GROSS AND MICROSCOPIC LESIONS OF EPIZOOTIC LYMPHANGITIS ON CARTHORSES IN CENTRAL OROMIA, ETHIOPIA

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of veterinary Science in Tropical Veterinary Pathology

By

Dereje Wakjira

Principal advisor; Dr. Hagos Ashenafi (DVM, MSc, PhD, Associate Professor)

Co-advisers; Dr. Assegedech Sirak (DVM, MSc, Pathologist) and

Dr. Nigatu Aklilu (DVM, MSc, Adj. Assistant Professor)

OCTOBER, 2015

BISHOFTU, ETHIOPIA

APPROVAL SHEET

Addis Ababa University
College of Veterinary Medicine and Agriculture
Department of Pathology and Parasitology

Title: Pathology of Epizootic Lymphangitis in Carthorses in Central Ethiopia, Oromia Regional State, Ethiopia

Submitted by: Dereje Wakjira _____ _____
Signature Date

Approved *for submittal to* thesis assessment committee by:

1. Dr. Hagos Ashenafi _____ _____
   Major Advisor Signature Date
2. Dr. Assegedech Sirak _____ _____
   Co- Advisor Signature Date
3. Dr. Nigatu Aklilu _____ _____
   Co- Advisor Signature Date
4. Dr. Yakob Hailu _____ _____
   Department chairperson Signature Date

APPROVAL AND SIGNATURE SHEET

Addis Ababa University
College of Veterinary Medicine and Agriculture
Department of Pathology and Parasitology

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: Dereje Wakjira, entitled '**Pathology of Epizootic Lymphangitis on Carthorses in Central Ethiopia**' and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Science in Tropical Veterinary Pathology

_____	_____	_____
Chairman	Signature	Date
_____	_____	_____
External Examiner	Signature	Date
_____	_____	_____
Internal Examiner	Signature	Date
Dr. Hagos Ashenafi	_____	_____
Major Advisor	Signature	Date
Dr. Assegedech Sirak	_____	_____
Co- Advisor	Signature	Date
Dr. Nigatu Aklilu	_____	_____
Co- Advisor	Signature	Date
Dr. Yakob Hailu	_____	_____
Department chairperson	Signature	Date

DEDICATION

This thesis manuscript is dedicated to my father, Wakjira Tike, and my mother Ilfinesh Disassa for nursing the author with affection and love and for their dedicated partnership.

STATEMENT OF AUTHOR

First, I declare that this thesis is my bonafide work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

Name: Dereje Wakjira Signature: _____

Date of Submission: _____

College of Veterinary Medicine and Agriculture,
Bishoftu

TABLE OF CONTENTS

TABLE OF CONTENTS (*Continued*)

ACKNOWLEDGEMENTS

I am highly indebted to my advisors Dr. Hagos Ashenafi, Dr. Assegedech Sirak and Dr. Nigatu Aklilu as without their keen support, encouragement, insight, guidance and professional expertise the completion of this work would have been impossible. I would also like to thank them for their friendly treatment and devotion of time during the work of this thesis research. My special appreciation also goes to Dr. Teshale Sori who had advised me on matters pertinent to the thesis.

I express my deep sense of gratitude and profound indebtedness to Prof. Gobena Ameni for his technical and financial support and for his valuable guidance, moral support, healthy criticism and inspiring guidance for the accomplishment of this manuscript.

It is my pleasure to acknowledge Addis Ababa University; College of Veterinary Medicine and Agriculture, Aklilu Lemma Institute of Pathobiology, Society for the Protection of Animals Abroad (SPANA) Ethiopia project and National Animal Health Diagnostic and Investigation Center (NAHDIC) as an organization in general and all staff members in particular for their genuine and cooperativeness during the research work.

I would like to express my heartfelt appreciation to Addis Ababa University; College of Veterinary Medicine and Agriculture Salalle campus for granting me a study leave, and it is my pleasure to specially acknowledge Mr. Geberew Tullu, the former managing director of Salalle campus and Dr. Dinka Ayana, Dean of College of Veterinary Medicine and Agriculture for their special concern and motivation in this study leave.

Finally I would especially like to convey my deepest gratitude and immense respect to my beloved family to their all rounded help and encouragement throughout my long term study and to their patience in my absence during this thesis research work.

LIST OF ABREVIATIONS

AGID	Agar-Gel Immunodiffusion
CF	Compliment Fixation
CSA	Central Statistics Authority
CVMA	College of Veterinary Medicine and Agriculture
EARO	Ethiopian Agricultural Research Organization
EZL	Epizootic Lymphangitis
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
GMS	Gomori Methen-amine-Silver stain
HCF	*Histoplasma Capsulatum variety Farciminosum*
HE	Hematoxylin and Eosin stain
ID	Immunodiffusion
IP	Incubation Period
NAHDIC	National Animal Health Diagnostic and Investigation center
OIE	Office International des Epizootics
PAS	Periodic Acid Schiff reaction stain
SPANA	Society for the Protection of Animals Abroad
USD	United States Dollar

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF APPENDIX

ABSTRACT

A cross sectional study was conducted in eight towns in central Oromia, Ethiopia, from March to August of 2015 with an objective of describing the pathological findings of topical and internal organs lesion in carthorses naturally infected with Epizootic lymphangitis and revealing the presence of the fungus within the lesions of internal organs. The study animals were local breed carthorses located in the study areas that are naturally infected with *Histoplasma capsulatum variety farciminosum*. Among the 70 disease suspected carthorses that were found in the study area 30 were purposively screened out. Field assessment, clinical observation, microscopical examination, isolation of the agent, autopsy examination, and histopathological examination were used for this investigation. Field Survey part of this study revealed presence of a gap on knowhow of the disease. In spite of the fact that 76% (76/100) of the interviewed carthorse owners were trained by SPANA, 44% of them still believe that the disease is caused by an evil-eye or "Buda".71% of them are accustomed to use both traditional and modern treatment methods. 37% of them believe presence of small and large nodular lesions which is found to be true that out of the 804 nodular lesions counted on this study 396 (49.3%) were small type (Female) and 408 (51.7%) large type (Male). Early and moderate stages of Epizootic lymphangitis are considered curable, however, the fate of carthorses with sever stage of the disease was euthanasia. Severely affected and abandoned carthorses were usually found staggering at the middle of highways. All the 70 disease suspected carthorses showed signs of the cutaneous forms of the disease and there were no clearly separated three forms, rather there were an extension and manifestation of the cutaneous form lesions in and around eye and respiratory organs. Giemsa stained smears of the contents of pyogranulomatous skin nodules and imprint smear of affected internal organs revealed yeast like organisms; showing that the disease. The topical body parts affected with lesion were skin and superficial lymphatics of skin of the body extremities. Nodular lesions were observed mostly in the limbs, lower trunk and head regions. Severely affected regions were the limb extremities, especially the hind limb extremities. Tick infested parts seems to be more vulnerable. The gross lesions detected on internal organs were few in number and even on few organs. Although, diverse types of lesions were detected on different organs, a mass of nodule on

mucosal part of trachea and a large nodular lesion of right lung together with diffused small granulomatous nodules of both lungs were the reportable findings of this study. Hematoxylin and Eosin stained histopathological sections reveled the characteristic pyogranulomatous lesion and many yeast-like organisms distended within macrophages. Those characteristic histopathological lesions were found both from topical and internal organ lesions. The inflammatory cells that infiltrate the tissue were macrophages, polymorphonuclear cells and plasma cells; macrophages being the predominant one. There were neither gross nor microscopic lesions detected within internal organs of some cases in this study; even in one organ. There was neither pyogranulomatous lesion nor yeast-like organisms detected from normal tissue sections of internal organs and from some non-nodular lesions of internal organs. Having such an open skin, their internal organs were found to be normal in pathologically point of view. The pathological lesions were restricted on the skin and most of the vital organs were found normal. Additionally skin has a great regeneration capacity and early and moderate stages of Epizootic lymphangitis are curable. Therefore all stages of EZL seem curable, if handled properly. The treatment may not be cost effective, however, euthanasia of carthorses in sever stage of Epizootic lymphangitis for welfare reason seems against the principle of welfare.

Key words: Epizootic lymphangitis, carthorses, pyogranulomatous lesion, necropsy, biopsy, histopathology

1. INTRODUCTION

Agriculture is the backbone of the Ethiopian economy in which the livestock sector has a lion's share and by far the role of Equines is significant. Despite the importance of the livestock sector, it has remained unexploited due to various technical and non-technical impairments (Asfaw, 1999; Gari *et al*., 2010). Among the major causes of such impairments is the prevalence of a large number of livestock diseases throughout the country (Hagos *et al*., 2010). The case of equines is not different from the overall livestock situation.

Ethiopia possess 2.75 million horses, 5.02 million donkeys and 0.63 million mules (Statistical Bulletin, 2011), which is 46% of all equines in Africa (FAO, 1996). Equines have a prominent position in the county's agricultural and transport systems since the transportation activities are performed by equids (Ameni, 2006a; Hadush *et al.,* 2008). They are mainly used as draught and pack animals and they are also used for ploughing in some parts of the country (Endebu, 1996; Ameni & Siyoum, 2002). Despite the significance of equines in general and horses in particular to the country's economy, the attention given to these species of animals has been quite negligent compared to other species of animals. This can be due to misconception of the owners about the hardiness and tolerance of equines against adverse effects including diseases and because they are not providers of meat and milk at least in Ethiopia (Feseha, 1993; Gari *et al*., 2010). The consequence of such misconception and negligence is the wide spread occurrence of diseases. One of such rampant diseases is epizootic lymphangitis.

Epizootic lymphangitis is one of the infectious diseases causing huge economic losses and low productivity in horses. It is particularly prevalent in carthorses in most parts of Ethiopia studied (Ameni, 2006a). For instance it occurs in 24.9% horses in Walliso (Asfaw *et al.,* 2012). Due to its significant impact on the livelihood of carthorse owners, it is considered a major disease of horses in many parts of Ethiopia. Data for economic losses incurred by epizootic lymphangitis is lacking but one conservative estimate showed that mortality associated losses of about $129 USD incurred per annum per owner (Hadush *et al.,* 2014).

Despite of its economic importance, EZL is a least studied disease in the world by large and in Ethiopia in particular. Little is known about EZL in general and regarding its pathology and its pathogenesis in particular; especially on pathology of EZL in internal organs. Thus, there is a need to conduct exhaustive study in order to improve the gap on knowhow of EZL in general and regarding its pathology in particular so that the economic impact of EZL will be resolved.

Based on the above background, this cross sectional study was designed with the following objectives:

- To describe the pathological findings of topical and internal organs lesion and

- To reveal the presence of the fungus with in the lesions of internal organs of the carthorses naturally infected with EZL in central Ethiopia.

2. LITERATURE REVIEW

2.1. Lymphangitis

The lymphatics are a type of tubular system that drains excess fluids and proteins from tissue and gradually routes it back into the main blood circulation. Lymphatic vessels are low-pressure vessels similar to veins that collect the fluid that surrounds cells and return it to the blood stream. Lymphatics are present practically everywhere in the body. The richness of the lymphatic plexuses in almost all tissues and their important role as drainage channels for interstitial fluid makes for almost inevitable involvement of lymphatics by any inflammation. In most instances, the lymphatic lesions are so small as to have no significance, but the consequence of lymphatic involvement may be the major presenting clinical sign or lesion.The most common sites that encounter lymphatic problems in the horse are their legs (Jubb *et al*., 2006; http://www.merckvetmanual.com/mvm/index.jsp).

Lymphangitis means inflammation of the lymphatic vessels that may be caused by infection or allergy which can occur anywhere in the body (Edward, 2005). Inflammation is the body's reaction to harmful or irritating stimuli, which could be anything from a burn, allergy, or cut, to a serious infection. Inflammation begins the process of healing. The typical signs of inflammation redness, heat, swelling, pain and sometimes lost function, are indicators of it. Because lymphangitis is an inflammation, the swelling is partly due to lymph vessels and lymph nodes adjacent to the affected area responding to the presence of a potential invader. Inflammation is frequently confused with infection; however infection is caused by a bacterium, virus or fungus, while inflammation is the body's response to it that will occur also in response to infection (Mc Gavin & Zachary, 2010).

Lymphangitis has been recognized in Ethiopia since 1968 (Solomon, 1980).There are a number of diseases conditions which causes lymphangitis, but here mentioned are the three more familiar causes for lymphangitis in horses. Those are Idiopathic lymphangitis, Ulcerative lymphangitis and Epizootic lymphangitis (Endebu, 1996; Ameni & Siyoum, 2002).

2.2. Epizootic Lymphangitis

Epizootic lymphangitis (EZL) is a contagious relatively common infectious disease of horses and other equids caused by the dimorphic fungus, *Histoplasma capsulatum variety farciminosum* (OIE, 2008; AL-Ani, 1999). It is a debilitating disease. Most cases of EZL are reported from horses (90%), and the remainder from mules and donkeys. EZL infection can occur in camels, cattle and dogs, (AL-Ani, 1999). EZL infection in humans has also been reported (AL-Ani *et al.*, 1988; Radostits *et al.,* 2006). Cattle are more resistant than equids (AL-Ani *et al.*, 1988).

Epizootic lymphangitis is a chronic granulomatous disease of the skin, lymph vessels, and lymph nodes of the limbs, chest and neck of Equidae. It is clinically characterised by a spreading suppurative inflammation of cutaneous lymphatic vessels, lymph nodes, and adjacent skin (Ameni & Siyoum, 2002; Negesse *et al.,* 2012).

Epizootic lymphangitis is a common infectious disease of horses in Ethiopia (Ameni, 2006a). It is a significant concern in the country, where the prevalence in carthorses is nearly 19%, and economic losses from this disease are high (Ameni & Siyoum, 2002; Ameni, 2006a). EZL is particularly prevalent in the study area (Ameni, 2006a): it is 24.9% for instance in Woliso (Asfaw *et al.,* 2012). It causes significant impact on the carthorse owners, that they considered it as the first and as a major disease of horses in Ethiopia. EZL causes an average loss of 129 USD incurred per annum per owner as a result of death of affected horses and an average 2.5 USD decline from the net profit per day due to reduced working performance of affected horses (Hadush *et al.,* 2014).

2.2.1. Aetiology

The causative agent of EZL is *Histoplasma capsulatum variety farciminosum (HCF)*. It is a thermally dimorphic fungal soil saprophyte. The mycelial form is present in soil, while the yeast form is usually found in lesions (Ameni, 2006b). *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.,* 1983b; Gabal & Hennager, 1983; Soliman *et al.,* 1986)

2.2.2. Epidemiology

The infection rate of EZL varies with the geographic area and the age of the animal. Horses under six years of age are most susceptible (Radostits *et al.,* 2006). 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, 1989). The disease is common in Ethiopia, especially in cart horses, affecting an average of 18.8% of horses in warm, humid areas between 1500 and 2300 meters above sea level (Ameni & Siyoum, 2002; Ameni, 2006a).

The incubation period (IP) 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 main loss results from the inability of animals to work for several weeks because of extremely painful lesions. The OIE classify it as a list B disease (OIE, 2008).

