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Research Article

Isolation and Molecular Detection of H. Capsulatum var. Farciminosum from infected Cart-horses and Assessment of its Economic Impact in Bishoftu, Ethiopia

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Abstract: Epizootic lymphangitis is a highly prevalent and economically important but less studied disease of equines of poor countries. An attempt to isolate, characterize, and molecular identification of Histoplasma capsulatum variety farciminosum from Epizootic lymphangitis-infected horses was made on three different media from 36 cases. A total of 43 samples: 22 unruptured aspirates, 10 open pus swabs, and 11 blood samples were collected cross-sectionally from Bishoftu. These study units were categorized into different categories of risk-factors to see their associations. The characterization was made with Giemsa and Lactophenol-blue stain, whereas molecular identification of the isolate was made with nested PCR. Additionally, for the assessment of economic impact and cart-horse owners' perceptions of the disease, 40 interview questionnaires were considered randomly. In this study, typical H. capsulatum yeast cells were recognized in 96.8% (31/32) of pus samples. 7 out of 22 aspirated pus samples were successfully isolated within 3-8 weeks. Neither blood nor swab samples gave a positive result for culture and the former additionally for direct microscopic examination. A significant statistical difference (χ^2=11.4, df=1, P=0.004) in the presence of yeast cells in the smear between clinical and non-clinical cases was detected. Economically, 50% (20/40) of the respondents each lost an overall, 73,105.13 ETB per-head of horse within three years of their experience and 95% of the respondents were able to describe the clinical features of this disease. So, understanding this impact, we recommend the government or other stakeholders to find the best control measures which includes vaccine production should be undertaken.

Keywords: Arthroconidia, Conidia, Epizootic lymphangitis, H. capsulatum var. farciminosum, Lactophenol blue stain, Mycelia, Sabouraud glucose agar, Yeast cell.

Introduction

Epizootic lymphangitis is an afebrile, non-contagious, debilitating disease of Equidae characterized by a spreading, ulcerating pyogranulomatous dermatitis and lymphangitis with a possible extension through lymphatic chords to the lung, and eyes leading to pneumonia and conjunctivitis [9]. Occasionally, systemic infection has also been reported but predominantly local. It is a highly pathogenic fungal infection caused by Histoplasma [17], which has been classified into antigenically indistinguishable three separate varieties and is endemic to many worlds [5]. Histoplasma capsulatum var. farciminosum is particularly the causative agent of Epizootic lymphangitis in horses [16]. It is a saprophytic and thermally dimorphic fungus mainly found in nitrogen and phosphate-enriched soils. The yeast form is pleomorphic cells measuring 3-5 µm long and 2.5-3 µm wide found singly or in groups, free or in phagocytes [17,26]. It contains an achromatic hazy centrally to marginally situated cytoplasm surrounded by a chromatid substance and some blue-staining basophilic substance filling the gap giving the cells the achromatic ring of malaria [31].

Equines in Ethiopia account 11.32 million; of which 2.01 million are horses. This is about 34.5% of total equines in Africa and 3.45% of total world equine populations [6]. These equine populations are engaged in several life-saving activities, especially for developing countries, even if their welfare and health conditions might be compromised by several diseases [28]. Currently, Epizootic lymphangitis is assumed to be highly prevalent in Ethiopia, where between 0-39 % of equids were reported to be infected, with the rate being dependent upon the region temperature, humidity, and activities of the equideas [8]. It is clinically indistinguishable from sporotrichosis, penicillinosis, and some bacterial skin infections.

In a survey by one researcher [24], the socio-economic impacts of EL were income generation loss, welfare compromise, environmental contamination, and a traffic jam which may result in a car accident. Despite these impacts, the disease is less confirmed by culture, and little is known about the isolated and recognized socio-economic impacts so did not get much attention from the government. In our country, only at ALIPB, successful isolation with convincing evidence of PCR and colony characteristics has been undertaken [22]. However, isolation of this pathogen not only serves as a gold standard for diagnosis of the case but also helps to know the biological characteristics of the isolate and acts as an initial step for designing effective treatment and other intervention mechanisms like vaccine production. So, understanding the characteristics of the isolate with confirmative diagnosis and its socio-economic impact helps to attract governments and researchers' attention to design effective diagnostic and control methods. Therefore, this study aimed to isolate and characterize the gross and microscopic characteristics of Histoplasma capsulatum var. farciminosum with molecular confirmations and assess the socio-economic impact and carthorse owners' perception of epizootic lymphangitis.

Materials and Methods

Study Area and Samples Collection

This study employed tangible data collection for characterization, isolation, and molecular identification of the causative agent of epizootic lymphangitis, the H. capsulatum var. farciminosum; and observational and face-to-face semi-structured and unstructured interview questionnaires serve to collect retrospective data for the assessment of the economic impact of EL and carthorse owners' perception and practice on EL. All of the data were collected from Bishoftu districts and were processed at the National Veterinary Institute (NVI) mycology laboratory. The district has a high population of horses which is mainly concentrated in the town for packaging purposes and optimum favorable ecological conditions for the occurrence of the disease. The main economic activities of this town are agriculture and trade. Horses are playing a major contribution in the packaging and transporting of equipment and people.

Study Population

The study population for this study was carthorses infected with epizootic lymphangitis and presented for treatment at Spana clinic in Bishoftu, Ethiopia. Additionally, randomly selected carthorse owners at their working sites were part of the interview questionnaires after they were informed of the objective of the study and assured of their data confidentiality and anonymity. The horses were categorized based on their history of treatment into treated or not treated and their lesions were also graded into three; mild, moderate, and severe based on the coverage of lesion distribution adopted from [20]. They are also categorized into three, based on body condition considering from a welfare point of view as well. The carthorse owners were asked for retrospective data and usual activities for the assessment of socio-economic impact and their practice related to the disease.

