood. Bad odor ruptured nodules were also observed with white to yellow pus discharging.

3.2 Microscopic Examination

Only 32 out of 191 cart-horses (16.7%) were at least with two un-ruptured nodules. On gram stain and microscopy, 93.75% (30/32) of the samples showed a positive microscopic futures of yeast phase of HCF characterized by Gram-positive yeast forms that are round to oval one edge wider and the other bluntly pointed in shape with a halo (unstained capsule-like) structure or with faint blue cytoplasmic space. They were occurring individually or in groups either free (Figure 2, green arrow) or intracellular within the macrophages (Figure 2, black arrow).

3.3. Isolation of HCF

Only one yeast form of HCF was isolated. PCR test conducted on DNA isolated from the culture and corresponding buffy coat and this culture cells was positive. On gram-stain, both from this culture cell and its corresponding pus smear showed typical yeast form of HCF. Culture and isolation was challenged by contamination and frequent electric power failure.

3.4. PCR Detection

HCF positive control DNA was obtained from Prof. Dr. Gobena Ameni, Aklilu Lema Institute of Pathobiology, Addis Ababa University. DNA preparations were amplified with primers P3/2R8 (first round) and then diluted 1 in 10 and subjected to a second round of PCR amplification with PCR primers F5/2R5 to ITS gene products (514 bp) indicative of the presence of HCF DNA. None of the tested amplicons of the first PCR, P3/2R8 primer pairs, were positive on gel electrophoresis except after a nested PCR by F5/2R5 primer pairs. Overall PCR identified as HCF cases were 44% (84/191); of which 26.2% (22/84) were apparently healthy but 73.8% (62/84) were clinically with suspected EL lesions (Table 3). Of the 70

cart-horses with clinical suspect of EL, 88.6% (62/70) were PCR positive while of 121 apparently healthy cart-horses, 18.2% (22/121) were PCR positive.

There was very strong evidence of a relationship between age and being positive for EL using PCR (X^2 = 32.31, df = 2, p < 0.001). Considering the PCR test, adult cart-horses were 9.66 times more likely to be positive for HCF than young age group (AOR=9.66, 95% CI: 3.206 - 29.109, p<0.001). Moreover, there was a statistically significant association between body condition score and occurrence of EL (X^2= 21.59, df = 2, p <0.001) (Table 2). Considering the PCR, cart-horses that have moderate and good body condition are 0.77 (AOR=0.77, 95% CI: 0.104-5.681, p=0.797) and 0.105 (AOR=0.105, 95% CI: 0.017-0.658, p=0.016) times less likely to be positive for HCF infection, respectively than of those that have poor body condition. Alternatively; cart-horses that have poor body condition were 1.299 and 9.523 times more likely to be positive for HCF using PCR than of those that have moderate and good body condition respectively. There was no evidence of relationship between study area and being positive for HCF using PCR (X^2 = 3.068, df = 3, p = 0.381). The odds ratio of being positive for HCF using PCR for cart-horse with EL lesion on clinical observation was found to be 30.309 than of apparently healthy (AOR=30.309, 95% CI: 11.172-82.228, p<0.001) (Table 3).

3.5. Diagnostic Accuracy of Clinical Observation and Gram stain

Taking PCR as a gold standard/reference test (13), the results obtained with the 191 buffy coat samples and 32 pus smears/gram stain of clinically suspected animals and the diagnostic tests were represented in a contingency Table (Table 4). Sensitivities and specificities of clinical observation and gram stain using PCR as reference test are shown in Table 5. The sensitivity and specificity of clinical signs respectively was 73.81% and 92.52 % while the sensitivity and specificity of gram stain was 99.67% and 50%, respectively. The test agreement between clinical observation and PCR was good (k = 0.675, 95% CI 0.570-0.78) while the test agreement between gram stain and PCR test was moderate (k= 0.467, 95% CI -0.160 - 1.000).

4. Discussion

Epizootic lymphangitis (EL) is repeatedly reported to be one of the deadly chronic diseases of cart-horses in many towns of Ethiopia (2;4;8;9)). Despite the high prevalence, to date there is no sound control measures; one of the shortcomings being lack of reliable diagnostic methods that detects early cases as well as subclinical carriers. This study aimed at evaluating molecular detection of EL infection using nested PCR as compared to classical diagnostic methods of microscopic examination and clinical signs.