The wounds caused by harness are reported as major predisposing factors of EZL in carthorses in Ethiopia (Asfaw *et al.,* 2012). In the endemic areas in certain regions of the world, the occurrence of seasonal dusty winds expose horses to the inhalation of dust and spores, leading to pneumonia (Jubb *et al*., 2006; OIE, 20008).

The mode of transmission of EZL includes transmission by direct or indirect contact of HCF with traumatized skin, by biting flies, by ticks or by inhalation of HCF (AL-Ani& Al-Delaimi, 1986; Morrow & Sewell, 1990; Ameni & Terefe, 2004). According to Singh, 1965 direct contact with infective materials through injured skin or through cutaneous abrasions is the most common mode of infection. 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).

Records also exist for the transmission of the disease from stallions to mares during copulation (Gillespie & Timoney, 1981).The possibility of experimental infection of

horses is reported by Ameni G. in which the IP is much longer in horse inoculated with mycelial organisms than that of with the yeast form (Ameni, 2006b).

2.2.3. Diagnosis

The clinical signs of EZL in horses in endemic regions are often the basis of diagnosis; however confirmatory tests should be followed. Several confirmatory tests have been described. Laboratory diagnosis of epizootic lymphangitis usually is by stained smears of the cutaneous exudate based upon demonstration of the typical yeast-like, double-contoured cells in pus collected aseptically from the lesion and confirmed by culturing the pathogen (Al-Ani & Al-Delaimi, 1986; Al-Ani *et al.,* 1998).

Microscopic examination of Giemsa or Gram-stained smears of pus/ swabs aspirated from a nodule reveal Gram- positive yeast forms with a halo (unstained capsule-like) structure (Ameni, 2007; Asfaw *et al.,* 2012). According to Ameni, 2007, it reveals hyaline septated and branched hyphae. In one study, direct microscopy yielded positive results in 79% of cases (AL-Ani, 1999). The yeast form of the organism appears in pus as a double-contoured oval or ovoid body, measuring 2.5 - 3.5 µm by 3-4 µm (AL-Ani, 1999).

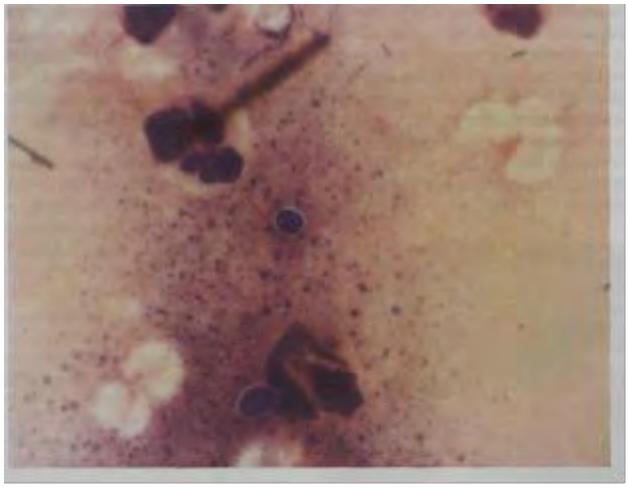


Figure 1. Gram-stain of HCF **(**AL-Ani, 1989).

Culture of HCF from body fluids or tissues is the "gold standard" for confirming the diagnosis but it may be impractical since it may be difficult and time-consuming to grow from body fluids and tissues. Moreover false-negative results are likely to occur (Gabal & Khalifa, 1983; Al-Delaimi & Khairallah 1984; AL-Ani, 1989). The fungus HCF can be cultured or isolated on special media such as Sabouraud dextrose agar but it dies quickly in specimens, unless these are collected in antibiotic solutions, refrigerated and cultured promptly (Radostits *et al.,* 2006; Asfaw *et al.,* 2012).

Serologic tests for the presence of antibodies in the blood are also the possible options, but they may not have a high degree of specificity for active disease. It may reflect past exposure or asymptomatic infection. Tube agglutination and passive haemagglutination tests have been reported to identify increased titers in horses with epizootic lymphangitis, (Gabal & Khalifa, 1983) which can be used as a practical screening test. A serum agglutination titer of 1: 80 or higher is reported to be positive (AL-Ani, 1989). Fluorescent antibody, AGID, and ELISA tests have also been described (Gabal *et al.,* 1983a; Gabal & Khalifa, 1983). ID and CF tests also have been reported as useful tests (Soliman *et al.,* 1985). Differential diagnosis includes glanders, strangles, ulcerative lymphangitis and sporotrichosis, especially when these diseases occur under the same environmental conditions (Quinn *et al.,* 1994). Histofarcin test could play a significant role in detecting early infection and in differential diagnosis (Ameni *et al.,* 2006a; Asfaw *et al.,* 2012).

2.2.4. Treatment, Control and Eradication

Intravenous dosing of iodide may be used particularly in endemic areas. The intravenous injection of 100 ml of sodium iodide of a 10% solution, repeated weekly for four weeks is recommended. Different antifungal drugs have also been used and successful treatment with amphotericin B has been reported (Richer, 1977; Gabal, 1984; Radostits *et al.,* 2006). Control and eradication of the disease is usually through elimination of the infection, through slaughter (culling) of infected horses and application of strict hygiene practices to prevent spread of the organism. There are also reports on the use of killed and live attenuated vaccines (Zhang *et al.,* 1985).

2.3. Pathology and Clinical Manifestations

There are three forms and three stages of the disease. The three forms are cutaneous (skin), ocular, and respiratory forms. The three stages are early, moderate and sever stages. Some horses are asymptomatic carriers of HCF. The form that the disease takes seems to depend primarily on the route of entry (Ameni & Siyoum, 2002). EZL causes painful skin lesions that lead to lameness and loss of use of working equids, if the route of entry is by contact of infected material with traumatized skin. It may cause sinusitis, pneumonia via inhalation of contaminated dust, it may cause rhinitis by contact with skin lesions, and it may cause keratoconjunctivitis if transmitted by flies (Ameni, 2007).

In all the three forms of the disease, severe inflammatory reaction, oedema, necrosis & pyogranulomatous nodular lesions are observed (Al-Ani *et al.,* 1998). Generally the granulomatous nodular lesions are usually confined to the skin, subcutaneous tissues, lymph vessels and lymph nodes. But in some cases, the lesions may extend to the underlying joints, resulting in arthritis, periarthritis or periostitis.

The granulomatous nodular lesions and abscesses may be found in the lungs, spleen, liver, testes and other internal organs in some cases (Jubb *et al*., 2006; Radostits *et al.,* 2006).The lesions usually heal spontaneously after 2–3 months, resulting in stellate scar formation (OIE, 20008).

The cutaneous form of the disease is the most frequent one, that epizootic lymphangitis is most commonly characterized by a cord-like appearance of the subcutaneous lymphatic and ulcerative cutaneous pyogranulomas (Gabal *et al.,* 1983; Ameni, 2007).HFC may cause sinusitis, and/or pneumonia via inhalation of contaminated dust, it may also cause rhinitis by contact with skin lesions, and it may also cause keratoconjunctivitis being transmitted by flies (El-Gundy *et al*., 1975; Jubb *et al*., 2006; Ameni, 2007).

Epizootic lymphangitis can also present as an ulcerating conjunctivitis, or rarely as a multifocal pneumonia. (Ameni, 2006: AL-Ani, 1999). When mucosal lesions occur,

most are confined to the upper respiratory tract and eyes (AL-Ani, 1999). Cutaneous lesions are also seen in the respiratory form of the disease but are accompanied by nasal discharge and severe coughing (Al-Ani *et al.,* 1998).

Typical lesions of epizootic lymphangitis are pyogranulomatous nodules that may be appreciated in lymphatic vessels. Pyogranulomatous nodules and the liquefied foci have also been found in the pleura, spleen, liver, testes, tunica vaginalis, and bone marrow (Khater *et al.,* 1968; Fawi, 1971; Jubb *et al*., 2006). Interstitial pneumonia may also be recognized. Although lesions may be present in other organs (Al-Ani *et al.,* 1998), the infection rarely becomes generalized in type (Ajello, 1968; Khater *et al.,* 1968; Addo, 1980).

2.3.1. Clinical Signs

Clinical signs of Epizootic lymphangitis are described based on the pathological lesions; otherwise the body temperature and general character of the animals are not changed. On the other hand most of the animals will lose their body condition (AL-Ani& Al-Delaimi, 1986; Radostits *et al.,* 2006; Ameni, 2007).

The cutaneous form of the disease, after which the disease was named, is the most common. Clinical signs are observed several weeks to 6 months after infection. The initial lesion is an open granulomatous wound along the course of a lymphatic vessel, which has a tendency to ulcerate, or to undergo alternating periods of discharge and closure for some weeks before healing with residual scar formation. Lesions are most common in the forelimbs, the chest wall, and the neck. In severe cases, skin over the entire body may be affected (Khater *et al.,* 1968; Guerin *et al.,* 1992; Ameni, 2007).

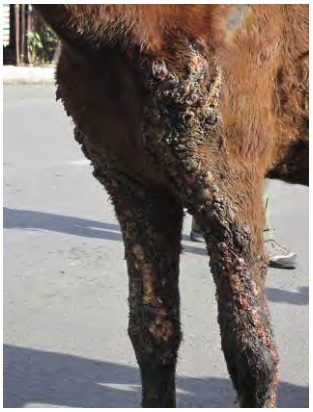


Figure 2. Cutaneous form of EZLin a carthorse. (Courtesy of Dr. Nigatu, SPANA, Ethiopia.) (ANDREW, 2014)

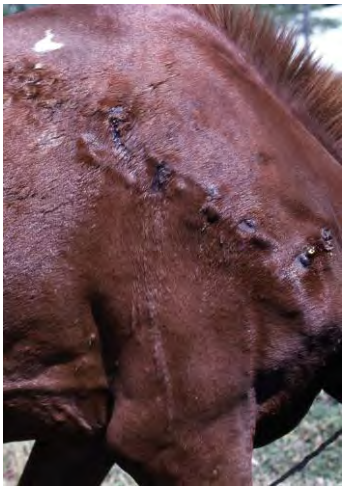


Figure 3. Cutaneous form of EZL in lymphatics of shoulder and neck of horse (Debra *et al.,* 2014).

The ophthalmic form of the disease is less frequent. Infection may occur as conjunctivitis or a naso-lachrymal infection (Fig. 2). Initial infection is characterized by a watery discharge from one or both eyes and some swelling of the eyelids, followed by the development of papules and ulcerating button-like growths on the conjunctiva and/or on the nictitating membrane (Singh, & Varmani 1966; AL-Ani& Al-Delaimi, 1986).

Figure 4. Ophthalmic form of EZL: A button-like growth on the eyelid (AL-Ani, 1999).

The pulmonary form of the disease is infrequent, which usually occurs as a late development in the cutaneous form of the disease (OIE, 20008). The nasal lesions are characterized by serous secretion, multiple small gray nodules, and or ulcers (Ameni, 2007). They are mostly confined to the upper respiratory tract. They usually found near the external nares and they may extend to the muzzle, or they may be found deep in the nasal cavity and in the pharynx, nasal sinuses, the larynx and the bronchi (Jubb *et al*., 2006).

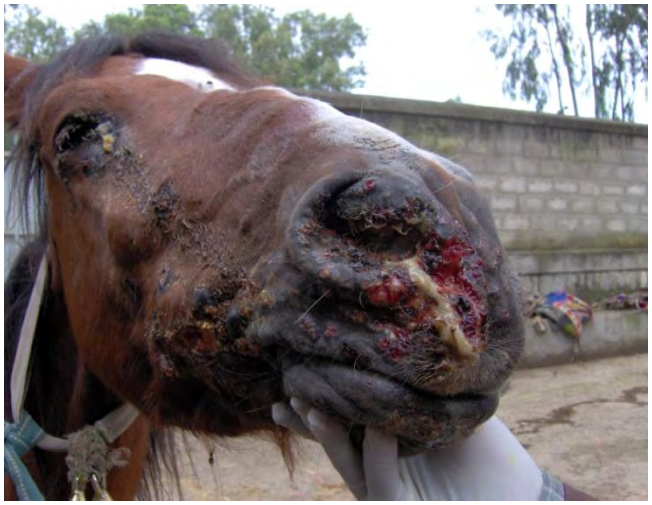


Figure 5. Pulmonary form of EZL

Some horses are asymptomatic carriers of HCF. They do not show clinical signs. They can be identified clinically by the identification of fibro-calcification of skin lesions at previous sites of infection, or based on serologic evidence of antibodies, and positive reactions to intra dermal tests. These methods do not distinguish exposure from chronic infection. (AL-Ani & Al-Delaimi, 1986). Such horses will give a positive result to an intra dermal sensitivity test and positive reactions to serological tests (Soliman *et al.,* 1985; AL-Ani, 1989).Six bacterial contaminant genera are isolated from lesions of epizootic lymphangitis and some of them are frequently found that they potentially contribute to the clinical signs, pathogenesis and to the severity of the disease, leading sometimes to death in severely infected horses (Hadush *et al.,* 2014).

2.3.2. Pathogenesis

After gaining entry through wounds, HCF 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. 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. Nodules occur wherever there is skin trauma (particularly under the harness and on the extremities). Horses that have a heavy systemic burden of fungi may succumb to pneumonia or failure of other affected organs. (AL-Ani, 1999; Jubb *et al*., 2006; Radostits *et al.,* 2006).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 occurred as the extension of the skin form, because the animals will scratch the skin lesions by their teeth and lips, thereby spreading it to the surrounding organs (Ameni, 2007).

2.3.3. Gross lesions

Gross lesions are manifested by pyogranulomatous, purulent discharge of thickened superficial lymphatic vessels and enlargement and inflammation of regional lymph nodes (AL-Ani, 1999). Regional lymph nodes are swollen, soft, and reddened and may contain purulent foci. Lymphatic vessels may be found distended with pus (Al-

Ani *et al.,* 1998). At necropsy, areas of the skin and subcutaneous tissue are thickened, and the skin may be fused to the underlying tissues. When the thickened skin is incised, it presents the lardaceous appearance of granulation tissue and it contains a number of small, yellow, purulent foci between which the lymphatics are dilated and filled with pus and serous fluid (Jubb *et al*., 2006).

In the early stages, the skin between the lesions remains normal and mobile, except in areas of extensive ulceration.The skin covering the nodules and the subcutaneous tissues may become thickened, fibrous, indurated, and firmly fused to the underlying tissues (Al-Ani *et al.,* 1998; Jubb *et al*., 2006; Ameni, 2007).In the early stages, the swollen nodes contain many small foci of softening, but later the foci coalesce and are heavily encapsulated and they may rupture to form ulcers (Jubb *et al*., 2006).

There is often thickening, or 'cording', of lymphatics, with the formation of pyogranulomatous nodules that have a thick, fibrous capsule (OIE, 20008). Nodules in the skin have a thick, fibrous capsule and the affected lymphatic vessels are usually thickened or distended. Both nodules and lymphatics contain purulent exudates (IVIS, 2006). The forelimbs, neck, and head are common sites to observe the Nodular and chronic suppurating lesions, however they are also observed on different body parts including the scrotal regions. In advanced cases, the nodules and ulcers may involve almost all body parts and may have unpleasant odor (Ameni, 2007).