Clinical Examination of the Cases

All carthorses presented to the clinic were clinically tested for the presence of any lesion suggestive of epizootic lymphangitis by inspection and palpation, especially for the presence of typical signs. Due attention was given to the lymphatic of the legs, nostrils, and eyes, and the whole skin during the physical examination of the horses. As a result, the horses were classified as healthy for EL (no detectable lesion) and clinically positive cases. The cases were confirmed when several ulcers, granulomatous nodules ran on the lymphatic trunk, and suppurative skin nodules with a yellowish-green color were recognized [7,26]. The samples were taken from both clinically suggestive and suspicious cases. The samples were blood, pus, and swabs with smears from the horses having open ulcers.

Sampling Techniques and Processing

From thirty-six cases of horses, a total of 43 samples; 22 aspirated pus samples, 10 swabs, and 11 blood samples were collected purposively and cross-sectionally (seven blood samples were collected from cases from pus samples already collected). Additionally, 40 semi-structured and unstructured interview questionnaires were considered. These questionnaires were collected from carthorse owners randomly at their packaging site, for the assessment of economic impact and carthorse owners' perception of EL. Verbal informed consent was considered from all participating owners before commencing sample collection. Un-ruptured nodules on horses were identified and thoroughly washed with soup and water and disinfected with 70% alcohol and with great care not to burst it and were shaved with a surgical blade, and the content was aspirated gently with a 16-gauge needle. The swabs were collected with sterile 10 cm long swabs from the open wound and kept in peptone water transporting media to avoid drying. All of the samples were ice-boxed and directly shipped to the National Veterinary Institute, Bishoftu, Ethiopia, for microscopic examination, isolation, and further processing.

Study Design

Microscopic Examination

Microscopic examination of Histoplasma capsulatum var. farciminosum was undertaken on pus (aspirated and impression smear) and blood samples to detect the yeast form of the fungus. For this, first methanol fixed uniform pus, and blood smears were made on a clean glass slide. The smears were dried by air and covered with 1:20 diluted Giemsa stain for 25 min. Without removing the stain, the same amount of buffer solution was poured drop by drop on the slide and remained for 4-5min. Then it was blown gently over the surface of the fluid to mix the buffer and the stain. Then finally, the slide was gently washed with flowing tap water and wiped with a clean and alcohol-moistened gauze tissue from the bottom to be examined under a 100x oil immersion objective lens after being air-dried by slanting [28]. The colony grown at 26°C was also taken onto a glass slide for smash smear preparation and underwent microscopic characterization of mycelium by lactophenol cotton blue and gram staining, and visualized under a 40x and 100x objective lens.

Culturing

Three different media were prepared for inoculation of the collected samples to harvest HCF. These media were SGA (Sigma-aldrich chemie gmbh riedstr 2 D-89555 Steinheim 497329970) and BHI (Sigma-aldrich chemie gmh) and PPLO (HimediaR, ref m267-500g). To guarantee the recovery, the media were added with penicillin and gentamycin at 0.1gm/1L concentration and contained 2.5% glycerol and 10% horse serum [29]. Media without antibiotics and/or glycerol was also inoculated. Each media was replicated, blocked, and prepared with slant and Petri dishes according to the standards and inoculated within days of the shelf life of the media. The inoculated media were incubated at 26°C, 37°C, and 5% CO_2 for about 8–12 weeks [32]. The growth of the mycelial form of H. capsulatum var. farciminosum was evident when dry, gray-white, granular wrinkled colonies were observed [26]. Typical colonies were collected and stained with Gram stain and Lactophenol blue for screening.

Any cultures suspected of Histoplasma capsulatum var. farciminosum with colony characteristics and staining were subjected to nested PCR for confirmation of their identity. Bacterial contaminated and early dried inoculates were undoubtedly considered culture-negative.

