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**EPIDEMIOLOGICAL INVESTIGATION OF TETANUS: A MATCHED CASE
CONTROL STUDY AND EVALUATION OF TREATMENT OPTIONS IN
EQUINES IN CENTRAL OROMIA, ETHIOPIA**

MVSc Thesis



By

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Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of
Veterinary Clinical Studies, MVSc Program in Veterinary Epidemiology

June, 2017

Bishoftu, Ethiopia

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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
Science in Veterinary Epidemiology

June, 2017
Bishoftu, Ethiopia

Addis Ababa University
College of Veterinary Medicine and Agriculture
Department of Clinical Studies

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Zerihun Asefa Dammessa** entitled: **EPIDEMIOLOGICAL INVESTIGATION OF TETANUS: A MATCHED CASE CONTROL STUDY AND EVALUATION OF TREATMENT OPTIONS IN EQUINES IN CENTRAL OROMIA, ETHIOPIA** and recommended that it be accepted as fulfilling the thesis requirement for the Degree of Master of Science in Veterinary Epidemiology

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DEDICATION

This thesis manuscript is dedicated to Mrs. Burkitu Yedate and Birkinesh Dammessa my Grandmother and aunt in appreciation for their unreserved help and love

SIGNED DECLARATION SHEET

First, I declare that this thesis is my original 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 a postgraduate (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.

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LIST OF ABBEREVATONS

ADARDO	Ada District Agricultural and Rural Development Office
BCS	Body Condition Scores
BW	Body Weight
CDC	Center for Disease Control and Prevention
CI	Confidence Interval
CNS	Central Nervous System
CSA	Central Statistical Agency of Ethiopia
CSF	Cerebrospinal fluid
DHWP	Donkey Health and Welfare Project
GABA	Gamma-amino butyric acid
GPS	Geographical Positioning System
IM	Intramuscular
IU	International unit
IV	Intravenous
Masl	Meter above sea level
MTN	Metronidazole
NVI	National Veterinary Institute
OR	Odds ratio
PPF	Procaine Penicillin G
Q12 h	Every 12 hours
SPANNA	The Society for the Protection of Animals Abroad
SPP	Species
TAT	Tetanus antitoxin
WHO	World Health Organization

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ABSTRACT

Although tetanus is considered as an important disease of equines in Ethiopia, little is known about its epidemiology, treatment options and impact on the livelihoods. This epidemiological study was carried out with the main objectives of identification of risk factors, evaluation of efficacy of different treatment options and description of spatiotemporal occurrence and clinical characteristics of the disease. A matched case control study was conducted to identify putative risk factors from cases admitted to the Donkey Sanctuary clinics in Bishoftu and Merkato, at the College of Veterinary Medicine and Agriculture of Addis Ababa University. The clinical case records of equines diagnosed with tetanus were retrieved between January 2008 and May 2017. For each case two controls were drawn from the same villages that cases were originated from. Matching was done for age and sex. Randomized control trial was used to evaluate the efficacy of procaine penicillin G, metronidazole and combination of both drugs on the survival and improvement in clinical signs. Isolation of *Cl. tetani* was done from soil samples collected from the animals' environment. Analysis of medical records of equines diagnosed with tetanus showed that the disease was spatially aggregated in Gerbicha 28.14 % (n=65) and Gicho Gerbabo 16.02 % (n=37) peasant associations of the Ada'a district. Temporally, the cases of tetanus were found to occur during the summer seasons. There was statistically significant association ($p < 0.05$) observed between the presence of wound and survival of equines affected with tetanus. The grade of tetanus at the time of presentation to clinics was also significantly associated with survival. Certain clinical signs (prognostic signs) were significantly associated with survival of equines diagnosed with tetanus. Besides, duration of illness before hospitalization and duration of hospitalization had significant effect ($p < 0.001$) on the survival. The results of matched case control study revealed that the presence of visible wound increases the risk of acquiring tetanus 10.35 times as compared to those animals which didn't ($p < 0.001$). Equines with good and moderate body conditions had respectively, 22.27 and 3.51 times higher risk of acquiring tetanus than equines with poor body conditions. The results of experimental study indicated that metronidazole injection caused highest survival (80 %) followed by combination of metronidazole and procaine penicillin G (66.67%) whereas

penicillin G alone showed lowest survival (33.33%). Better improvement of clinical signs of tetanus was also observed in donkeys treated with metronidazole than the other treatment groups. The present study showed that tetanus remains an important disease with higher case fatality and highlights a considerable welfare and economic significance in equines. Probable recommendations to limit the risk factors including management, prevention of wound and education of owners are suggested. Furthermore, epidemiological studies with the elucidation of the use of metronidazole are warranted.

Key words: Ada'a, Matched Case Control, Equine, Risk factors, Tetanus, Treatment Options

1. INTRODUCTION

Tetanus is an acute, non-contagious infectious disease caused by a gram-positive, spore-forming and exotoxin producing anaerobic bacterium, *Clostridium tetani* (*Cl. tetani*) in many animal species and humans (Cvetnić, 2002; Quinn *et al.*, 2002; Andrew and Jack, 2006). The organism gains entry to the body via wounds predominantly punctured type. Although deep penetrating wounds, such as punctures of the hoof capsule, are more liable to permit proliferation of *Cl. tetani*, superficial wounds can also provide suitable anaerobic conditions (Kay and Knottenbelt, 2007). The anaerobic condition is required for the spore to vegetative form, for replication and production of complex exotoxins such as tetanolysin and tetanospasmin (Turton *et al.*, 2002; Peter *et al.*, 2017). Tetanolysin causes damage to viable tissue, lowering its redox potential and creating favorable conditions for the spread of the bacterium (Cook *et al.*, 2001), whereas tetanospasmin enters the circulation and binds irreversibly to receptors on the motor nerve endings and this in turn leads to a spastic paralysis (Attygalle and Rodrigo, 2004). The disease is characterized by generalized muscular rigidity and spasms, hyperesthesia, convulsions, respiratory arrest, and death (Peter *et al.*, 2017; Cook *et al.*, 2001). The incubation period varies from 3 days to 4 weeks; with occasional cases occurring as long as several months after the infection is introduced. The initial clinical signs manifest as spasm of the head muscles, resulting in trismus, lockjaw and prolapse of the third eyelid up on touching eyeball, (Peter *et al.*, 2017). In the later stage, the third eyelid protrusion may be permanent without resting to normal position (Green *et al.*, 1994). The spontaneous protrusion of the third eyelid is regarded as pathognomonic sign of the disease (Roper *et al.*, 2007). With progression of the disease, spasms involve the neck and the esophagus, making swallowing difficult (Brook, 1970). Ears erect and immobile, and the tail-head is held elevated. In very severe cases, the affected animals adopt a sawhorse stance with serious dyspnea, inability to ingest food, stiff neck, and sudoresis, before becoming recumbent (Johntson, 1987). Signs of the general infection syndrome and spasms of the extremity musculature develop concurrently, making movement difficult or impossible (Peter *et al.*, 2017). Death is mainly due to spasm of the respiratory musculature (Johntson, 1987).

Diagnosis of the disease is based on wound history and characteristic of clinical signs (Kay and Knottenbelt, 2007; Van der Kolk and Veldhuis Kroeze, 2013) and isolation of the bacterium and less commonly by serological assays (Roper *et al.*, 2007). Tetanus usually affects a single animal, but outbreaks have been sporadically described in farm animals, especially in association with serial injections (Driemeier *et al.*, 2007).

Since routine vaccination of equines against tetanus was started, the occurrence of fatal cases has declined dramatically in the northern hemisphere (Green *et al.*, 1994). In contrast, equids living in developing countries where vaccination is often not performed are still frequently affected by tetanus (Kay and Knottenbelt, 2007). It continues to be associated with a high mortality, ranging from 58 % to 80 % (Green *et al.*, 1994; Smith, 2002; Reichmann *et al.*, 2008; Van Galen *et al.*, 2008) with case fatality of 68% (Van Galen *et al.*, 2008). In humans, tetanus is estimated to cause 309,000 deaths each year, with the overwhelming majority of cases occurring in neonates (Vandelaer *et al.*, 2003). Damjan *et al.* (2015) reported an overall mortality of 45.3 % (19/42) from twenty-year retrospective study of tetanus in horses in Croatia. In similar studies Kay and Knottenbelt (2007) reported an overall survival rate of 46% (n=56) in working donkeys suffering from tetanus in Morocco. In Ethiopia, Bojia *et al.* (2006) reported survival of 87.5% (21/24) in retrospective analysis of medical records of donkeys for the years 2003 to 2005 at DHWP. More recently, Ayele *et al.* (2015) found an overall survival rate of 63.3% and mortality of 33.3% from medical records of 45 working donkeys between 2008 and 2009 at DHWP clinic of Addis Ababa University, College of Veterinary Medicine and Agriculture.

Although vaccination has been considered effective strategy for the control of tetanus, in developing countries antibiotic treatment of affected equines serves as alternative to control the disease. Treatment goals include interrupting the production of toxin, neutralizing the unbound toxin, controlling muscle spasms and appropriate supportive management. Procaine penicillin G at a maximum dose (2–3 times the normal dose) of 20,000 IU/kg bw IM for seven days is recommended and supposed to inhibit further multiplication of *Cl. tetani* at the site of infection and thus further toxin production

(Seifert, 1996; Peter *et al.*, 2017). Historically, penicillin G was the drug of choice in tetanus treatment because of its excellent anaerobic activity; however, it may potentiate the effect of tetanus toxin by inhibiting the type-A (GABA) receptor for γ -amino-n-butyric acid. As a result, metronidazole is now considered the first line therapy and is advocated in human treatment of tetanus (Roper *et al.*, 2007; Gibson *et al.*, 2009). Ahmadsyah and Salim were the first to compare penicillin G and metronidazole, and showed a reduction in mortality of metronidazole group (7% compared to 24% in penicillin treatment group) (Ahmadsyah and Salim, 1985). In veterinary practice, metronidazole is now being used for treatment of tetanus cases in small animals (Linnenbrink and McMichael, 2006), but no reports of its use or effectiveness in equines have been published.

Tetanus can be successfully prevented by prophylactic measures, and consequently is rarely encountered by equine clinicians in developed countries where vaccination regimes are well established. However, the disease remains a major problem and is considered enzootic in many countries of the developing world (Reichmann *et al.*, 2008).

Despite the increasing incidence in the tetanus cases and associated mortalities in Ethiopia, apart from few case studies described above, a thorough epidemiological study on the identification of putative risk factors is missing. Moreover, presences of conditions that predispose animals to tetanus are widely prevailing in the country. For instance, donkeys are engaged in excessive physical works; in several parts of the country, the prevalence of wound is high in donkeys due to poor husbandry practice such as beating, improper tethering and harnessing and other situations that cause injuries to donkeys (Morka *et al.*, 2014). The distribution of these factors is taught to vary from place to place since the environmental factors responsible for injuries and wounds vary geographically. Empirical evidence from DHWP clinic showed that distribution of tetanus to be aggregated to certain peasant associations of Ada'a district (DWHP, 2012). The aggregation of the cases to certain peasant association could be due to unknown or unidentified prevailing risk factors in those areas. Since Ethiopia does not provide tetanus toxoid, treatment and management of cases remained the only solution. The treatment

options have also been limited to procaine penicillin and management support. However, metronidazole was shown to be effective in reducing mortality in humans and small animals, for cases that were admitted to the clinic but this has not been tested in equines. We hypothesized that its use as an alternative drug would be better for tetanus management approach. This study was, therefore, designed with the following objectives:

- To identify the risk factors for occurrence of tetanus through a matched case – control study
- To compare therapeutic response of donkeys to Procaine penicillin G, Metronidazole and combination of Procaine penicillin G and Metronidazole
- To describe the spatiotemporal occurrence and clinical characteristic of the disease

2. LITERATURE REVIEW

2.1. Description of the Agent

Clostridium tetani is a Gram-positive rod (approximately 2–5 μM long and 0.5 μM wide) and is typical of the toxigenic, sporulating bacteria alongside other genera of bacteria that are strictly anaerobic. When sporulating, the vegetative organism develops a spherical spore at one end of the cell, giving rise to the characteristic morphologic appearance of “drumsticks” in smears. The organism is a motile and forms spore that can persist in soil for many years. The spores are extremely resistant to many standard disinfection procedures, including steam heat at 100°C (212°F) for 20 minutes but can be destroyed by heating at 115°C (239°F) for 20 minutes. The spores are very resistant to chemicals; even acidified phenol takes 2 h or more to kill them under the most favorable circumstances (Quinn *et al.*, 2002; Andrew and Jack, 2006; Peter *et al.*, 2017). Destruction of spores requires autoclaving for 12 minutes at 121°C or prolonged exposure to iodine, hydrogen peroxide, formalin, or glutaraldehyde (Wassilak *et al.* 2004).

