ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

MVSc THESIS

Epizootic Lymphangitis (EL) in Oromia Region, Ethiopia: Isolation of *Histoplasma Capsulatum Var Farciminosum* and Other Co-Infecting bacteria from Characteristic Lesions of EL and evaluation of dovyalis abyssinicia *in vitro*

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LIST OF ABBREVIATIONS

ALIPB	Aklilu Lemma Institute of Pathobiology
BCS	Body Condition Score
BHI	Brain Heart Infusion Agar
E. *coli*	*Escherichia coli*
EH	Equine Histoplasmisis
EL	Epizootic Lymphangitis
ELISA	Enzyme Linked Immuno Sorbent Assay
EMB	Eosine Methylene Blue
HCF	*Histoplasma capsulatum variety forciminosum*
MCK	MacConKey Agar
MIC	Miminum Inhibitor Concentration
MSA	Manitol Salt Agar
NA	Nutrient Agar
NMSA	National Metreorological Service Agency
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
SDA	Sabouraud Dextrose Agar
SPANA	Society for the Protection of Animals Abroad
TSA	Tryptic Soy Agar
XLD	Xylose Lysine Deoxycholate

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ABSTRACT

A cross sectional study was conducted in eight towns in Oromia regional state, Ethiopia, from December 2019 to May 2020. The *Histoplasma capsulatum var farciminosum* and other co-infecting bacteria from characteristic lesions of Epizootic Lymphangitis were iaolated and an effect of Dovyalis *abyssinicia* on isolated mycelia *in-vitro* was evaluated. In this study 85 equines suspected with Epizootic Lymphangitis were investigated with clinical observation. In laboratory, the result of *Histoplasma capsulatum var farciminosum* was isolated (56.47%) from unrupture nodules of pus. The pus sample was also examined under giemsa staining to identify *Histoplasma capsulatum var farciminosum*. The minimum inhibition concentration of the Dovyalis *abyssinicia* plant was determined by agar dilution assay. The results showed that Dovyalis *abyssinicia* had inhibitory effect at ranges of 0.15625mg/ml minimum inhibitor concentration on mycelia. A culture media with no antifungal agent and media containing ketoconazole served as negative and positive control, respectively. Various bacteriological media (Nutrient Agar, Blood Agar, Mannitol Agar, Mackonkey Agar, Edward Agar and Eosin Methylene Blue Agar) were used to isolate contaminants bacteria associated epizootic lymphangitis lesions at Aklilu Lemma Institute of Pathobiology, Addis Ababa University. In concerning of isolation of co-infecting bacteria from three site sample (Holeta, Sebeta and Bishoftu), *Staphylococcus species (82.14%), Corynebacterium species (57.14%), Micrococcus species (50.00%), Aeromonus species (50.00%), Klebsiella species (46.42%), Streptococcus species (42.86%), Proteus species (35.71%) and E. coli species (35.71%)* were identified. The current study revealed the presence of various contaminant bacterial species which may aggravate an EL and compromise the progress of treatment. The methanol extract of Dovyalis *abyssinicia* showed strong *in vitro* growth inhibitory activity against mycelia.

Key Words: *Co-infecting bacteria, EL, HCF, Dovyalis abyssinicia*

1. INTRODUCTION

Horses provide different services to the community including ploughing transportation, racing and pack especially in developing countries (Pritchard *et al.*, 2005). However, the major works output expected from horses and the consequent benefits gained by the society are affected by equine diseases. Epizootic lymphangitis (EL) is a neglected chronic disease of horses and other Equidae caused by a dimorphic fungal agent called *Histoplasma capsulatum var farciminosum* (HCF) (Kasuga *et al.*, 1999). The common clinical characteristic of the disease, as its name indicates, is inflammation of lymphatic system of the host affecting the subcutaneous lymph nodes and the lymphatics of the neck, chest and legs (Ameni, 2007; Maxie, 2015; Radostits *et al.*, 2006). The typical cutaneous signs of disease is suppurative, ulcerating, and spreading pyogranulomatous, multifocal dermatitis and lymphangitis in horses (Hawi, 2019; Jagema and Jarso, 2016). In addition, the disease is also characterized by ulcerating conjunctivitis of the palpebral conjunctiva, or rarely as a multifocal pneumonia. Generally, in most of the cases the lesions are nodular and granulomatous (Maxie, 2015). Epizootic lymphangitis has a serious negative impact on the livelihoods of equine owners especially those using their animals for pulling carts and the disease is also affects the welfare of animals (Hawi, 2019; Nigatu and Abebaw, 2010). In terms of its global occurrence, it is most common in tropical and sub-tropical regions such as countries bordering Mediterranean areas, north and horn parts of Africa, and in regions of Asia and Russia (Al-Ani, 1999). The mode of transmission of the disease was contact with infective materials through injured skin. Various epidemiological reports showed that EL is a very common disease of equines in Ethiopia particularly in humid and sub-humid areas affecting mostly horses, on average 18.8% in this area (Ameni, 2006), but there are reports of the disease affecting mules and donkey (Ameni, 2006; Ameni and Terefe, 2004).

HCF is thermally dimorphic fungus presenting itself in mycelia and yeast forms. The mycelia form is found in soil at broader temperatures ranging from 5-30^{o}C and its yeast form grows at 37^{o}C in host (pathogenic in lesion) species (Selim *et al.*, 1985). In earlier times HCF was known as a separate fungal species called *Histoplasma farciminosum*, but molecular studies revealed that it is sub-species of *Histoplasma capsulatum* (Ueda *et al.*, 2003; Weeks *et al.*, 1985). Transmissions of a HCF occurs through direct contact with infective materials through injured skin or through cutaneous abrasions is the most common mode of infection (Selim *et al.*, 1985).

The diagnosis of EL is based on characteristic clinical signs and laboratory confirmation through demonstration of the organism in smear (Radostits *et al.*, 2006). Similar to other fungal diseases, cases of EL are difficult to treat with very poor prognosis particularly at the chronic level and treatment failure is very common. In regarding to treatment of EL intravenous sodium iodide or Amphotericin B and local treatment of skin lesions by incising nodules, draining the pus and infusing with 4% iodine tincture followed by oral potassium iodide (30g for a horse of 200-250Kg) daily for 5days and then every other day for a further 3-4 weeks (Hawi, 2019). This treatment, devised by the society for the protection of Animals aboard (SPANA) clinics, was reported to result in recovery rate of 85% (Aklilu and Zerfu, 2010). But in Ethiopia, medicinal plants are widely used in treating many infectious animal diseases including EL. An *in-vitro* study conducted by (Mesfin, 2012) indicated that fresh garlic extract has some degree of growth inhibitory effect on the mycelia form of HCF, (Negesse, 2012) has been evaluated of berries of *Phytolacca dodecandra* (Endod) and (Hawi, 2019) has been investigated of berries of *Phytolacca dodecandra* and *Curcuma longa* for growth inhibition of HCF and treatments of cases of EL in Ethiopia and that was effective. The high chance of infected wounds to harbor diverse population of microorganisms can be one of the contributing factors for the treatment failure and availability of limited research on medicinal plants with EL disease. Study focusing on identifying the diversity of the microorganism associated with EL lesions is also quite limited and most of the studies are rather focusing isolating HCF from the lesions (Mideksa *et al.*, 2017). However, presence of co-infecting

microorganism particularly secondary bacterial complications can play a great role in the clinical management of the disease in terms of treating EL disease. Therefore, this study was designed to isolate HCF from EL and some major contaminants bacteria. So objectives of the study are:-

- Isolation and identification of HCF from EL clinical cases in horses involving acute and chronic lesions and to evaluate effects of *Dovyalis abyssinicia.*
- Identification and isolation of potential co-infecting aerobic bacteria from EL lesions.

2. LITERATURE REVIEW

2.1. Etiology of EL

Epizootic lymphangitis is caused by thermally dimorphic saprobic fungal soil agent called *Histoplasma capsulatum variety farciminosum* (HCF). The organism was first demonstrated in the pus by Revolt in 1873 but the first successful isolation of the fungus was reported in 1886 in Japan (Mahendra, 2012). The mycelial form is generally present in soil and can be cultured at room temperature (26ºC) on SDA agar. While the yeast form is usually found in tissues of animal lesion or pus in nodules that grows on BHI with enriched blood at temperature 37ºC which appears as small oval buds of 2-4µm (Ameni, 2006; OIE, 2019). Macronidia are 8-15µm and are thick walled with tubercles or projections. The micronidia are 2-4µm and are smooth walled (lowa state University, 2009).

The yeast form of the organism is present in large numbers in well-established lesions, and appears as pleomorphic ovoid to globose structure, approximately 2–5µm in diameter, presented both extracellular and intracellular in macrophages and giant cells. Fungus are usually surrounded by a ‘’halo’’ when stained with Gram stain, haematoxylin and eosin, Giemsa, periodic acid–Schiff reaction or Gomori methenamine–silver stain (OIE, 2018).

HCF is highly resistant to the effects of physical and chemical agents. It may survive for up to ten weeks in non-sterile water at 26°C (Gabal *et al.*, 1983; Gabal & Hennager, 1983; Soliman *et al.*, 1986). The fungus persists for months in moist and warm conditions. HCF is susceptible to 1% solutions of sodium hypochlorite, 20% phenol, 2% glutaraldehyde, isopropyl alcohol and formaldehyde. Its spores and yeast cells are inactivated when held at temperatures above 40 ºC for extended periods of time.

Growth is inhibited at PH below 5 and above 10. Dry conditions also facilitate the inactivation of the spores. *Histoplasma capsulatum* is present primarily in nitrogen rich soils with a PH ranging 5-10 and moist soils at temperatures between 18 to 37 ºC can support the growth of the fungus. Organisms are known to survive excess of 10 years in soil (lowa state University, 2009).

2.2. Pathogenesis

HCF enter the host body through wounds or skin trauma (particularly under the harness) and, invades subcutaneous tissue, sets up a local granuloma or ulcer and disseminates through the lymphatics to regional lymph nodes or in severe cases, to other organs and form nodules. The spread of infection along the lymphatic vessels causes chronic inflammation and thickening of the vessel walls. Nodular lesions develop in the skin along the lymphatics and in the lymph nodes. These lesions eventually ulcerate and drain a thick, mucopurulent material containing yeast cells (Hawi, 2019). Horses that have a heavy systemic burden of fungi may succumb to pneumonia or failure of other affected organs. The ocular form of the disease results from inoculation of the organism into the eye, likely by biting flies (Radostits *et al.*, 2006). Both conjunctivitis and rhinitis may occur as the extension of the skin form, because the animals will scratch the skin lesions by their teeth and lips, there by spreading it to the surrounding organs (Ameni, 2007; Radostits *et al.*, 2006).

At HCF is entering in the host cell, it exposed to defense system (phagocyte) which is engulfed by phagocytosis actions. In contrast to host cells, *Histoplasma capsulatum var. farciminosum* opposing the action of phagocytosis (mainly attack the macrophage, leukocyte and dendrite) (Mittal *et al.*, 2018). This killing condition of HCF in the host cell may occur in both extracellular and intracellular (Sil and Andrianopoulos, 2015). *HCF* is almost exclusively found as an intracellular pathogen. The species was so named by Samuel Darling because he observed it within phagocytic cells: ‘histo-’ because the

microbe was located within histiocytes (a general term for tissue phagocytes), and 'plasma' because he believed the microbe was a parasite-like creature. To secure intra-cellular residence, *H. capsulatum* must first overcome extracellular obstacles to infection (De Sanchez and Carbonell, 1975; Holbrook *et al.*, 2011).