Out of 191 observed cart-horses 84 (44%) were identified as HCF cases using nested PCR which was higher than the clinical prevalence reported in various areas of the country; overall prevalence of 26.2% in

Debre Zeit, Mojo and Nazareth (2); 21% in Bako and Ejaji towns (8); 18.8% in 28 town of Ethiopia (4), 24.9% in (18) and 12% in Mekelle (19).

The DNA isolated from buffy coat of the 70 presumably EL positive cases, 88.6% (62/70) were PCR positive which was much higher than the performance of the test (63%; 17/27) on DNA isolated from whole blood samples of presumably EL positive animals (13). Considering the intracellular nature of HCF particularly in neutrophils and macrophages, the buffy coat centrifugation could allow aggregation of the infected cells and probably the yeast fungi in the buffy coat layer. Subclinical infection by HCF was reported in 18.2% (22/121) of the apparently healthy cart-horses. This is comparable with the findings of (13), in the highland horses of Ethiopia. This proves the importance of PCR in the epidemiology of the EL, especially for screening purposes while moving horses from endemic to disease-free zones.

Considering the PCR result, adult cart-horses were 9.66 times more likely to be infected by HCF than younger age group (AOR = 9.66, 95% CI 3.206–29.109, $p < 0.001$). This finding contradicts the finding of (4) where horses under six years of age were most susceptible. The high prevalence in adult aged cart-horses could be due to the work related exposure to wounds and other abrasive materials that could potentially favor entry of the soil borne HCF (20). Practically horses aged above four are engaged in full work. Assembling of those adult horses in the cart-horse station during work favors the transmission of HCF by biting flies (8) and the frequent use of similar bedding, harnessing and saddlers for all owned cart-horses favor rapid disease transmission (21).

Carthorses that have poor body condition are 1.299 and 9.523 times more likely to be positive for HCF using PCR than of those that have moderate and good body condition, respectively (AOR = 0.105, 95% CI: 0.017–0.658, $p = 0.016$). This finding was consistent with the findings of other studies in central Ethiopia (4;18;21). Carthorses with poor condition tend to acquire EL because they are prone to wound infliction, which then attracts flies that increase the chance of infection.

The clinical features and microscopic appearances of the yeast forms of EL observed in the present study were in agreement with previous report (2;5;8). In agreement with (2;14) there were not clearly separated three forms of EL but the cutaneous form of EL were the dominant cases.

In this study, we evaluated the diagnostic accuracy of clinical observation in comparison with nested PCR primer sets for diagnosis of HCF infection developed by (13). Clinical signs used for diagnosis of EL showed a sensitivity of 73.81% and specificity 92.52%. The test agreement between PCR and clinical signs was good ($k = 0.675$, 95% CI 0.57–0.78). The lower sensitivity may be explained by due to the presence of asymptomatic carrier and pre-clinical stage of the infection in which the incubation period ranges from several weeks to six months (5) that was positive using PCR but experiences no symptom to be detected using clinical observation. Yeast cells were present in 93.75% (30/32) of the stained smears, which was much higher than 52% reports of (13). This variation could be explained by difference in stages of the disease.

5. Conclusion

The study indicated that EL is one of the major health threats of working horses. As compared to previous studies, PCR amplification from buffy coat DNA was more appropriate. Molecular prevalence of EL in both presumable EL cases and apparently healthy animals was high. The nested PCR could be potentially useful in disease control and regulatory authorities particularly in equine trade and prevention of unintentional introduction of HCF. The culture and isolation experiment indicated that isolation of HCF is possible but external factors have to be controlled. We recommend in resource poor settings, currently diagnosis of diseases is based on clinical signs, should be supported by microscopic examination of gram stain smears from pus samples. However, early stages or potential carriers are left undetected and remain risk for the herd. Commercializing such technique for routine use is far from practice. Therefore, use of PCR as screening test during movement or purchase and future researches on production of vaccine is recommended.