Figure 6. Gross appearance of EZL lesions on the legs (Photo: Stephanie L. Church/TheHorse.com)

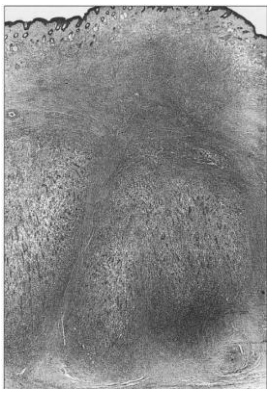


Figure 7. Intradermal node (Jubb *et al.,* 2006).

The conjunctival form of the disease may begin quite frequently on the conjunctiva or nictitating membrane, producing at first a small papule and a serous conjunctival discharge (Jubb *et al*., 2006). Serous discharge from the infected eyes of mule is observed by Ameni, 2007. The papules ulcerate to form flat, button-like growths of granulation tissue, the eyelids become severely swollen, and the inflammation extends to the tissues of the forehead (Jubb *et al*., 2006). Multiple, small, gray–white nodules or ulcers with raised borders and granulating bases may be apparent on the nasal mucosa, and on the conjunctiva and cornea. Several purulent foci may be apparent on cut section (AL-Ani, 1999).Sometimes the infection spreads into the facial tissues, seen as small nodules. If cases go untreated, secondary infections and severe ocular disease can occur.

The pulmonary lesions may be solid granulomatous areas or they may be liquefied with pus-like contents (Jubb *et al*., 2006).Nasal infection is usually accompanied by a mucopurulent discharge that may be bloodstained. On the nasal mucosa, the lesions begin as yellow flat papules or nodules on the nasal mucosa and these soon break down to form craterous granulating ulcers that bleed easily. When ulcerative lesions are present on the nasal mucosa, there is suppurative regional lymphadenitis (Jubb *et al*., 2006). Nodules and abscess could occur in the lungs in respiratory form of the disease (Ameni, 2007).

2.3.4. Histopathology

In Haematoxylin and Eosin (H&E) stained histological sections, the appearance of the lesion is quite characteristic that it consists of pyogranulomatous inflammation with fibroplasia. Langerhans giant cells are common (Radostits *et al.,* 2006; Ameni, 2007; OIE, 20008). (Fig. 8 & 9) histologically, granulomas are characterized by the presence of many large macrophages, often containing yeast cells that are seen after staining (Al-ani, 1999). In the greater majority of nodules, the most prominent infiltrated cells are mononuclear cells, but some nodules have predominantly polymorphonuclear cells (Al-ani *et al.,* 1998). These lesions may also occur in the lungs (Week's *et al.,* 1985; Rippon, 1988).

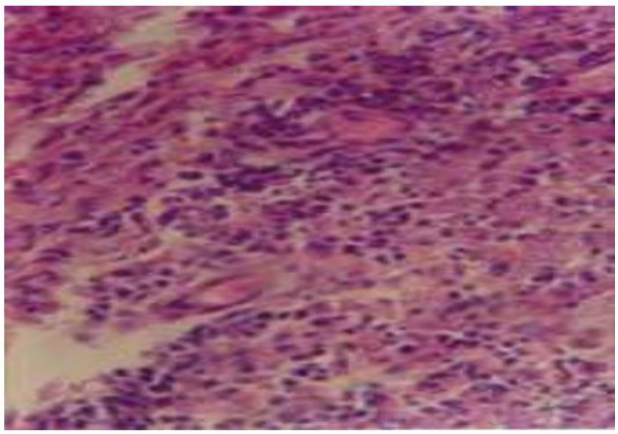


Figure 8. Microscopic appearance of EZL lesions (Ameni, 2007)

The presence of numerous HCF, some of which show budding, both intra- and extracellularly in tissue sections stained with H&E stain is of diagnostic value (Radostits *et al.,* 2006). There is some indication that the number of HCF in tissue sections increases with chronicity. Although the fungus can be demonstrated by H&E, special stains with PAS or GMS stain may be of value (AL-Ani, 1999).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Oromia Regional State, central Ethiopia in eight towns (Adama, Mojo, Bishoftu, Dukam, Akaki, Sabata, Tafki and Walliso). The first five towns are situated in east direction, while the last three are situated in south-west direction with about 100 km radius from Addis Ababa, the capital city of Ethiopia.

Clinical observations, selection of study animals, collection of biopsy sample and other field activities were accomplished in veterinary clinics located within the study towns. Postmortem examinations were carried out at Sabata laboratory to which humanely euthanized horses were transported. The postmortem investigations the histopathology work was carried out in the pathology laboratory of NAHDIC at Sabata, Ethiopia.

3.2. Study Animals

The study animals considered in this study were carthorses located in the study areas that are naturally infected with *Histoplasma capsulatum* variety *farciminosum* (*HCF).* Clinically infected carthorses 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. This was further confirmed by microscopic examinations of samples from the clinically infected horses. Both biopsy and necropsy methods were used for sample collection.

Table 1: Distribution of sampled carthorses

Carthorses sampled	Body condition score			Severity stage of EZL			Total
	A	b	C	1	2	3	
Only by biopsy	3	10	9	5	8	9	22
Both by biopsy and autopsy	1	4	8	0	2	6	8
Total	4	14	12	6	12	18	**30**

Key: a→good; b→moderate; c→poor body condition score

1→early; 2→moderate; 3→ sever stage of EZL.

Seventy clinically infected local breed carthorses were found using the field surveys and out of the 70 carthorses 30 were purposively sampled based on the giemsa staining technique result of FNA samples and accessibility of cases. Among the 30 selected carthorses, only biopsy samples were collected from 22 of them, whereas both biopsy and autopsy samples were collected from 8 of them based on accessibility of cases for autopsy.

An attempt was made to purposely include all the three forms and the three stages of the disease in the selected animals, however, only the cutaneous forms of the disease were found. Although the three stages of the disease were included in the selected animals, the number of carthorses sampled from early case were small, only 5, (Table 1) due to problem of accessibility i.e. unwillingness of the owners to allow for biopsy sample collection.

The 30 selected carthorses were assigned into 3 categories as cases with good, moderate and poor body condition (see Appendix 4& Table 1). And once again they were also grouped into 3 categories as early, moderate and sever stage of the disease severity. Out of the 8 carthorses necropsied, only 2 cases were at moderate and 6 were at the last stage; there were no cases necropsied at early stage (Table 1).

3.3. Study Design

A cross-sectional study design with purposive sampling method was used to identify carthorses showing typical clinical signs of EZL from March to August 2015. Field surveys for clinical cases were carried out with the help of carthorse owners' association leaders. The leaders were used as the source of information to get the clinically positive carthorses. The veterinary clinics of study areas were used as the sample collection spot.

Careful and systematic clinical examination of carthorses in the study areas were done in order to identify animals with a clinical signs of EZL and then confirmatory laboratory examination was performed followed by sample collection. During field surveys records of observed signs, body parts involved, number of topical nodules or ulcers, body condition of the selected carthorses and severity of the disease were recorded.

3.3.1. Field surveys for clinical cases

The field surveys were carried out to look for clinically sick carthorses and 70 clinically infected horses were found across all the study areas. During the field surveys an informal interview was carried out with carthorse owners and carthorse association leaders. The interview was focused on the way to find sick carthorses, the knowhow they have about the disease and the solution they experienced for the disease. It was performed in two phases. In the first phase, an open questioner with four questions was administered for 25 persons selected randomly. In the second phase, a closed questioner with five questions was interviewed for 100 persons selected randomly. Randomization was achieved by considering every 10^{th} arrival carthorse owner as selected entity. The closed questioner was prepared based on the information gathered from the open questioner (Appendix 4). Additionally an informal epidemiological assessment was also carried out.

3.3.2. Microscopic examination

Samples were collected using FNA (fine needle aspiration) method from un-ruptured nodules and they were used for microscopic examinations to identify the positive carthorses. Before collection of samples the nodules were washed with soap and water, shaved, and disinfected with alcohol. The contents of the nodules were aspirated with sterile needles and syringes, and used for the preparation of smears for microscopic examination. The smears were fixed with methanol, stained with Giemsa stain for the identification of the yeast form of *HCF*. Examination was made using 40 x magnification followed by oil immersion at 100 x magnification until the fungus was demonstrated in the clinical specimens as described by Asfaw *et al.,* 2012.

3.3.3. Isolation of the agent; HCF

An attempt for isolation HCF was executed according to Ameni (2007); OIE (2000). The lung tissue sample was taken from a nodular lesion on the lung, it was grinded with pistol and mortar and inoculated aseptically onto slants of Sabouraud Dextrose Agar (SDA, Oxoid) containing chloramphenicol (0.5g/ liter) and enriched with 2.5% glycerol. Incubation was made at 26 ^{0}C and 5% CO_2 for 8 weeks. The media were checked periodically for the presence of growth of mycelial form of HCF.

3.3.4. Sample collection

Photograph of superficial gross lesions were taken from selected carthorses and then biopsy samples were collected for histopathological examination.

3.3.4.1. Biopsy sample collection

Biopsy samples were collected in a welfare manner using a ring block infiltration with lidocaine. The nodule were first washed with soap and water, shaved, and disinfected with alcohol just before biopsy sample collection to prevent contamination, as *HCF* is also a saprophyte. Four mm tissues were incised from cutaneous lesions using a sharp surgical blade and placed in 10% formalin. The samples were collected from active nodular lesions including both normal and pathological parts (Appendix 1.).

3.3.4.2. Autopsy sample collection

The carthorses that were purposively selected or purchased for autopsy were first euthanized and then transported to NAHDIC postmortem hall (Figure 9.). Euthanasia was under taken under the supervision of SPANA, Ethiopia project staffs. The carthorses were euthanized by fully anaesthetizing with 20 ml thiopental sodium IV injection and then killed by IV injection of potassium chloride. Topical (skin) nodule samples were collected just before euthanasia after full anesthesia dose is reached. Necropsies of euthanized horses were undertaken according to the procedure by Dennis and Joanna (2006) (Annex 1).

Figure 9. Euthanasia (A) and transportation of carcass to postmortem hall (B)

Euthanized horses were examined thoroughly for gross pathological lesions which includes lesion distribution, contour, texture, shape, size and color (Table 2.), then data were recorded and Photograph of gross lesions were taken. Although examinations for gross pathological lesions were undertaken on the whole carcass, special attention was given to the pleura, lung, upper respiratory tracts, eye, spleen, liver, testes, and tunica vaginalis.

Table 2: Detailed records of autopsied cases

Case No	Age	Body condition	No of B. parts involved	Severity of EZL	No of topical nodules	No of topical ulcers	Total No of topical lesions	No of small nodules	No of large nodules	Total
I	14	Moderate	5	Severe	22	28	50	32	18	50
II	16	Moderate	4	Severe	29	32	61	41	20	61
III	14	Moderate	3	Severe	24	26	50	27	23	50
IV	15	Good	2	Moderate	6	11	17	0	17	17
V	14	Poor	5	Severe	17	23	40	12	28	40
VI	14	Moderate	4	Severe	18	23	41	28	13	41
VII	16	Poor	5	Severe	23	26	49	34	15	49
VIII	15	Poor	2	Moderate	11	13	24	15	9	24
Total					150	182	**332**	189	143	**332**

Autopsy samples were collected from the internal lesions of those carthorses and additionally, from normal tissues of autopsied cases in which no gross lesion were found. Tissues with lesions were cut to the size of 4mm with a sharp blade and put in

the universal bottle containing 10% buffered neutral formalin. The volume of the formalin was adjusted to be ten times larger than the size of the tissue sample according to Talukder (2007) (Appendix 2).

3.3.4.3. Histopathology techniques

Both the biopsy and autopsy samples were submitted and stored in sterile manner at NAHDIC pathology laboratory until they were tested by histopathology. Tissues samples were trimmed and put in to plastic tissue cassettes and then processed using an automatic tissue processer.

Within the automatic tissue processer the followings were performed. Firstly, the specimen were fixed in 10% buffered neutral formalin(Formalin-I for2 hours & Formalin-II for hours), secondly, dehydrated in ascending grades of alcohol1 (70 % for1 hour, 95 % for 1 hour, 100% I for1 hour, 100% II for2 hours & 100% III for 2 hours), thirdly, cleared with xylene (Xylene-I for 1:30 hours, Xylene-II for1:30 hours & Xylene-III for 1:30 hours) and finally, impregnated with molten paraffin wax(Paraffin-I for12 hours & Paraffin-Il for 3 hours). Totally the tissue specimens were processed within the automatic tissue processer for 20 hours & 30 minutes and finally tissue specimens embedded with paraffin wax named as tissue blocks were removed from the machine.

Then the tissue blocks were sectioned at a thickness of 4 to 5 μm using a semi-automatic microtome machine, tissue ribbons were spread on warm water bath and tissues were attached to albumenized glass slides. Then the slides were incubated in incubator at 60 ^{o} C to avoid paraffin wax. The sectioned tissues were then deparaffinised in three changes of xylene (Xylene-I, Xylene-II & Xylene-III), and then rehydrated in descending grades of alcohol(100% I, 100% II, 100% III, 95 %, &70 %) and stained with H&E stain, mounted with DPX, covered with cover slip and finally examined at 4x, 10x, 40x and 100 x magnification under light microscope for the presence of microscopic lesions and/or the yeast form of *HCF* on the tissue samples and finally photographs of the slides were taken.

3.4. Statistical Analysis

Data collected from the study animals were stored in MS excel spread sheet and analyzed with SPSS version 17. Accordingly, the gross as well as histopathological lesions and findings were described using qualitative methods. One-way ANOVA analysis was performed by taking body condition score of the carthorses as an independent variable and number of ruptured, unruptured, small and large nodules as dependent variable at 95% confidence interval. And once again the same procedure was repeated by taking severity stages of EZL as an independent variable. Additionally multiple comparisons were also performed between the factors of independent variables.

4. RESULTS

4.1. Results of Field Surveys

The results of informal interview are summarized in Table 3. Regarding the knowhow they have about the disease, 76% of the interviewed carthorse owners have taken at least one training on EZL from veterinarians of SPANA. However, 44% of them still believe that the disease is caused by an evil-eye or "Buda" and it attacks carthorses with good body condition. Thirty seven percent of the interviewed carthorse owners also believe that the disease is of two types, namely 'male' and 'female' types, having a character of small and large nodular lesions, respectively. As their belief, the female type is the severe type (Annex 4 & Table 3).

Table 3: Results of closed questionnaires in percentile

Questions	Yes or to traditional healers (%)	No or to veterinarians (%)	I don't know or Both methods (%)	Total (%)
Do you get at least one training on EZL?	76	24	-	100
Do you think that EZL is caused by an evil-eye?	44	56	-	100
Do you believe EZL has two types of lesions?	37	35	28	100
Do you think EZL is a curable disease?	64	36	-	100
Where do you go for treatment of EZL?	14	15	71	100

See annex 4, for the full questionnaires.