2.2. Epidemiology

2.2.1. Occurrence

Tetanus occurs in all parts of the world and is most common in closely settled areas under intensive cultivation. Tetanus occurs sporadically throughout the world, the prevalence in unprotected populations correlating with the numbers of spores in the soil. *Cl. tetani* is often abundant in equine feces but it occurs in the gut of other herbivores and in the soil. The numbers in soils vary greatly, but the organism is especially abundant in warm areas with rich, well-cultivated soils. No agreement exists about whether the presence of *Cl. tetani* in the soil depends on the soil type or not. With an increasing population of humans and animals and intensification of land use, the amount of organisms is mounting; they are more frequent in ploughed fields than on pasture. It can multiply

within the soil in symbiosis with other anaerobes and/or aerobes; therefore, tetanus must be considered as a Saprozoonosis (Seifert, 1996; Andrew and Jack, 2006; Peter *et al.*, 2017).

2.2.2. Source of infection and transmission

Cl. tetani organisms are commonly present in the feces of mammals (including humans), especially in horses, and in the soil contaminated by these feces (Peter *et al.*, 2017; Roper *et al.*, 2007). The bacterium is present in the gastro-intestinal tracts of 5–9% of healthy horses (Wilkins *et al.*, 1988). Surveys in different areas of the world showed that it is present in 30-42% of soil samples. However, manure-treated soil and soil rich in organic matter may contain large numbers of spores (Seifert, 1996; Peter *et al.*, 2017). The portal of entry is usually through deep puncture wounds but the spores may lie dormant in the tissues for some time and produce clinical illness only when tissue conditions favor their proliferation. For this reason, the portal of entry is often difficult to determine (Peter *et al.*, 2017). In horses, the umbilical cord in neonates, retained placenta, puncture wounds of the foot, and surgical wounds are known to be frequent portals of entry for *Cl. tetani* infection (Kay & Knottenbelt, 2007). Of these, puncture wounds of the hooves are common sites of entry in horses. Introduction to the genital tract at the time of parturition is the usual portal of entry in cattle. A high incidence of tetanus may occur in young pigs following castration and in lambs following castration, shearing, docking, vaccinations, or injections of pharmaceuticals. Docking by the use of elastic band ligatures is reported to be especially hazardous in sheep and goats. Neonatal tetanus occurs when there is infection in the umbilical cord associated with insanitary conditions at parturition (Peter *et al.*, 2017). Previous studies have found that infection is most commonly acquired when a wound is contaminated by soil containing bacterial spores (Wilkins *et al.*, 1988; Newman, 1996).

2.2.3. *Survival of Cl.tetani in the environment*

The spores remain infectious for years particularly in warm climates where the soil is rich in organic matter, and are capable of surviving in dried earth for years because they are highly resistant to environmental changes. *Cl. tetani* cannot survive in normal tissues, but requires anaerobic conditions typically provided by devitalized tissues resulting from trauma (Mayhew and MacKay, 1983; Newman, 1996).

2.3. Distribution of the disease in humans and animals

The global incidence of tetanus in humans has been estimated to be approximately one million cases per year (Thwaites & Farrar, 2003; Bleck, 2005). Approximately 400 000 of the global tetanus cases are neonatal tetanus (Dietz *et al.*, 1996). The disease remains to be endemic in 90 countries of the world. Mortality associated with tetanus greatly varies among countries or continents. Of all death recorded, 80% of them occur in Africa and South East Asia (Whitman *et al.*, 1992). However, mortality can be as high as 100% in the absence of medical treatment (Blencowe *et al.*, 2010). The occurrence of tetanus cases in equines based on few retrospective studies is summarized in Table1.

Table 1: Overview of reports on the tetanus from various countries

Country	District or state	Year	No. of cases (species)	Mean age affected (years)	Case fatality	Treatment/ antibiotic used	Type of study	References
Croatia	Zagreb	1990-2009	42 H	5.8	45.3 %	PPF	RS	Damjan <i>et al.</i> , (2015)
USA	California	1970-1990	20 H	3.43	75%	PPF	RC	Green <i>et al.</i> , (1994)
Ethiopia	Bishoftu, CVMA	2008-2009	45 working D	9.04	33.3%	PPF	RS	Ayele <i>et al.</i> , (2015)
Morocco	charity clinic in Morocco	2003-2004	56 equids (25 D, 20 M and 11 H)	9.73	54%	PPF	RS	Kay and Knottenbelt (2007)
Belgium	Liege		31 (30 H and 1D)	-	68%	-	RS	Van Galen <i>et al.</i> , (2008)

D=Donkey, H= Horse, M=Mule, RS=Retrospective study, RC=Review of case, PPF=Procaine Penicillin G

2.4. Pathogenesis

Proliferation of the *Cl. tetani* is accompanied by release of a complex toxin composed of tetanospasmin, tetanolysin and a non-spasmogenic toxin called tetanus toxin (Seifert, 1996). Tetanus toxin is one of the most potent toxins ever identified, with a minimum lethal dose of less than 2.5 ng/ kilogram of body weight in humans (Van der Kolk and Veldhuis Kroeze, 2013). The toxin production can occur when spores germinate to the vegetative form under suitable anaerobic environment as occurs in deep puncture wounds or injuries producing necrotic tissue in susceptible hosts (Rings, 2004). The organism does not disseminate from the initial site of proliferation, but they produce tetanolysin and tetanospasmin in sufficient quantity when the level of oxygen in the tissue is markedly low. Tetanolysin promotes local tissue necrosis that facilitates spread of bacteria and resistance to macrophages of *Cl. tetani* during the early steps of wound tetanus, since tetanolysin is able to form pores and to induce membrane damage in macrophages (Francisco *et al.*, 2016). More importantly, of the mentioned exotoxins, tetanospasmin is the most relevant for the pathophysiology of the condition and it blocks the inhibitory nerve impulses by interfering with the release of neurotransmitters and therefore, tetanospasmin is responsible for the spastic paralysis that occurs in affected animals (COOK *et al.*, 2001).

Toxin production may occur immediately after introduction if the accompanying trauma has been sufficiently severe, or if foreign material has also been introduced to the wound, or may be delayed for several months until subsequent trauma to the site causes tissue damage. The original injury may be in apparent by then. Once produced tetanospasmin diffuses to the systemic circulation, and binds to gangliosides GD1b and GT1b on the membranes of local nerve terminals. If toxin load is high, some may enter the bloodstream from where it diffuses to bind to nerve terminals throughout the body. The toxin is then internalized and transported intra-axonally and retrogradely to the cell body. Transport of tetanospasmin occurs first in motor and later in sensory and autonomic nerves. Once in the cell body, it can diffuse out so affecting and entering nearby neurons will happen. When spinal inhibitory interneurons are affected symptoms occur.

Furthermore, retrograde intraneural transport occurs with toxin spreading to the brainstem and midbrain. This passage includes retrograde transfer across synaptic clefts by a mechanism which is unclear (Cook *et al.*, 2001). Then, tetanospasmin inhibits the release of the neurotransmitters mainly gamma-aminobutyric acid (GABA) and prevents the Ca²⁺ dependent release of glycine from CNS neurons in victims of tetanus (Spencer, 2000; Roder, 2004b; Peter *et al.*, 2017). The lack of inhibition by glycine results in unchecked muscle contraction, largely of the powerful extensor muscles of the limbs and the masseter muscles. The stereotypical sawhorse stance and “lockjaw” result within 5–10 days of wound infection. Less severe signs include elevation of the nictitating membrane, which may be the earliest indication of toxicosis in the horse, the most sensitive species (Coleman, 1998). When the toxin reaches the post-synaptic sites where it exerts its effect, it cannot be neutralized by antitoxin and is only removed by gradual degradation. The action of tetanus toxin within neurons persists for several weeks; the mechanism of functional recovery remains unclear (Wassilak *et al.*, 2004; Roper *et al.*, 2007). Death is usually caused by respiratory arrest from dysfunction of the tetanic diaphragm or aspiration pneumonia secondary to dysphagia or increased airway secretions (Rings, 2004; David, 2012).

2.5. Zoonotic importance

Tetanus is a disease common to man and animals. Some authors ascribe the role of reservoir to animals (McComb, 1980; Benenson, 1990), but it is more likely that the disease agent derives from the soil. Therefore, tetanus must be considered as a saproozoonosis (Seifert, 1996) and that it is present in the digestive tract of herbivores and omnivores only transitorily and does not multiply there (Smith, 1975; Wilson and Miles, 1975). Nevertheless, domesticated animals can disseminate toxigenic strains of *Cl. tetani* by means of their feces, in cultivated as well as uncultivated areas. It is estimated that there are approximately one million human tetanus cases per year in the world, with a fatality rate varying from 20 to 50% (Demócrito *et al.*, 2000), thus constituting an important public health problem, especially in many countries of Latin America, Africa, Asia and Oceania (Ogunrin, 2009).

2.6. Diagnosis and Diagnostic Techniques

Early diagnosis and therapeutic intervention is imperative for any chance of survival. Despite advances in understanding of the pathophysiology, there is no readily available diagnostic test that detects the presence of the toxin. The diagnosis is based on the wound history and the characteristic clinical signs (predominantly protrusion of the nictitating membrane in horses eventually following lifting of the head) (Green *et al.*, 1994; Van der Kolk and Veldhuis Kroeze, 2013).

2.6.1. Clinical findings

Typical symptoms of tetanus include increased general muscular rigidity evolving to a complete tetanic state, frequently with a fatal outcome (Linnenbrink and McMichael, 2006). The clinical signs are similar in all animal species. In the initial stage of the disease, there is characteristic stiffness of muscles, accompanied by tremors. There is trismus with restriction of jaw movements, prolapse of the third eyelid, stiffness of the hind limbs causing an unsteady, straddling gait, and the tail is held out stiffly, especially when backing or turning (Appendix V). Retraction of the eye and prolapse of the third eyelid - a rapid movement of the third eyelid across the cornea followed by a slow retraction - is one of the earliest and consistent signs (with the exception of sheep) and can be exaggerated by sharp lifting of the muzzle or tapping the face below the eye. Additional signs include an anxious and alert expression manifested by an erect carriage of the ears, retraction of the eyelids and dilation of the nostrils and hyperesthesia with exaggerated responses to normal stimuli. The affected animals may continue to eat and drink in the early stages but mastication is soon prevented by tetany of the masseter muscles, and saliva may drool from the mouth. If food or water is taken, attempts at swallowing are followed by regurgitation from the nose. Constipation is usual and urine is retained, partly as a result of inability to assume the normal position for urination. The rectal temperature and pulse rate are within the normal range in the early stages but may rise later when muscular tone and activity are further increased. As the disease progresses, muscular tetany increases and the animal adopts a 'sawhorse' posture (Fig. 1).

Uneven muscular contractions may cause the development of a curve in the spine and deviation of the tail to one side. There is great difficulty in walking and the animal is inclined to fall, especially when rising up from recumbency. Falling occurs with the limbs still in a state of tetany and the animals can inflict severe injury on themselves. Once down it is almost impossible to get a large animal to its feet again. Tetanic convulsions begin in which the tetany is still further exaggerated. Opisthotonos is marked; the hind limbs are stuck out. Cases of tetanus depend largely on keeping the animal alive through the critical stages (Peter *et al.*, 2017).

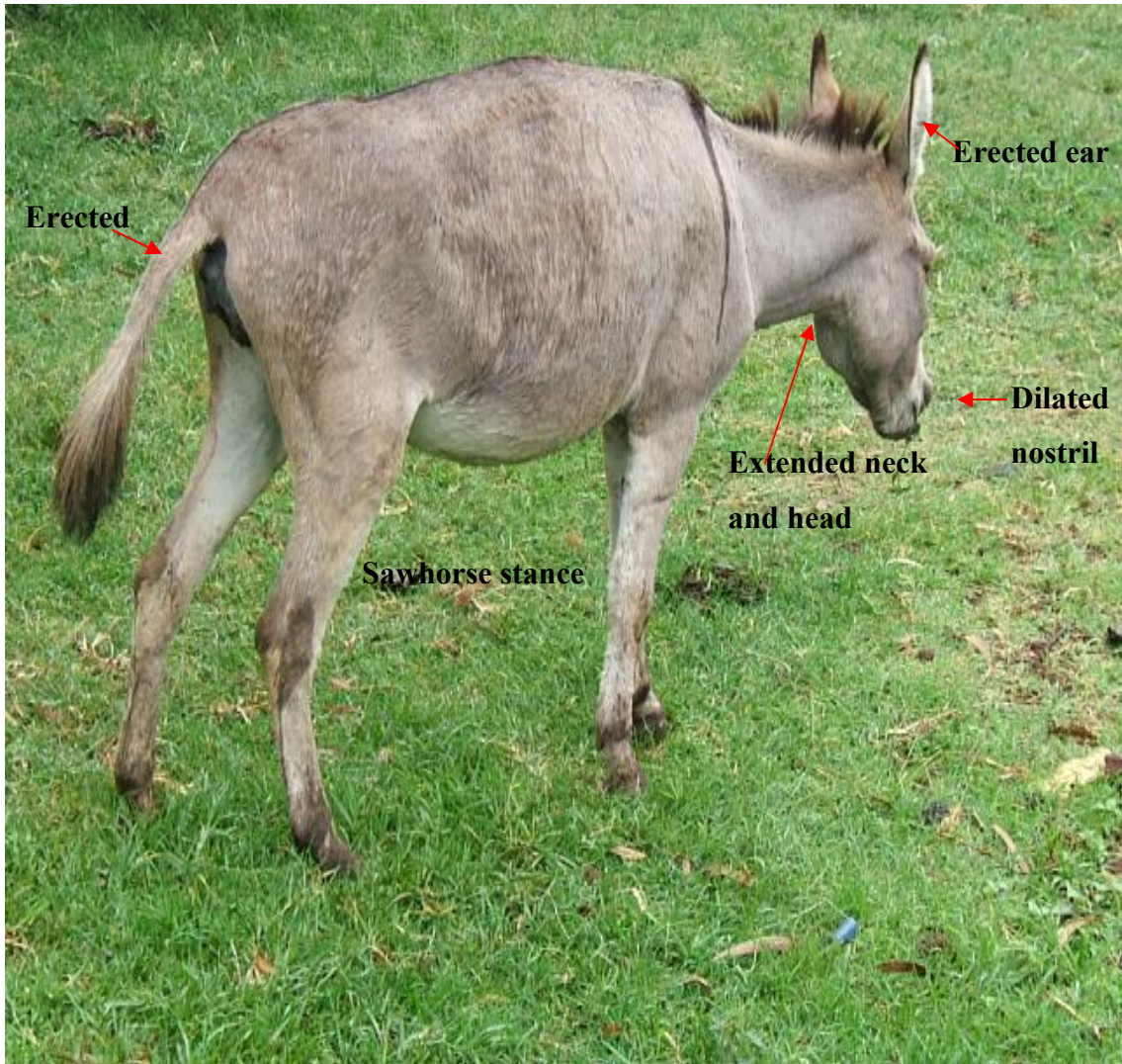


Figure 1: An adult donkey affected with tetanus. Note the typical stiffness and extended neck, sawhorse stance, erected ears and elevated tail. Photo by DHWP

2.6.2. Direct microscopy

Gram-stained smears of material from a wound may reveal the characteristic 'drumstick' sporing forms of *Cl. tetani* (Quinn *et al.*, 2002). However, gram staining of wound aspirates is limited diagnostic value. Sporulated and vegetative forms of *Cl. tetani* appear similar to other anaerobic bacteria.