2.3. Epidemiology

2.3.1. Host range

Histoplasma capsulatum is found in humans and animals, including horses, cattle, sheep, dogs, cats, chickens and rats (lowa state University, 2009). The disease is more common in the tropics and subtropics and is endemic in north, east and north-east Africa, and some parts of Asia, including some countries bordering the Mediterranean Sea, India, Pakistan and Japan (Ajello, 1968; Al-Ani, 1999). Exposure to dust or soil for prolonged period of time as well as activities which disturb bird and bat guano increase the risk of infection and have also been considered indicators of localized out breaks (Benedict and Mody, 2016). Clinically EL affects mainly horses, mules and donkeys (Scantlebury *et al.*, 2016). The disease is common in Ethiopia, especially in cart horses and was reported to affect an average of 18.8% of horses in warm, humid areas between 1500 and 2300 meters above sea level (Ameni, 2006). An incubation period of the disease is from 3 weeks to 12 months (Ajello, 1968). It causes considerable debility but low mortality that doesn't usually exceed 10% to 15% (Jubb *et al.*, 2006). The major loss results from the inability of animals to work for several weeks because of extremely painful lesions. There are no breeds, sex and age differences in EL indicating that all group of horses are affected equally (Ameni, 2006).

2.3.2. Transmission and risk factors

Even if the exact route of entry of HCF is not known (Endebu and Ronger, 2003), the different route of transmission are suggested according to clinical signs and sites of entry as cutaneous, ocular, respiratory and asymptomatic form (Singh, 1965; Pal, 2007). Hence equines those are prone to lesion become directly or indirectly the destination of HCF through traumatized skin (Hawi, 2019). The wounds caused by harness are reported as major predisposing factors of EL in carthorses in Ethiopia (Asfaw *et al.*, 2012). The skin form of the disease occurs when traumatized skin is contaminated with infected soil (Timony *et al.*, 1988; Aiello and Mays, 1998; Gilbert, 1998) or fungal spores of infected skin, pus, nasal, ocular excretion or contaminated harness, whip, grooming equipment, bedding, feeding, watering utensil and as well as biting flies and ticks transmit the agent mechanically (Pal, 2007; Al-Ani, 1999). The conjunctival form of the disease is believed to be due to the biting flies especially of musca and stomoxys species that may indicate organism is isolated from alimentary tract of biting flies (Brilhante *et al.*, 2016). In the endemic areas in certain regions of the world, the occurrence of seasonal dusty winds exposes horses to the inhalation of dust and spores, leading to pneumonia (Jubb *et al.*, 2006; OIE, 2018). Spread of infection can also occur by indirect contact through contaminated objects such as grooming tools, feeding and watering utensils, and harnesses and through wound dressings (Jubb *et al.*, 2006). Wounds from harnessing materials, sharing of harnessing and other materials among cart horse owners, tick bite and confinement of cart horses at place of work are found to be a major predisposing factor (Asfaw *et al.*, 2012). Sexual transmission was also reported (Al-Ani, 1999). The possibility of experimental infection of horses was reported by Ameni in which the incubation period is much longer in horse inoculated with mycelial organisms than that of with the yeast form (Ameni, 2006).

2.3.3. Morbidity and Mortality

Morbidity is high when large numbers of animals live together such as in military stations and racing area where as mortality is generally low and can be up to 10-15 % (Pal, 2012).

2.3.4. Geographic Distribution

From historic reports, EL is reportedly common in the tropics and subtropics being endemic in north, east and north-east Africa, and some parts of Asia, including some countries bordering the Mediterranean Sea, India, Pakistan and Japan (Ameni, 2006; AL-Ani, 1999). The disease is common in Ethiopia, particularly in cart horses in warm and humid areas between 1500 and 2300 meters above sea level that favor condition for mycelia from of HCF, but was nil or low in cold an in dry and windy towns (Ameni, 2006; Ameni & Siyoum, 2002).

2.3.5. Prevalence

The disease causes a major economic loss especially in carthorses mainly in endemic areas in Ethiopia. The highest prevalence (39%) was recorded at Mojo while the lowest (0.0%) was recorded in five towns:- namely, Agaro, Bokoji, Debre Berhan, Dinsho and Sagure (Ameni & Siyoum, 2002). Another study conducted in various part Ethiopian in 28 towns, the average prevalence was reported to be 18.8% (Ameni, 2006). In similar studies, the prevalence in 390 cart horses was found to be 24.9% (97/390) in Woliso (Rahel *et al.,* 2012). Besides horses, the prevalence of EL has been reported to be 32.84% (132/402) in cart mules (Demeke *et al.,* 2018).

Table 1: Prevalence of EL in horses and mules in some parts of Ethiopia

Location	Number of examined	Prevalence (%)	Species	Source
East and northeast part of Ethiopia (28 towns)	1982	18.8	Horses	Ameni (2006)
Western (Bako and Ejaji)	309	21.0	Mules	Ameni and Terefe (2004)
Central Ethiopia (Bishoftu and Akaki)	1100	10.4	Horses	Endebu and Roger (2003)
Central Ethiopia (Bishoftu, Modjo, Nazareth)	2907	26.2	Horses	Ameni and Seyoum (2002)

2.4. Diagnosis

The clinical signs of EL in horses in endemic regions are often the basis of diagnosis. Several confirmatory tests have been described. The peculiar confirmatory tests of EL especially in endemic region are microscopic examination of yeast in lab by giemsa and gram stain, serological tests, skin hypersensitivity test and molecular techniques (Ameni, 2006 and Hawi, 2019).

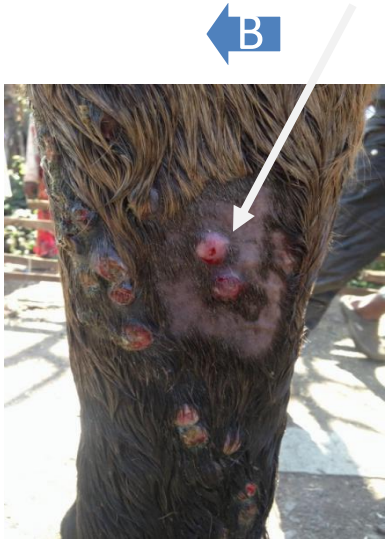
2.4.1. Clinical examination of HCF

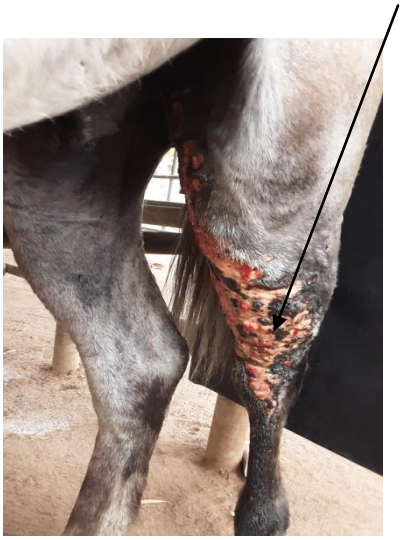
The major clinical signs of EL in horses in endemic regions are spreading, suppurative, ulcerating pyogranulomatous lesion particularly in the neck, legs and chest which can be used as an indication for cutaneous form of the disease. Ulcerating conjunctivitis is the common sign for ocular form and purulent nasal discharge, pyogranulomatous lesions around the nares supported with the above-mentioned signs can be considered indicators of pneumonic form of the disease (Hawi, 2019). The lesion is most commonly seen on the skin, subcutaneous tissue, lymph vessel and nodes. It is characterized by thinking with freely movable pyogranulomatous nodules of lymph node and open granulomatous wounds the course lymphatic vessels (Ajello, 1968; Al-Ani, 1998; Gilbert., 1998). Clinical diagnosis of EL is however, limited to detection of clinical cases and it is not

suitable for detection of early cases and carrier animals. Hence clinical diagnosis is not suitable for screening and isolation of positive animals so as to prevent spread of the fungus.

Ocular lesions from Ginir town

Cutaneous nodule on front leg from Bishoftu





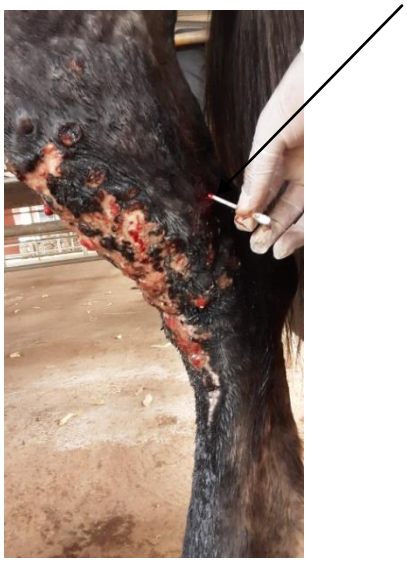
C

D

Rupture nodules on front leg from Holeta

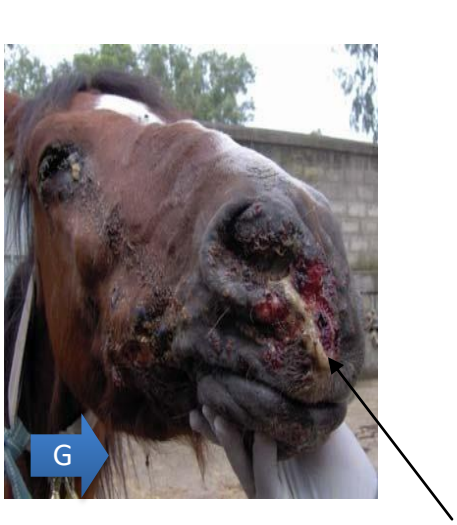
E

Swab sample from hind leg from Sebeta



F

Source: Ginir, Holeta, Sebeta, Asella and Bishoftu towns, Oromia.



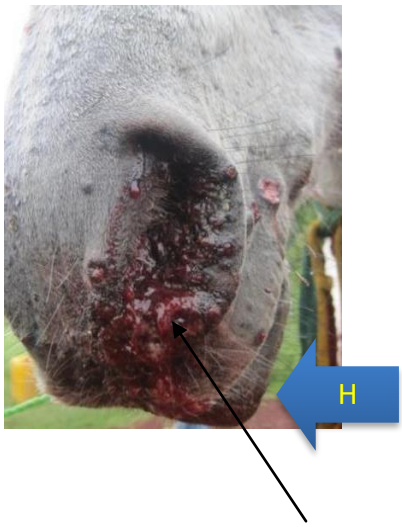


Figure 1: Typical lesions of Equine Histoplasmosis B, C, D, E and F = Cutaneous form, G and H = Respiratory form and A = Occular form (Source SPANA, 2006; Ethiopia, Field examination).

2.4.2. Direct smear examination and culture techniques of HCF

Diagnosis is usually based upon demonstration of the typical yeast-like, double-contoured cells in pus collected aseptically from the lesion and confirmed by culturing the pathogen. When stained using Gram's staining HCF appear gram-positive, ovoid, pleomorphic ranging from 2–5 µm in diameter which can occur singly or in groups (Hawi, 2019). It is observed in smears prepared from purulent materials. The typical morphological appearance of the yeast form of HCF can be visualized using Giemsa or Periodic Acid–Schiff staining. For morphological identification of HCF sterile needle aspiration (FNA) pus collected form un-ruptured nodules and superficial lymph nodes are used. Smears prepared from these samples can be stained with modified Giemsa (MayGrunwald-Giemsa, Merck KGaA, Darmstandt, Germany) and examined microscopically (Rahmeto *et al.,* 2017).