Molecular Detection

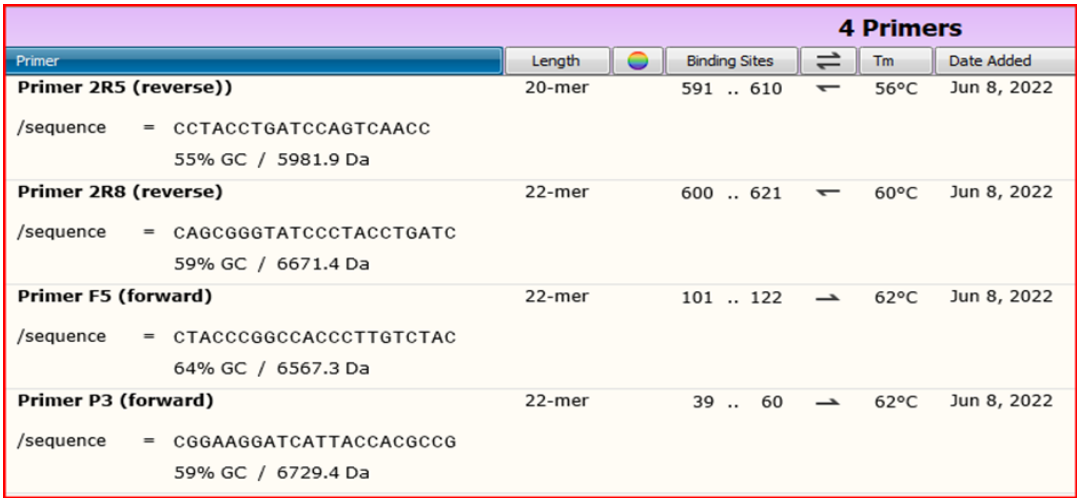
DNA Extraction

Qiagentm Dneasy blood and tissue DNA extraction kit (Qiagen GmbH, Dusseldorf, Germany) supplies closed protocols consisting of proteinase K, lysis Buffer, DNeasy silica membrane, ethanol (96.97%), wash Buffer1, wash Buffer2, and elusion Buffer. Suspensions of the fungal colony were made in a 2ml micro-centrifuge tube by the addition of 1 ml of phosphate buffer saline (PBS). The suspension was agitated, vortexed, and then centrifuged for 10 min at 7500 rpm and resuspended in 180µl of 2 mM EDTA solution for incubation at 37°C for 30 min. DNA extraction was performed according to the DNeasy Blood & Tissue extraction kit. 20µl of proteinase K (Qiagen, Germany), 200µl enzymatic lysis buffer, and 200µl fungal cellular pellet from the suspension were added to a 1.5ml micro-centrifuge tube and followed per [18,13] recommendations.

DNA Amplification and Visualizations

For amplification of the extracted DNA, a nested PCR protocol targeting the internal transcribed spacer (ITS) sequence region coding for ribosomal RNA of HCF genes was designed by [23] based on the Ajellomyces capsulatus 5.8S rRNA gene of ITS1, and ITS2. The first pair of primers replicating 587bp corresponding to 39th to 60th and 604th to 625th nucleotide position was P3, 5′-CGGAAGGATCATTACCACGCCG-3′ and 2R8, 5′-CAGCGGGTATCCCTACCTGATC-3′ respectively as indicated in Figure 1. For the first round PCR run, each reaction mixture volume of 24µl containing 3µl RNase-free water, 2µl P3-Primer, and 2R8-Primer, 5µl 10X dream Taq buffer, 1.5µl DNA Taq polymerase, 5µl dNTP, and 5µl DNA Template was set in thermocycler PCR (Applied biosystem) with 94°C for 7min initial denaturation and 35 cycles of denaturation at 94°C for 1min, annealing at 49°C for 2min, and elongation at 72°C for 2min, followed by a one cycle final extension at 72°C for 7min.

For more specification and increasing sensitivity, the second set of pair of PCR primers corresponding to 101th to 122th and 594th to 614th, having the ability to bind internally to the first PCR product multiplying 514 nucleotides bp, was designed as 5F, 5′- CTACCCGGCCACCCTTGTCTAC-3′ and 2R5, 5′-CCTACCTGATCCAGTCAACC-3′ respectively as indicated again in. The PCR running program for the second round was the same as that of the first, but the annealing temperature was raised to 55°C for 1 min and it is the first PCR product that served as a DNA template in the second PCR run. For visualization, a 2% agarose gel well was prepared with 5µl intercalating gel red dye. The second PCR product was mixed with 5µl DNA loading dye and 10µl of this mixture was dispensed into these wells and electrophoresed for 1hrs at 120V. Finally, the gel was radiated with UV radiation and captured with a UV camera.



4 Primers

Primer	Length	Binding Sites	Tm	Date Added
Primer 2R5 (reverse))	20-mer	591 .. 610	56°C	Jun 8, 2022
/sequence = CCTACCTGATCCAGTCAACC 55% GC / 5981.9 Da				
Primer 2R8 (reverse)	22-mer	600 .. 621	60°C	Jun 8, 2022
/sequence = CAGCGGGTATCCCTACCTGATC 59% GC / 6671.4 Da				
Primer F5 (forward)	22-mer	101 .. 122	62°C	Jun 8, 2022
/sequence = CTACCCGGCCACCCTTGTCTAC 64% GC / 6567.3 Da				
Primer P3 (forward)	22-mer	39 .. 60	62°C	Jun 8, 2022
/sequence = CGGAAGGATCATTACCACGCCG 59% GC / 6729.4 Da				

Figure 1: Primers with their binding sites on a complete sequence of ITS1, 5.8S, and ITS2 Gene (gene bank accession number U18363) and their melting temperature.

Questionnaire Survey

Observational and face-to-face semi-structured and unstructured interview questionnaires containing retrospective data from three years (2021, 2022, and 2023 G.C) were collected for the assessment of the economic losses of EL which were estimated per cart owner and knowledge and practice related to it. These questionnaires (Questionnaire file) were interpreted in to local language orally. Data concerning mortality, treatment length, number of families that depend on its income, and feeds that the horse consumes per day were employed to assess the economic impact of EL. This questionnaires did not include treatment cost and welfare issues as the owners has no idea about it. The second part of the questionnaire contains questions relevant to disclose the knowledge and practices of the respondents related to EL. For this, open questions enable them to express their views concerning their knowledge about EL and other diseases, measures that would be taken to protect the horse from EL were asked.

Calculations for economic loss due to EL:

TFC (p.h) = FC (p.d) × length of duration they fed without work. If the infected horse dies, the Overall cost/head=mortality cost +TFC for the length of feeding a horse before its death. Where, TFC=total feed cost; FC=feed cost; ph= per head; p.d= per day.

All monetary losses were recorded in Ethiopian Birr (ETB), which has a mean exchange rate of 0.019 USD for the study year.

Statistical Analysis

All the data collected from the sample collection site and laboratories were coded and inserted into an MS Excel spreadsheet and analyzed with Stata/SE version 14.0 statistical software. This study used descriptive (mean, standard deviation and proportion calculated from the frequency distribution table were used as descriptive statistics) as analytical techniques.