6. Declarations

Ethics approval and consent to participate

The study has passed through the Animal Ethics and Experimentation review process of the Research and Community Services Council Office of the College of Veterinary Medicine, Mekelle University, Ethiopia (CVM/EXAP-02/5 Feb 2018). Sampling was made after getting verbal consent from the owners. Considering the less invasive nature of the sampling procedure, verbal consent was enough and approved by the ethics review committee. Willing owners restrained their horses. Skilled veterinarian did the Blood sampling.

Consent for publication

Not applicable. No someone else data is incorporated in this publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Contribution of authors

BH, MM, HT, NA, AT, HK, and BA have conceived study, collected samples and processed in laboratory. BED, GG, and GA have collected data and collected samples. BH, MM, and HT analyzed data and wrote the draft manuscript. All have read and approved the final manuscript.

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Abbreviations

COR crude odds ratio

DNA Deoxyribonucleic acid

EDTA Ethylenediaminetetraacetic acid

EL Epizootic lymphangitis

HCC Histoplasma capsulatum var. capsulatum

HCF *Histoplasma capsulatum var. faciminosum*

Kms kilometers

masl meter above sea level

mm millimeter

PCR Polymerase chain reaction

rpm revolution per minute

US$ United States Dollar

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Tables

Table 1. Sample type and number per each study area

Study town	Number of horses sampled	Sample type		
		Carthorse with EL lesion		Apparently Healthy
		Buffy coat	Pus*	Buffy coat
Mekelle	61	36	25	25
Kombolcha	52	6	1	46
Bati	36	11	4	25
Kamisse	42	17	2	25
Sub-Totals	**191**	**70**	**32**	**121**

*Pus samples for culture and isolation, and gram stain depends on the number of carthorses with un-ruptured EL lesions.

Table 2: PCR results and their association with age, body condition, study area and clinical condition

Variables		PCR result		X^2	df	p value
		+ve	-ve			
Age	young	5	34			
	adult	79	73	32.31	2	<0.001
Body condition	poor	7	2			
	moderate	23	7	21.59	2	<0.001
	good	54	98			
City	Mekelle	35	26			
	Bati	16	20	3.07	3	0.381
	Kamisse	18	24			
	Kombolcha	15	37			
Clinical condition	with suspected EL lesion	62	8			
	apparently healthy	22	99	89.18	1	<0.001

Table 3: Odds ratio for risk factors associated with the occurrence EL

COR= Crude Odds Ratio, CI= Confidence Interval, B= intercept and AOR= Adjusted Odds Ratio

Variables		COR	95% CI	P	B	AOR	95% CI	P value	B
Age	Young	1				1			
	Adult	7.359	2.73–19.83	<0.001	1.996	9.660	3.21-29.11	0.00	2.269
Body condition	Poor	1				1			
	Moderate	0.939	0.16–5.59	0.945	-0.06	0.770	0.10-5.68	0.797	-0.262
	Good	0.157	0.03–0.79	0.024	-1 .84	0.105	0.02-0.66	0.016	-2.253
Clinical condition	apparently healthy	1				1			
	with EL lesion	3.552	14.62-83.18	<0.001	3.552	30.309	11.17-82.23	<0.001	3.411

Table 4. Contingency table with results of all buffy coat samples tested in the PCR, the Clinical signs and gram stain

pos= positive, neg=negative, N= number of tested samples

PCR	Clinical sign (N=191)		Gram stain (N=32)	
	Pos	Neg	Pos	Neg
Pos	62	22	29	1
Neg	8	99	1	1

Table 5: Accuracy and agreement of clinical observation and gram stain with PCR as reference test

Result		Clinical observation (n=191)
Sensitivity		73.81%
Specificity		92.52 %
Accuracy	AUC	0.832
	95% CI	0. 768-0. 895
	p	<0.001
Agreement	*k*	0.675
	95% CI	0.570-0.78
	p	<0.001