Regarding the solution they experienced for the disease, 71% of the interviewed carthorse owners are accustomed to use both traditional and modern treatment methods. The latter is used to be carried out by veterinarians of SPANA, while the former is used to be carried out by local personals called “Beka” who uses medicinal plants. Sixty four percent of them believe that the disease has a probability of being cured if treated at early stage in either of the treatment methods. Early and moderate stages of EZL are considered curable and SPANA is accustomed to treat such animals, however, the fate of carthorses with sever stage of the disease is euthanasia both for the sake of welfare and disease control.

According to the carthorse owners, after repeated trial of both treatment methods the owners of affected carthorses are accustomed to abandon the uncured carthorses. Some of the owners of abandoned carthorses, especially those who accept the false believe, neither allow euthanasia nor want to be responsible for the abandoned carthorse. Severely affected and abandoned carthorses were usually found staggering at the middle of highways (Figure 10). There were some carthorses that had the gross lesions of EZL that found together with the normal at carthorse gathering stations. They were found at different stages of the disease, however, in routine work as the normal carthorses. There were some owners that were observed while using even severely affected carthorses.

Figure 10. Abandoned carthorses staggering at the middle of highways

4.2. Clinical Observations

All the 70 EZL suspected carthorses showed signs of the cutaneous forms of the disease. There was no a distinct case with signs of ocular and or respiratory form observed rather there was a manifestation respiratory signs in some cases with the cutaneous forms of the disease. Over all the observed clinical signs were loss of body condition or emaciation (Figure 11, B), reduced appetite, restlessness, scratching of the lesions (Figure 11, A), lameness, purulent nasal discharge, coughing, and dyspnea. Pungent smell from ruptured nodules or ulcers was observed almost in all cases that had at least one ruptured nodule. The bad odor was also observed in some unruptured nodules while aspirating pus sample but it was not observable from distances as in case of the ulcerated lesions. The body temperature was in a normal range (37.5 - 38.5) as fever is uncommon in EZL.

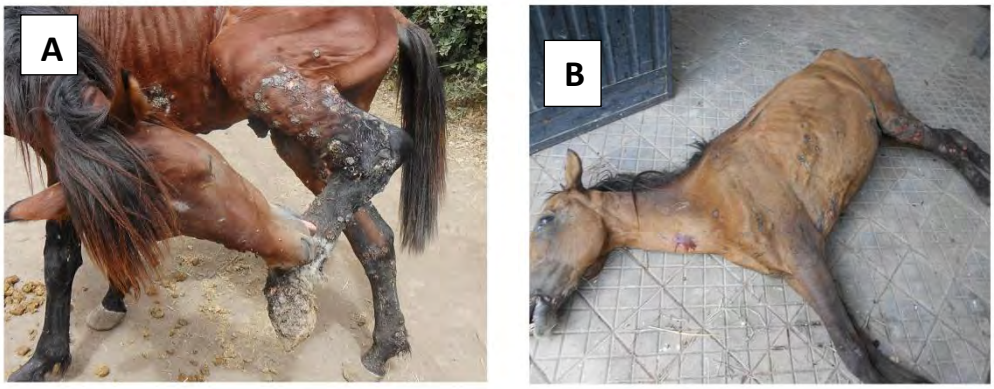


Figure 11. Scratching the lesions (A) and emaciation (B)

4.3. Microscopic Examination Result

Giemsa stained smears of the contents of pyogranulomatous skin nodules(Figure 12) and imprint smear of affected internal organs(Figure 13) revealed yeast like organisms that are round to oval in shape with about 2 -4 μm in diameter. They were found either with unstained transparent or with faint blue cytoplasmic space; either free or intracellularly within mono-nucleated leucocytes mostly being phagocytized within the macrophages (Figure 12 and 13).

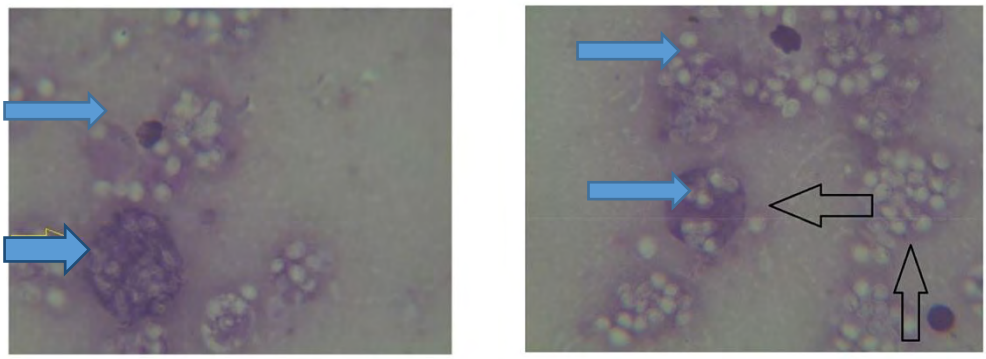


Figure 12. Giemsa stained smears of FNA samples of skin nodules

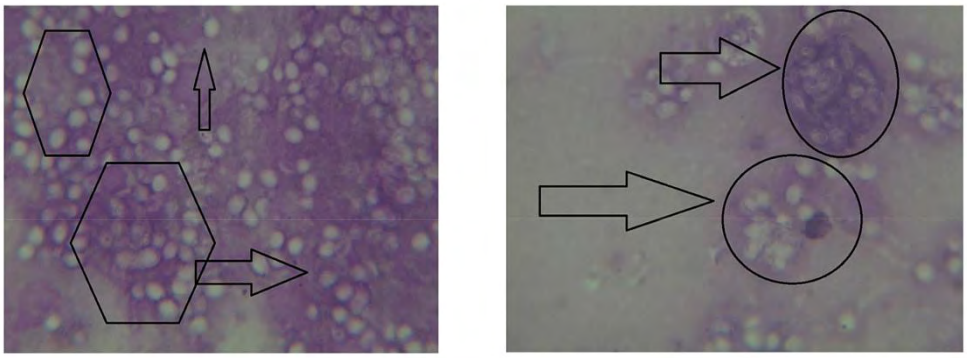


Figure 13. Giemsa stained smears of imprint smear sample from internal organs

The attempt executed for isolation the agent, *HCF*, was not successful. The media were checked periodically for the last eight weeks for growth of mycelial form and no growth was detected until the eighth week of inoculation.

4.4. Macroscopic Examination Result /Gross Pathology/

The body parts affected with lesions was skin and superficial lymphatics of skin of the body extremities. Nodular lesions were observed on different body parts including the limbs, chest wall, face, neck, abdominal region, pennies, and cervical regions. But they were observed mostly in the limbs, lower trunk and head regions (Figure. 14). Severely affected regions were found to be the limb extremities, especially the hind limb extremities. Tick infested parts seems to be more vulnerable (Figure. 15).

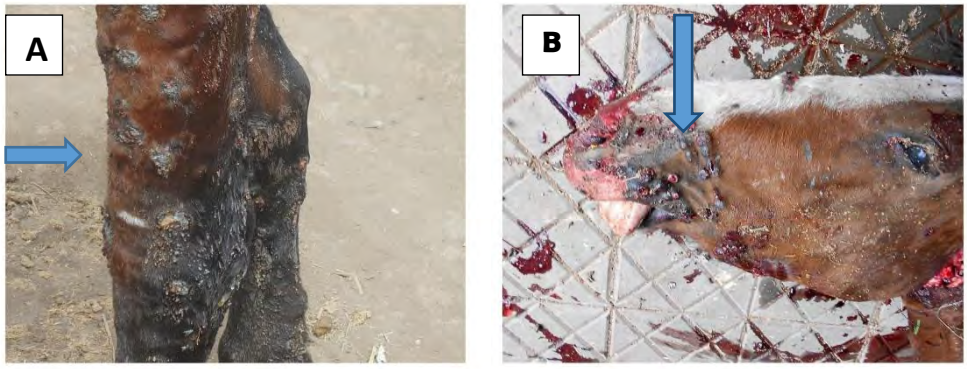


Figure 14. EZL lesions on limbs (A) and face (B)

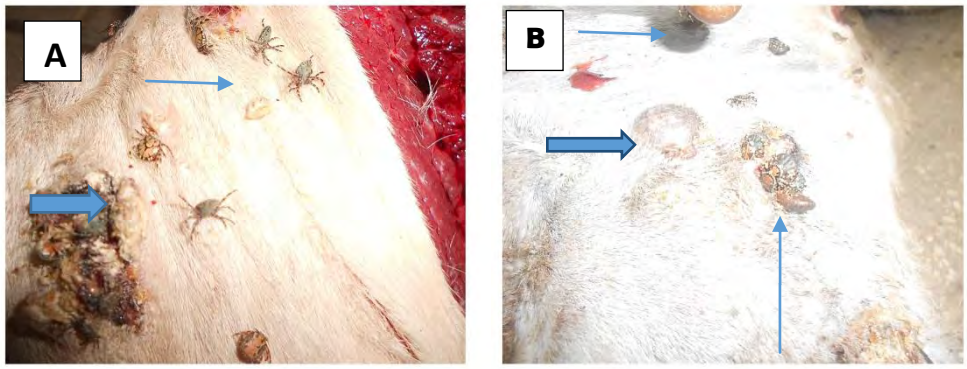


Figure 15. Tick infested body parts being more vulnerable

The common and consistent pathological findings observed almost in all cases of necropsied carthorses in general include congestion, hyperemia, enlargements of superficial regional lymph nodes, and distention of superficial lymph vessels. Those lesions were also common pathological findings for topical and internal organs

4.4.1. Topical gross lesions

There were mixed types of lesions in affected body parts which includes unruptured nodular lesion, ruptured nodules that form ulcers and scar tissues. In some cases, the nodular lesions were found to be small and inconspicuous (Figure14-B) while in others large and prominent (Figure16&Figure14-A). There were coalescing ulcers with purulent discharge of yellow color from ulcers. There were cords like thickening of superficial lymphatics of skin with or without further skin involvement (Figure16).

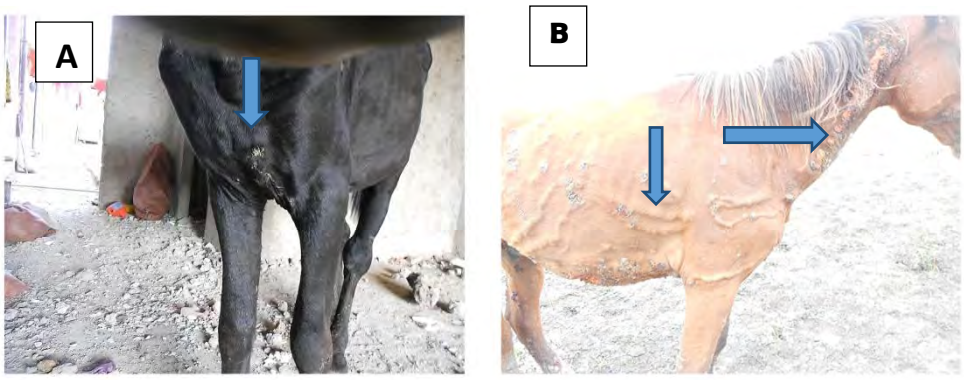


Figure 16. Large (A) and small (B) nodules; cord like thickening of lymphatics (B)

In severe cases, ruptured nodules were observed to be arranged in a line following affected lymphatic vessels of skin (Figure16-B). Some un-ruptured nodules were flabby with softened and pointed tip(Figure15-B).Freely moveable intra dermal nodules were also observed, they were few in number 2 to 3 and localized in some anatomical region.

Skin ulcers with cycles of granulation and partial healing together with new eruptions of lesion were also observed. In some cases the surrounding skin was edematous or thickened, hard and painful, while in others the skin over the nodules was fixed to the underlying tissues. In few cases the regional lymph nodes were found to be grossly normal, while in most of the cases regional lymph nodes were found to be enlarged and edematous. Some of the enlarged lymph nodes were firm and dry (Figure16).

4.4.2. Gross lesions in internal organs

The gross lesions in internal organs were few; only in five cases out of the eight carthorses examined for necropsy. Generally diverse types of lesions were detected on trachea, lung, liver, small intestine, cecum, and colon. There were enlargements of internal regional lymph nodes but there were no grossly observable distended internal lymphatic vessels.

There were also some exceptionally observed lesions like mass of nodule on mucosal part of trachea of case three (Figure17), typhlitis with hyperemia on case six (Figure19), liver cirrhosis and enteritis on case seven (Figure 20), and a large nodular lesion of right lung surrounded by diffused small granulomatous nodules of both lungs of case eight (Figure 18), are the reportable findings of this study.

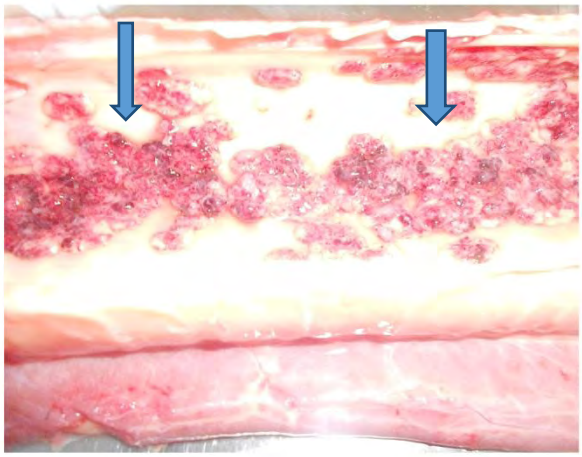


Figure 17. Nodular masses on upper part of tracheal mucosa

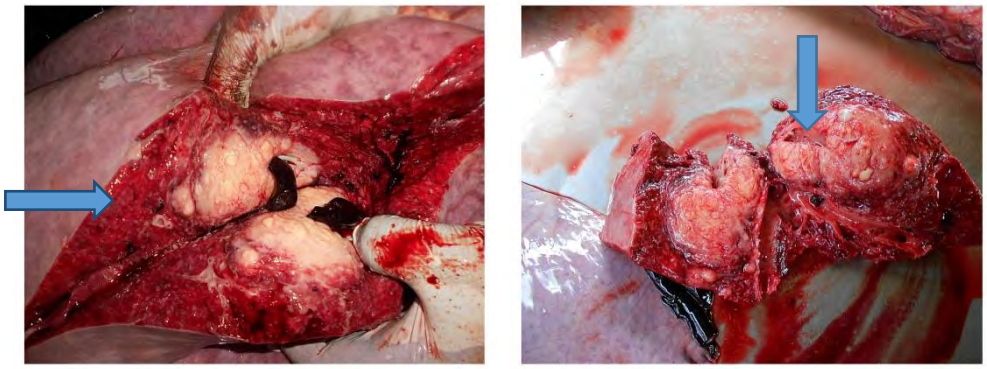


Figure 18. A large granulomatous nodule on lung

Particularly the tracheal nodular mass and the large nodular lesion of right lung of case three and case eight (Figure17 &Figure18) respectively are lesions with special consideration of this study which might be directly associated with EZL. Otherwise most of the internal organs of carthorses necropsied in this study were found to appear normal on gross examination.