2.6.3. Isolation of *Cl.tetani*

Isolation of *Cl. tetani* is difficult because of low concentration of wound contaminating organisms and the strict anaerobic conditions required for culture (David, 2012; Van der Kolk and Veldhuis Kroeze, 2013). However, bacteriologic culture and identification of *Cl. tetani* adapted from methodology described in the *manual of clinical microbiology* (Murray *et al.*, 1995) indicated possibility of isolation of an organism from punctured wound, feces and soils. Deep wound puncture swabs are usually inoculated into pre-oxygen reduced blood agar plates at the bedside. These agar media often contain 10% sheep blood in a blood agar base and are incubated anaerobically for a minimum of 30 minutes before use. The swabs are then immersed into a thioglycollate medium. All cultures have to be examined for growth of *Cl. tetani* at 24 and 48 hours after inoculation. To aid in the isolation of a pure culture of *Clostridium* species and reduce contaminants, the broth is heated to 80°C for 10 minutes and re incubated at 37°C for a further 2 days. *Clostridium* species are identified by gram film (Gram-positive rods with characteristic position of spores), colony morphology, and swarming properties on solid media (Murry *et al.*, 1995; Hatheway, 1990). Isolation of *Cl. tetani* from a wound is not a reliable diagnostic indicator. A biological assay for pre mortem diagnosis is possible by injecting infective material into mice and observing for the onset of clinical signs (Kay and Knottenbelt, 2007).

2.6.4. Identification of bacteria and its toxin

2.6.4.1. Colonial morphology

Cl. tetani forms colonies on the surface of agar medium only in anaerobiosis. Motile strains swarm over the entire surface of the agar, yielding a transparent film. Discrete colonies (2 to 5 mm) can be obtained by cultivation on media containing (3–4%) stiff agar. On blood agar, colonies are slightly raised, semi-translucent, and gray, with an irregular margin and surrounded by a narrow zone of hemolysis. *Cl. tetani* grows fairly

well on the usual media containing peptones or tissue extracts (Quinn *et al.*, 2002; Francisco *et al.*, 2016).

2.6.4.2. Biochemical reactions

Most of the usual biochemical tests used for identification of *Clostridium* spp. are negative, as no carbohydrates are acidified, and there is neither proteolysis nor production of lipase and lecithinase. Gelatin is liquefied slowly (2 to 7 days). The peptone used in the basal medium is considerable important when evaluating the ability of *Cl. tetani* to liquefy the gelatin. H₂S and indole are usually produced (Francisco *et al.*, 2016).

2.6.4.3. Toxin identification

The toxin present in an animal's serum in filtrate from cooked meat broth or thioglycollate medium can be demonstrated in laboratory animals and identified by neutralization or protection tests using specific antitoxin at least 2 hours before inoculation with the material containing toxin. The control mice show typical signs of tetanic spasm in the region of inoculation (Quinn *et al.*, 2002).

2.7. Risk factors

The epidemiological variability of tetanus is related to management practices. Tetanus affects omnivorous and herbivorous animals. However, there is wide variation in the susceptibility of animal species to tetanus toxin. Horses, nonhuman primates, and swine are highly susceptible, with cattle, sheep, goats, and humans less so and dogs and cats quite resistant (Murray, 2010; Peter *et al.*, 2017). In contrary to this, Wernery *et al.* (2004) and Driemeier *et al.* (2007) have reported that small ruminants and humans are known to be the most susceptible species next to horse. Susceptibility between sex and breeds has not been reported. The most commonly reported risk factor, in a series of affected horses, was the absence of vaccination (Van Galen *et al.*, 2008). It is likely that thin, parasitized donkeys also have compromised immune systems, making them more susceptible to the disease (Kay and Knottenbelt, 2007). It has been suggested that

donkeys have better survival rates than horses. Young animals are affected more often and more severely by tetanus than older animals (Van Galen *et al.*, 2008).

2.8. Differential diagnosis

Fully developed tetanus is so distinctive clinically that it is seldom confused with other diseases. The muscular spasms, the prolapse of the third eyelid, and a recent history of accidental injury or surgery are characteristic findings. However, in its early stages or mild forms, tetanus may be confused with the following conditions or diseases:

- meningitis
- brain trauma
- tetany (hypocalcaemia)
- myopathy
- severe lameness and especially with reference to the protrusion of the nictitating membrane hyperkalaemic periodic paralysis (HYPP) and other channelopathies should be considered (Van der Kolk and Veldhuis Kroeze, 2013; Peter *et al.*, 2017).

2.9. Prevention and control

2.9.1. Treatment

The treatment of tetanus has several starting points: neutralization of unbound toxin, antibiotic application, and treatment of wound, symptomatic treatment and providing supportive care or nursing (Seifert, 1996; Roper *et al.*, 2007; Peter *et al.*, 2017). Recovery from clinical tetanus is dependent on the gradual replacement of affected gangliosides by normal metabolic processes, because the binding of tetanospasmin to gangliosides in the CNS is irreversible (Mayhew and MacKay, 1983). Therefore, treatment of tetanus is necessarily symptomatic and supportive. It is important to remember that any external stimuli may provoke extreme reactions in the patient, making treatment difficult and potentially dangerous for the clinician (Pearce, 1994).

2.9.1.1. Neutralization of unbound toxin

The efficacy of antitoxin is limited as it has no effect on tetanospasmin already bound to gangliosides; however, it is an accepted part of the standard treatment protocol because it is believed that antitoxin prevents continued binding of gangliosides and so prevents aggravations of clinical signs (Pearce, 1994). Published data revealed that doses and routes of administration of tetanus anti-toxin (TAT), varied from as high as 2.5 million IU given once to as low as 220 IU/kg bwt, IV, q12 h. One investigation reported better outcomes with 2500 IU administered subcutaneously everyday for 3 days (Green *et al.*, 1994), whereas Pearce (1994) suggested 5,000 to 10,000 IU given intravenously every day for three days was sufficient to neutralize circulating toxins. Still it is debatable whether the higher doses are more effective; indeed, it has been suggested that tetanus antitoxin may be neither beneficial nor economically viable in the treatment of tetanus in working animals in the developing countries (Kay and Knottenbelt, 2007). These antibodies will bind the tetanus toxin before it can exert an effect on the neuromuscular junction. For intrathecal administration (spinal injection), either into the atlanto-occipital or lumbosacral space, positive results have been reported with the administration of 5–10,000 IU of TAT directly into the subarachnoid space. It is recommended that an equal volume of cerebro-spinal fluid (CSF) be removed before injecting the TAT (Cynthia *et al.*, 2015). In human cases of tetanus, following the onset of clinical signs, intrathecal administration of anti-tetanus immunoglobulin is associated with higher survival rates (Kabura *et al.*, 2006), although this has not been demonstrated in equids (Steinman *et al.*, 2000). Administration of antitoxin into the subarachnoid space under general anesthesia has been advocated in the treatment of both equine tetanus and human neonatal tetanus, but its advantages are questionable and studies to assess its efficacy have produced conflicting results (Neequaye and Nkrumah, 1983; Green *et al.*, 1994). Similarly, a meta-analysis done by Kabura *et al.* (2006) indicated that intrathecal administration was superior to the intramuscular route with respect to survival.

2.9.1.2. Antibiotic application

Penicillin is active against the vegetative form of *Cl. tetani* and is indicated in all cases of tetanus (Green *et al.*, 1994). It is also valuable in controlling aerobic contaminants that help to establish the anaerobic environment necessary for toxin production at the wound site, and can control secondary infections such as pneumonia which may complicate tetanus cases (Mayhew and MacKay, 1983). The standard protocol described in the literature recommends an initial dose of 35,000 IU/kg of sodium benzyl penicillin given intravenously, followed by intramuscular injection of 20,000 IU/kg of procaine penicillin 12 hours later. This should be followed by injections of procaine penicillin every 8 to 12 hours for a minimum of five days. However, the frequency of injections can be reduced to once a day when clinical signs start to regress (Pearce, 1994). Additionally, penicillin ointment may also be applied to the wound itself. Penicillin acts as a competitive antagonist to GABA, and in high doses may cross the blood brain barrier and cause CNS excitability (Bloch *et al.*, 2001). In patients suffering from tetanus, it is possible that, this will exacerbate clinical signs by worsening the effects of tetanospasmin at GABAergic neurons. A case of penicillin induced haemolytic anaemia has been reported in a horse following treatment for tetanus (Step *et al.*, 1991). In treatment of human tetanus cases, Metronidazole is considered the first line therapy. This is because Metronidazole is rapidly bioavailable and causes fewer spasms than administration of Penicillin. This may be explained by the action of penicillin at GABAergic synapses and may therefore potentiate the effect of tetanus toxin, which could increase muscle rigidity (Ganesh *et al.*, 2004).

2.9.1.3. Treatment of the wound

The site of bacterial proliferation should always be searched or explored; any wound found must be actively treated. Devitalized tissues should be removed and the wound left open; *Cl. tetani* is an obligate anaerobe and will be killed by this treatment, thereby eliminating the source of the toxin. However, wound cleaning and debridement should only be done after antitoxin has been administered, because debridement, irrigation with

hydrogen peroxide, and the local application of Penicillin may facilitate the absorption of the toxin (Peter *et al.*, 2017). Moreover, it has been suggested that antitoxin serum injected around the wound may inhibit the spread of toxin from infected tissues, although this should not be necessary if the wound is meticulously cleaned and debrided (Pearce, 1994). Hydrogen peroxide 1% is useful for cleaning wounds as it increases oxygen tension which results in the inhibition of obligate anaerobes (Greene, 1998).

2.9.1.4. Symptomatic treatment

Treatment of the muscular rigidity and spasms in tetanus is of vital importance, since this feature of the disease often interferes with respiration and is a likely cause of death (Bleck, 2005; Amare *et al.*, 2012). Rigidity and spasms also cause severe pain, which stimulates muscle activity. Muscle relaxation is customarily achieved by administration of muscle relaxants. Sedatives are used to diminish the response to external stimuli and thereby control muscular spasms and rigidity. This makes nursing easier and safer, and reduces the risk of self-inflicted injuries during tonic muscle contractions. Under the standard protocol described in the literature, acepromazine is given intravenously or intramuscularly at a dose of 0.05-0.1 mg/kg (Pearce, 1994). This dose may need to be repeated as often as treating affected animals with 0.05-0.2 mg/kg diazepam by slow intravenous injection (Kay and Knottenbelt, 2007). Muscle relaxants are now rarely used and thus heavy sedation alone may provide adequate neuromuscular relaxation (Bloch *et al.*, 2001). Due to the need for repeated injections, the use of sedatives is only appropriate where hospitalization facilities are available.