Figures

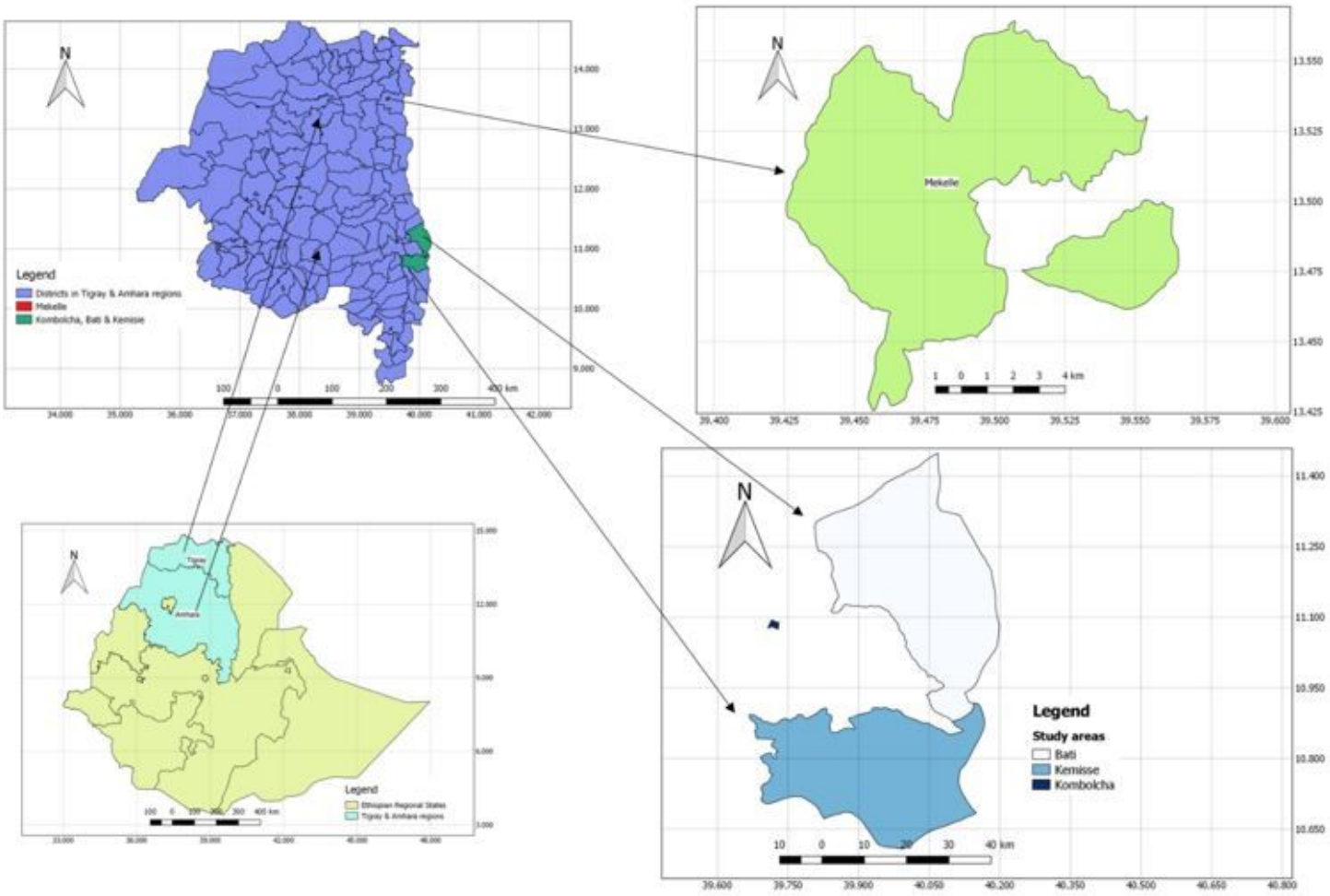

Figure 1

Map of study areas.