There was no gross lesion detected on internal organs of case one, two and four, except for the mentioned common lesions. The carthorses of case one and two were in sever stage of the disease and in moderate body condition while the case four carthorse was in moderate stage of the disease and in a good body condition(Table 2).

The raised nodular masses were located on the upper part of trachea on its mucosal part. The lesions were multifocal coalescing in distribution (Figure17). They were firm in consistency, white to pale in color and with about 2-3 mm diameter of each individual nodule. There was neither grossly observable external lesion on nasal and oral opening of this animal nor clinically observable respiratory problem. Except for this lesion the other internal organs of this animal including the lung were found to appear normal on gross examination. The animal was in moderate body condition and in severs stage of the disease (Table 2).

The typhlitis was characterized by a diffused hyperemia on both mucosal and serosal surfaces of the cecum (Figure19). Additionally there were multi focal calcification on liver (Figure19) and multi focal pin head sized hemorrhage on the diaphragm of this animal. There was also an ulcer on the penis (Figure 23). The animal was found to be in a moderate body condition and in sever stage of the disease (Table 2).

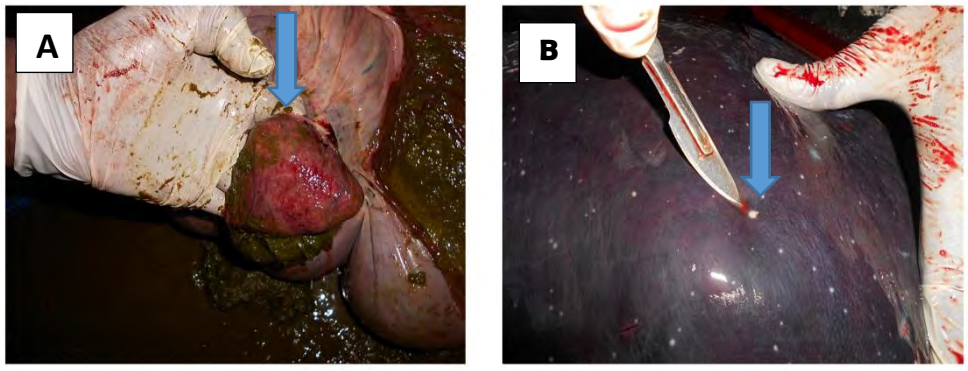


Figure 19. Typhlitis (A) and multi focal calcification on liver (B)

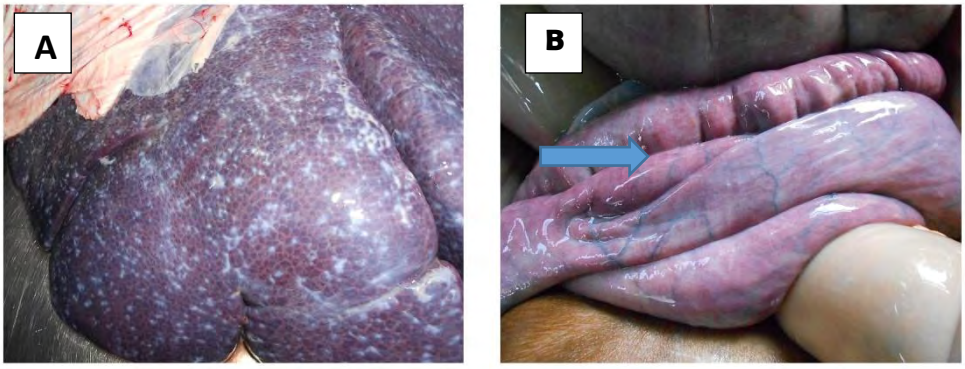


Figure 20. Liver cirrhosis (A) and enteritis (B)

The large granulomatous nodule was located on the cranial lobe of right lung. It was large in size with about 3 cm diameter, white to pale in color and hard in consistency (Figure18). On both lungs there were diffused small granulomatous nodules which appear on palpation as small sands buried within the lungs (Figure 21). They were many in number observed on entire parts of the lung, small in size with about 1mm diameter, white to pale in color and hard in consistency. The mediastinal lymph nodes of the respiratory system were found being enlarged. Enteritis with diffused hyperemia of duodenum (Figure 21) and arthritis (Figure 22) on the left stifle joint characterized by a coalescing granulomatous nodule was also observed on this case. There was an ulcer on the penis (Figure 22). The mesenteric lymph nodes in the intestine were found being enlarged. The animal was found to be in a poor body condition and in moderate stage of the disease (Table 2).

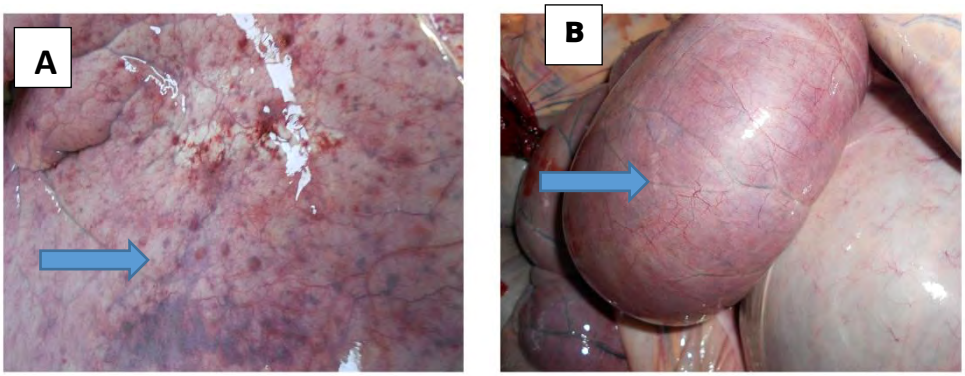


Figure 21. Diffused small granulomatous nodules on lung (A) and enteritis (B)

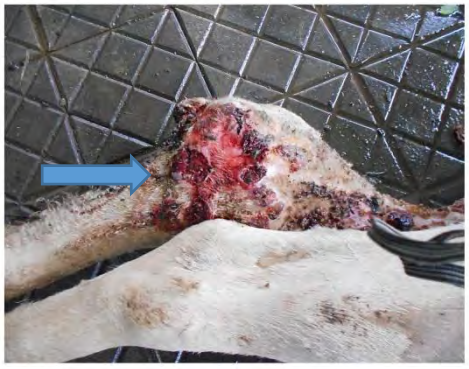


Figure 22. Arthritis on the left stifle joint

Figure 23. Ulcer on penis

4.5. Histopathological Findings

Tissue specimens collected for histopathology from biopsy and necropsy revealed pyogranulomatous (Figure 27, 28-A & 31-B)and granulomatous (Figure 24& 26) lesions with mononuclear infiltration(Figure 24) accompanied with, oedema (Figure 27), congestion (Figure 29-A) haemorrhage (Figure 29-B) and many yeast-like organisms within macrophages (Figure 30-A, 33 & 34-B). Also there were necrosis (Figure 28- B) observed in the microscopic lesions. There were tissue reactions in different organs (Figure 25 & 26). Those histopathological examination results were found both from samples collected by biopsy and autopsy or both from topical and internal organ lesions.

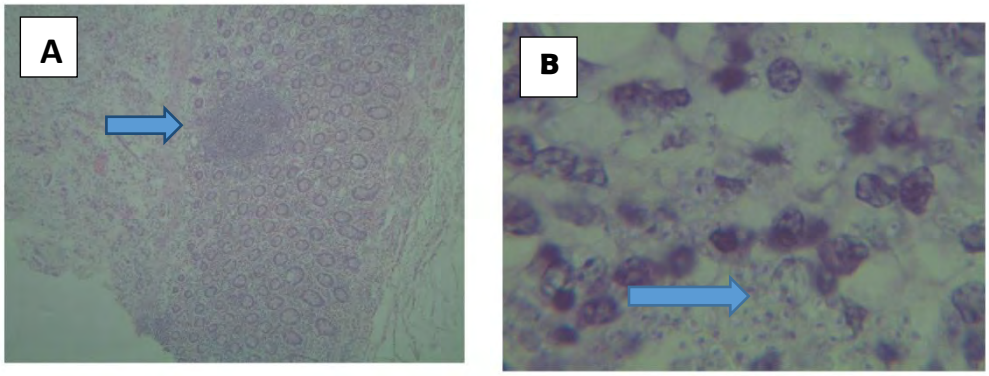


Figure 24. Granuloma & yeast-like organisms on cecum (4x; A & 100x; B)

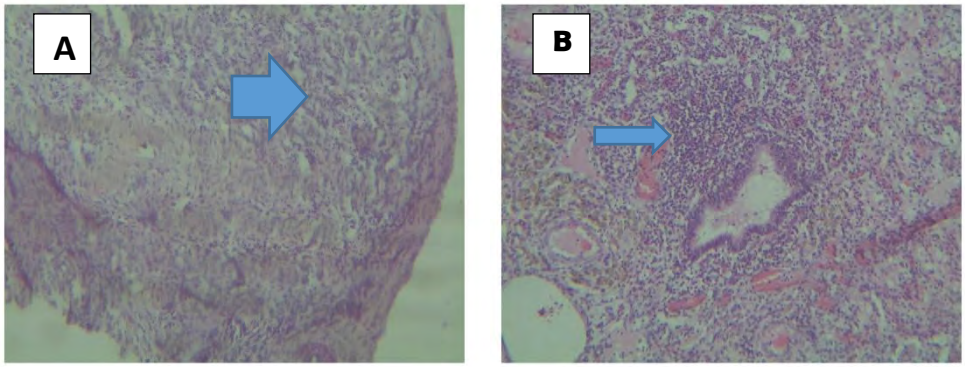


Figure 25. Tissue reaction on lung & around bronchioles (4x; A&10x; B)

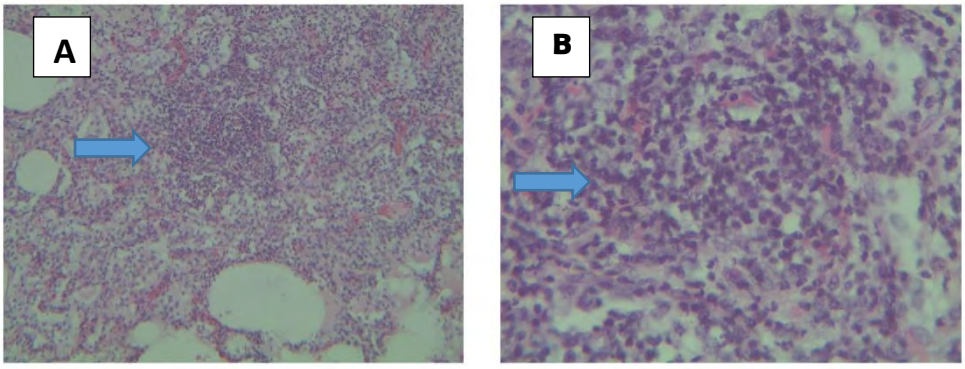


Figure 26. Tissue reaction with granuloma on lung (10x; A & 40x; B)

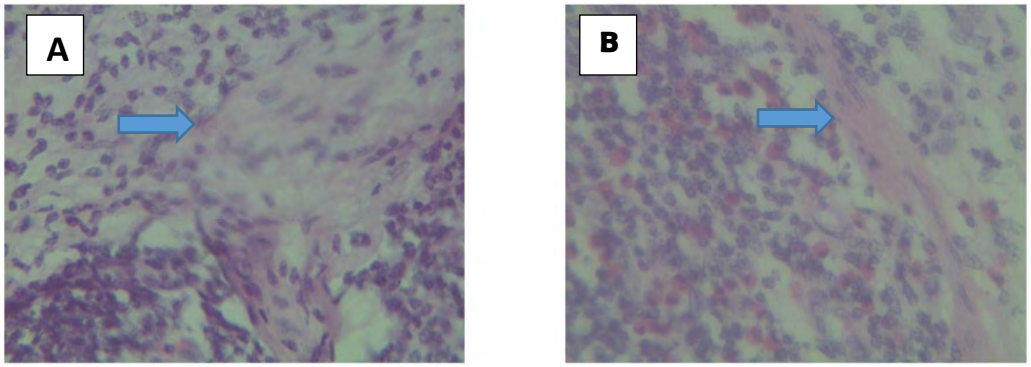


Figure 27. Oedema & pyogranuloma on lymph node (A) and cecum (B) (40x)

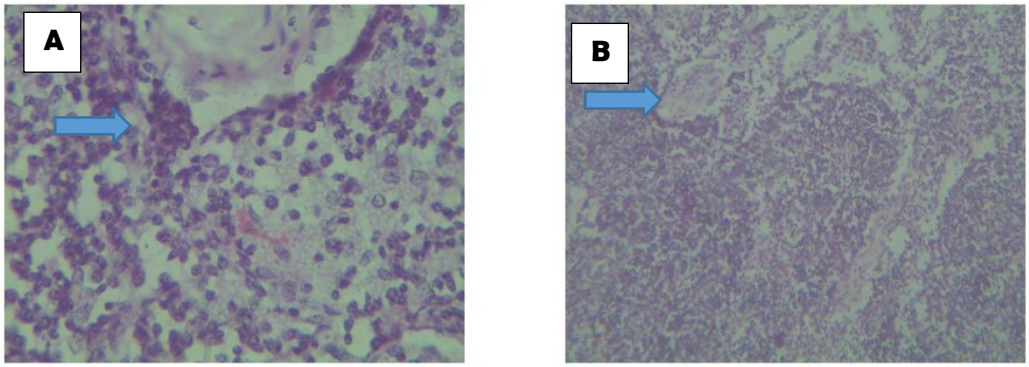


Figure 28. Pyogranuloma (A) & necrosis with pyogranuloma (B) on lymph node (40x; A & 10x; B)

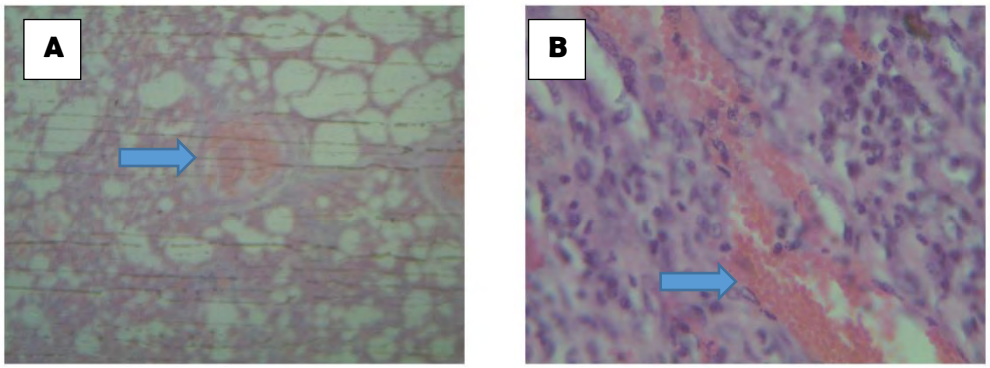


Figure 29. Congestion (A) & haemorrhage (B) (4x; A&40x; B)

The inflammatory cells that infiltrate the tissue were macrophages (Figure 30-A) epithelioid cell (Figure 30-B), polymorphonuclear cells (neutrophils and basophils)

(Figure 31-A) and plasma cells (Figure 32). The leucocytes were observed in most of the sections. The predominant infiltrating leucocyte type observed were macrophages, with a less common presences of polymorphonuclear cells and plasma cells.