2.9.1.5. Providing supportive care

Supportive care is arguably the most important aspect of the treatment protocol for tetanus. If the source of the toxin is eliminated, the patient's hydration and nutritional status can be maintained, and self-inflicted injuries are prevented, mild to moderate clinical signs will tend to resolve over time as the toxin fixed to the gangliosides of the CNS is removed by metabolic processes. External stimuli should be minimized by

housing the animal in a quiet, dark stable and well bedded quarters with nonslip flooring and plenty of room to avoid injury if convulsions occur. Placement of an indwelling jugular catheter will reduce the stress of repeated intravenous injections (Mayhew and MacKay, 1983; Peter *et al.*, 2017). The bedding should be sufficient to allow the animals to rest comfortably and prevent self-inflicted injuries, but not so deep as to impede movement around the stable (Pearce, 1994). Feed should be highly palatable to encourage voluntary intake, offered *ad libitum*, and preferably soft and moist to aid prehension and mastication. Water and feed containers should be elevated to trough height. The hydration and nutritional status of dysphagic animals can be maintained by nasogastric intubation and/or intravenous infusion with acetated Ringers solution (Kay and Knottenbelt, 2007). Care must be taken not to traumatize the pharynx and esophagus when the tube is passed repeatedly. Manual evacuation of the rectum and urinary catheterization may be necessary and these may relieve the animal's discomfort (Peter *et al.*, 2017). In extreme cases, slings may be used to support a recumbent animal or one which is likely to fall over and injure itself, but it has been argued that this is rarely justified from a welfare perspective and euthanasia is probably indicated in such cases (Pearce, 1994). As general, nursing, plus penicillin, ataractic drug and antitoxin for an average of 14 days, can deliver something like a 50% recovery by an average of 27 days, but the cost is high (Peter *et al.*, 2017).

2.9.2. Prognosis of disease

Few studies exist about factors affecting the outcome of equids suffering from tetanus (van Galen *et al.*, 2008). The prognosis for survival is reported to be dependent on several factors including the dose of clostridial organism inoculated, the size, age, and immune status of the animal, and the availability and duration of aggressive treatment and supportive care (Mayhew and MacKay, 1983; Green *et al.*, 1994). A history of prophylactic vaccination with tetanus toxoid is strongly associated with survival (Green *et al.*, 1994). In their study of a mixed population of 56 equids presented to a charity clinic in Morocco, Kay and Knottenbelt (2007) reported that the severity of clinical signs at presentation is the only clinical factor significantly associated with prognosis. Case

fatality rates reported in the literature are variable and often based on small sample sizes. Green *et al.* (1994) reported a fatality rate of 75% in a case series of 20 horses referred to the Ontario veterinary college-veterinary teaching hospital, Canada. Van Galen *et al.* (2008) report a fatality rate of 68% in a case series of 31 equids (30 horses and one donkey) admitted to the equine clinic of the Liege university, Belgium. In the only case series of working equids yielded by an internet search, Kay and Knottenbelt (2007) reported a fatality rate of 54% in a case series of 56 equids (25 donkeys, 20 mules and 11 horses) at a charity clinic in Morocco. Cases that were presented with advanced spastic muscular contractions, well-developed trismus, or lateral recumbency, any of which carry a grave to hopeless prognosis, were often considered candidates for immediate euthanasia (Pearce, 1994). Moreover, dyspnoea, recumbency, and the combination of dysphagia, dyspnoea, and recumbency were considered indicators of a poor prognosis in a retrospective study of 31 cases presenting with clinical tetanus to a clinic in Belgium (Van Galen *et al.* 2008). In general, cases with a good prognosis for recovery are those in which mild clinical signs have appeared over a number of days. In comparison, the prognosis of generalized human tetanus is strongly predicted by the incubation and onset periods. Short incubation and onset periods correlate with increased disease severity and higher mortality. Autonomic dysfunction also predicts high mortality, especially if it manifests early in the disease course (Brauner *et al.* 2002; Roper *et al.* 2007).

2.9.3. Control

Natural infection and recovery from tetanus does not lead to protective humoral immunity in equines because of the very low dose of toxin required to induce clinical signs (Mayhew and MacKay, 1983). Tetanus can be prevented efficiently through active immunization. At the same time, care should however be taken to prevent circumstances in animal management which favor injuries in animals during castration and hoof trimming which is appropriate for the development of the infection (Seifert, 1996; Peter *et al.*, 2017).

2.9.3.1. *Passive immunity*

Short-term prophylaxis can be achieved by the injection of tetanus antitoxin. The immunity is transient, persisting for only 10-14 days (Peter *et al.*, 2017). Tetanus antitoxin should be administered prophylactically to unvaccinated equids following injury or surgery, at a minimum dose of 3,000 IU. Toxoid can be administered at the same time as antitoxin, but not mixed in the same syringe or at the same injection site (Mayhew and MacKay, 1983). Foals from unvaccinated mares, or those that did not receive colostrum, can be protected at birth with 3,000 IU antitoxin serum given subcutaneously, repeated at four to six weeks of age, and then toxoid course started at three months (Pearce, 1994). However, tetanus antitoxin is no longer routinely given to newborn foals because of the potential for serum hepatitis (Tom *et al.*, 2008).

2.9.3.2. *Active immunity*

At present, vaccines are produced as formalin and /or glutaraldehyd-toxoids. The vaccine is available (toxoid-bound to aluminium hydroxide, aluminium phosphate or calcium phosphate) and induces protective immunity for 2-4 years and therefore, booster vaccination is needed only every 2-4 years. Combined vaccines which also contain toxoids from other clostridia are commercially available. As a standard, 150 IU of tetanus toxoids are applied to all animal species and boosted after 4 weeks (Seifert, 1996; Peter *et al.*, 2017). A challengeable immunity will have been achieved 10-14 days after the booster vaccination. Brood mares are commonly given a booster one month before foaling, and then their foals begin the toxoid course at three to four months of age. However, there is evidence that maternal antibodies acquired by foals born to mares vaccinated shortly before parturition significantly inhibit the antibody response of the foal to primary vaccination until it is 6 months of age and that primary vaccination should be delayed until that age (Peter *et al.*, 2017).

3. MATERIALS AND METHODS

3.1. Description of study areas

The study was conducted on medical case records of the Donkey Sanctuary clinic also called the Donkey Health and Welfare project clinics (DHWP) and the Society for the Protection of Animals Abroad (SPANNA) at the College of Veterinary Medicine and Agriculture, Addis Ababa University in Bishoftu and Merkato DHWP clinic in Addis Ababa (Fig. 2).

Tetanus cases admitted to the DHWP clinics were used for experimental study. Soil samples were collected from selected peasant associations of Ada'a district with high and low tetanus cases.

The Donkey health and Welfare clinic of the College of Veterinary Medicine and Agriculture is located in Bishoftu town, Ada'a district of Oromia regional state. The clinic provides fee free clinical and animal owners' education service to surrounding communities from various peasant associations (commonly called Kebele) found in the district (Fig. 3). Geographically, Ada'a district is found in Oromia regional state in east Shoa zone 45km southeast of Addis Ababa, located between longitudes 38°51' to 39°04' East and latitudes 8°46' to 8°59' North. Most of the land (90%) is plain midland ranging between 1600 to 2000 meters above sea level. The district is characterized by sub-tropical climate and receives 860 mm rainfall/annum. The main rainy season occurs between mid-June and September, followed by a dry season that might be intercepted by the short rainy season in February and March. Mean annual temperature ranges from about 8–28°C. The mean relative humidity is 61.3%. Black clay vertisol is the dominant soil type, with good soil fertility but with water logging problems in those areas where the land slope is below 8%. Farming system is mixed crops–livestock production system. Major crops grown are teff, wheat (mainly bread variety) and pulses. Chickpea is the main pulse crop grown in the district and used as a crop rotation to wheat and teff crops. Irrigated horticultural crops represent a newly emerging business in areas where small-scale irrigation has been identified as potential venture. Livestock population is estimated to be

371864. The equine population is estimated at 36107. Of this donkeys accounts 80.45% of the equine population and provides a transportation of agricultural commodities in the area (ADARDO, 2013).

Addis Ababa is the capital city of Federal Democratic Republic of Ethiopia, and it has an area of 51,000 hectare in the central highlands with an average altitude of 2000-2560 meters above sea level. Addis Ababa has ten administrative sub cities based on geographical settlement. These are Addis ketema, Akaki-kaliti, Arada, Bole, Gulele, Kirkos, Kolfe-keraniyo, Lideta, Nefas silk and Yeka (Fig. 4). The area is characterized by bimodal rainfall with an average of 1100 mm, the highest percentage of rainfalls during the long rainy season from June to September. The short rainy season is from February to April. Addis Ababa has an estimated human population of 3.15 million (CSA, 2007). Despite of modern transportation facility in Addis Ababa, donkeys remain valuable animal in transporting of different commodities. There is an estimated of ten thousand donkeys found in Addis Ababa and predominating male donkeys. Majority of them involve in transportation of grain from Mesalamiya to different areas of the city and now days they are also fully engaging in transporting of construction materials around CMC and other places. The existing data showed that donkeys were presented to Merkato DHWP clinic from Addis Ababa sub cities and districts of surrounding Addis Ababa namely Welmera, Mulo and Sululta districts of Oromia special zone. Both Addis Ababa and Ada'a district are surrounded by hills and mountains that could lead flow of soil into catchment area or basin during rainy season.

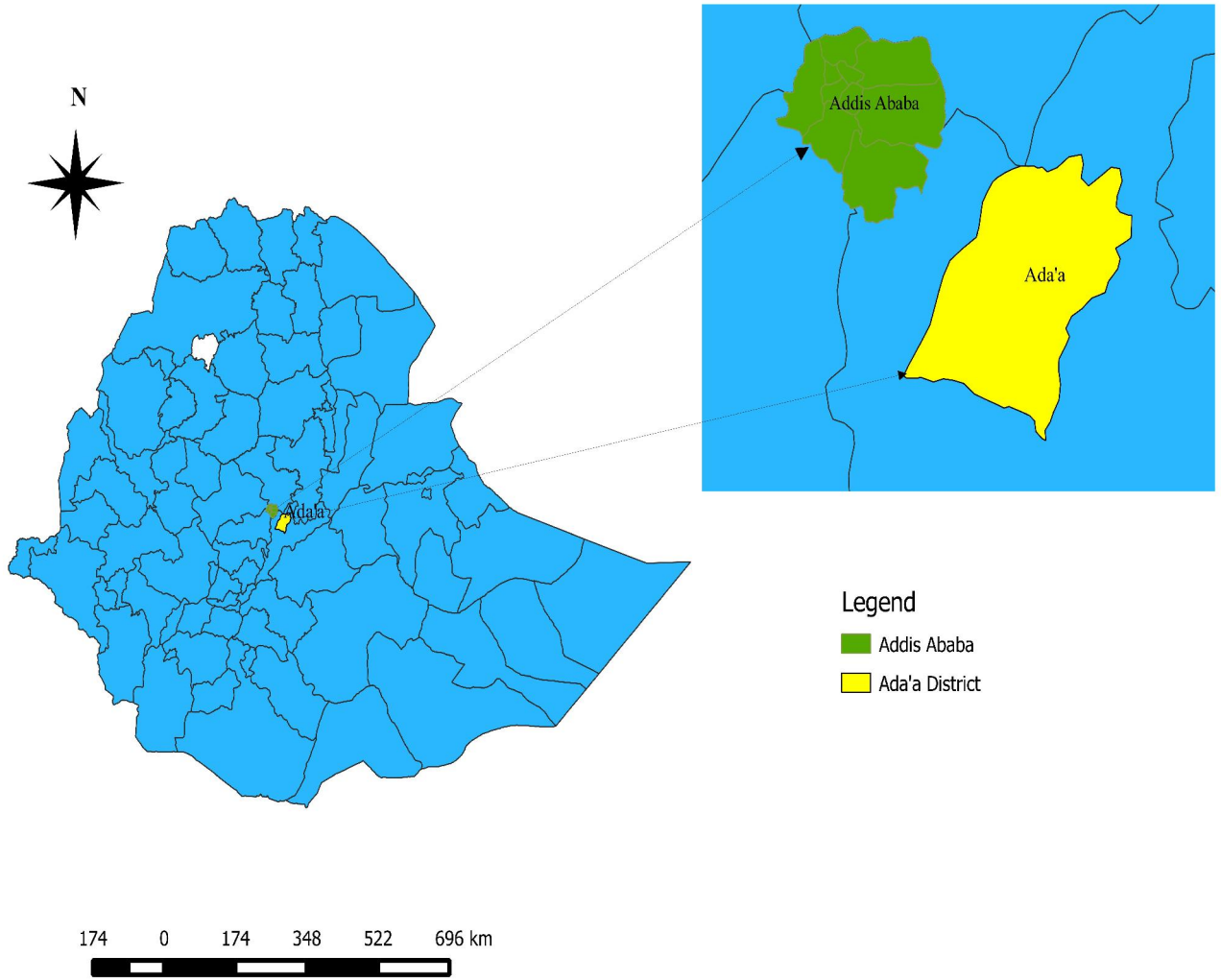


Figure 2: Study Areas: yellow color (Ada'a district) and green color (Addis Ababa).