Infection with HCF can also be revealed by the presence of multinucleated Langhans giant cells in histological sections stained with haematoxylin and eosin (basophilic mass surrounded by a halo), Periodic acid–Schiff stain and Gomori methenamine silver stain. The appearance of the lesion is quite characteristic and consists of pyogranulomatous inflammation with fibroplasia (Ameni, 2006; OIE, 2018). Electron microscopy has also been used for examination of skin biopsy samples immediately prefixed in phosphate buffered 2% glutaraldehyde solution at 4°C and post-fixed in 1% osmium tetroxide. Uranyl acetate and lead citrate stained sections has been used to study the fine internal structures of the organism, including the cell envelope, plasma membrane, cell wall, capsule and inner cell structures (Al-Ani, 1999).

2.4.3. Isolation of HCF

Culture of HCF from body fluids or tissues is considered the 'gold standard' for confirmation of the diagnosis of EL but it is difficult and time-consuming to grow from

body fluids and tissues. HCF has been cultured and isolated using Sabourauds Dextrose Agar (SDA) with 2.5% glycerol (Ameni *et al.*, 2006). But it dies quickly in samples, unless these are collected in antibiotic solutions and refrigerated and cultured promptly (Asfaw *et al.*, 2012). HCF can also be isolated on other fungal media including mycobiotic, BHI agar with 10% horse blood, and pleuropneumonia like organism (PPLO) media with 2% dextrose and 2.5% glycerol (pH 7.8). The HCF organisms grow slowly and colonies develop in approximately 2 to 8 weeks at 26°C. The colonies are dry, granular, wrinkled and grayish-white, becoming brown as they age (lowa state University, 2009). The typical colony of HCF is found in figure 2

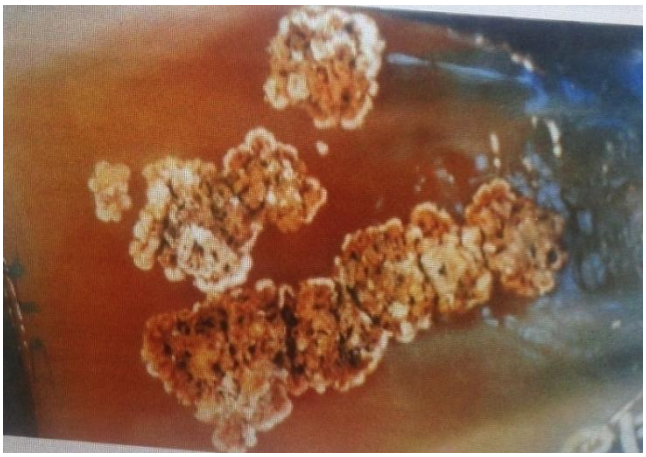


Figure 2. Colony morphology of HCF, grown on mycobiotic agar after 42 days of incubation at 26°C (Al-An, 1999).

2.4.4. DNA Extraction

The SDS-CTAB-chloroform-isoamyl alcohol (modified method) was employed for the extraction of DNA (Umesha *et al.*, 2016). To briefly describe: 200mg of mycelia powder was taken and transferred to 2 ml Eppendorf tube. 500 µl of SDS CTAB chloroform

isoamyl alcohol (S-CCI) extraction buffer (250-mM Tris–HCl (pH 8.0), 20mMEDTA (pH8.0), 200MNaCl, 10% CTAB, 0.15 % SDS) was added and vortexes and boiled for 10 min at 50 °C then centrifuged at 10,000 rpm for 10 min. After the centrifugation the upper liquid part were aspirated out carefully and one volume of chloroform: isoamyl alcohol (23:2) was added then mixed for 1 min and centrifuged at 10,000 rpm for 5 min. The aqueous phase was recovered and mixed with one volume of ice-cold isopropanol, and the tubes were turned upside down for 1min to precipitate the DNA. The tubes were again centrifuged at10, 000 rpm for 2 min to recover the pellet and washed with 500µl of absolute ethanol and then centrifuged at 10, 000 rpm for 1min. DNA was air dried and resuspended in 200 µl deionized or TE buffer for further use.

2.4.4.1. Polymerase Chain Reaction and Gel-Electrophoresis

A pair of primers (ITS1 and ITS4) supplied from Bioneer Corporation (8-11 Munpyeongseon-ro, Daedeok-gu Daejeon, 34302, Republic of Korea) were used for PCR reaction. The forward primer was ITS1 with sequence of TCC GTA GGT GAA CCT GCG G while the reverse primer was ITS4 with a sequence of TCC TCC GCT TAT TGA TAT GC (White *et al.,* 1990). Each reaction mixture contained 50ng/µl of template DNA and 10-pmol concentrations of PCR primers ITS1 and ITS4 added to BioMix red (Bioline Reagents Limited, UK) in a 25µl reaction volume, as follows: 12µl Bio-Mix red, 2µl forward primer and 2µl reverse primer, 8µl H2O, and 1µlDNA template. The PCR was performed in the thermal cycler (Eppendorf Mastercycler) programmed for the first denaturation at 95^{o}C for 5 min followed by 35 cycles of the succeeding steps denaturation at 95^{o}C for 45 seconds, annealing at 58^{o}C for 1 min., and extension at 72^{o}C for 1 min and a final extension period of 72^{o}C of 7 min. The PCR products were electrophoresis

through 1.8% Agarose (Sigma Chemical Co., St. Louis, Mo.) dissolved in Tris-borate-EDTA buffer(0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA [pH 8.4]). Electrophoresis was conducted at 100 V for 45 min, with 5 in l of each PCR amplicon and loading dye in each well. The bands were visualized with a UV trans-illuminator after gel red staining.

2.4.5. Serological tests

Serological tests such as direct and indirect fluorescent antibody tests, Enzyme Linked Immunosorbent Assay (ELISA) and Passive Heamaglutination tests have been used as diagnostic tools for detection of antibodies in animals infected with HCF (Al-Ani, 1999; OIE, 2018). However, these tests may not have a high degree of specificity for active disease finding and may reflect past exposure and asymptomatic infection (Hawi, 2019). Antibodies usually develop at or just after the onset of clinical signs. Tube agglutination and passive hemagglutination tests have been reported to identify increased titters in horses with EL, which can be used as a practical screening test. Indirect fluorescent antibody technique has been used as an aid to the diagnosis of infections by HCF in horses, using yeast phase cells of the organism as antigen. An enzyme linked immune-sorbent assay was also evaluated for the detection of antibody in sera of equine naturally infected with HCF. In that study ten sera from infected horses were examined and all were proved positive (Gabal and Mohammed, 1985).

2.4.5. Skin hypersensitivity test

Skin test has been applied for the detection of cell-mediated immunity (OIE, 2018) manifested as skin thickness. Histofarcin test could play significant role in detecting early infection and in the differential diagnosis of EL. This technique is suitable for routine diagnosis of large number of animals screening. This consist of interadermal injection of

0.1ml of soluble antigen prepared from the mycelia form of HCF an increase the thickness of skin more than 4mm within 24 to 48 hr after injecting 0.2mg/ml of antigen can be regarded as positive result (Ameni *et al.*, 2006 and Hawi, 2019).

2.4.6. Molecular diagnosis

The nested PCR were used as a diagnostic test for HCF cultures of clinical samples from infected horses. Two-stage nested amplification was done to manage the clinical diversity within the clinical samples. The diagnostic reliability of the nested PCR protocol was much superior to that of conventional microscopy or the sole reliance on clinical signs. In all cases, sequencing of the 514-bp amplicons has been shown to demonstrate a presence of HCF DNA, and this was further confirmed by sequencing of a large sample of clones (Scantlebury *et al.*, 2016).

Table 2. Comparative performance of diagnostic methods for diagnosis of EL (OIE, 2018).

Purpose						
Method	Freedom from	Movement control	For eradication	Clinical sign	Surveillance	Immune status
Antigen identification						
Direct smear	-	-	-	+	++	-
Culture	-	-	++	+++	+++	-
PCR	++	++	++	++	++	-
Detection of immune response						
FAT	n/a	n/a	++		++	-
Indirect ELISA	n/a	n/a	++	++	++	-
Passive HA	n/a	n/a	++	++	++	-
Histofarcin test	n/a	-	++	++	n/a	++

Key: +++= recommended method, validated for the purpose shown; ++=suitable method but may need further validation; += may be used in some situation, but cost, reliability, or other factors severely limits its application; - =not appropriate for this purpose; n/a= purpose not applicable. PCR=polymerase chain reaction; FAT=Fluorescent antibody test, ELISA=Enzymelinked immunosorbent assay; HA=haemagglutination.

2.5. Differential diagnosis

EL is clinically confusing with other disease but the final diagnosis is by laboratory confirmation. The diseases which could be listed as a differential diagnosis include ulcerative lymphangitis caused by *Corynebacteria Pseudotubeclosis*, indolent ulcer caused by *Rhodococcus equi*, strangles caused by *S. equi*, Sporotricosis caused by *S. schaki*, Histoplasmosis caused by *Histoplasma capsulatum var. capsulatum*, crytococcosis, sarciod and cutaneous form of glanders also called farcy, which is caused by Burkholderia mallie (Al-Ani, 1999; Gilbert, 1998; OIE, 2018; Ruberson, 2007).

2.6. Treatment of HCF

There is no complete chemotherapy for EL. The recurrence of clinical signs has been reported in treated patients. The early case of the disease can be managed by surgical excision of the nodules on the affected parts and dressing of the wound with 4% tincture of iodine. The infected animals can be treated with potassium iodide orally 0.096gm/Kg body weight and sodium iodide by intravenous injection of 100ml repeated weekly for four weeks (Al-Ani, 1999; SPANA, 2006; Mahendra, 2012). As a drug of choice the infected horses were treated with an intra venous injection of Amphotericin B at a dose of 0.2mg/kg body weight three times on alternate days and treating the lesion with 4% iodine solution for several days (Al-Ani, 1999). Ketoconazole can also be given orally at a daily dose of 10mg/kg (Plumb, 1999). But the outcome depends on the stage of disease, dosage rate and length of treatment (Getechew and Hadush, 2007).

As one studies investigated on clinical response of EL by Birhanu *et al.* (2007) Iodides, *Phytolacca dodecandra* and Pen-strip for their therapeutic value on cases of EL, a combination of sodium Iodide and Pen-strip had significantly higher therapeutic value as compared to other remedies (Table 3). The comparison on treatment response between the early stage and advanced stage of the disease showed that treatment during early stage provided better response (Birhanu *et al.*, 2007).

Table 3. Clinical response of EL to treatment options (Birhanu *et al.*, 2007).