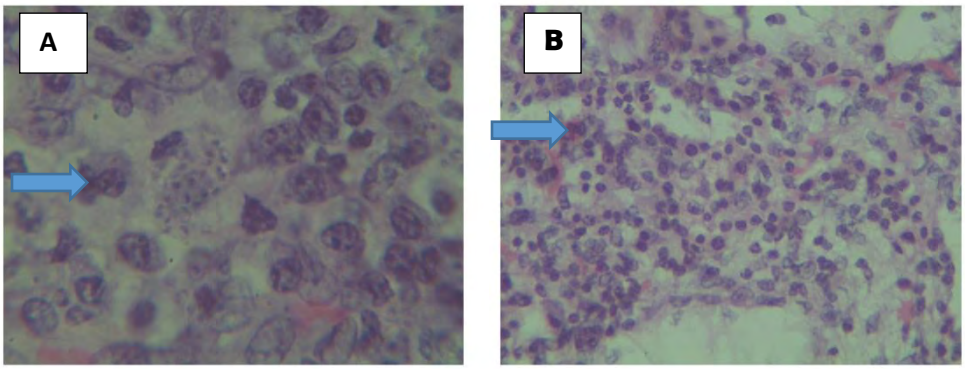


Figure 30. Distended macrophages (A) and foamy cells (B) (100x; A&40x; B)

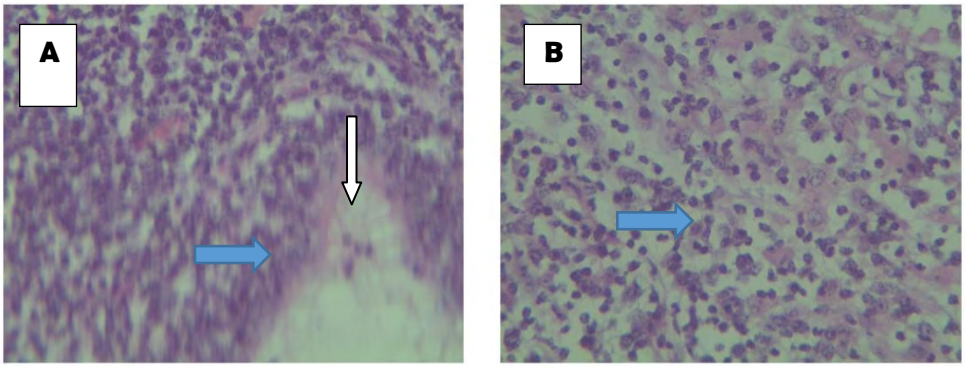


Figure 31. Neutrophil in and around alveoli (A) & pyogranuloma on lymph vessel (B) (40x)

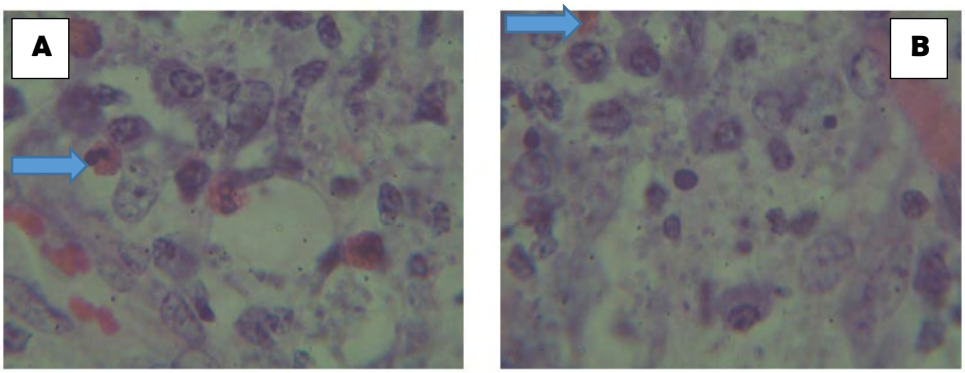


Figure 32. Plasma cells (100x)

There were many yeast-like organisms seen in slides prepared from sections of active nodular tissue that were observed under oil immersion(Figure 33& 34-B). They were found both extracellularly and intracellularly phagocytized within macrophages in tissue sections stained with H&E. The yeast like organisms occurred individually or in groups. They were pleomorphic with round to oval shaped basophilic masses, varying from about 2–4 μm in diameter. There were also some budding yeasts that were budding from one end. The cytoplasm in HE-stained preparations was stained faint blue and some yeast forms had unstained transparent cytoplasmic space.

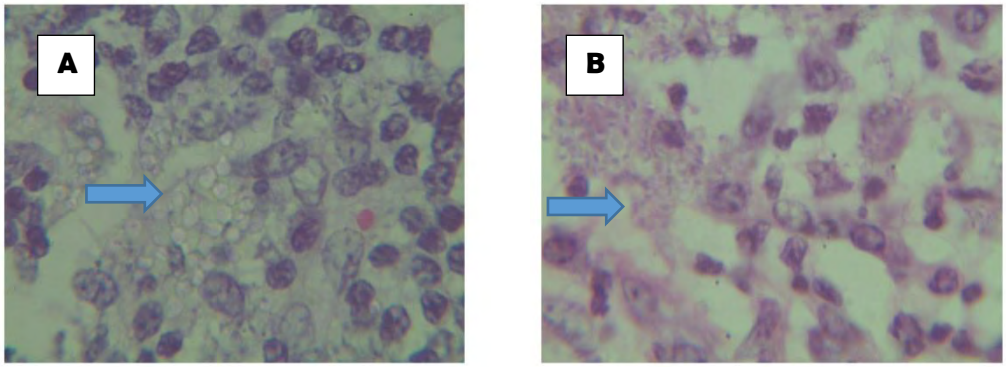


Figure 33. Yeast like cells in Lymph node (A) & trachea (B) (100x)

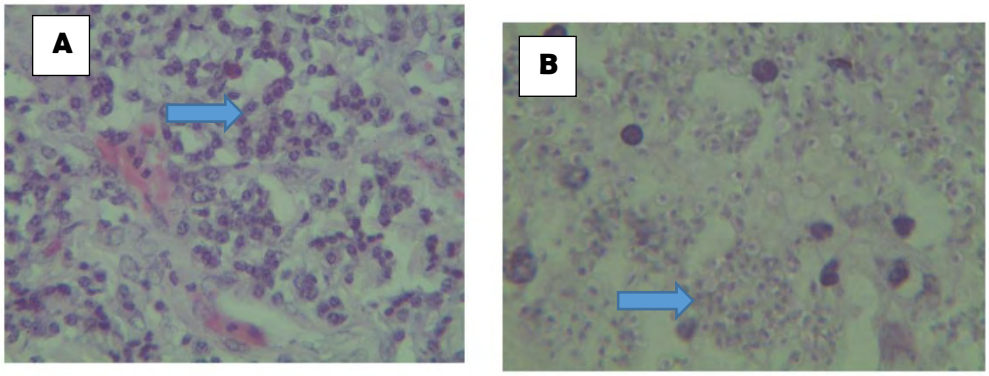


Figure 34. Pyogranuloma (A) & yeast like cells in skin (B) (40x; A& 100x; B)

The nodular lesions of skin and enlarged regional lymph nodes revealed the characteristic lesion pyogranulomatous inflammation with numerous round to spherical yeast-like organisms (Figure 34).The Histopathological findings on tissue

samples from lesions of internal organs also revealed the characteristic pyogranulomatous inflammatory reaction together with numerous round to spherical yeast-like organisms. The typical microscopic lesions were found within tissue samples from lesions of raised nodular masses on the trachea of case three, from lesions of cecum of case six, and from lesions of the large granulomatous nodule and arthritis of case eight.

There was neither pyogranulomatous lesion nor yeast-like organisms detected in slides prepared from normal tissue sections of internal organs of case one, two and four. Both the pyogranulomatous inflammatory reaction and the yeast-like organisms were not detected from lesions of liver of case five and from lesions of the swollen liver and enteritis of case seven.

4.6. Statistical Analysis Results

Number of topical nodules (all types; unruptured, ruptured and scar) tend to increase both with body condition score of the patient and severity stages of the disease; being more in carthorses with poor body condition and sever stage of the disease (Table 4).Similarly number of topical nodules (both types; small and large) tend to increase both with body condition score of the patient and severity stages of the disease; being more in carthorses with poor body condition and sever stage of the disease (Table 5).

Table 4: Distribution of unruptured nodule, ruptured nodules &scar tissues

Types of Lesions	Body Condition of Animal			Severity Stages of EZL			Total number of lesions
	A	B	C	1	2	3	
Unruptured Nodules	13	159	182	14	67	273	354
Ruptured Nodules	20	202	228	23	93	334	450
Scar (healing) tissues	14	92	58	6	84	74	164
Total	47	453	468	43	244	681	968

Key: a→good; b→moderate and c→poor body condition score.

1→early, 2→moderate and 3→ sever stage of EZL.

Table 5: Distribution of the small and large nodules

Types of Lesions	Body Condition of Animal			Severity Stages of EZL			Total number of Lesions
	a	B	C	1	2	3	
Small Nodules	5	199	192	12	67	317	396
Large Nodules	28	162	218	25	93	290	408
Total	33	361	410	37	160	607	**804**

Key: a→good; b→moderate and c→poor body condition score.

1→early, 2→moderate and 3→ sever stage of EZL.

Statistical analysis result revealed that the number of ruptured, unruptured and large nodules were significantly influenced ($p = 0.011$, 0.02 & 0.013 respectively), while the number of small nodules was not significantly influenced ($p = 0.074$) by the body condition score of the carthorses (Table: 6). It also revealed that the number of all types of nodules were significantly influenced by the severity stages of EZL ($p = 0.0$ for all) (Table: 7).

Table 6: Influence of body condition score on types of nodules

		Sum of Squares	Do	Mean Square	F	Sig.
Ruptured Nodules	Between Groups	596.571	2	298.286	5.350	0.011
	Within Groups	1505.429	27	55.757		
	Total	2102.000	29			
Unruptured Nodules	Between Groups	431.169	2	215.585	4.521	0.020
	Within Groups	1287.631	27	47.690		
	Total	1718.800	29			
Small Nodules	Between Groups	679.693	2	339.846	2.874	0.074
	Within Groups	3193.107	27	118.263		
	Total	3872.800	29			
Large Nodules	Between Groups	482.105	2	241.052	5.161	0.013
	Within Groups	1261.095	27	46.707		
	Total	1743.200	29			

Table 7: Influence of severity stages of EZL on types of nodules

		Sum of Squares	Do	Mean Square	F	Sig.
Ruptured Nodules	Between Groups	1657.767	2	828.883	50.379	0.000
	Within Groups	444.233	27	16.453		
	Total	2102.000	29			
Unruptured Nodules	Between Groups	1279.500	2	639.750	39.320	0.000
	Within Groups	439.300	27	16.270		
	Total	1718.800	29			
Small Nodules	Between Groups	1949.767	2	974.883	13.688	0.000
	Within Groups	1923.033	27	71.223		
	Total	3872.800	29			
Large Nodules	Between Groups	1047.767	2	523.883	20.340	0.000
	Within Groups	695.433	27	25.757		
	Total	1743.200	29			

The multiple comparisons were executed to know the exact factor at which the difference occurred at 95% confidence interval. The factors were namely good, moderate and bad body condition scores and early, moderate and sever severity stages of EZL.

There were significant differences between good & poor body condition scores of the carthorses, between early & sever and between moderate & sever severity stages of EZL ($p < 0.05$) and the result was found to be consistent for the number of all types of lesions. There were also significant differences observed between good and moderate ($p < 0.05$) body condition scores of the carthorses for the three types of lesions namely ruptured, unruptured and small nodular lesions. However, there were no significant difference between good and moderate ($p = 0.248$) body condition scores for the large type of nodular lesion.

There was no significant difference observed between moderate & poor ($p > 0.05$) body condition scores of the carthorses for the three types of lesions namely ruptured,

unruptured and small nodular lesions. However, there was significant difference between moderate & poor (p = 0.021) body condition scores for the large type of nodular lesion.

Table 8: P values of factors for multiple comparisons

Type of nodule	multiple comparisons P values for factors of body condition score			multiple comparisons P values for factors of severity stages of EZL		
	Between good and moderate	Between good and poor	Between moderate and poor	Between early and moderate	Between early and sever	Between moderate and sever
Ruptured	0.034	0.003	0.131	0.044	0.000	0.000
Unruptured	0.048	0.006	0.172	0.089	0.000	0.000
Small	0.045	0.026	0.680	0.360	0.000	0.000
Large	0.248	0.009	0.021	0.134	0.000	0.000

There was no significant difference observed between nearly and moderate (p > 0.05) severity stages of EZL for the three types of lesions namely unruptured, small and large nodular lesions. However, there was a significant difference between early and moderate (p = 0.044) severity stages of EZL for the ruptured types of nodular lesion.

5. DISCUSSION

The false believe that 44% of the interviewed carthorse owners believe as if the disease was caused by an evil-eye or "Buda" may have great impact on the control of the disease, since they may not apply the proper prevention and control measures. They may not handle or feed their carthorses properly, since they believe as if the disease attacks carthorses with good body condition.

One of the most important finding of the field assessment part of this study is assumed to be the information gathered on the presence of small and large nodular lesions. It is found to be true that during clinical observations out of the 804 nodular lesions counted on this study 396 (49.3%) were small type (F) and 408 (51.7%) large type (M). This difference could be due to the difference in strains of HCF or it may not be. Of course both types of lesions were found both on the same animal and separately on different animals, but the occurrence of both types of lesions on the same animal might be due to a mixed infection.

The information gathered on the presence of traditional treatment methods that carried out by local personals called "Beka" who uses medicinal plants is also of value; if further study is to be considered. The case of abandoned carthorse requires a due attention, because firstly it is against the welfare of animals and secondly abandoned carthorses are potential causes for car accidents. The reason why severely affected and abandoned carthorses were usually found at center of main roads of vehicle is assumed to be to reduce the fly population that feeds on the open wounds of the skin. Carthorses that were found with gross lesions of EZL mixed with the normal can be the potential sources of infection for the healthy ones. This may have great impact on the control of the disease and it is against welfare of animals.

In agreement with (Ameni, 2007; Ameni & Siyoum, 2002) there are not clearly separated three forms of EZL, rather an extension and manifestation of EZL lesions in and around eye and respiratory organs. Case four and eight of this study can be a good example for this. They were considered as the cutaneous form of EZL based on their

clinical manifestation, but the gross and microscopic lesions of internal organs indicate respiratory form of EZL.