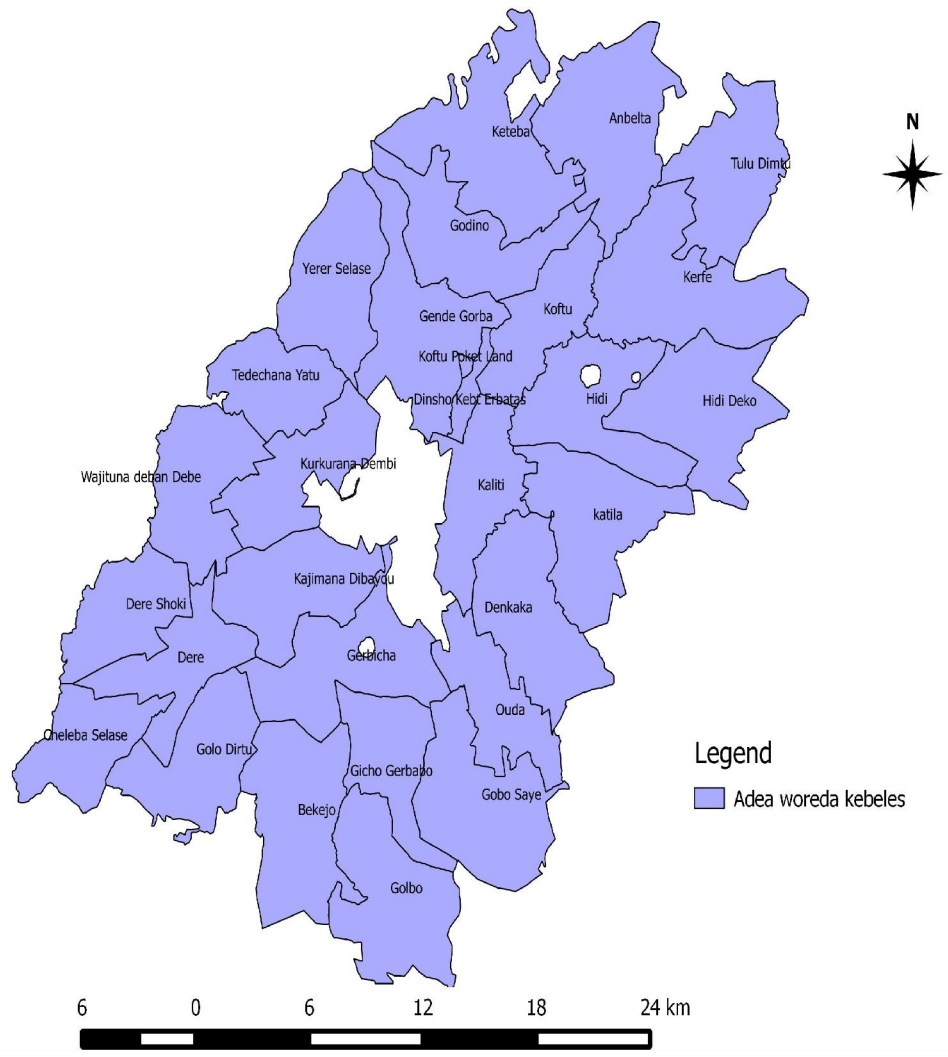


Figure 3: Map of Ada's district showing the various peasant associations

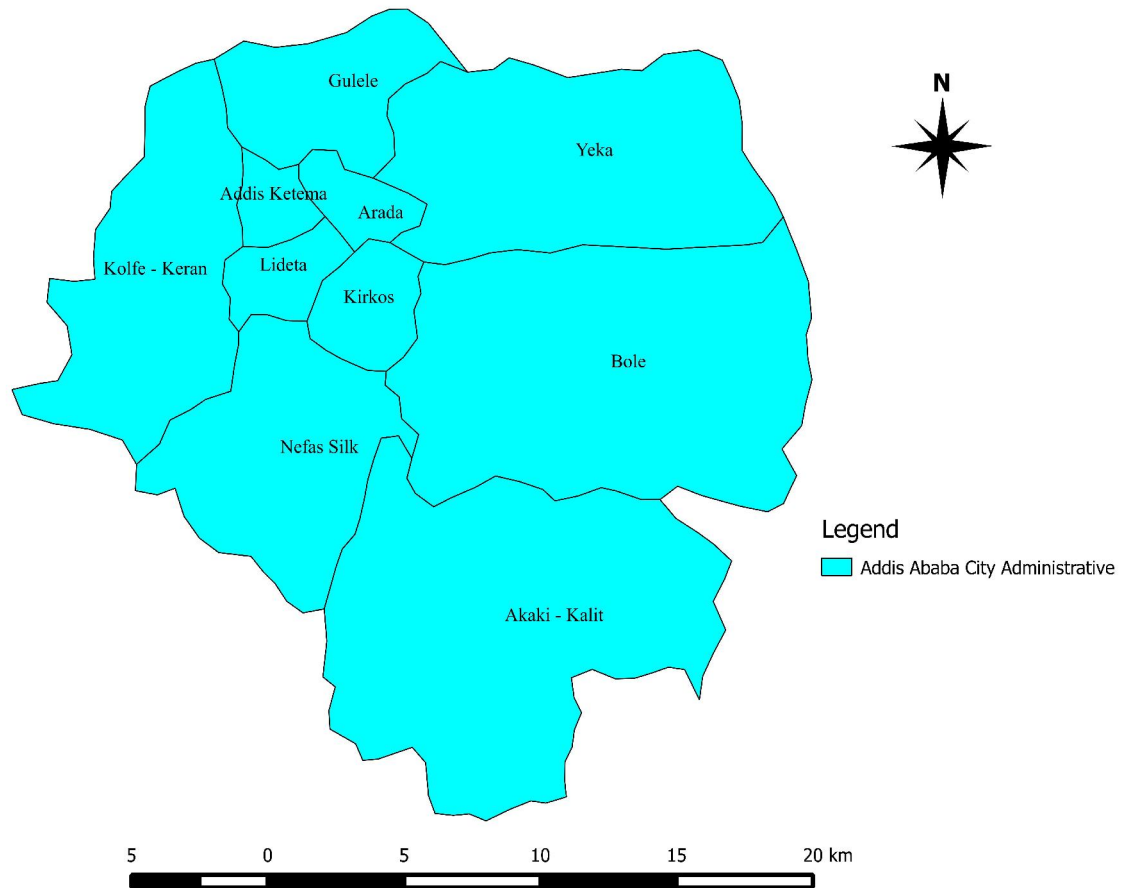


Figure 4: Map of administrative sub-cities of Addis Ababa

3.2. Study methodology

3.2.1. Study design

In this study, two study designs namely case control for the identification of putative risk factors and randomized control experimental design for the clinical trial.

3.2.1.1. Case control study

The medical records of 274 equines (257 donkeys, 15 horses and 2 mules) diagnosed with tetanus cases from 2008 to 2017 were retrieved. Available and required information

including spatiotemporal occurrences of tetanus and other risk factors were collected from the DHWP clinical records (Appendix I). Based on the outcome, cases were divided into survivor and non-survivor groups to determine prognostic guideline. Since data on the survival status of 13 donkeys were not available, they were excluded from the study. For matched case-control, a case definition for equine tetanus was formulated as owners complaint of retention of urine, donkeys, mules or horses with physical signs of skeletal muscle stiffness demonstrated by raised ear, tail and head, difficult in movement, limited or no jaw movement, prolapse of the third eyelid and retention of urine. All equines admitted to DHWP and SPANA clinics from 2008 to 2016 were which clinically diagnosed as tetanus cases. Tetanus cases that died were also considered cases. Whilst those equines admitted just for deworming or other health disorders drawn from the same area were considered as non-cases, and hence controls. Epidemiological data were gathered for both cases and controls.

Matched case-control study

Selection of cases

Medical records of donkeys with clinical diagnosed of tetanus that were presented to the DHWP clinics of Bishoftu and Addis Ababa between January 2008 and May 2017 were enrolled. Additionally, due to unavailability of data before 2011, only medical records of horses presented with history of tetanus to SPANA clinic from 2011 to May 2017 were used in this study. Information on the attributes of animals such as age, sex, species, presence and sites of wounds, duration before hospitalization, grade of tetanus, treatment protocol, duration of hospitalization, survival status and other factors were collected from the records.

Control selection

Two equines that were not diagnosed with tetanus were selected for every tetanus case. Accordingly, 548 equines (514 donkeys, 4 mules and 30 horses) served as control. Selection of controls was carried out from equines presented to the same clinics from the same areas and during the same study periods as cases. Whenever it was not possible to obtain controls from the same place as cases, equines were recruited from nearest peasant association. All controls were matched with cases with respect to age and gender before analysis of the data.

3.2.1.2. Experimental study

For the experimental study, each donkey presented to DHWP of Bishoftu and Addis Ababa between October 2016 and May 2017 with history tetanus were clinically examined and classified according to the grade of tetanus described in the literature (Kay, 2006) (Table 2). Donkeys were grouped into three age categories. Donkeys under two years of age were classed as young, those in range of two to ten years were classed as adult and those greater than ten years were classed as old. These age classes were based on the age of first work, productive age and the life span of Ethiopian donkeys (Yoseph *et al.*, 2001; Svendsen, 1997). Bodyweights were estimated using the Ouassat and Pearson (1997) nomogram for working donkeys where as body condition score was done as described by Svendsen (1997) (Appendix III). Then, each graded cases were randomly allocated to one of the three treatment groups (procaine penicillin G, metronidazole, combination of the procaine penicillin G + metronidazole). The route of administration and dosage of all drugs were determined as recommended by Svendsen (1997). Pulse rate (beats/min), respiratory rate (breaths/min), rectal temperature (°c) were measured immediately before treatment on first day and daily for four consecutive days after treatment was provided. Changes in clinical signs were recorded daily for five days (Appendix II). The route, dose and frequency of administration of the three treatments are presented as follows:

Treatment 1: Procaine Penicillin G 20,000 IU/kg, q12h, IM for 5 days

Treatment 2: Metronidazole 15mg/kg, q12h, IV for 3-5days

Treatment 3: Procaine Penicillin G 20,000 IU/kg, q12h, IM plus Metronidazole 15mg/kg, q12h, IV for 5days.

Additionally, after treatment on first day until observation of outcome, donkeys were placed in quite, non-agitating environment (such as a dark and deep bedded room) and ears were plugged with cotton to reduce external stimuli.

Table 2: Clinical grading of tetanus based on the assessment of clinical signs observed as described by (Kay, 2006)

Grade	Description	Criteria
1	Mild clinical signs	Slightly stiff gait but still walking and eating without difficulty
2	Moderate clinical signs	Limbs stiff and walking with difficulty, trismus and generalized muscle spasm, animal still capable of eating and drinking
3	Severe clinical signs	Capable of maintaining an upright posture but incapable of walking, difficulty eating
4	Terminal clinical signs	Animal recumbent, incapable of eating

3.2.1.3. Isolation of bacteria from soil

In order to identify occurrence of *Cl.tetani* in animal environment, soil samples were collected from area with high cases of tetanus (Gerbicha, Gicho Gerbabo and Kajimana Dibayou) and low cases of tetanus (Oude and Hidi) in the peasant associations of Ada'a district. Point data (Geographical coordinates) was taken by GPS apparatus from soil sampled areas (Appendix IV). Soil samples collected from animal's environment were transported and processed at NVI. Collection of soil samples was made following bacteriological standard safety conditions described by CDC (2009) using anaerobic transport media (Murray *et al.*, 1995; WHO, 2007). Gram stain and spore staining (Appendix X) were done for all cultures of soil samples prepared on VF (Viande Et Foie)

broth media (Appendix VIII). Biochemical tests were done for selected samples (based on availability of media) as described by (Merchant and packer, 1967) and details of the materials and reagents used, procedures and interpretation of the results are presented in appendix IX

3.2. Study population

The study populations were equines admitted to DHWP and SPANA clinics between 2008 and 2017. Equines that did not have tetanus cases and admitted on the same date as of the cases from the same locality were purposively recruited as controls. Epidemiological investigation to identify the risk factors of exposure was carried out retrospective (Thrusfield, 2005). Additionally, soil samples were collected from selected Ada'a peasant association with high and low cases of tetanus in the last eight years. For experimental study, all donkeys presented with history of tetanus to DHWP from October 2016 to June 2017 irrespective of their age, sex and body condition were closely examined for the case of tetanus. Those clinically found with typical signs of tetanus were used for treatment options of experimental trial. Accordingly 11 donkeys were used for experimental clinical trial.

3.3. Sample size and sampling method

Non-probabilistic sampling approach was used for both case control and experimental studies. Records of equines with tetanus at DHWP and SPANA clinic were selected purposively as cases (Thrusfield, 2005). Accordingly, the medical records of 274 equines (257 donkeys, 15 horses and 2 mules) diagnosed with tetanus cases from 2008 to 2017 were obtained. Control equines were selected purposively from list of equines admitted for other than tetanus cases after matching of the date of admission, gender and age as that of the cases. A total of 548 equines in the ratio of one case to two controls were included in the study as control.

3.4. Ethical considerations

The study was approved by the Addis Ababa University, college of veterinary and agriculture animal research ethical review committee Ref.no:VM/ERC/11/06/09/2017 (Appendix XIII).

3.5. Data management and analysis

The collected epidemiological data were stored in Microsoft Excel spreadsheet MS office (2007) program. The data were imported to STATA version 13 (Stata Corp., 2013). Associations between survival and different parameters were analyzed by the Chi-square test. Mean and standard deviation was calculated for continuous variables: mean rectal temperature, pulse and respiratory rates were calculated for each treatment group by averaging the data obtained from all animals in-group every day for five consecutive treatment days. The effects of duration before hospitalization and duration of hospitalization on the survival of equines affected with tetanus were analyzed using two-sample t-test in Stata. The map presenting spatial distribution of tetanus cases was generated using QGIS version 2.0.1 (2013). Multivariable conditional logistic regression analysis was used to identify risk factors of occurrence of tetanus in matched case control study. A significance level ($P < 0.05$) and confidence level of 95% was considered statistically significant.