	Early		**Advanced stage**		**Total**
Treatment group	Number cured	(%)	Number cured	(%)	Number (%) cured
Endod +penstrip	3 (60)		3 (60)		6 (60)
Penstrip	2 (40)		0 (0)		2 (20)
Sodium iodide + penstrip	5 (100)		3 (60)		8 (80)
Potassium iodide	3 (60)		1 (20)		4 (40)
Endod	3 (60)		3 (60)		6 (60)
Potassium iodide + penstrip	3 (60)		2 (40)		5 (50)
Control	0 (0)		0 (0)		0 (0)

2.7. Bacterial contaminant isolated from EL Lesion

Bacterial contaminant present everywhere may infect and create complication with equine EL lesion. This disease is rarely responding to treatment a common antifungal and antibacteria. One of the reason in addition to the chronic nature of the disease and could also be due to the complication of the lesion with different bacteria (Hadush *et al.*, 2007; Hadush, 2004). The bacterial pathogenesis leading to leukocyte recruitment and lymphocytosis requiring an efficient anti biotherapy coupled to antifungal drugs. Bacterial infection of wound generally clinically comprises two identifiable elements which include regional inflammation that is excessive for the type, size or age of wound and purulent exudates from or within the wound (James, 2014). Hence in order to provide effective treatment the identification of bacteria invaders from the lesion and then

determination of their drug susceptibility pattern are of paramount importance. One report indicates that study was conducted and isolated bacteria invaders contaminating the lesion of EL and also drug sensitivity test on isolated bacteria. As the study conducted around Mekelle, sample collection done from 24 cart horses and at different anatomical sites of infection from different type of lesion closed, open, and mild sever lesions in sterile conditions (Hadush *et al.,* 2007).

The dominant bacteria isolates from confirmed cases of EL in closed and open lesions of equines observed in 24 cart horses at Mekele town. The bacteria genera (*Bacillus, Aeromonus, Klebsiella* for bacilli group and *Staphylococcus, Micrococcus*) where isolated from different stages and forms of EL lesions. Broadly the majority of the bacteria isolated from closed EL lesions were bacilli where as in open lesions cocci, mainly *Staphylococcus* species were predominant but bacilli species were also frequently identified in open lesions. An *Aeromonus* predominantly isolated from the closed lesion and frequency seen in open lesions of horse histoplasmosis, so it will require attention during treatment of the disease (Hadush *et al.,* 2014). Other study was also confirmed that the swelling is due to a restriction of lymphatic flow because of bacterial complication like *Staphylococcus, Streptococcus, Pasturella, Pseudobacterium, Fusobacteria, Actino bacillus and Nocardia* (Mideksa *et al.,* 2017).

Another study reported that the isolation of Streptococcus and Corynebacterium as well as pseudomonas are also observed (Hadush, 2004; Ameni and Fontaine, 2003; Amen and Tilahun, 2003). This organism are important wound pathogen and pus forming organisms of domestic animals including horse, pigs, sheep's and cows incase of polluted environment they are invade and pathogen of soft tissue infection (Janda and Abbott, 2010). They are infecting skin and form skin nodules as well as lesion (Young and Barr, 1981; Mideksa *et al.,* 2017). Klebsiella is also gram negative bacterium that colonizes and cause for wound infection and ulcerogranulomatous and characterized by beefy red ulcer and create necrosis. Proteus is another gram negative bacterium widely distributed in nature. They have clinical significance in creating of wounds and urinary tract

infection. They also cause abscessiation and rarely exist in animal tissue (Rauprich *et al.*, 1999). Coryneabacteria a gram positive polmorphic bacteria that cause for ulcerative lymphangitis in horse. The bacteria enter to the horse through abrasion or wounds on the skin and transmitted to other via flies. These bacteria affect lymphatic vessels of hind limbs that create formation of nodule and abscess with greenish discharge (Pascoe and Knottebelt, 1999; Mideksa *et al.*, 2017). Some bacteria such as Staphylococcus, Bacillus and Aeromonas that are frequently found in the EH lesions can potentially contribute to the pathogenesis and to the severity of the disease leading sometimes to death in severely infected horses. In other study, the abscess is the hallmark of Staphylococcal infection, which consists of a fibrin wall surrounded by inflamed tissues enclosing a central core of pus containing organisms and leukocytes. From this focus of infection, the organisms may be disseminated hematogenously, even from the smallest abscess (Loir *et al.*, 2003; Smith, 2007). As study indicated in a total 340 bacterial isolates were identified from all the equine wound and skin samples. Pseudomonas *aeruginosa* and Enterococcus *faecium* were the most predominantly isolated bacterial species from equine wound and skin samples respectively (Mideksa *et al.*, 2017).

Staphylococcus was the most commonly isolated genus in both rupture and rupture EL lesion. Bacteria cultured from chronic and acute wounds showed significantly higher biofilm forming potential than bacteria isolated from skin. This paper highlights preliminary evidence supporting the presence of biofilms and a high microbial diversity in equine chronic wounds. The presence of biofilms in equine wounds partly explains the reluctance of many lower limb wounds to heal. Non-healing limb wounds in horses are a well documented welfare and economic concern (Mideksa *et al.*, 2017). Another study indicated that 90% of horse wounds are colonized by Staphylococcus species, which it significant wound pathogen in animals as well as humans. *S. aureus* and other species are heavily involved to difficulty in wound infection healing in equines (Theoretic *et al.*, 1999; Carnwath *et al.*, 2014; Tobiasc *et al.*, 2016). Wound management of equine patient can be very difficult due to bacterial infection (James, 2014; Mideksa *et al.*, 2017).

Another study reported that the presence of gram negative bacteria in wounds is aggravated the severity of infection besides other criteria and cause for the delaying of wound healing. So severely infected wound would not heal easily because defective immune function that leads the bacterial to proliferate and contaminant Bacteria infect the wounds via exudates or by environmental contaminants. The identified wound pathogen were found in study before, *Staphylococcus spp, Streptococcus spp, Actiobacter, E.coli, Proteus Vugaris and Pseudomona aeroginosa* (Nagoba *et al.,* 2013; Mediksa *et al.*, 2017).

Other study also indicated that the most implicated bacteria in lymphangitis and cellulitis in horse are coagulase positive and negative *Staphylococcus spp, Streptococcus spp* gram negative aerobic organism and aerobic bacteria are involved (Mideksa *et al.*, 2017). Bacillus strains were susceptible to all antibiotics except that sensitivity to Streptomycin is moderate. According to Hadush (2004) report, the bacillus species were susceptible to aminoglycosides, particularly gentamycine. Aeromonas where susceptible to gentamycin and amoxicillin but resistant to tetracycline. Micrococcus was highly susceptible to all antibiotics except tetracycline. As other study indicated that the yeast form and mycelia form of *Histoplasma capsulatum* treated by the combination of chloramphenicol was more effective against the yeast phase of *H. capsulatum* than the mycelial phase. In contrast, clotrimazole was more effective against the mycelial phase (Gabal and Mohammed, 1985; Mideksa *et al.*, 2017).

3. MATERIALS AND METHODS

3.1. Description of Study area

The study was conducted in Ambo, Holeta, Bishoftu, Sebeta, Asella, Bale Robe and Ginir towns of Oromia regional state, Ethiopia. Ambo, the town and district is located in the west part of the central highland of Ethiopia, in the West Shewa Zone of the Oromia Region state, west of Addis Ababa, 117.7 km from Addis Ababa. This town has a latitude and longitude of 8°59′N 37°51′E and an elevation of 2101 meters. The climate in Ambo is warm and temperate. In winter, there is much less rainfall than in summer. The average annual temperature is 18.0°C in Ambo. The rainfall here averages 1012 mm with relative humidity of 56% (21^{O}C) (Prabu *et al.*, 2011). Holeta is located in Oromia Regional State, Oromia Special Zone Surrounding Finfinne and central Ethiopia. Holeta town has a latitude and longitude of 9°3′N 38°30′E/ 9.050°N 38.500°E/ 9.050; 38.500 and an altitude of 2391 meters above sea level. It experiences a bimodal pattern of rainfall with the long rainy season extending from June to September and a short rainy season during March and April (Shiferaw *et al.*, 2003). However, there may be rains in any months of the year from small amount of clouds, letting additional moisture for the area. The least amount of rain fall occurs in November and the average rain fall is 1134 mm. The mean annual temperature of the area is about 14.3°C with a maximum of 24.5°C recorded from January to May and minimum of 1.6°C which is recorded during December (National Meteorological Services Agency). As report indicated, the district has an estimated number of 14,000 donkeys, 1,400 horses and 700 mules (Chaburte *et al.*, 2019). Bishoftu is located in Adea district, Oromia regional state, Ethiopia. It is located at 45 km away from the capital city, Addis Ababa. Bishoftu town is found at 1900 meter above sea level and receiving an annual rainfall of 1115mm with two rainy season: March to May (short rainy season) June to September (main rainy season). The annual average maximum and minimum temperature are 10ºC and 8.5ºC respectively with relative humidity of 61.3% (NMSA, 2007). Sebeta is town located in the Oromia special zone surrounding Finfinne of the Oromia region state, Ethiopia. It is located at 22.9 km away from the capital city,

Addis Ababa this city has a latitude and longitude of $8^0$54'40''N $38^0$37'17''E/ 8.951111^{0}N 38.62139^{0}E. Sebeta town is found at 2356 meter above sea level and receiving an annual rainfall of 1073mm with two rainy season: March to May (short rainy season) June to September (main rainy season). The annual average maximum and minimum temperature are 25ºC and 5ºC respectively (NMSA, 2007). Asella is located in Arsi zone, Oromia regional state, Ethiopia. It is located at 159 km away from the capital city, Addis Ababa; this city has a latitude and longitude of $7^0$57'N $39^0$7'E/ 7.950^{0}N 39.117^{0}E. Asella town is found at 2430 meter above sea level and receiving an annual rainfall of 1147mm with two rainy season: March to May (short rainy season) June to September (main rainy season). The annual average temperature is 15.1ºC (NMSA, 2007). Robe is located in Bale zone, Oromia regional state, Ethiopia. It is located at 402.7 km away from the capital city, Addis Ababa; this city has a latitude and longitude of $9^0$36'N $39^0$08'E/ 9.600^{0}N 39.133^{0}E. Robe town is found at 2435 meter above sea level and receiving an annual rainfall of 941 mm with two rainy season: March to May (short rainy season) June to September (main rainy season). The annual average temperature is 17.5ºC (NMSA, 2007). Ginir is also located in Bale zone, Oromia regional state, Ethiopia. It is located at 502.1 km away from the capital city, Addis Ababa; this city has a latitude and longitude of $7^0$08'N $40^0$42'E/ 7.1333^{0}N 40.700^{0}E. Ginir town is found at 1986 meter above sea level and receiving an annual rainfall of 1147mm with two rainy season: March to May (short rainy season) June to September (main rainy season). The annual average temperature is 15.1ºC (NMSA, 2007).

3.2. Study animals

The study animals considered in this study were horses located in the study areas that naturally infected with HCF. Clinically infected horses were identified and included in this study at each study sites. Horses with detectable lesions on inspection and palpation were considered infected during the course of clinical examination. The total animals sampled are eighty five equines.

3.3. Study design and sampling method

A cross-sectional study design method was used to identify horses showing typical clinical signs of EL from December 2019 to March 2020 through field surveys to identify clinical cases in association with cart horse owners. The veterinary clinic of the study areas were used as sample collection sites. The cases have been identified through systematic clinical examination of horses. During sampling clinical examination and interviews of the owners, relevant related information were recorded including: the site, clinic name, owner's name and address, animal identification, observed clinical signs, body parts involved, status of animals, number of topical nodules or ulcers of the selected horses.

3.4. HCF Isolation

The H*istoplasma capsulatum var. farciminosum* isolate was obtained from Aklilu Lemma Institute of Pathobiology, Addis Ababa University. The isolate was then sub cultured into SDA agar slants (2.5% glycerol and 0.005% chloramphenicol) and incubated for 21 day at 26^0C.