Results

Case Categories of this Study

The frequency distribution of epizootic lymphangitis cases in this study revealed that most of the cases were old cases following a prolonged time of treatment regimen 86.11% (31/36). Only fewer new cases appear per week than old cases. Usually, these old cases were horses with moderate and severe lesions. Additionally, the below Table 1 and Figure 2 show, about 55.56% of the cases were in a state of poor body condition, and 50% were in severe condition.

Table 1: Frequency distribution of cases by body condition.

Body condition	Freq.	Percent	Cum.
poor	20	55.56	55.56
moderate	10	27.78	83.33
good	6	16.67	100.00
Total	36	100	

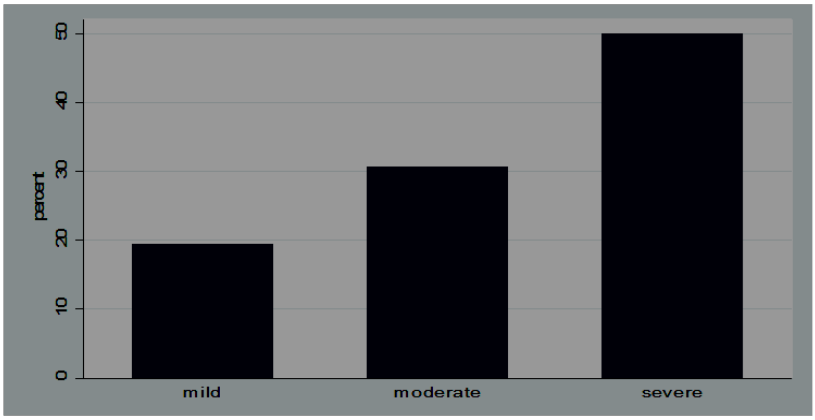


Figure 2: Bar graph showing the percentage of different lesion grades.

Microscopic Characteristics of Histoplasma

Direct Giemsa Stain Characteristics

Smears were prepared from a total of 32 pus samples (open and closed), and 11 blood samples from 36 cases of horses (in which from 4 horses, only blood was collected). Yeast cells were apparent in 96.8% (31/32) pus (impression and thick) smear preparations, revealing pleomorphic yeast cells; one or both sides pointed, oval to round measuring 1-5 µm in diameter having an achromatic unstained central cytoplasm surrounded by a chromatid substance some resembling a signet ring as indicated in Figure 3 A and B and Figure 5.

However, none of the blood samples gave positive results for yeast cells. In comparison to immature closed nodules, open ulcers, and chronic mature nodules have a high population of fungal yeast cells. Frequently, one pole was more pointed than the other. The cells were found singly or in groups, free or phagocytized by macrophages or other leukocytes. But the free were very large in number. In some cases, the cytoplasm of the cells may be very small and invisible; giving the yeast cells a bean shape with or without an unstained zone resembling a capsule. Upon exposure to room or lower temperature, within twenty-four hours or more, these cells became enlarged and transformed into a sac-like structure containing elongated mycelia. This structure was discovered even in immediately processed pus. However, such pus samples were taken from chronic and highly matured nodules without active vascularization and exposed ulcers.

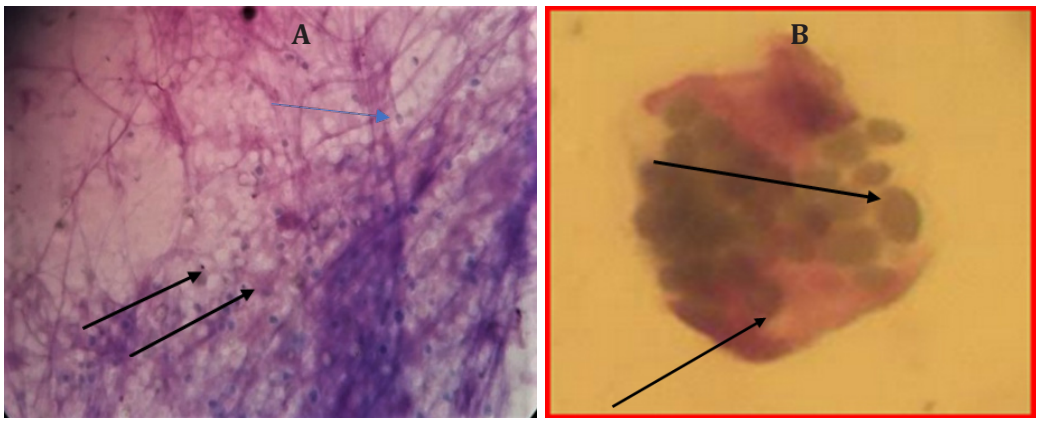


Figure 3: A) Histoplasma capsulatum var. farciminosum yeast cells with flaky mycelia from aspirated nodule after 24 hrs. B) Macrophage nuclei and surrounded bean-shaped yeast cells from immediate smear preparation from aspirate containing flakes of blood with Giemsa under 100X.

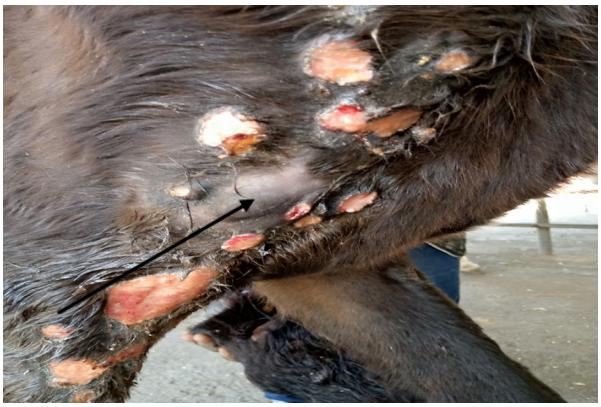


Figure 4: Shaved and disinfected mature nodule ready for sampling on the horse.