4. RESULTS

4.1. Results of descriptive analysis

4.4.1. Demographic characteristics of study population

The study population has comprised of records of 274 that were diagnosed for tetanus cases at the clinics of DHWP (Bishoftu and Merkato) and SPANA, College of Veterinary Medicine and Agriculture, Addis Ababa University from between 2008 and 2017. Of 274 equines diagnosed with tetanus, 257(93.80%), 2(0.73%) and 15(5.47%) were donkeys, mules and horses, respectively. Among the study animals, 5 (1.82%) were young, 186 (67.8%) were adult and 83 (30.29%) were old. One hundred and eighty one (66.06%) were males whereas 93 (33.94%) of them were females.

4.4.2. Spatial and temporal occurrence of tetanus cases

Of all tetanus cases recorded, 84.30% (n=231) and 15.70 % (n=43) were from Ada'a district and Addis Ababa, respectively. Of 29 peasant associations in Ada'a district, tetanus was recorded in 17 i.e it has occurred in 58.62% of the peasant association. Of these, Gerbicha peasant association had the highest share of cases 28.14 % (n=65), followed by Gicho Gerbabo 16.02 % (n=37), Kajimana Dibayou 14.72% (n=34) and Bishoftu town 9.1% (n=21) whereas the lowest cases were recorded in Ganda Gorba, Gobo Saye and Oude (Fig. 5). In Addis Ababa tetanus was recorded in four of the ten sub cities, which is equivalent to 44.44% of the city. Over all 43 cases of tetanus were recorded in Addis Ababa, the majority of which were diagnosed in Kolfe-keraniyo 62.8% (n=27) followed by Addis Ketema 16.28% (n=7) (Fig. 6).

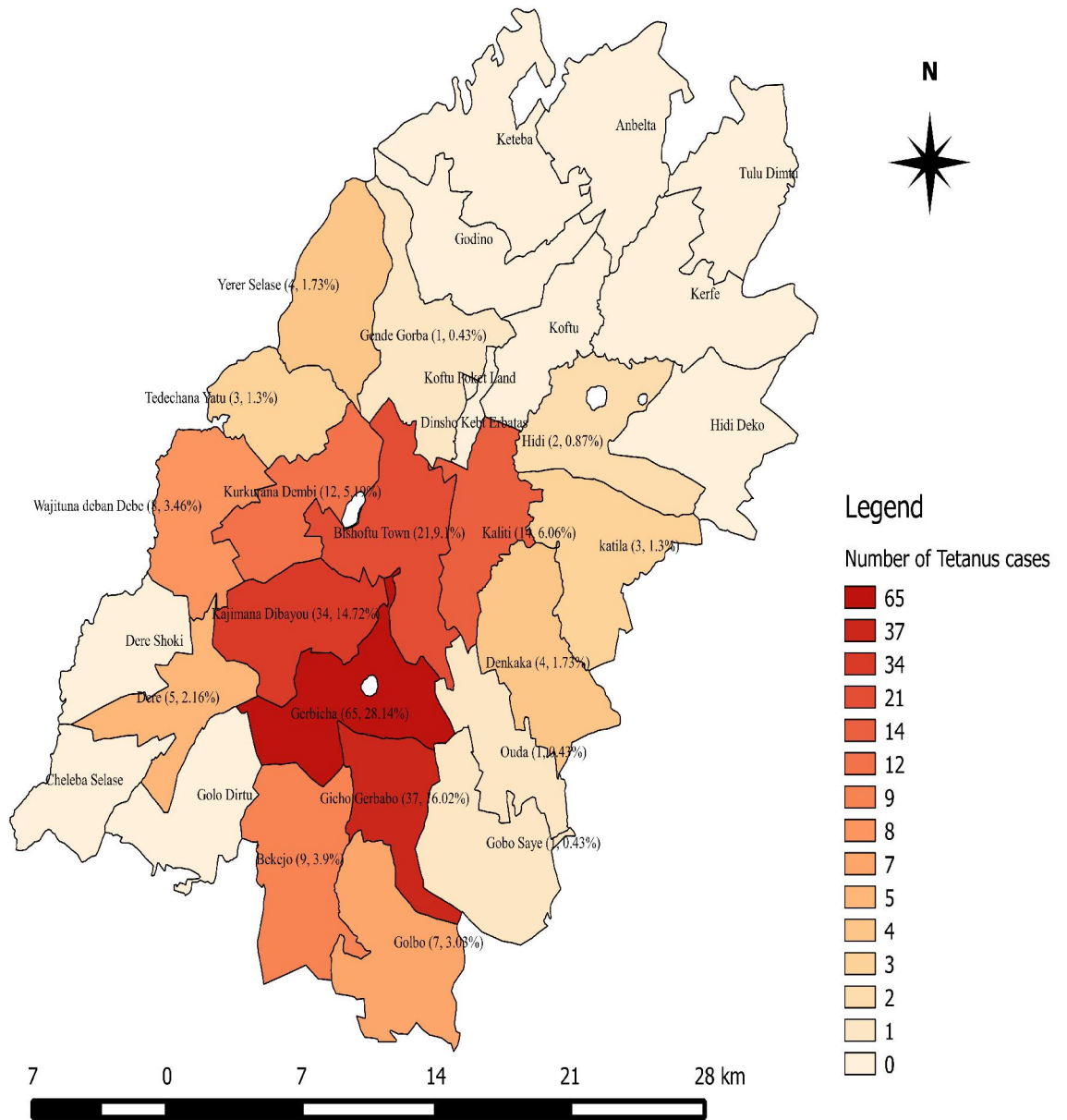


Figure 5: Occurrence of tetanus cases in different peasant associations of Ada'a district between 2008-2017

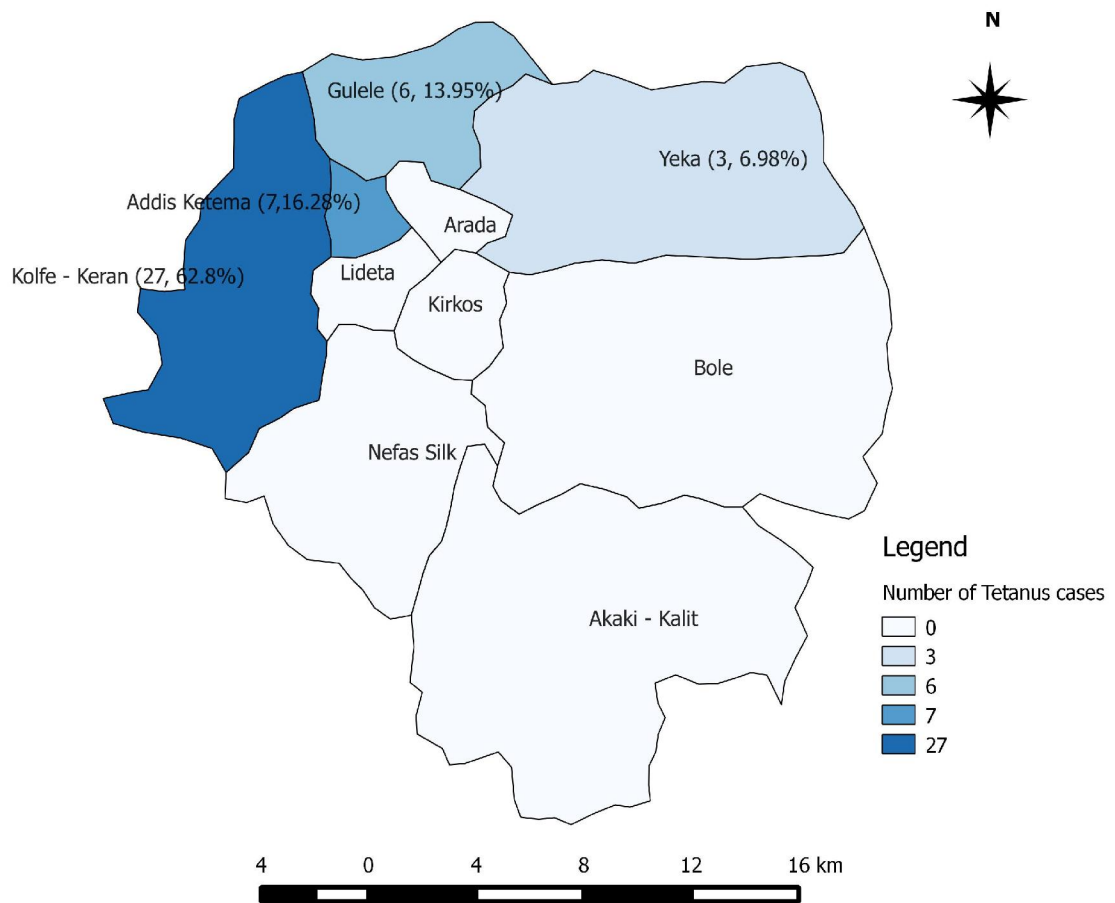


Figure 6: Occurrence of tetanus cases in various sub cities of Addis Ababa between 2008-2017

Of 274 equines diagnosed for tetanus cases, 155 (56.57%) survived, 79(28.83%) of them had died and 27(9.85%) were euthanized while the status of 13(4.74%) of them was unknown. The overall case fatality during the study period indicated 40.61% (106/261) including the 27 animals subjected to euthanasia. The highest case fatality 61.90% (13/51) was recorded in 2010 while the lowest case fatality was recorded in 2008 (Fig. 7). However, the differences observed among years was not statistically significant ($p>0.05$). During the study period, the case fatality percentage due to tetanus ranged from 28.13 % to 61.9 %. Tetanus cases were recorded in all months although the highest cases were recorded in summer season (August and July). The lowest number of cases was recorded

in winter season (February and December) as depicted in Fig. 8. There was no statistically significant difference observed between factors such as age and sex of animals, use of tetanus anti toxin (TAT) and status of wound disinfection and the survival of animals. Most cases of tetanus were associated with wounds 64.37% (168/272). Punctured wound was the most common type of wound recorded among equines with visible wound 67.26% (93/168). The commonest sites of wounds were the hoof, around fetlock, the back and neck. About 50% of the equines with tetanus had more than two wounded areas on their body. Interestingly, different foreign materials were recovered from punctured wound affecting hoof such as thorns, nails, stones and wires (Fig. 9) (Appendix VI).

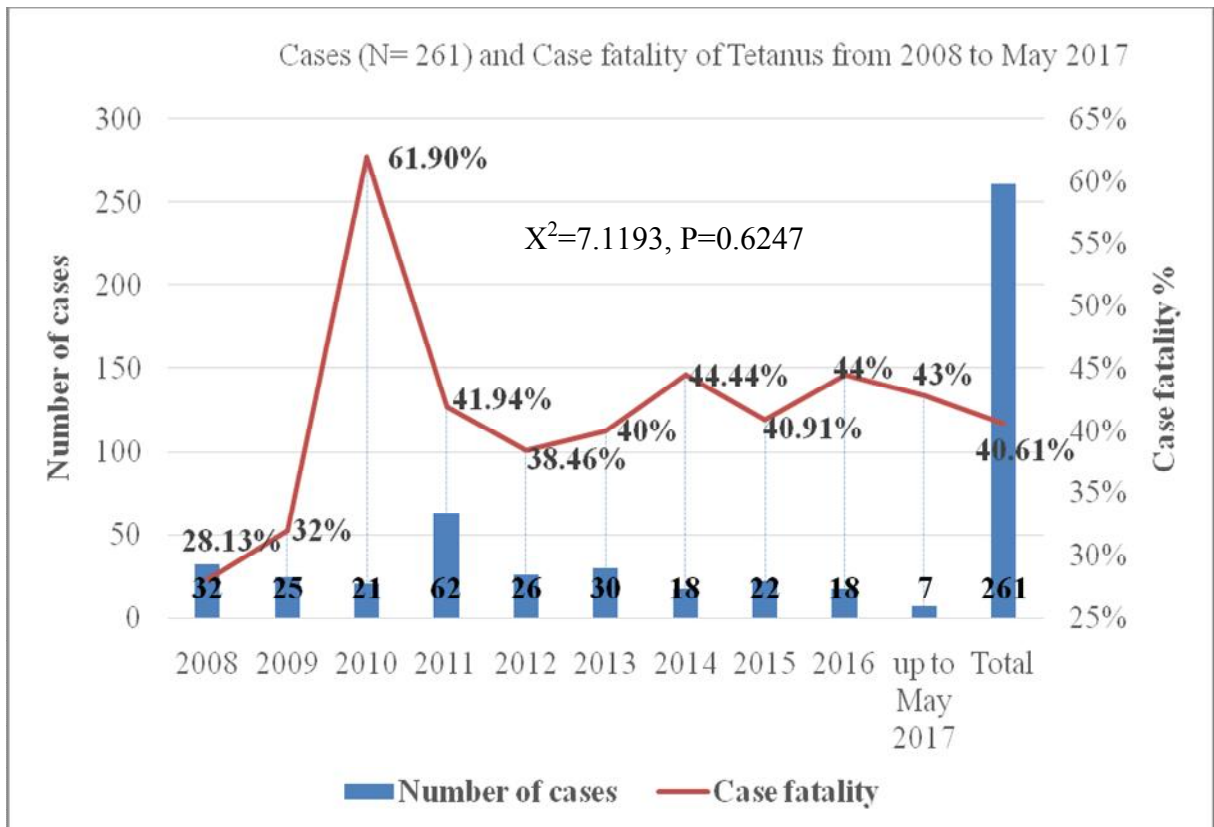


Figure 7: Temporal distribution of equine tetanus cases and case fatality between 2008 and 2017 (n=261)