3.5. Sample collection, processing and laboratory examination

The sample processing and laboratory examination was conducted at Aklilu Lemma Institute of Pathobiology, Addis Ababa University. The cross-sectional study was used for an isolation of HCF and potential aerobic co-infecting bacteria and an experimental study design method was used for *Dovyalis abyssinicia* was used. The HCF isolates used in this study were obtained from acute and chronic EL cases in cart horses and cart donkeys in different towns in Oromia, Ethiopia. Eight towns (Ambo, Holeta, Bishoftu, Asella, Robe, Gasera, Sebeta and Ginir) were purposively selected based on the number

of cart horses found and prevalence of EL reported from previous studies (Ameni, 2006). Pus samples were collected from un-ruptured palpable nodules using sterile syringe and needle after the nodules were washed with soap and water, shaved and disinfected with alcohol for HCF isolation and swab sample were collected without disinfected to isolated co-infected contaminant bacteria. The aspirated content of the nodules were used for the preparation of smears for microscopic examination and cultured on different fungal media (Getachew *et al.*, 2007). Media were prepared according to the rules prescribed on the bottle container. Media were weighed, dissolved using heat and shakers, autoclaved at 121ºC for sterilizing and cool in water bath at 50°C and dispensed in to sterilized universal bottle and wait to cool and for BHI agar, enriched with 7% defibrinated horse blood as well as Chloramphenicol (0.5g/L) for SDA agar preparation. Once the medium were prepared and ready to use aseptically collected pus sample was inoculated under laminar air flow on different plates tubes to avoid contaminants on sterile media and incubated in incubator at 37ºC in inverted position for yeast growth and at room temperature for mycelial growth. The aspirated pus samples were also cultured on two Sabeourads Dextrose Agar (SDA) impregnated with Fortified Chloramphenicol to identify the mycelia form incubated at room temperature and yeast at 37°C. The inoculated media are kept at 26°C for 3-8 weeks as it has slow growth in case of fungal mycelial agent by Carter, (1991), Getechew *et al.*, (2007) and Asfaw *et al.* (2012). Mycelial form of HCF was isolated after incubation at 26°C for about 8 weeks. The growth of dry, grey-white, granular wrinkled colonies on the agar suggested the growth of the mycelial form of HCF. The typical colonies were harvested, stained with Gram stain for confirmation of morphological features of HCF.

Also the same collected samples were hygienically dispensed with four quadrant strike techniques on nutrient agar media for less fastidious bacteria growth and on blood agar (Oxoid, U) for fastidious bacteria isolate and to check heamolysis. Macconkey agar (Oxoid UK), was used to isolate the gram negative bacteria and the isolates were further sub cultured on Eosin Methylene blue (EMB) and Xylose Lysine Deoxycholate (XLD) agar. All agar plates were incubated aerobically at 37°C for 24 to 48 hours to identified

bacteria colonies. *Staphylococcus* and *Aeromas* were isolated from Manitol salt agar (Oxoid UK), *Streptococcus* isolated from Blood agar and sub cultured on Eduward media (Oxoid UK), *E. coli* isolated from Eosin Methyl Blue (Oxoid UK), *Klebsiella* and *Proteus* isolated from XLD agar. An isolation and identification of aerobic bacterial contaminants was done by gram stain, colony characteristics and use of selective media.

3.6. Collection of medicinal plant

Both fresh leaf and root of *Dovyalis abyssinica* was collected from Bishoftu, Mojo and Dera towns of Oromia regional state, Ethiopia. The sample was cleaned, chopped to small piece and air dried at Aklilu lemma institute of Pathobiology laboratory to avoid direct sunlight that could degrade some of the compounds in the sample. The part of collected plant were then spread out and regularly turned over to avoid fermenting and rotting for four weeks till they dried. The dried sample was ground to fine powder using electrical grinder. The powder was then weighed, packed and labeled in sample bags and stored at room temperature. Powder was preserved in zip lock bags at room temperature.

3.7. Preparation of Plant Extract

The dried, ground sample was obtained by direct dipping of dry, powdered plant material at 1:10 ratio in 80% methanol for overnight on an orbital shaker. The extract was first filtered by gauze on a sterile beaker to prevent plant body and pure filtration was done using Whatman filter paper (Number 1, diameter 6mm, Whatman ltd, England). The extraction techniques were repeated three times for exhaustive extraction. Methanol was then evaporated from the filtrate by using vacuum rotaryvaporator at 50°C and the extract allowed remaining in a micro oven at 40°C for one week for complete drying. The dried stock powder was kept in deep freeze.

Three serial dilutions of the extract were prepared using Dimethylsulphoxide (DMSO) for Dovyalis *abyssinica* and final concentrations of 10mg/mL, 5mg/mL, 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.3125mg/mL and 0.15627mg/mL were obtained that mixed each with 9mL SDA agar media. As a standard of treatment (positive control), dilution of ketoconazole was done at the concentrations of 0.8µg/mL, 0.4µg/mL, 0.2 µg/mL, 0.1µg/mL, 0.05µg/mL, 0.025µg/mL and 0.0125µg/mL. Cultures of HCF with no antifungal treatment served as negative control.

3.8. **Preparation and inoculation of media and *Dovyalis abyssinicia***

Different concentrations of the *Dovyalis abyssinica* extract was added to sterilize universal bottles using micro pipette. SDA media was prepared and nine mililiter of the media was transferred to universal bottles having concentration of plant extract and allowed to solidify after labeling. A well grown colony collected from sub-culture of a mycelial form of the HCF was suspended in a saline water until turbidity matches 0.5 McFarland standard (which is equivalent to 0.4x104– 5x104cfu/mL) and uniformly streaked all over the agar surface by a sterile swab and incubated at 26°C. Positive control was prepared by culturing a HCF colony on SDA media with ketoconazole (standard) and negative control is prepared by culturing HCF colony on SDA free of any antifungal agent. The growth of HCF was monitored at least once per week.

3.9. Determination of minimum inhibitory concentration (MIC)

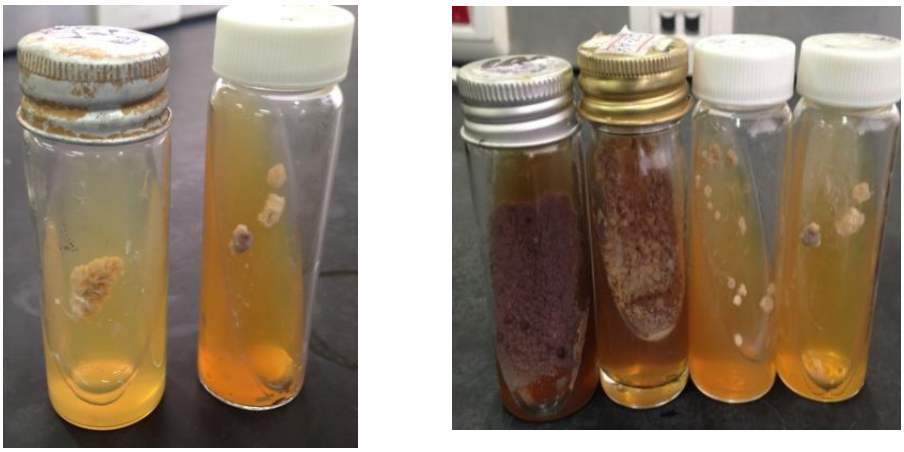
The minimum inhibitory concentration was defined as the lowest concentration of drug that completely inhibited visible growth. Each concentration of the plant extracts was mixed with SDA agar. The isolated HCF was cultured on each media containing the various concentrations of plant extract. The cultures were then incubated for 8-12 weeks. At the end of 12 weeks of incubation, growth of HCF was inspected on each culture. The

minimum concentration that inhibits the growth of HCF was considered the minimum inhibitory concentration (MIC) of *in-vitro* growth of HCF colony.

4. RESULTS

4.1. Isolation of HCF from field samples

The HCF isolate which was obtained from Aklilu Lemma Institute of Pathobiology, AAU was examined with gram staining and sub cultured in which the mycelial form of the colony grows slowly under aerobic conditions at 26^0C on SDA media and BHI media, enriched with 2.5% glycerol and 0.005% chloramphenicol for its activeness and showing characteristic morphology (white grey to brown) of the target HCF colony. HCF is usually surrounded by a 'halo' when stained with gram staining. During this study no active case of EL was observed at Robe and Gasera towns for active HCF colony isolation and identification. The typical colonies of HCF were identified as shown in figures 3 from samples were collected from Ambo, Holeta, Bishoftu, Asella, Ginir and Sebeta towns making a total of 85 isolates.



Figures 3. Colonies of HCF after 8-12 weeks of incubated at 26^0C on SDA and BHI agar

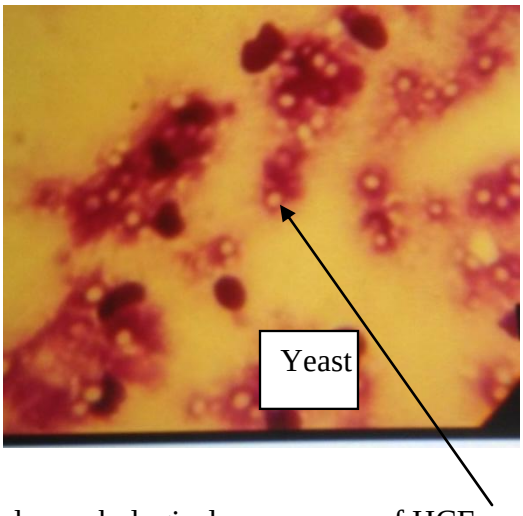


Figure 4. Typical morphological appearance of HCF yeast in Gram stained smear

4.2. Result of polymerase chain reaction

Representative sub-samples of eleven isolates of the mycelial cultures were confirmed by PCR using ITS1 and ITS4 primers. The gel image below shows the presence of 600bp bands that indicate HCF positive isolates on PCR (Figure 6).

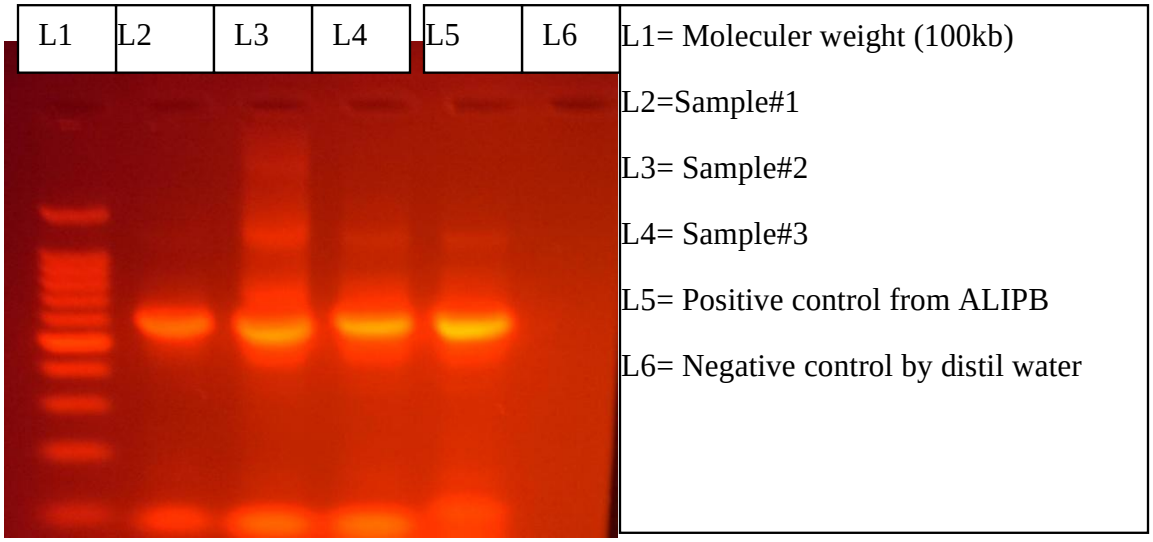


Figure 5: Representative gel image of PCR amplification of suspected HCF isolates

4.3. *In vitro* Anti HCF effect of the *Dovyalis Abyssinica*

Ten representative HCF mycelia isolates were selected from each of four sites (Bishoftu, Holeta, Ginir and Asella) for evaluation of anti-HCF effects of the Dovyalis *abyssinica* leaf and bark species. HCF growth was not observed in media containing the methanol extract of Dovyalis *Abyssinica* (Koshim) showed a good anti-HCF effect by inhibiting the growth of HCF at concentration ranging from 10mg/mL to 0.15625mg/mL. The MIC of the Dovyalis *Abyssinica* against HCF was 0.15625mg/mL (Figure 3) and that of the positive control was 0.025µg/mL (Figure 3), while HCF grows on all the media with no antifungal agents (negative control). The results of the agar dilution assays are summarized in below.