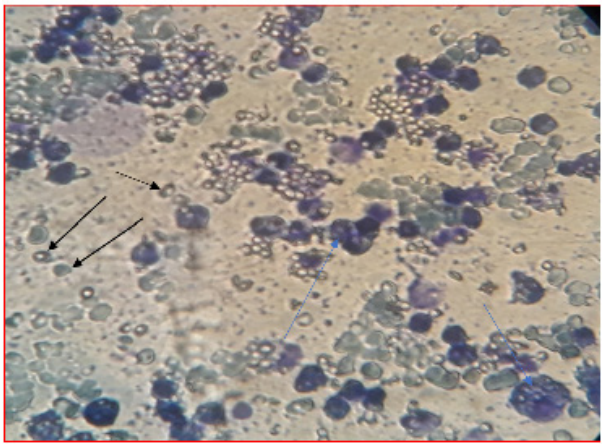


Figure 5: Yeast cells with chromatid and some blue staining basophilic substance surrounding a central to marginal achromatic cytoplasm in macrophage (blue arrow) and free (black arrow) by Giemsa stain under 100X.

Histoplasma Capsulatum var. Farciminosum Isolate

Successful isolation of the fungus was attempted from 31.8% (7/22) of aspirated unruptured nodules which were confirmed by nested PCR as indicated in Figure 8. However, none of the swabs and blood samples gave positive culture upon incubation for up to eight weeks. Regardless of antibiotics in use, all swab samples were occupied by bacteria and early-growing fungi before HCF grows. Interestingly the jugular blood samples were culture sterile upon incubation for up to four weeks on all media. Additionally, this study showed that open ulcers are not important for the isolation and purification of this fungus. The in vitro recovery of yeast form was unattained by this study upon incubation at 37°C for three weeks with weekly transfer to fresh media.

The growth of the isolate on all of the three media supplemented with horse serum, antibiotics, and glycerol was very slow and appeared only after three to eight weeks of incubation at 26°C. On PPLO agar medium the fungus started to appear only as minute grey flakes after 4-8 weeks of incubation at 26°C and seemed to be inhibited. Whereas on SGA and BHI the characteristics of the isolate were pasty to dry, grey-white, granular, convoluted, and wrinkled cerebriform mycelial colonies which appeared within 3–8 weeks of incubation and turned brownish with aging (see Figure 6: A and B). PPLO was least in luxurious colony recovery even with incubation for 9 weeks. BHI and SGA supplemented with horse serum and antibiotics were equally important. Very interestingly all media without glycerol were negative for growth.

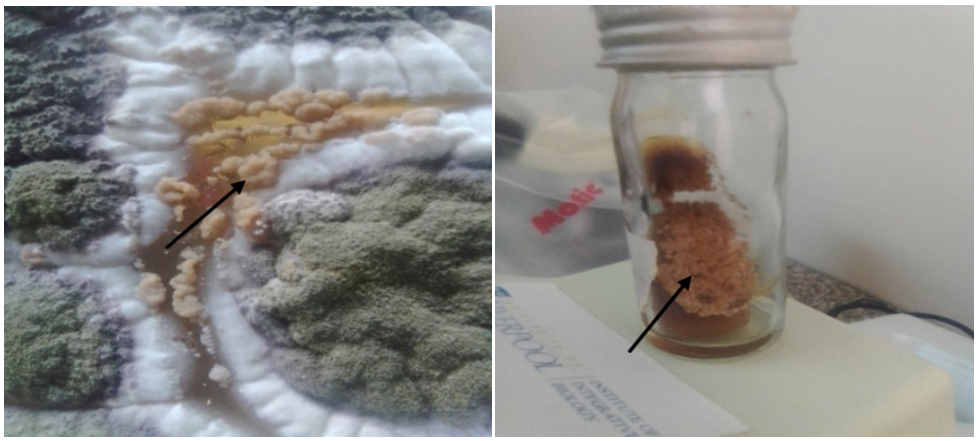


Figure 6: A) Convoluted, pasty, and folded grey-whitish to a brown primary culture of five weeks age on SGA. B) A 28-day subculture of primary culture on BHI which is a dry and granular colony.

Lactophenol blue staining indicated a variety of conidia, including chlamydospores and some blastoconidia but predominantly arthroconidia with short hyaline, branching, septate hyphae, and mycelium in 46.5% (20/43) of the isolated samples which were suggestive of the isolate of interest and were further subjected to PCR confirmation for HFC. The large round double-walled macroconidia that are often observed in H. capsulatum var. capsulatum are also present as indicated in Figure 7. However, the macroconidia had no several tuberculations unlike that of H. capsulatum variety capsulatum. On the other hand, the gram staining of the colony revealed eosinophilic staining and solid round budding bodies. The mycelia were not stained at all.

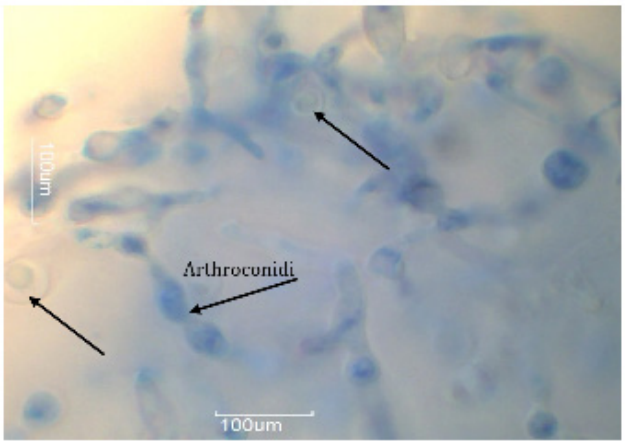


Figure 7: Arthroconidia characteristics of double-walled blastoconidia of HCF by Lactophenol blue staining under 100X.