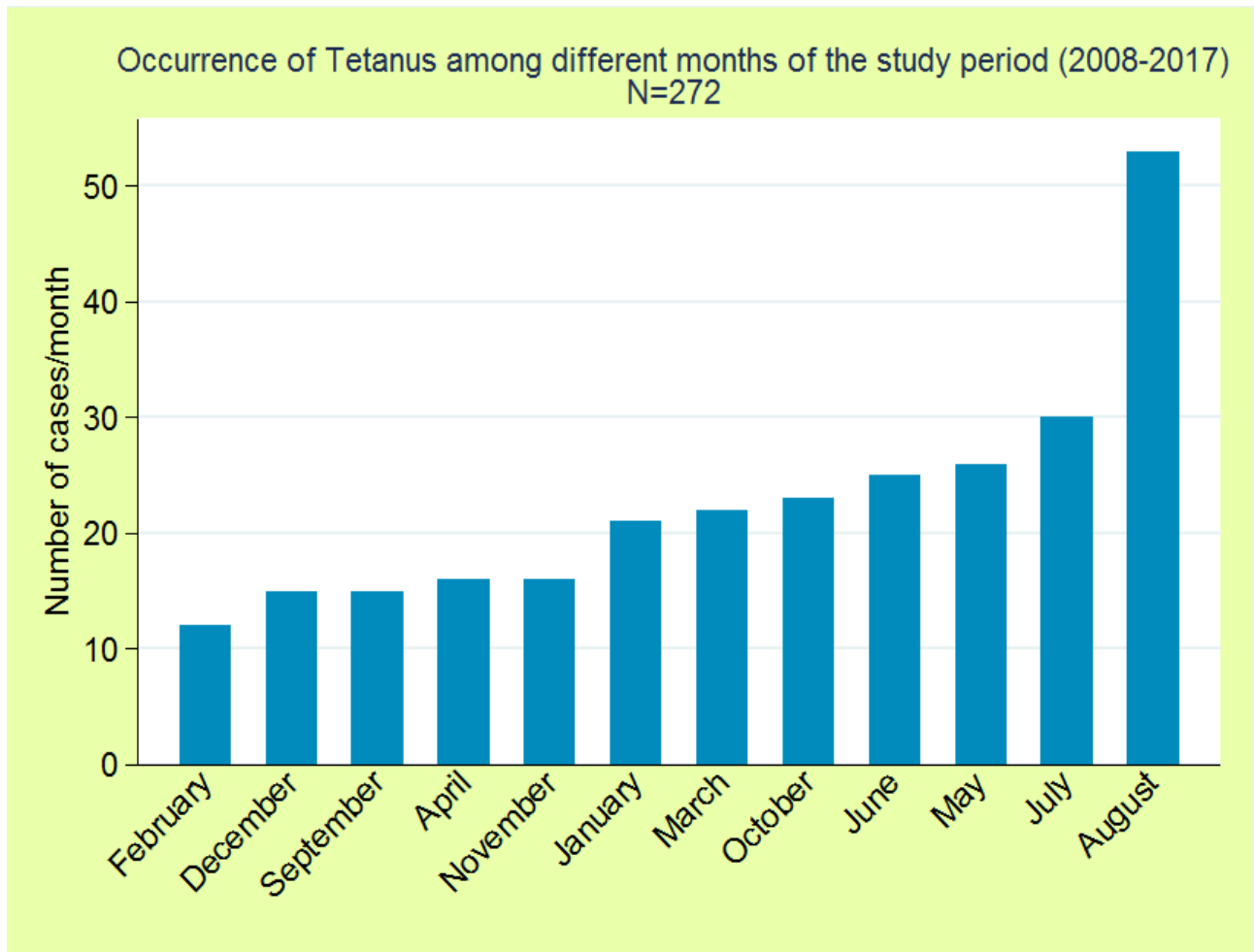


Figure 8: Distribution and occurrence of tetanus by months between 2008 and 2017



Figure 9: Foreign materials from hoof of donkeys affected with tetanus A. Thorn (red arrow) B. Wire (yellow arrow) (photo by DHWP).

4.4.3. Clinical characteristics and factors affecting survival of equines diagnosed with tetanus

There was statistically significant association ($p < 0.05$) between the presence of wound and survival status. Mortality was higher in equines that had wounds than those without wound. The grade of tetanus at the time of diagnosis was also significantly associated with survival. Survival was very low in equines diagnosed with grade 3 and 4 tetanus (Table 3). The pulse rates of equines diagnosed with tetanus was on average 58.87

whereas the respiratory rate and rectal temperatures were on average 36.09 and 37.45, respectively. No statistically significant difference observed with values of pulse rate, respiratory rate and temperature between equines that survive tetanus and those which did not survive (Fig. 10).

Table 3: Effect of various factors on the survival status of equines diagnosed with tetanus (n=261)

Variables	Survivors N (%)	Non- survivors N (%)	Total (%)	X ²	P-value
Age					
Young (<2yrs)	3(60.00)	2(40.00)	5(1.92)		
Adult (2-10yrs)	45(55.56)	36(44.44)	81(31.03)	0.7175	0.6986
Old (>10yrs)	107(61.14)	68(38.86)	175(67.05)		
Sex					
Male	68(39.31)	105(60.69)	173(66.28)	0.3632	0.547
Female	50(56.82)	38(43.18)	88(33.72)		
Wound					
Present	81(48.21)	87(51.79)	168(64.37)	24.402	0.0001
Absent	74(79.57)	19(20.43)	93(35.63)		
Grade of Tetanus					
Grade 1	46(88.46)	6(11.54)	52(19.92)		
Grade 2	90(65.22)	48(34.78)	138(52.87)		
Grade 3	19(29.23)	46(70.77)	65(24.90)	53.452	0.0001
Grade 4	0(0)	6(100)	6(2.30)		
TAT					
Yes	41(63.08)	24(36.92)	65(24.90)		
No	114(58.16)	82(41.84)	196(75.10)	0.4886	0.485
Wound disinfection					
Yes	90(60.00)	60(40.00)	150(61.22)	0.0268	0.870
No	56(58.95)	39(41.05)	95(38.78)		

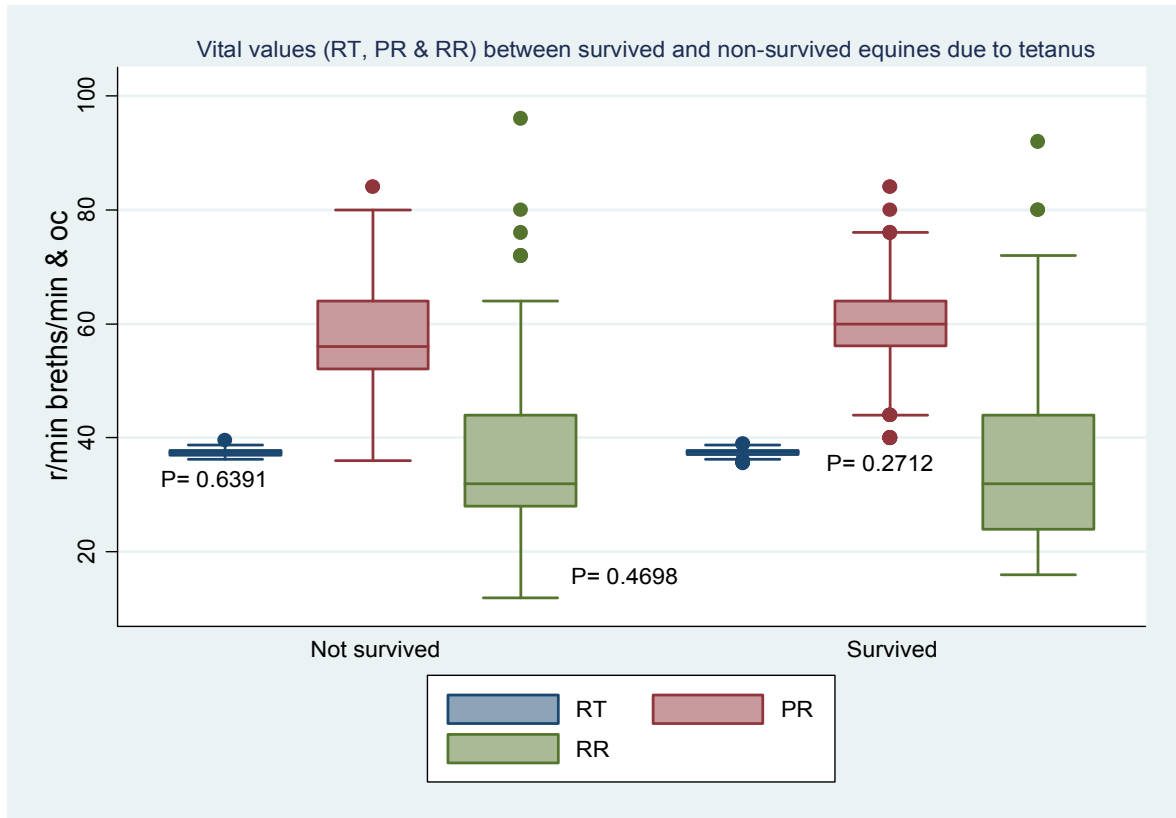


Figure 10: Values of vital signs in equines that survive tetanus and those which did not survive

The result of the retrospective investigation showed that stiff gait was the most frequently observed clinical sign (93.1%) followed by stiffening and erection of the ears (86.21%) and pulling back ears (81.61%). The result of clinical signs at presentation in affected equines is depicted in Fig. 11. There was significant association between survival status and clinical signs of tetanus such as constipation and urine retention, saw horse stance, locked jaw, flared nostrils, prolapsed 3rd eyelid, retracted lips, dysphagia, recumbency and opisthotonus. Other clinical signs appeared to have no significant effect on the survival of equines affected with tetanus (Table 4). Non-survivors had significantly higher clinical signs of constipation and urine retention, saw horse stance, locked jaw, flared nostrils, Prolapsed 3rd eyelid, lips retracted, dysphagia, recumbency and opisthotonus when compared to survivors (Table 4).

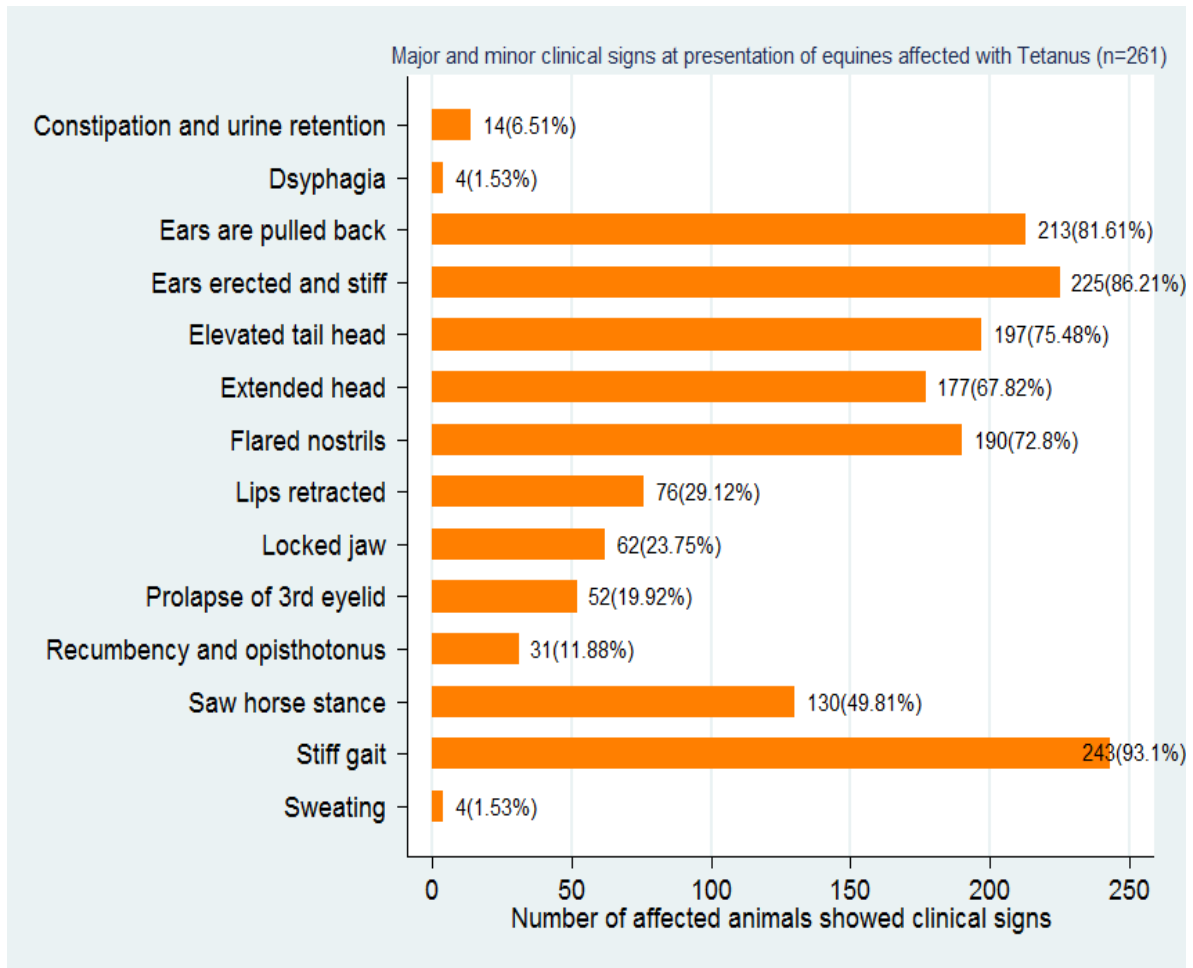


Figure 11: Frequency of clinical signs observed in equines affected with tetanus (n=261)