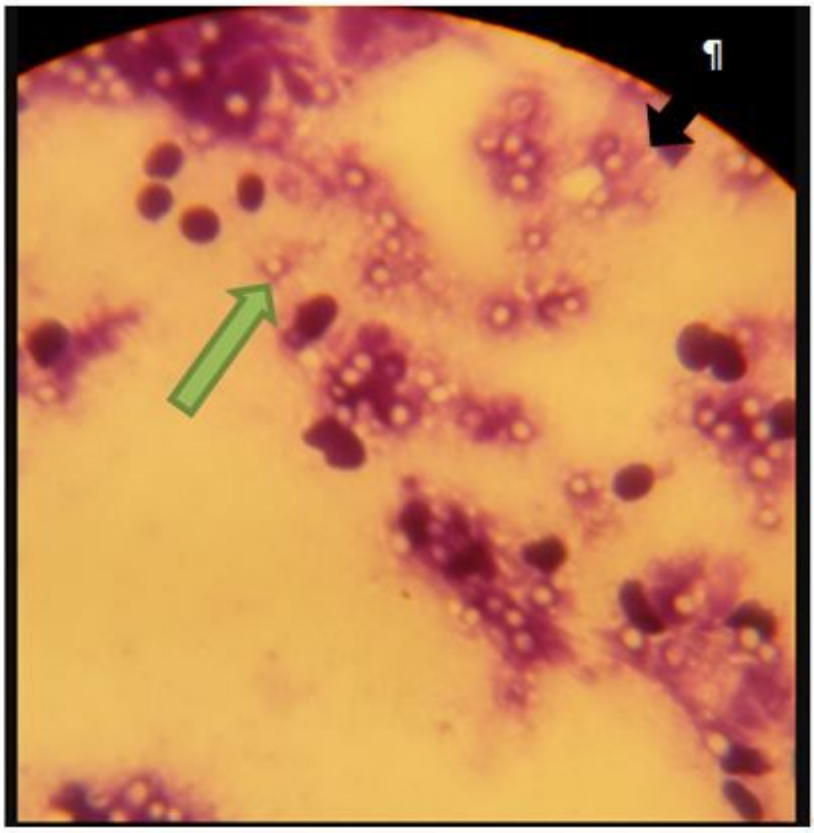

Figure 2

Gram stained smears of pus samples from Closed EL nodules

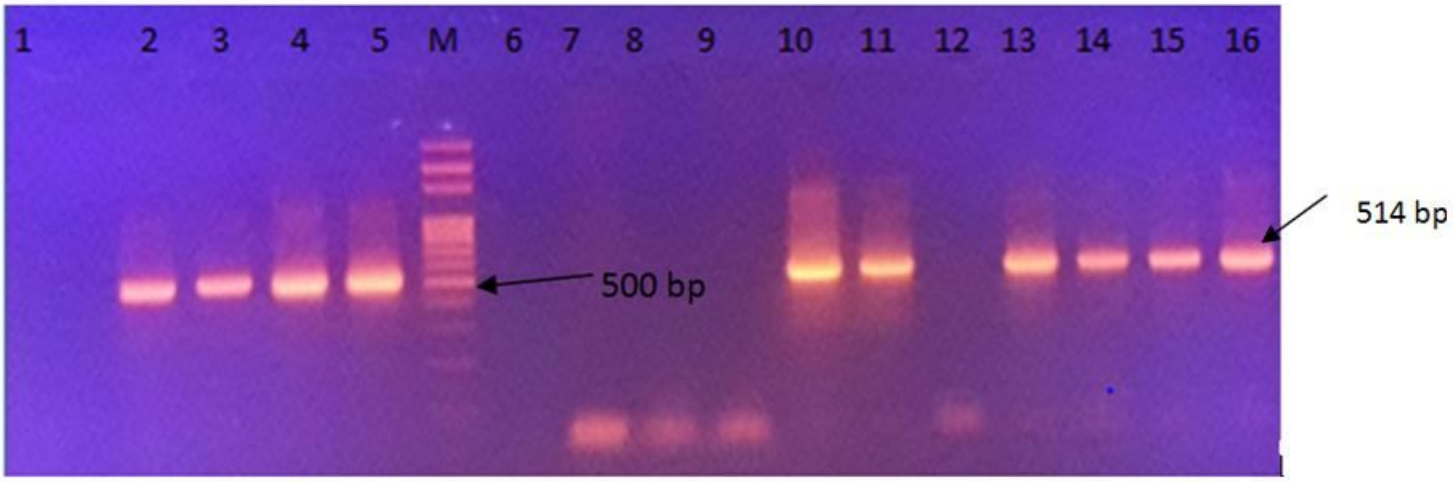

Figure 3

Gel electrophoresis of nested PCR amplification products obtained from DNA extracted from horse buffy coat samples.