Figure 6: Selected medicinal plant (Dovyalis *Abyssinica, Koshim local name)* part.

Table 4: Growth of HCF in different concentration of Dovyalis *Abyssinica* and ketoconazole

Dovyalis Abyssinica		**Ketoconazole**	
Concentration	**Growth of HCF**	**Concentration**	**Growth of HCF**
10mg/mL	-	0.8µg/mL	-
5mg/Ml	-	0.4µg/mL	-
2.5mg/mL	-	0.2µg/mL	-
1.25mg/mL	-	0.1mg/mL	-
0.625mg/mL	-	0.05mg/Ml	-
0.3125mg/mL	-	0.025mg/mL	-
0.15625mg/mL	+	0.0125mg/mL	+
0.078125mg/mL	+	-	-

Key: **-** = *No growth observe* **+** = *Growth observed*

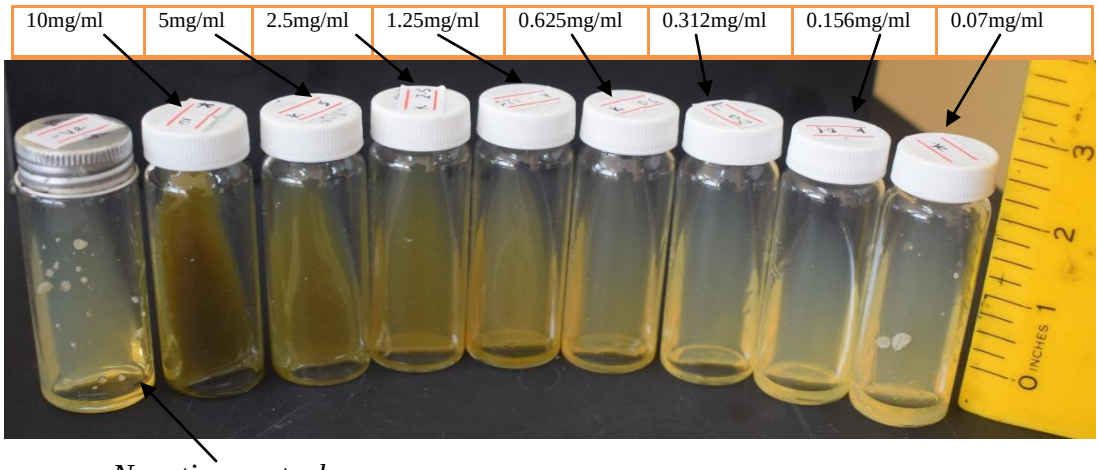


Figure 7: MIC of the plant extracts of Doyvalis *abyssinicia*

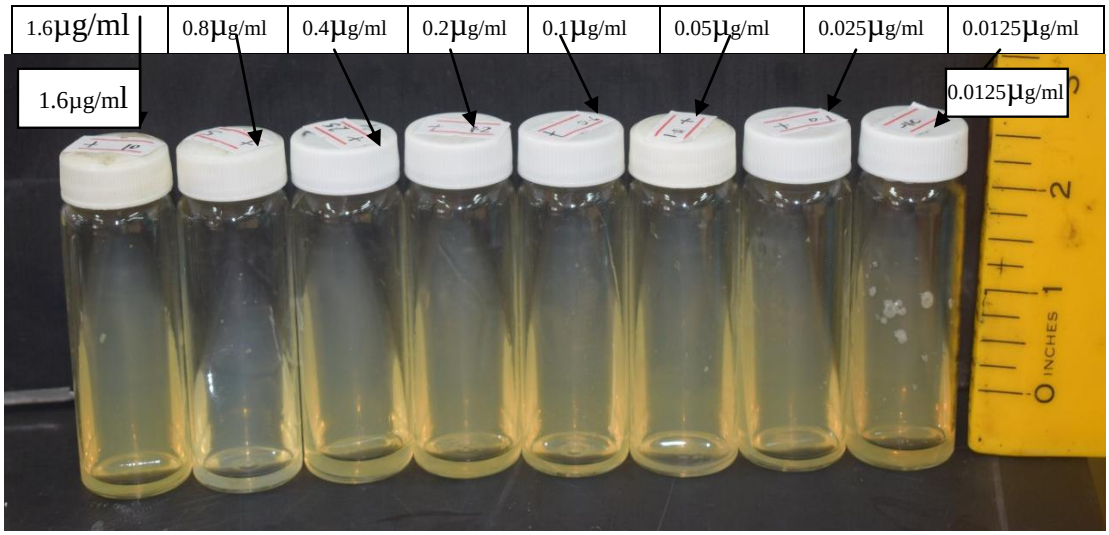


Figure 8: MIC of the plant extracts of ketoconazole

Table 4. The number of isolates of HCF used to evaluate the *in vitro* effects of Dovyalis *Abyssinica*

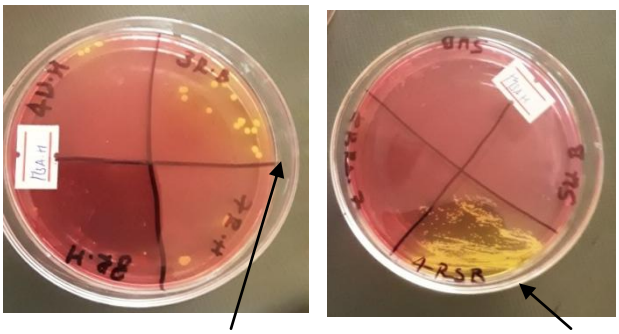
Site of sampled	Cases number	HCF isolated	Evaluated HCF	Response of Dovyalis *Abyssinica*
Ambo	2	2	-	-
Holeta	18	5	2	Effective
Sebeta	14	7	-	-
Bishoftu	33	10	2	Effective
Asella	20	13	3	Effective
Robe	-	-	-	-
Gasera	3	1	-	-
Ginir	11	9	3	Effective

4.4. Bacteria isolates from horses with EL nodules with swabs

Different pyogenic bacterial genera particularly gram positive and gram negative bacilli such as *Klebsiella, proteus, Aeromonus, pseudomonas and E coli* were isolated. Besides, gram positive cocci such as *Staphylococcus spp, Micrococcus spp and Corynebacterium* were isolated from acute and chronic stages of EL lesions. The common isolated bacteria from rupture and unrupture nodules of cases of EL lesions of horses were *Staphylococcus species, E. coli* and *Micrococcus* both from treat and untreated. Also many genera of bacteria were isolated from a single sample on different bacteriological media. The following figures were indicated that an isolated bacteria genera.

4.4.1. Macroscopic appearance of bacteria on different media and microscopic examination of under gram stain

Staphylococcus isolates



Figures 9: *Golden yellow pigmented colony on mantoli salt agar*

Klebsiella isolates

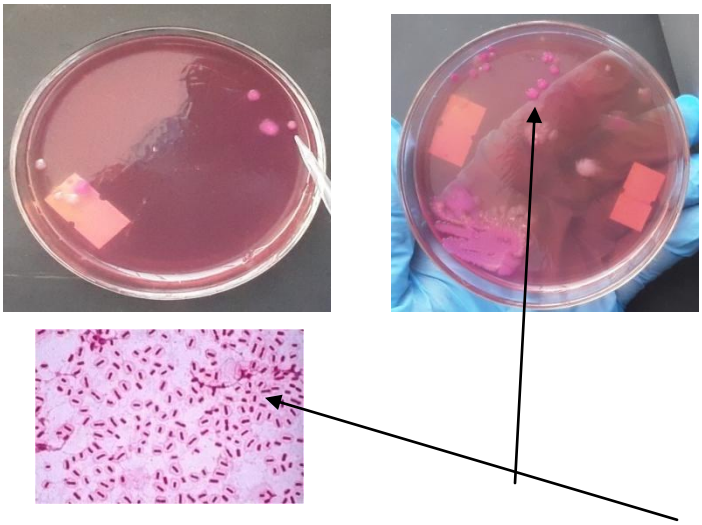
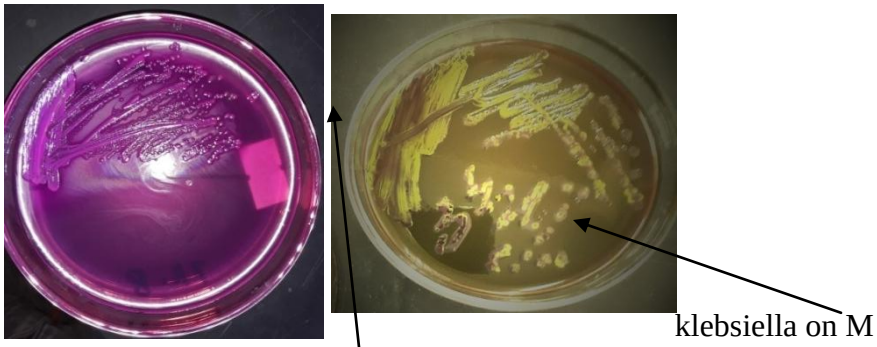


Figure 10: *Pink colony on MacConkey and gram staining of Klebsiella*

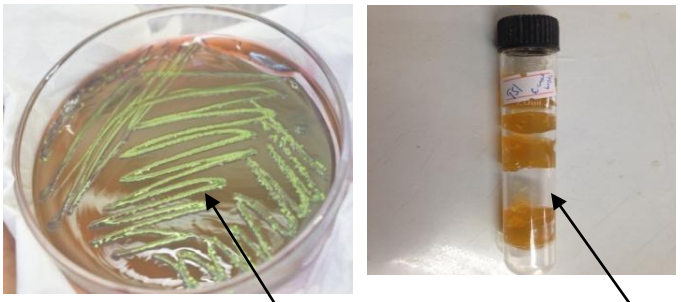
Escherichia coli isolates

Figures 11:



E. *coli* on MacKonKey agar

klebsiella on M

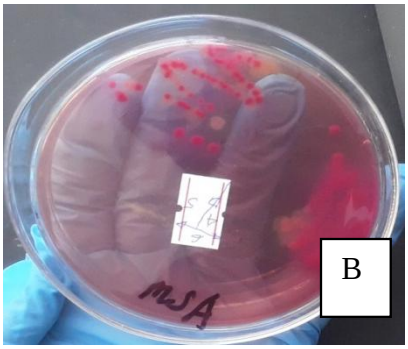


E. coli agar

Biochemical test

Distinctive green metallic sheen colony of E. coli on EMB agar

Aeromonas isolates



Figures 12: *Aeromonas colony on MSA*

Corynebacterium isolates

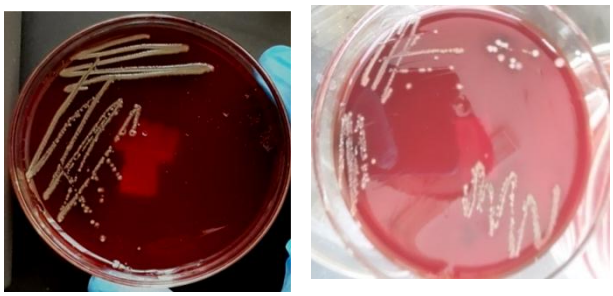
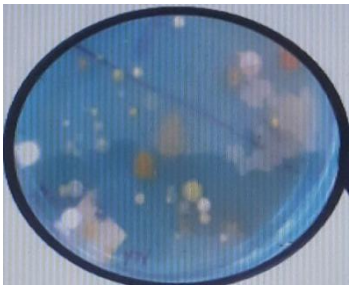


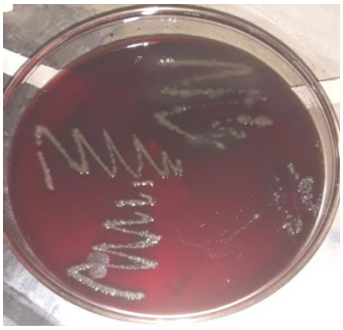
Figure 13: ***Colony of Corynebacterium grown on blood agar and showing no heamolysis***

Micrococcus isolates

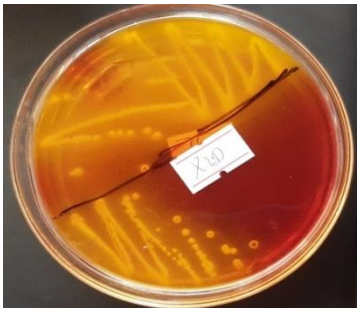


Figures 14: Colony of micrococcus growth on Nutrient agar

Streptococcus species that hemolysis the Blood agar



Pretous species



5. DISCUSSION

From 85 examined pus samples, 48 (56.47%) of the mycelia form of the HCF was grown on mycobiotic agar (SDA) enriched with 2.5% glycerol with addition of Chloramphenicol. An isolates of HCF was also checked by colonies morphology, sub culturing and gram staining techniques (Weeks *et al.,* 1985; Selim *et al.,* 1985; Radostits *et al.,* 1994). An interesting investigation that encountered while sub culturing the HCF colony was well visible growth has been seen three weeks even if the growth relatively slow and most isolates requires from three to eight weeks for development of characteristic colonies (Al-Ani, 1999). The HCF colony produce moderate growing, white to buff meat (brown), dry, granular, wrinkled, cerebriform colonies, convoluted grey white and become yellow to dark brown colonies at 26^0C so that it was grown with this characteristics on SDA agar (with 2.5% glycerol and 0.005% Chloramphenicol) (Deanna *et al.,* 1997; Ameni and Siyoum, 2002; Awad 1960). As a result of shortage and very expensive drug of EL disease in Ethiopia: the best solution of prevention, treatment and control method by using early detection of the disease, identification of the source of EL, minimize and manage the causes and transmission of disease. In this study we provided the preliminary *in vitro* effects of Dovyalis *abyssinicia* on HCF. This study also demonstrated that D. *abyssinicia* have strong anti-HCF effect at various concentrations *in vitro.*

The recent study also indicated that some different bacterial genera were isolated from almost samples collected from acute unrupture lesions of EL. *Klebsiella, proteus, pseudomonas, Aeromonus, Corynebacterium* and *E. coli* were an isolated gram negative bacilli group and *Staphylococcus, Micrococcus, Streptococcus* were cocci group from samples of acute and chronic lesions of EL. The current finding is closely similar with Hadush *et al.* (2014) and Mediksa *et al.* (2017) who reported majority of currently identified bacteria from EL lesions.

The difference between Hadusha, (2014) and Mediksa *et al,* (2017) might be due to the difference in wound type from the samples were collected; in the recent study also types of samples collected to investigate the origin of bacterial genera may observed (swab sample from rupture lesions). So in current study indicated that most bacteria general were colonized the EL lesion from environment and very few such as proteus was colonize from external. It was well documented that infected lesions harvest diverse populations of microorganisms that can be difficult to identify and fail to respond to antibiotic treatment, resulting in chronic non-healing wounds (Westgate *et al.,* 2011; Nagoba *et al.,* 2013; Carnwath *et al.,* 2014). The most commonly isolated bacteria from the EL lesions were *Staphylococcus species* (82.14%) and *Corynebacterium* (57.14%) followed by *Aeromona* (50.00%), *Micrococcus* (50.00%), *Klebssiella* (46.42%), *Streptococcus* (42.86%) were isolated. E. *coli* and *Proteus* being the least frequent. The dominance of *Staphylococcus*, *Micrococcus* and *Streptococcus* may be due to the fact that those bacteria were commonly found as skin flora of animals where easily to invade the infected skin tissues. But the rest one were isolated in environment and mud area or in water part (Aeromonas) where it may get probability of invading the skin lesions of EL. So, in severe cases highly abundant due to the lesion or wounded skin part increased and attack. In line with this, several reports indicated that *Staphylococcus* was the most commonly isolated genus in wounds and skin sample (Theoretic *et al.,* 2012; Carnwath *et al.,* 2014; Tobiasc *et al.,* 2016; Mediksa *et al.,* 2017).

Other study also supported that, the most dangerous bacteria in EL and cellulitis in horses are coagulase positive and negative *Staphylococcus* s*pecies*, *Streptococcus species* and gram negative aerobic bacteria. The current finding also agree with the positivity with Wilson (2001) and Westgate *et al.* (2011) finding in which *Pseudomonas* were the most predominantly isolated bacterial species from equine wound and skin samples respectively. The presence of gram negative bacteria including *E. coli*, *Proteus vugaris* and *P. aeroginosa* in lesions aggravate the severity of infection besides to other criteria and cause for the delaying of wound healing (Bessa *et al.,* 2015; Nagoba *et al.,* 2013).

The study supported that in advanced cases, the nodules and ulcers may have unpleasant odor in advanced case Ameni (2007) and welling of the lymphatic vessel is due to bacterial complication restrict lymph vessel. From this angle the organisms may be disseminated hematogenously, even from the smallest abscess could ability to aggravating the EL (Loir *et al.*, 2003; Smith, 2007). These indicated that the presence of bacterial contaminates in lesions of EL infected horses.

Some limitations of the study

There were different limitations faced in this research: first, the long incubation period needed to grow HCF resulted in overgrowth by fast growing fungal contaminants and limited our capacity for timely harvesting and processing of pure colonies of the fungus. Second, shortage of bacteriological media and material availability to isolate and identify the bacterial species. Despite all these limitations, I provided a preliminary finding that will play a significant role in treating and controlling EL in the future.

6. CONCLUSION AND RECOMMENDATIONS

The result of the recent study indicated that, HCF and co-infecting bacteria were significantly associated with EL lesions. HCF was particularly prevalent in carthorses in most parts of humid and warm area, Ethiopia. The bacterial isolated includes *Staphylococcus, Micrococcus, Streptococcus* as a gram positive bacilli and other gram negative bacilli such as *Klebsiela, Aeromonas, Corynebacterium, Pseudomonas* were frequently exist in the EL lesions hence E. *coli* and *Proteus* were rarely present. The occurrences of co-infect bacterial increased along the advancement of the lesion of EL. This research reflected that co-infect contaminant bacterial have their own contribution in enhancing pathogenecity and severity of the EL, which may lead to chronic non healing wounds. However, in this study medicinal plant indicate that Dovyalis *abyssinicia* had strong inhibitory effect on the growth of the mycelia form of HCF *in vitro and* inhibited the growth of HCF at concentrations ≥ 0.3125mg/ml.

Recommendation

Based on the above conclusions the following recommendations forwarded

- The molecular diagnostic techniques should be under taken for isolation and identification of bacterial contaminants of EL to species level and antimicrobial susceptibility test should be provided as well as further investigation could be investigated on EL in order to develop practical and sustainable control and prevention of the diseases.
- During EL treatment, consideration should be taken about the presence of bacterial and their consequence on potential complications.
- Anaerobic bacteria were not isolated due to shortage of laboratory material so it needs further study.
- Further studies on Phyto-chemical analyses should be conducted on Doyvalis *abyssinicia* and the active chemical ingredient on HCF mycelia should be identified.

- Further investigation of the Doyvalis *abyssinicia* concentrations *in vitro* trials should be repeat and corrected as result of toxicity on HCF growth.
- *In vivo* studies should be conducted so that the safety margin, toxicity and cure rates will be known in order to use them commercially.

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ANNEXES

Annex 1. Procedure for Sample collection

1. Clinically confirmed positive horses were identified
2. Areas with unruptured nodules were detected
3. The nodule and its surrounding was thoroughly washed with soap and water and disinfected with 70% alcohol
4. With a great care not to burst the nodule it was shaved with surgical blade and content of nodule was gently aspirated with a sterilized needle.

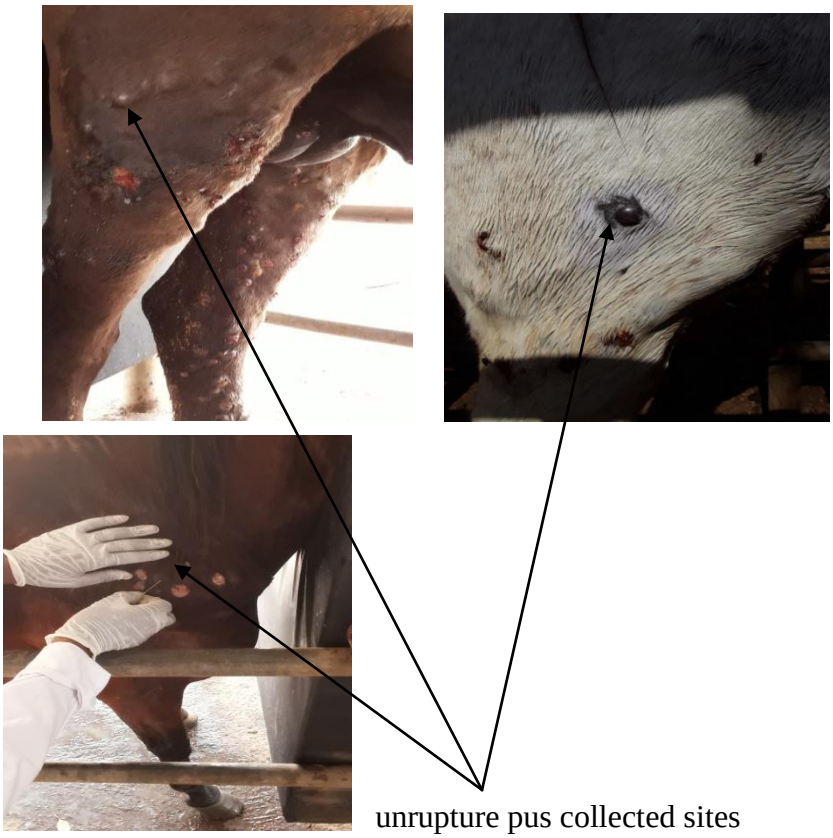
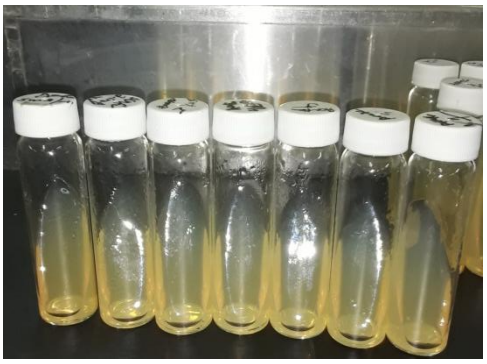


Figure: *Shaved unruptured, washed and disinfected nodule ready for Content aspiration*

Annex 2. Procedure for preparing of SDA medium and inoculation

1. A 65g powder Sabourauds dextrose agar was added to 1000ml of sterilized distilled water and boiled on a heater until the mixture dissolved completely and uniformly.
2. The media was then autoclaved at 121^{o}C for 15 min and cooled.
3. After cooling 0.5g/L chloramphenicol and 2.5% glycerol was added and gently mixed
4. The agar was immediately poured to the bottles and kept in a slant position.
5. A drop of contents of pus sample was put on the slant and strikes with sterilized wire loop gently.