The clinical signs, topical gross lesions and FNA results of EZL of this study are in agreement with previous studies (Ameni, 2007 & Al-Ani *et al.,* 1998). One of the observed clinical signs, scratching of the lesions is considered as a risk factor for the formation of ulcers that play a great role on the dissemination of EZL. In agreement with previous studies (Al-Ani & Al-Delaimi, 1986; Al-Ani *et al.,* 1998) there might be a chance for transmission of EZL through coitus, since the lesion is detected on penis and it is transmitted by direct and indirect contacts.

The pungent smell from ruptured nodule or ulcers has its own negative impact on management of such animals. It may make both the owners and professionals ignorant to infected carthorses. As fever is uncommon in EZL, the body temperature of almost all the cases were in the normal range (37.5-38.5) at least on the spot at which it was taken. However since the first line of defense (skin) is damaged, immunity is compromised and secondary bacterial contaminants has been isolated from infected carthorses in previous study (Hadush *et al*., 2008), it would be logical to expect hyperthermia in at least some of the cases. The reason for the absence of fever is not clear, but it is beyond the scope and objective of this study.

The more affected and severely affected body parts were the limb extremities especially the hind limb extremities which could be due to vulnerability for fly attack and mechanical injury. In agreement with Ameni & Terefe, 2004tick infested body parts were found to be more vulnerable since they have more nodules and ulcers than the non-tick infested body parts.

Considering nodular skin lesions, thickening of the lymphatics, enlargements of superficial regional lymph nodes, and distention of superficial lymph vessels as the common and consistent lesions of EZL is in agreement with previous studies (Ameni, 2007 &Al-Ani *et al.,* 1998), however, considering congestion, hyperemia and haemorrhage as the common and consistent lesions of EZL is unique for this study. This could be due to the reflection of PME in this study in which the animals were

euthanized by chemicals. Those lesions may arise due to absence of bleeding or due to effect of the used chemicals.

In agreement with previous studies, (Ameni, 2007 and Al-Ani *et al.,* 1998) the pyogranulomatous chronic inflammatory process, and the presence of many yeast-like organisms within and out of macrophages in tissue samples is considered as the main histopathological findings of EZL in this study. But in previous studies the microscopic lesions were defined only in tissue samples from skin. In this study they were found both from topical and internal organ lesions.

The Nodular thickenings of the mucosa and submucosa may result from infiltration of lymphocytes, plasma cells and macrophages. The presence of mixed types of lesions in affected body parts are indicators for presence of continues new eruptions and healing process. The inflammatory cells that infiltrate the tissue were macrophages, polymorphonuclear cells (neutrophils and basophils) and plasma cells and in this study macrophages were found to be the dominant leukocytes this is also in agreement with previous studies (Ameni, 2007 & Al-Ani *et al.,* 1998). The reasons for this could be the chronic nature of the disease and since EZL is infection of the reticuloendothelial system.

There was a strong association between numbers of topical nodules of all types with severity stages of the disease as well as the body condition score of the carthorses. The number of nodular lesions of skin were highly influenced both by the body condition score of the carthorses and severity stages of EZL. They tend to rise as the body condition of the carthorses deteriorates and as severity stages of EZL rise. The number of yeast-like organisms and distended macrophages increases with chronicity of EZL.

The yeast form of HCF that were found in giemsa stained smears from FNA samples, in imprint smear of affected internal organs and in histopathological tissue samples shows that the disease and the lesions (both topical and internal) are caused by EZL. They were found together with pyogranulomatous lesions in tissue samples both from topical and internal gross lesions. Based on this we can consider that most of internal

gross lesions in EZL infected carthorses have an association with the disease which could be direct or indirect association. The large nodular lesion of right lung of case eight and tracheal nodular mass of case three of this study were confirmed to be typical lesions of EZL since the yeast is detected both in the imprint smear from lesions and in histopathological slides.

Only the yeast forms were found in infected tissues, since HCF is a thermally dimorphic fungus in which the yeast form is usually found in lesions (Ameni, 2006b). They were found either with unstained transparent or with faint blue cytoplasmic space which may be due to strain differences (Endebu,). The yeasts that had a distorted cytoplasm are indicatives of immunological process. In contrast to previous studies, there were no capsular morphology appreciated in this study which could be due to the differences in the used methods giemsa in this study and gram in others. Although it was not convenient to do PAS, the morphology of the fungus would have been appreciated clearly if PAS was used. Since EZL is an intracellular mycotic infection of the reticuloendothelial system the yeasts that were found free extracellularly could arise by rupture of distended macrophages; some of them were seen budding.

Since the gross lesions detected on internal organs were few in number and distribution and neither pyogranulomatous lesion nor yeast cells were detected in normal tissue sections, it seems the distribution of HCF is restricted on skin. Moreover both pyogranulomatous lesion and yeasts were not detected in histopathological slides from normal tissue sections of carthorses with systemic form of EZL; for instance carthorse of case number 8 of this study.

The absence of gross and histopathological lesions on the muscles under severely affected skins and observed cord like thickening of superficial lymphatics of skin can be considered as an evidence for the passage of HCF through lymphatic vessels. An attempt of detecting HCF from blood samples were not successful and no such report was found in literatures. Based on this we can say that there is no hematological distribution in EZL and the distribution of the disease through lymphatic vessels is restricted in to limited anatomical regions due to the anatomical nature of lymphatic

vessels and due to the presence of some restricting ability of regional lymph nodes in this passage. There is a distinct anatomical separation between superficial and deep lymphatic vessels that may not allow the passage of HCF in to deep lymphatic vessels from superficial lymphatic vessels. The reason why gross lesions detected on internal organs were few could be due to the restriction of HCF within the lymphatic vessels and due to the presence of large number of leucocytes ready to fight secondary invaders. The nonspecific or non-nodular lesions detected on this study in different organs could be caused by secondary invaders through the open skin.

There were neither gross nor microscopic lesions detected within internal organs of the three carthorses' cases of this study; even in one organ. Their internal organs were found to be normal in pathologically point of view, having such an open skin.

Generally this study revealed that most of the gross and microscopic lesions of EZL including that of the sever stage were restricted on the skin and most of the vital organs were found to be normal. Additionally skin has a great regeneration capacity and early and moderate stage of EZL are curable. Therefore all stages of EZL seems curable including the sever stage of EZL, if the proper treatment is given in an intensive care system. If the skin lesions of early and moderate stage of the disease are curable (reversible), why not for the sever stage of EZL since skin is a highly regenerative organ. It is only a matter of cost, time and commitment of professionals to resist the pungent smell of EZL.

The fate of carthorses with sever stage of EZL was observed to be euthanasia for welfare reason. The treatment of sever stage of EZL may not be cost effective in the cost benefit analysis, however, euthanasia of carthorses in sever stage of EZL for welfare reason seems against the principle of welfare.

6. CONCLUSION AND RECOMMENDATION

Epizootic lymphangitis is one of the infectious diseases causing huge economic losses and low productivity in horses. It is particularly prevalent in carthorses in most parts of Ethiopia studied (Ameni, 2006a). Due to its significant impact on the livelihood of carthorse owners, it is considered a major disease of horses in many parts of Ethiopia. Data for economic losses incurred by epizootic lymphangitis is lacking but one conservative estimate showed that mortality associated losses of about $129 USD incurred per annum per owner (Hadush *et al.,* 2014). Despite of its economic importance, EZL is a least studied disease. Little is known about EZL in general and regarding its pathology and its pathogenesis in particular; especially on pathology of EZL in internal organs. This study revealed presence of a gap on knowhow on EZL. Carthorse owners still believe that EZL is caused by an evil-eye or "Buda" due to lack of knowledge. We were thinking as if there are clearly separated three forms of EZL, while the disease is only of cutaneous type with an extension and manifestation of EZL lesions in and around eye and respiratory organs. This study showed the presence of two types of lesions namely small and large nodular lesions. Even though they are few in occurrence, nodular lesion of lung and trachea are the typical gross lesions of EZL found in this study that are confirmed by the presence of yeasts detected both in the imprint smear from lesions and in histopathological slides. This study revealed that most the gross and microscopic pathological lesions of EZL were restricted on the skin and mostly the vital organs were found to be normal.

Based on the above conclusion the followings are recommended

- Large scale training on the knowhow of EZL for carthorse owners has to be programmed and implemented in a sustainable way.

- More researches have to be done on pathology, pathogenesis, immunology and treatment of EZL to fill the gap on the knowhow of EZL.

- The medicinal plants that the local healers use has to be investigated in depth.

- The presence of different strains of HCF that may cause the two types of lesions small (F) and large (M) nodular lesions also needs to be investigated.

- A pilot project for trial of the treatment of sever stage of EZL that considers the proper treatment with an intensive care system has to be designed.

- Attention should be given to the management of the abandoned carthorses and there should be rules and regulations on the welfare of them and responsibility of abandoned carthorse owners.

7. REFEERENCES

Addo P. (1980). A review of epizootic lymphangitis and ulcerative lymphangitis in Nigeria: *misnomer or misdiagnosis. Bull. Anim. Hlth Prod. Afr.,***28**, 103-107.

Ajello L. (1968). Comparative morphology and immunology of members of genus Histoplasma. *A review. Mykosen,* **11,** 507-514.

Al-Ani F. & Al-Delaimi A. (1986). Epizootic lymphangitis in horses: clinical, epidemiological and hematological studies. *Pakistan vet. j.,***6,** 96-100.

Al-Ani F. (1999). Epizootic lymphangitis in horses: a review of the literature. *Rev. sci. tech. off. int. Epiz.,****18(3),*** *691–699.*

Al-Ani F., Ali H. &Banna H. (1998). Histoplasma farciminosum infection of horses in Iraq. *Vet. Arhiv.,***68**, 101-107.

Al-Ani F., Hassan F. & Al-Juborri K. (1989). Sero-diagnosis of epizootic lymphangitis in horses by passive haemagglutination test. *J. Iraqi Vet.,* **7,** 1-16.

Al-Ani F., Khalifa K., Ali A.., Hassan F., Al-Abbassy S., Redha Y. & Al-Zubaidi I. (1988). Epizootic lymphangitis in horses: mice inoculation studies. *Pakistan vet.j.,***8**, 5-8.

Al-Delaimi A. & Khairallah A. (1984). Application of fluorescent antibody test in the diagnosis of the epizootic lymphangitis. *Haryana Vet.,* **23,** 123-125.

Ameni G. & Siyoum F. (2002). Study on histoplasmosis (epizootic lymphangitis) in carthorses in Ethiopia. *J. Vet. Sci.,***3,** 135-139.

Ameni G. & Terefe W. (2004). A cross-sectional study of epizootic lymphangitis in cart-mules in western Ethiopia. *Preventive Vet. Med.,***66,** 93–99.

Ameni G. (2006a). Epidemiology of Equine Histoplasmosis (Epizootic lymphangitis) in cart horses in Ethiopia. *Vet. J.,***172,** 160-165.

Ameni G. (2006b). Preliminary trial on the reproducibility of epizootic lymphangitis through experimental infection of two horses. Short Communication. *Veterinary J.,***172 (3),** 553–555.

Ameni G. (2007). Pathology and clinical manifestation of epizootic lymphangitis in Cart Mules in Ethiopia. *Journal of Equine Science,***18(1),** 1-4.

Ameni G. Terefe W. & Hailu A. (2006). Histofarcin test for the diagnosis of epizootic lymphangitis in Ethiopia: development, optimization and validation in the field. *Veterinary J.,***171**, 358–362.

Asfaw R, Ameni G. & Mahendra P. (2012). Prevalence of Epizootic Lymphangitis in Cart Horses in Southwest Shewa of Oromia Region, Ethiopia. *Int. J. Livest. Res.,***2 (3),** 146-151.

Asfaw W. (1999). Message from Federal Veterinary Services Team. *Ethio Vet. Epidemiol. News let.,***1**, 1.

Dennnis, M. & Joanna, M. (2006). Clinical textbook for veterinary technicians. 5th Ed., Elselver pub.

EARO. (1999). National Animal Health Research Program Document. Ethiopian Agricultural Research Organization, Addis Ababa, Ethiopia. Pp.: 1- 46.

Edward B. (2005). Black's veterinary dictionary. 21st Ed. A & C Black Publishers Limited. 38 Soho Square, London. Pp.: 430

El-Gundy M., Shokeir A., Wasfey I., Ahmed K., Elbedeiwy A., Abou-Gabal M. & El-Rehewy M. (1975). Histoplasmosis of the eyes of donkeys. An electron microscopic study. *Assiut Vet. med. J.,***2,** 235-239.

Endebu B. (1996). Epidemiology of epizootic lymphangitis in Ethiopia: Retrospective analysis and cross-sectional study and treatment trail at Debrezeit and Akaki. DVM thesis, Faculty of Veterinary Medicine, University of Addis Ababa, Ethiopia. Pp.: 1-30.

F.A.O. (1996). Animal production and Health Yearbook. Food and Agricultural Organization of the United Nations. Rome.

Fawi M. (1971). Histoplasma farciminosum, the etiological agent of Equine Cryptococcal pneumonia. *Sabouraudia*, **9,** 123-125.

Feseha G. (1993). Use of Equines in Ethiopia. In: Proceedings of the Fourth National Livestock Improvement Conference. Addis Ababa, IAR, Ethiopia. pp 51–57.

Gabal M. & Hennager S. (1983). Study on the survival of *Histoplasma farciminosum* in the environment. *Mykosen.,***26,** 481-484.

Gabal M. & Khalifa K. (1983). Study on the immune response and serological diagnosis of Equine histoplasmosis (Epizootic lymphangitis). *Zentralbl. Veterinärmed. B.,***30,** 317-321.

Gabal M. (1984). The effect of Amphotericin B, 5-Fluorocytosine & Nystatin on *Histoplasma farciminosum* in vitro. *ZentralblVeterinärmed, B.,***31,** 46-50.

Gabal M., Bana A. &Gendi M. (1983a). The fluorescent antibody technique for diagnosis of equine histoplasmosis (Epizootic lymphangitis). *ZentralblVeterinarmed B.,***30(4)**, 283.

Gabal, M., Hassan, F., Said, A. & Karim, K. (1983b). Study of equine histoplasmosis and characterization of *Histoplasma farciminosum*. *Sabouraudia***21,** 121–127.

Gari F., Hagos A., Alemu T., Bruno M., Goddeeris & Filip C. (2010). Comparative diagnosis of parasitological, serological and molecular tests in dourine-suspected horses. *Trop anim. Health Prod.,***42(8)**, 1649-1654.

Gillespie J. & Timoney J. (1981). Hagan and Bruner's infectious diseases of domestic animals, 7th Ed. Cornell University Press, London. Pp.: 390-391.

Guerin C, Abebe S. &Touati F. (1992). Epizootic lymphangitis in horses in Ethiopia. *J. Mycol. Méd.,***2,** 1-5.

Hadush B., Ameni G., Medhin G. (2008). Equine histoplasmosis: Treatment trial in cart horses in Central Ethiopia. *Trop. Anim. Hlth. Prod.,***40,** 407-411.