PCR Confirmation

Molecular confirmation of the isolate was being started from the preparation of isolate suspension and DNA extraction from a colony and continued to the PCR reaction process. The results of nested PCR analysis of this variety as indicated in Figure 8. showed the presence of an intended gene in 16.3% (7/43) of the isolated colonies. Thirteen isolates were negative, whereas, twenty-three were discarded as negative before lactophenol and some by lactophenol blue staining before PCR.

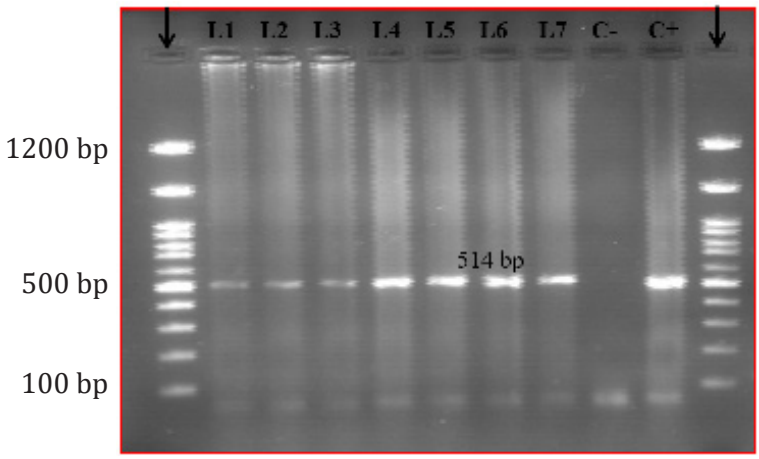


Figure 8: Gel electrophoresis result of second PCR product from the colony of Histoplasma capsulatum var. farciminosum.

This nested PCR amplifies 587bp at first and 514bp on the second PCR run for the amplification of ITS1 and ITS2 genes of HCF. L (left and right) is a 1200bp DNA ladder; Lane (L1-L7) are DNA extract of Hcf isolates from this study; C- Negative control; C+ is a positive control.

Socio-economic Impact and Carthorse Owner Perception

The questioned attendants for this study have an average of three years of experience. From a survey of 40 attendants of cart owners, on average, 85% (34/40) of the owners had lost one carthorse due to EL per three years which was about 14,932.33 ± 972.2 ETB per head per three years, and only about 25% of these attendants had other work besides it. On a median, the number of families that depend on the income generated from carthorse was four. Additionally, 75% (30/40) of these attendants fed infected horses with decreased workforce for an average of 14±7.3 weeks but only 33.3% (10/30) were recovered. The average feed and water cost per day per head was found to be 153.6 ±25.77 ETB. Table 2 shows the total average feed and water cost per head for the above length of periods was about 15,052.8 ETB. If it was on work, the average daily income from carthorse service would be 440±71 ETB, which was around 43,120 ETB losses per hospitalization time. Overall, 50% of the attendants each lost 73,105.13 ETB on average per head per average of three years.

This study did not include welfare issues and treatment costs, because the attendants had no information on them, as it is given free by the Spana project in Bishoftu.

Table 2: Average economic losses due to epizootic lymphangitis per animal per owner.

Variables	Economic loss/cart owner/ carthorse
	Mean
Mortality loss	14,932.33
Days without work/ decreased workforce	14 wks
Feed and water cost per day	153.6
Total feed costs/ mean hospitalization time	15,052.8
Income generation loss per day	440
Income loss/ mean hospitalization time	43,120
Total	**73,105.13**

Regarding the knowledge of carthorse owners about horses' diseases and practices, only 5% (2/40) of them did not know and describe any type of horse disease. Epizootic lymphangitis is known as Biche in Afaan Oromoo whereas, Nideft in Amharic. About 55% (22/40) of the attendants know three and more than three diseases and about 30% (12/40) know two diseases and about 10% (4/40) know only 'Biche'. The most known diseases by carthorse owners were Biche (95%) followed by African Horse Sickness (AHS) (75%), Colic (65%), and anuria and tetanu (5%).

This study also revealed their practice to protect their carthorse from a disease, especially 'Biche', and the measurements they will take when a carthorse is infected with a disease as shown in Figure 9 and Figure 10. For this, 90% (36/40) of the carthorse owners wash their horses with 'hiddi', 'Nimi leaf', soaps, and 'endode'. About 55% (22/40) prefer proper harnessing practices to avoid soreness, 25% (10/40) respond as isolation and avoiding contact is best to prevent the disease, and 20% (8/40) seek vaccination even if there is no commercial vaccine for epizootic lymphangitis, 5% (2/40) burn the lymphatic cord surrounding the lesions where the disease started. However, only 25% (10/40) of these attendants disagree with using Biche' infected horses. This agrees with the results from observation during the interview in which 75% of 'Biche' infected were encountered at work. Additionally, during this study many horses were abandoned on the street.