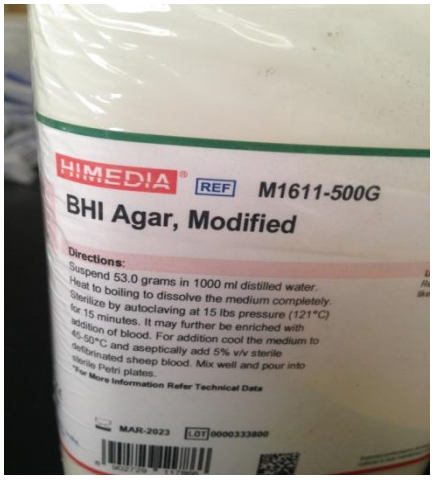


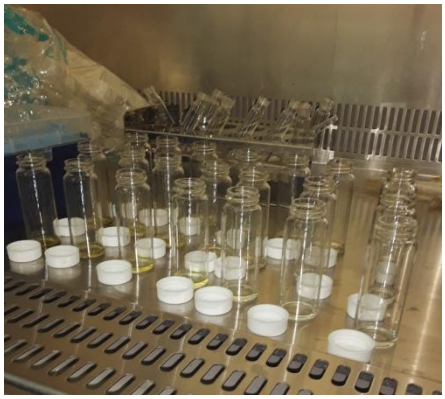
Principle of SDA

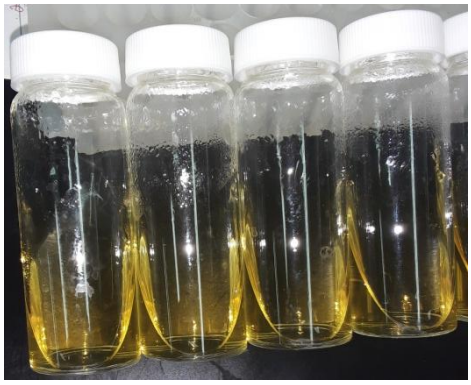
Peptone (Enzymatic Digest of Casein and Enzymatic Digest of Animal Tissue) provide the nitrogen and vitamin source required for organism growth in SDA. Dextrose is added as the energy and carbon source. Agar is the solidifying agent. Chloramphenicol and/or tetracycline may be added as broad spectrum antimicrobials to inhibit the growth of a wide range of gram-positive and gram-negative bacteria. The neutral pH of the Emmons modification seems to enhance the growth of some pathogenic fungi, such as dermatophytes.

Annex 3: procedure for preparing of BHI medium and inoculation

1. A 53.0g powder Brain Heart Infusion agar was added to 1000ml of sterilized distilled water and boiled on a heater until the mixture dissolved completely and uniformly.
2. The media was then autoclaved at 121^{o}C for 15 min and cooled.
3. After cooling 0.5g/L chloramphenicol and 2.5% glycerol was added and gently mixed, as well as it enriched with addition of 10% horse blood
4. The agar was immediately poured to the bottles and kept in a slant position.
5. A drop of contents of pus sample was put on the slant and strikes with sterilized wire loop gently.



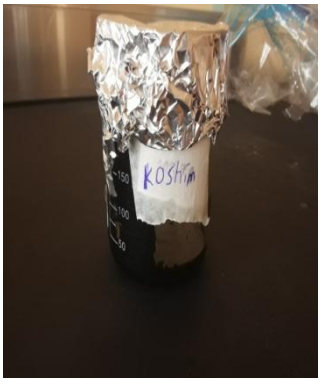




Annex 4: Procedure for plant extraction

1. Collected plant were transported to the laboratory and allowed to air dry under shadow (not sun).
2. Dried plant part were ground by an electric motor grinder and dissolved in 80% methanol at 1:10 concentration.
3. The solution was allowed to stay on an orbital shaker for 3 days and it was filtered by gauze and watman filter paper, this process was repeated three times for exhaustive extraction of particles.
4. Finally methanol was evaporated by rotary vapor and solution was kept in an oven at 40°C for around a week to dry.




Annex 5: Bacteriological Media Preparation

A/ Blood agar Preparation

1. Suspend 40.0 gram of the powder in 1 liter of distilled water.
2. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121^0C for 15 minutes. Cool the base to 45-50^0C and add 5% sterile defibrinated blood.

B/ MacConkey agar Preparation

1. Suspend 51.5g in 1 liter of distilled water.
2. Bring to boil completely.
3. Sterilize by autoclaving at 121^0C for 15 minutes.

C/ Mannitol salt agar Preparation

1. Suspend 111 gram in 1 liter distilled water and heat to boiling to dissolve completely.
2. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (1210C).
3. Cool to 45 – 450 C and dispense in to Petri dishes.

D/ Nutrient agar Preparation

1. Suspend 28 gram in 1 liter of distilled water.
2. Bring to boil to dissolve completely.
3. Sterilize by autoclaving at 1210C for 15 minutes

E/ Edwards medium (modified)

A selective medium for the rapid isolation of Streptococcus.

1. Directions Suspend 41g in 1 liter of distilled water.
2. Bring to the boil to dissolve completely.
3. Sterilize by autoclaving at 115°C for 20 minutes.
4. Cool to 50°C, add 5-7% of sterile bovine or sheep blood, mix well and pour plates.

Description: *A selective medium for the rapid isolation of Streptococcus. Crystal violet or gentian violet and thallium salts have long been used in selective media for streptococci*

F/ Eosin Methylene Blue Agar (MODIFIED) (EMB) preparation

An isolation medium for the differentiation of the E. *coli.*

1. Suspend 37.5g in 1 liter of distilled water.
2. Bring to the boil to dissolve completely.
3. Sterilise by autoclaving at 121°C for 15 minutes.
4. Cool to 60°C and shake the medium in order to oxidise the methylene blue (i.e. restore its blue colour) and to suspend the precipitate which is an essential part of the medium.

G/ MacConKey Agar

An isolation gram ***negative bacteria*** and adjusted as required to meet performance standards Directions

1. Suspend 52g in 1 liter of distilled water.
2. Bring to the boil to dissolve completely.
3. Sterilize by autoclaving at 121°C for 15 minutes.
4. Dry the surface of the gel before inoculation.

H/ XLD Agar

XLD Agar is a selective differential medium for the isolation of Gram-negative enteric pathogens from fecal specimens and other clinical material.

Preparation of XLD Agar

1. Suspend 55 grams of dehydrated medium in 1000 ml purified or distilled water.
2. Heat with frequent agitation until the medium boils. Note: ***Do Not Autoclave.***
3. Transfer immediately to a water bath at 50°C.
4. After cooling, pour into sterile Petri plates.

Note: *It is advisable not to prepare large volumes, which will require prolonged heating and may produce precipitate. Degradation of xylose, lactose and sucrose generates acid products, causing a color change in the medium from red to yellow. Lysine decarboxylation in the absence of lactose and sucrose fermentation causes reversion to an alkaline condition and the color of the medium changes back to red.*

Typical colonial morphology on XLD Agar is as follows:

- *Salmonella Typhi* – Red Colonies, Black Centers
- *Proteus vulgaris* – Yellow Colonies
- *Salmonella choleraesuis* – Red Colonies
- *Shigella sonnei* – Red Colonies
- *Shigella flexneri* – Red Colonies
- *Escherichia coli* – Large, Flat, Yellow Colonies; some strains may be inhibited
- *Enterobacter/ Klebsiella* – Mucoid, Yellow Colonies
- *Pseudomonas aeruginosa* – Pink, Flat, Rough Colonies
- Gram-positive bacteria – No growth to slight growth

Annex 6

Primary identification tests and gram's stain (Carter, 1991)

- Gram‟s reagent - Crystal violet
- Gram‟s iodine (mordant)
- Ethanol 95%
- Counter – stain (carbon fuchsine / safranin)

Procedure: *From a fresh colony take a loop full of bacteria and emulsify it in a small drop of water or saline on the slide. This should be a thin, not milky, suspension or it will not stain properly. Air dries the slide.*

1. Fix the smear by passing on Bunsen burner
2. Pour crystal violet for 1 minute
3. Flood with tap water
4. Pour Gram's iodine (Mordant) for 1 minute
5. Flood with Tap water
6. Decolorize with 95% Ethanol alcohol for 5-10 seconds
7. Rinse with tap water
8. Pour safarenin (counter stain) for 1 minute
9. Rinse with Tap water
10. Examine the slide under 100x oil immersion microscope

Interpretation

- *Gram positive bacteria appear blue/ violet*
- *Gram negative bacteria appear red/ pink*

Annex 7: Types of Medias and type of microbial

	Selected media were been used	Pyogenic and fungus agent
1	Sabouradus Dextrose Agar Brain Heart Infusion agar	 For fungus
2	Nutrient Agar media	Less fasitidius
3	MacConkey	Gram negative
4	Manitol salt agar	Staphylococcus, Aeromona isolates
5	Blood agar	For fastidius and to check heamolysis
6	Edwards media agar	Streptococcus isolates
7	Eosin and ethylene blue (EMB) agar	E. *coli*
8	Xylose, lactose H2S (XLD) Agar	*Klebssiella and Proteus*

Source (Quinn, 2002)

Annex 8: Colony characteristic of bacterial contaminants and HCF

Contaminants of EL lesion	Colony Characteristics of each contaminants on their selective media
Staphylococcus	Yellowish golden round smooth , glistening on MSA
Crynebacteria	Small White dry non heamoltic colony on sheep blood agar
Micrococcus	Shiny and white convex colony but often pigmented on nutrient agar
Streptococcus	Translucent, mucoid colony on Eduward agar
Proteus	Gray wavy swarming growth over agar (due to cell growth), on blood agar brown colony and have foul smell but on MacConkey pale colony with irregular edge on blood agar.
Pseudomonas	Yellowish green, flat or gray, yellowish is due to pigment called pyoverdin on blood agar
Aeromonas	On agar plate their colony Smooth convex, rounded have Buffy colored and not disintegrated in fridge on MSA
E. coli	Distinctive metallic sheen on EMB
HCF	Dry, granular wrinkled/lined, cerebriform, convoluted grey white colony and, dark brown(up on aging)

Quinn, 2002