Hadush B., Biratu D., Taddele H., Tesfaye D., & Ameni G. (2014): Bacterial contaminants isolated from lesions of equine histoplasmosis in cart horses of Mekelle town, northern Ethiopia. *Revue Méd. Vet.,***165 (1-2),** 25-30

Hagos A. Getachew A., Büscher P., Goddeeris B., & Filip Claes F. (2010). Serological and parasitological survey of dourine in the Arsi–Bale highlands of Ethiopia. *Trop Anim Health Prod*., **42**, 769–776

IVIS (2006): Epizootic Lymphangitis: Proceedings of the 9th International Congress of World Equine Veterinary Association Jan. 22 - 26, 2006. Marrakech, Morocco. 419. Available on line at http://www.ivis.org/

Jubb K., Kennedy P. & Palmer N. (Eds.) (2006). Epizootic lymphangitis. In Pathology of Domestic Animals, Vol. 3, 5th Ed, Grant M. & Wayne F. Eds.: Academic Press, New York, USA. Pp.: 98-102.

Khater A., Iskander M. & Mostafa A. (1968). A histo-morphological study of cutaneous lesions in equine histoplasmosis (Epizootic lymphangitis) in Egypt. *Vet. med. Assoc.,* **28,** 165-174.

Mc Gavin & Zachary, (2010): Acute Inflammation. In Pathologic Basis of Veterinary Disease, 4th Ed, Mark R. Eds.:Mosby. Elsevier publisher. Pp.: 10-62. Available on line at http://evolve.elsevier.com/McGavin/vetdisease

Morrow A. & Sewell M. (1990). Epizootic Lymphangitis. In Handbook on Animal Diseases in the Tropics, 4th Ed, Sewell, M. &Brocklesby, D. Eds.: Bailliere, Tindall, London. Pp.: 364-367.

Negesse M., Eyasu M., Nigatu A. & Ameni G. (2012).Evaluation of berries of Phytolacca dodecandra for growth inhibition of Histoplasma capsulatum var. farciminosum and treatment of cases of epizootic lymphangitis in Ethiopia. *Asian Pacific Journal of Tropical Biomedicine,* 505-510

OIE. (2008). Epizootic lymphangitis: Chapter 2.5.4. In Manual of diagnostic tests and vaccines for terrestrial animals. Office International des Epizooties, Paris: Available at http://www.oie.int/eng/normes/mmanual/2008/pdf/2.05.04.

Quinn P., Carter M., Markey B. & Carter G. (1994). Veterinary clinical microbiology, 1st Ed. Wolfe Publishing Company, London. Pp.: 407.

Radostits O., Gay C., Hinchcliff K. & Constable P. (2006). Epizootic Lymphangitis. In Veterinary medicine: A Textbook of The Diseases of Cattle, Horses, Sheep, Pigs and Goats. 10th Ed. Saunders; Elsevier: London. Pp.: 1478 -79.

Richer F. (1977). La lymphangiteépizootique. Revue générale de la maladieet observations cliniquesenRépublique du Sénégal. [Epizootic lymphangitis. Review of the disease and clinical observations in Senegal.] PhD thesis, Écolenationalevétérinaire, Maisons-Alfort, 89 pp.

Rippon J. (1988). Medical Mycology, 3rd Ed. WB Sounders Company, Philadelphia. pp. 417–419.

Selim S., Soliman R., Osman, K., Padhye, A. & Ajello, L. (1985). Studies on histoplasmosis farciminosi (Epizootic lymphangitis) in Egypt: Isolation of *Histoplasma farciminosum* from cases of histoplasmosis farciminosi in horses and its morphological characteristics. *Eur. J. Epidemiol.,***1 (2),** 84–89.

Singh T. & Varmani B. (1966). Studies on epizootic lymphangitis: A note on pathogenicity of *Histoplasma farciminosum* (Rivolta) for laboratory animals. *Indian J. vet. Sci.,***36,** 164-167.

Singh T. (1965). Studies on Epizootic lymphangitis. I. Modes of infection and transmission of equine histoplasmosis (Epizootic lymphangitis). *Indian J. Vet. Sci.,* **35,** 102–110.

Singh T. (1966). Studies on Epizootic lymphangitis: Clinical cases and experimental transmission. *Indian j. vet. Sci.,***36,** 45-59.

Singh T., Varmani B. &Bhalla N.P. (1965). Studies on epizootic lymphangitis. II. Pathogenesis and histopathology of equine histoplasmosis. *Indian J. vet. Sci.*, **35,** 111-120.

Soliman R., Saad M. &Refai M. (1985). Studies of Histoplasmosis Farciminosii (Epizootic lymphangitis) in Egypt. III. Application for a skin test (Histofarcin) in the diagnosis of epizootic lymphangitis in horses. *Mykosen,***28,** 457-461.

Solomon H. (1980). Animal Health Review in Ethiopia (1972–1979). Addis Ababa: Department of Veterinary Service Division, Ministry of Agriculture.

Statistical Bulleitin.(2011). Federal Democratic Republic of Ethiopia,Central Statistical Agency.Report on livestock and livestock characteristics.Volume 2.Addis Ababa, Ethiopia.Talukder, S. (2007). Histopathology techniques: Tissue processing and staining. Available on line athttp:// www.talukderb.com

The Merck Veterinary Manual (2000). 8th Ed.Epizootic Lymphangitis. In Fungal Infections. Available on line at http://www.merckvetmanual.com/mvm/index.jsp:

Weeks R., Padhye A.& Ajello L. (1985). *Histoplasma Capsulatum* var. farciminosum. *Mycopathologia***77 (6),** 964–970.

Zhang W., Wang Z., Liu Y., Zhang D., Liang P., Fang Y., Huang Y. & Gao S. (1986). Attenuated vaccine against Epizootic lymphangitis of horses. *Chin. J. vet. Sci. Technol.,* **7,** 3-5.A

8. APPENDICES

Appendix: 1. Steps for Biopsy Sample Collection

1. Observation and Palpation to search for un-ruptured nodules
2. Washing the nodules with soap and water
3. Shaving the nodules
4. Disinfecting the nodules with alcohol
5. Aspirating the contents of the nodules with sterile needles and syringes
6. Fixing the smears with methanol
7. Staining the smears with Giemsa stain
8. Examining the smears with light microscope
9. Photographing of superficial gross lesions
10. Infiltrating of un-ruptured nodules with lidocaine by ring block
11. Incising 4 mm tissues sample from cutaneous lesions
12. Placing tissues sample in to 10% formalin

NB

Samples should be collected from active nodular lesions

Samples should be collected including normal and pathological parts

The volume of formalin should be 10X that of the tissues sample

Appendix: 2. Necropsy Procedure

1. The animal is placed in left lateral recumbency (on the left side).
2. A midline incision is made beginning at the right maxilla and extending cranially to the mandibular symphysis.
3. The incision is continued in the opposite direction caudally as a median or paramedian incision passing between the mammae and around the penis, prepuce, and scrotum to the perineum.
4. The upper forelimbs are reflected by dissection between the scapula and the ribs.
5. Fat, fascia, and superficial muscles are reflected back together with the skin.
6. Skin of the ventral aspect of the neck and throat is reflected.
7. Abdominal skin is reflected, and the hind limbs are reflected by extending the incision into the coxofemoral (hip) joints.
8. The animal is now placed in dorsal recumbency (on its back).
9. Skin incisions are extended down the cranial medial aspects of both rear legs, and the skin is reflected.
10. As they are exposed in the dissection, superficial organs are examined and samples from these organs are collected: lymph nodes (mandibular, superficial cervical, pre scapular, axillary, inguinal, and popliteal), mammary glands, testes, and skin.
11. Next, the three major body cavities (peritoneal, pleural, and pericardial) are opened.
12. All organs are examined in situ, and any abnormalities are noted.
13. The abdomen is opened by making a midline incision from the sternum to the symphysis pubis and making incisions laterally from the sternum along both caudal costal margins.
14. The abdominal wall is then reflected laterally to expose the abdominal cavity.
15. The diaphragm is now punctured to check for negative pleural pressure, and the diaphragm is cut away from the ventral and lateral rib cage.
16. The ventral rib cage is removed by cutting the ribs bilaterally (on both sides) midway between the costochondral junction and the vertebral column.

Appendix: 3. Sample Questionnaire distributed to carthorse owners

Open questionnaires

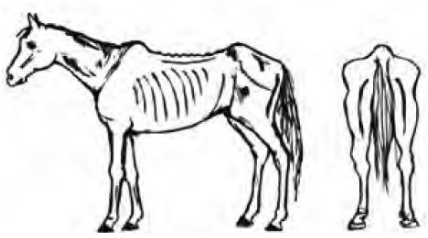
1. What do you know about EZL?
2. Who or what is the source of your knowhow?
3. What solution measure do you take, when your carthorse have lesions of EZL?
4. Whose carthorse have lesions of EZL at this time?

Closed questionnaires

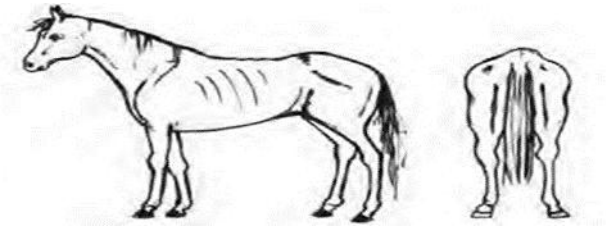
1. Do you get at least one training on EZL?
   1) Yes 2) No
2. Do you think that EZL is caused by an evil-eye or "Buda"?
   1) Yes 2) No
3. Do you believe that, EZL has two types ('male' and 'female' types) of lesions?
   1) Yes 2) No 3) I don't know
4. Do you think EZL is a curable disease if treated at early stage?
   1) Yes 2) No
5. Where do you go for treatment of EZL?
   1) To traditional healers 2) To Veterinarians 3) To both

Appendix: 4. Horse body condition scoring

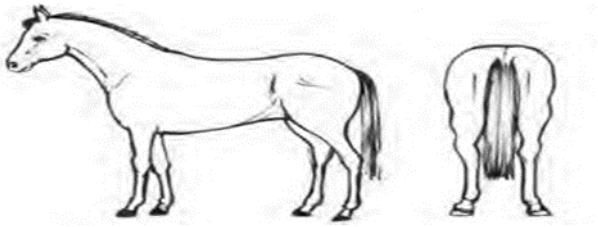
Body Condition Score One = /Sunken rump + Prominent poverty line in hind quarters cavity under tail + Ribs prominent + Prominent backbone and croup + Ewe neck, narrow and slack/



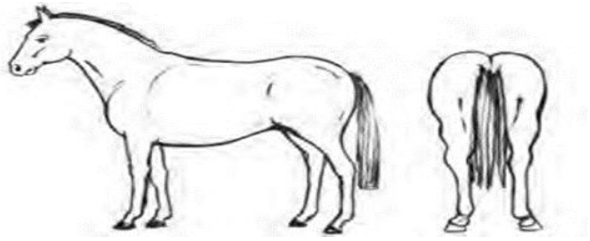
Body Condition Score Two = /Flat rump on either side of backbone + Poverty line still visible + Ribs just visible + Narrow but firm neck Backbone covered/



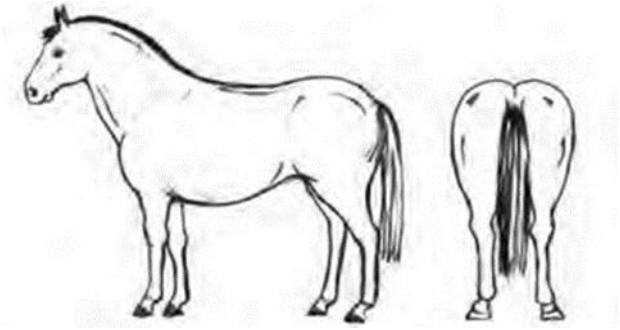
Body Condition Score Three = /Rounded rump + Ribs just covered but easily felt +No crest, firm neck/



Body Condition Score Four = /Well-rounded rump + Gutter along back + Ribs and pelvis hard to feel + Slight crest on neck



Body Condition Score Five =/ Very bulging rump + Deep gutter along back + Ribs Buried + Marked crest on neck +Folds and lumps of fat /



It was taken from Queensland government manual /Queensland primary industries and fisheries/ animal health and disease investigation center: animal body condition scoring guide pdf (pp, 3 of 4).

It was taken with some modifications considering the local breeds' body condition. In this study; body condition score one is considered as poor, body condition score two is considered as moderate and all body condition scores three, four and five were considered as good body conditions

Appendix: 5. Chronological order of what were done during the research work

1. Getting information through
- Field survey
2. Clinical observation, Inspection and palpation of suspected carthorses
3. Detail recordings about
- Observed signs
- Body parts affected with lesion
- Number of topical nodules
- Number of topical ulcers
- Severity of the disease
- Body condition of the selected carthorses
4. Laboratory works
- Pus samples Collection
- Washing nodules with soap and water,
- Shaving nodules
- Disinfecting nodules with alcohol.
- Aspirating contents of nodules with sterile needles and syringes
- Preparation of smears
- Fixing with methanol,
- Staining with Giemsa stain
- Microscopic examinations
5. Selection of positive carthorses for biopsy sample collection
6. Taking photograph of superficial gross lesions
7. Convincing owners of selected animals for biopsy sample collection
8. Biopsy sample collection with a welfare manner
- By using ring block infiltration with lidocaine or
- By collecting sample just before euthanasia after full anesthesia dose is reached
- By incising 4mm tissues from cutaneous lesions
- By fixing tissue sample in 10% formalin
9. Purposive selection of carthorses for euthanasia based on
- Stage of the disease
- Form of the disease

- Willingness of the owner
- Accessibility of the case

10. Euthanasia of horses by

- Anaesthetizing with 20 ml thiopental sodium IV injection and
- Killing with IV injection of potassium chloride

11. Transport of carcass to NAHDIC postmortem hall
12. Necropsy of euthanized carthorses
13. Examining for gross pathological lesions including the

- Lesion distribution
- Lesion contour
- Lesion texture
- Lesion shape
- Lesion size and
- Lesion color

14. Autopsy samples collection from the lesions by

- Cutting tissue samples with lesions to the size of 4mm
- Putting tissue samples in the universal bottle
- Fixing tissue samples with 10% BNF

15. Submitting and storing of both the biopsy and autopsy samples in a sterile manner
16. Histopathology works

- Trimming tissues samples
- Fixing in 10% BNF
- Dehydrating in ascending grades of alcohol
- Clearing with xylene
- Impregnating with molten paraffin wax
- Sectioning at five micrometers
- Spreading on warm water bath
- Attaching tissues to albumenized glass slides
- Incubating slides in incubator at 60 ^{o}C
- Deparaffinizing in three changes of xylene
- Rehydrating in descending grades of alcohol
- Staining with H&E stain

- Mounting with DPX cover slip
- Examining at 4x, 10x, 40x and finally with oil immersion (100 x magnification)

17. Taking photographs of the slides

Thank you!