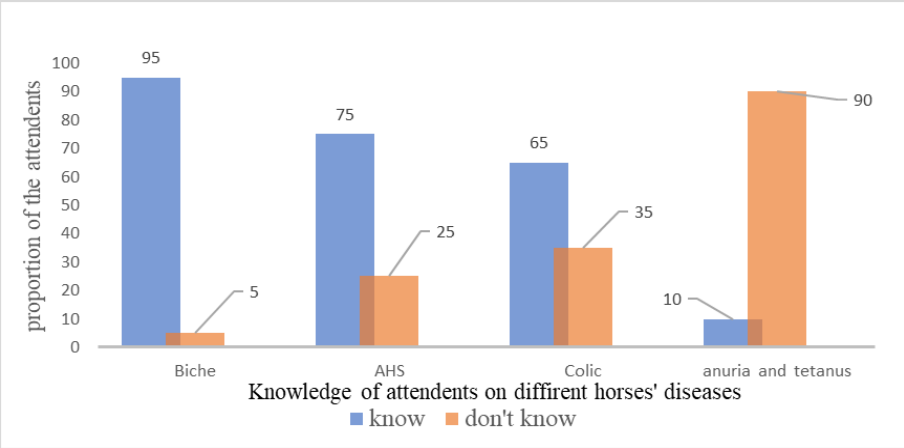


Figure 9: Bar chart indicating the proportion of carthorse owners and their knowledge of different diseases.

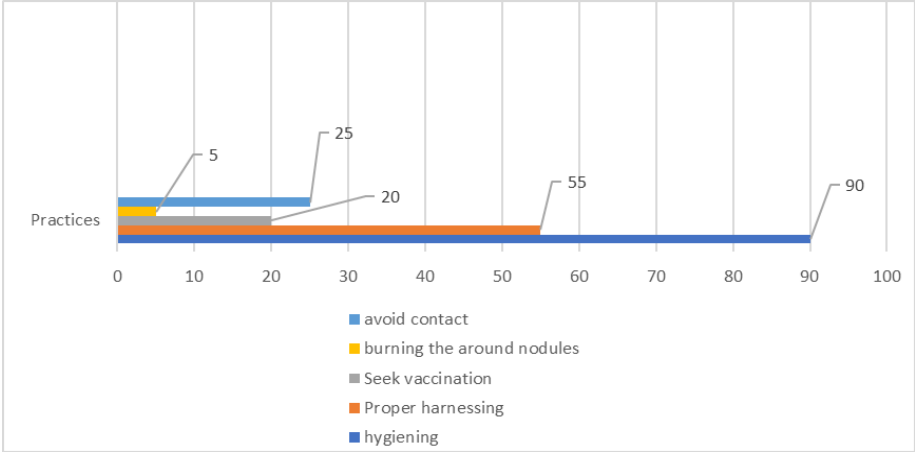


Figure 10: Bar graph showing the practices of the carthorse owner to prevent EL.

Discussion

Epizootic lymphangitis is one of the health and welfare issues of the Ethiopian equine population with an overall prevalence of 16.67% [1]. It is caused by dimorphic fungi Histoplasma capsulatum variety farciminosum [26]. This study characterized the nature of this disease, the microscopic and macroscopic nature of this strain with molecular confirmation, and finally the impact of this disease. The high number of old (86.11%%), poor body condition (55.56%), and severe (50%) cases observed in this study is due to the chronicity and debilitating nature of the disease and poor response of the disease to the available treatments, and frequent follow-up needed [22]. This was in turn in agreement with the present study which indicates the insignificance of the treatment effect on the presence of yeast cells in the pus smears. Spana project in Ethiopia adopted animal sedation and incision of all nodules with topical 4% iodine tincture treatment during this study as an available drug of choice (personal observational). As anti-fungal drugs are not available in veterinary clinics in Ethiopia, and the ineffectiveness of the available choices, the final fate of affected horses is to be left outdoors to die [8]. This may be the reason why several debilitated horses infected with EL were abandoned and observed on the street.

In different parts of epizootic lymphangitis endemic countries of sub-Saharan Africa, the diagnosis is highly dependent on clinical signs and detection of yeast cells in pus smear [14,12,21].Similar to the present study, one researcher [3] got a significant difference (P=0.001) between clinically confirmed and healthy cases with the probability of being PCR positive for clinically confirmed, 30.309 times than that of clinically healthy cases. A good agreement (k=0.8732, SE=0.1653, P=0.0000) between clinical signs and microscopic examination was observed in this study compared to [3] which was (k=0.675) from a comparision of microscopic examination and PCR. The combination of these two diagnostic tests solidifies the tentative diagnosis. However, body condition and grade of a lesion have no significant effect according to this study, which contradicts the findings from [10,3]. This may be due to the insensitivity of direct microscopic diagnosis compared to PCR, which is highly sensitive and can detect healthy cases.

Microscopical examination of the pus indicated several free and intracellular yeast cells. The pleomorphic, one or both sides pointed, 1-5 µm in diameter yeast cells, which have achromatic unstained central cytoplasm surrounded by a chromatid substance and some blue staining basophilic substance filling out the oval or round outline are a typical Histoplasma cell which agrees with an early report from [19]. Congruently to a report by [30], some of these preparations have a hyaline, branched but less segmented and long hyphae when delayed at room temperature. In such cases, the number of typical yeast cells diminishes and starts to disappear. This was surrounded by an unstained zone of a refractile capsule [2] which [7] called it cell wall. However, this was not consistent throughout this study. From immature nodules containing a flake of blood, there was a high probability of acquiring several macrophages engraved with several oval yeast cells.

The Histoplasma yeast cells were absent on the smears from all blood samples. The same finding was recorded by [28]. However, this opposed [15], findings on human peripheral blood smear and culture for H. capsulatum var. capsulatum which showed the presence of many typical H. capsulatum human strain and colony cultures, and [21] PCR findings on horses' peripheral blood for H. capsulatum var. farciminosum, even if the latter did not check for direct smear and culture from a blood sample. So, we propose the unsuitability of peripheral blood samples for direct detection of H. capsulatum var. farciminosum as its probability of being smear and culture positive for HCF is 0.379 times less than that of pus (95% CI= 0.316-0.455, P= 0.000).

The Lactophenol blue staining as a variety of conidia with short hyaline, branching, septate hyphae, and mycelium, confirmed the growth of Histoplasma. These findings are consistent with the findings of [12,2,30,31] even if [30] failed to indicate the large round double-walled macroconidia, which were consistent in this study. Unlike the reports from [32], the tubercles on the outer walls of the macroconidia were absent in this study. Rather smooth-walled microconidia which were consistent in their study and tubercles at the tips of short arthroconidia and sometimes from sides were depicted. The eosinophilic staining, and solid, round bodies from gram staining of the colonies in this study were also reported by [4].

The growth of pasty to dry, grey-white, granular, convoluted, and wrinkled cerebriform mycelial colonies indicated typical colony characteristics of Histoplasma. Similar results from [1,30,32] confirm the present finding. The growth of this fungus was demonstrated to be very slow and only a few grew. This finding is parallel with the statement of [30], in which one specimen showed no visible growth either at 25 or 37°C, despite a long incubation period of 5 months and the demonstration of yeast cells in the clinical material before cultivation.

The minimum incubation period recorded for a colony to appear in this study was three weeks at 26°C on SGA and BHI, and more delayed and inhibited on PPLO. This finding was in agreement with [30,32] findings, even if [30] reported the superiority of PPLO over several mediums he used including SDGA and BHI without difference in incubation time. On the other hand, in primary cultures incubated on Sabouraud's agar and Hartley blood agar at 37°C, the minimum colony appearance time was 12 days and showed the superiority of Hartley blood agar over Sabouraud's agar without difference in colony appearance time [14].

The two-stage nested PCR primer sets developed by [23] for Histoplasma capsulatum var. capsulatum cultures can detect genes for H. capsulatum var. farciminosum from clinical or cultured samples [1,28]. For all, the detection of the Histoplasma gene of 514 bp from an isolate with nested PCR (Figure 7) confirmed the present findings regarding an isolate. Either primary culturing or subculture to purify this fungus was demonstrated to be very slow and less attainable in a clinical sample from a contaminated and open specimen. This may be due to the incubation periods needed for the growth of this fungus, which permits the luxurious growth of rapidly growing fungi unless selective media is used.

For the cart-horse owner, the income from providing a cart-horse service was often the sole means of supporting themselves and their family [22], in only 25% of the attendants disclosed that to had other work besides being carters in this study. EL is known to lower the daily income of households [24]. According to this study, the huge economic loss for carthorse owners was from income generation loss during the hospitalization of EL-infected horses. The other losses on the carthorse owners are feed and water costs during hospitalization and losses due to mortality. These reports are three times more than the reports from Gonder [25] which may be due to the inflation of the current market situation and the difference in the study area. The reason for huge economic loss from the absence of income generation, and feed and water costs during hospitalization was due to the chronic nature of this disease and decreased workforce as indicated by [25] emphasizing greater than one-month clinical course.

Most respondents in this study (95%) were able to describe the clinical features and precautions needed to protect their horses from epizootic lymphangitis and about 55% of the attendants knew more than three diseases of the horse including African horse sickness and colic. This was because of the proximity of this study area to the Spana project which serves and trains without charge, and the abundance of the disease. From their practice, 90% of these respondents strictly claimed the importance of hygiene with good harnessing to protect against shearing. For this, they wash an animal and sore with different herbals of claimable efficacy; which but, need further study for its confirmation of efficacy. Regardless of their knowledge of the diseases, most of the respondents claimed the use of epizootic lymphangitis-infected horses. This is in agreement with my observation during this study, in which several horses with typical EL clinical pictures were presented at a workplace. They reasoned that the condition would get worse if the horse did not work.

Conclusion

In conclusion, Epizootic lymphangitis is a well-known chronic disease and welfare issue of equines with paramount economic loss to the carthorse owners. Even if it is difficult to isolate H. capsulatum var. farciminosum due to its long incubation periods and lack of optimum conditions for its preference, it is possible to isolate and detect it. Swab and blood samples showed unsatisfactory results for isolation. Where BHI and SGA were equally important for the isolation of this fungus, PPLO agar had some inhibitory effect on the isolate. Different factors influenced the presence of yeast cells in the smear. These include the presence of clinical signs and the type of samples. On the other hand, body condition, grades of a lesion, and available treatment history did not affect the presence of yeast cells in the smear. Considering this the government and researcher should directed towards best control measure of vaccine production against this disease.

Abbreviations

- ALIPB: Aklilu Lema Institute of Pathobiology
- ASS: Agricultural Sample Survey
- BCS: Body Condition Score
- BHI: Brain Heart Infusion agar
- CI: Confidence Interval
- EDTA: Ethylene Diamine Tetra Acetic acid
- EL: Epizootic Lymphangitis
- ETB: Ethiopian Birr
- HCF: Histoplasma Capsulatum var.Farciminoosum
- ITS1: Internal Transcribed Spacer Sequence 1
- ITS2: Internal Transcribed Spacer Sequence 2
- MS: Microsoft
- NVI: National Veterinary Institute
- PCR: Polymerase Chain Reaction
- PPLO: Pleuropneumonia like Organism agar
- SE: Special Edition
- SGA: Sabaoroud Glucose Agar
- TFC: Total Feed Cost
- UV: Ultra Violet

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