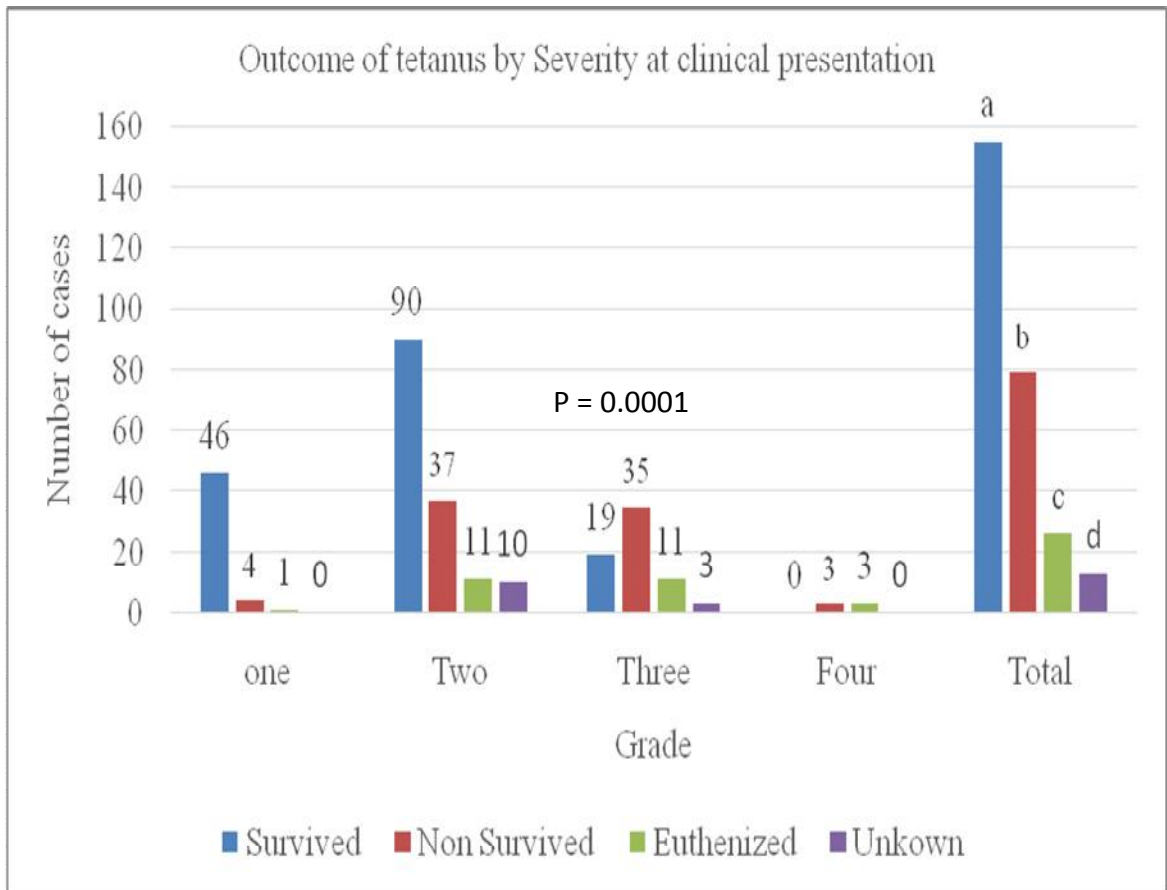
Table 4: Comparison of the common clinical signs between survivors and non-survivors

Clinical signs	Survivors N (%)	Non survivors N (%)	Total (%)	X ²	P-value
Ears pulled back					
yes	125(58.69)	88(41.31)	213(81.61)	0.236	0.6269
No	30(62.50)	18(37.50)	48(18.39)		
Saw horse stance					
yes	66(50.77)	64(49.23)	130(49.81)	7.975	0.0047
No	89(67.94)	42(32.06)	131(50.19)		
Ears erected and stiff					
yes	134(59.56)	91(40.44)	225(86.21)	0.019	0.8897
No	21(58.33)	15(41.67)	36(13.79)		
Stiff gait					
yes	142(58.44)	101(41.56)	243(93.10)	1.321	0.2505
No	13(72.22)	5(27.78)	18(6.90)		
Flared nostrils					
yes	103(54.21)	87(45.79)	190(72.80)	7.759	0.0053
No	52(73.24)	19(26.76)	71(27.20)		
Extended head					
yes	104(58.76)	73(41.24)	177(67.82)	0.091	0.7636
No	51(60.71)	33(39.29)	84(32.18)		
Elevated tail head					
yes	114(57.87)	83(42.13)	197(75.48)	0.768	0.3807
No	41(64.06)	23(35.94)	64(24.52)		
Constipation and urine retention					
yes	6(35.29)	11(64.71)	17(6.51)	4.376	0.0364
No	149(61.07)	95(38.93)	244(93.49)		

Table (continued)

Locked jaw					
yes	17(27.42)	45(72.58)	62(23.75)	34.454	0.0001
No	138(69.35)	61(30.65)	199(76.25)		
Prolapsed 3 rd eyelid					
yes	23(44.23)	29(55.77)	52(19.92)	6.184	0.0129
No	132(63.16)	77(36.84)	209(80.08)		
Dysphagia					
yes	0	4(100)	4(1.53)	5.940	0.0148
No	155(60.31)	102(39.69)	257(98.47)		
Sweating					
yes	2(50.00)	2(50.00)	4(1.53)	0.148	0.7001
No	153(59.53)	104(40.47)	257(98.47)		
Recumbency and opisthotonus					
yes	4(12.90)	27(87.10)	31(11.88)	31.515	0.0001
No	151(65.65)	79(34.35)	230(88.12)		

Of 274 equines diagnosed with tetanus, 52 (18.98%), 148 (54.01%), 68 (24.82%) and 6 (2.19%) of them had mild (Grade 1), moderate (Grade 2), severe (Grade 3) and terminal (Grade 4), respectively including animals with unknown status. Survival was statistically significantly associated with the grade of tetanus ($p = 0.0001$). Among equines having grade 1, tetanus 88.46% survived. Among those diagnosed with grade 2 tetanus 65.22% survived. Survival dropped to 29.23% in equines with grade 3 tetanus while none of equines with grade 4 tetanus survived (Fig. 12). The mean duration of illness prior to hospitalization and duration of hospitalization were significantly associated with survival of equines affected with tetanus. The longer the hospitalization period the higher chance of survival whereas the longer duration before hospitalization the lower chance of survival (Fig. 13)



*Letters with different superscripts on bar graph indicates significant difference

Figure 12: Relationship between grades of tetanus and survival status of affected equines

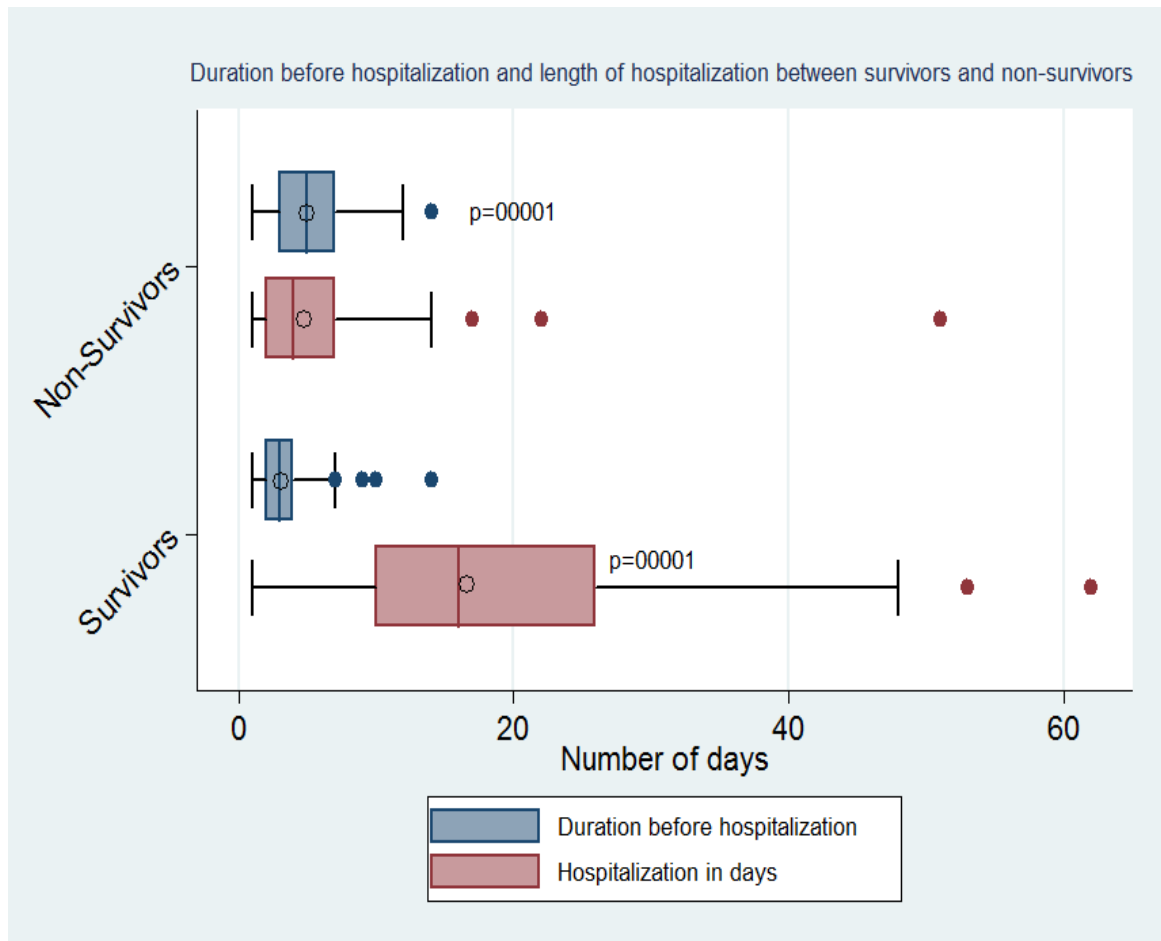


Figure 13: Effect of duration before hospitalization and duration of hospitalization on the survival of equines with tetanus

4.2. Results of matched case-control study

The results of conditional logistic regression analysis showed that body condition scores and presence of visible wound were statistically associated with occurrence of tetanus in equines whereas gender and age of animals did not show significant association since cases were matched 1:2 with controls with respect to age and sex (Table 5). Animals with visible wound had 10.35 times odds of acquiring tetanus as compared to those animals which did not have wounds ($p < 0.001$). Animals with good body condition had 22.27 odds of acquiring tetanus than those having poor body condition ($p < 0.001$). Animals

with moderate body condition had 3.51 odds of acquiring tetanus than those having poor body condition ($p < 0.001$).

Table 5: Results of conditional logistic regression analysis on effects of risk factors on occurrence of tetanus

Variable	Cases=274 n (%)	Control=548 n (%)	Adjusted OR [95% CI]	P-value
Age	8.40±0.26	8.30±0.17	1.03[0.92-1.16]	0.573
Sex				
Female	93(34)	202(36.86)	Ref	
Male	181(66)	346(63.14)	1.18[0.67-2.0]	0.556
BCS group				
Poor	28(10.21)	114(20.8)	Ref	
Good	10(3.64)	4(0.73)	22.27 [4.02-123.48]	0.000
Moderate	236(86.13)	430(78.47)	3.51 [2.00-6.14]	0.000
Presence of visible wound				
No	95(34.7)	436(79.56)	Ref	
Yes	179(65.3)	112(20.43)	10.35[6.70-16.00]	0.000

Ref=reference

4.3. Isolation of *Clostridium tetani* from soil

Out of ten soil samples collected from five peasant associations of Ada'a district where tetanus found in high and low occurrence (Fig. 14). Up on Gram staining smear of soil culture, ten samples were found with gram-positive rods; they appeared in single or in clusters (Appendix XII). *Clostridium tetani* morphology; round, terminal and distending spores which give the typical appearance of drumsticks were observed in 94% (6/10) of soil sampled following spore staining (Appendix XII). Soil isolates of *Cl.tetani* showed a higher presence in tetanus aggregated areas. Fewer soil samples from lower tetanus cases

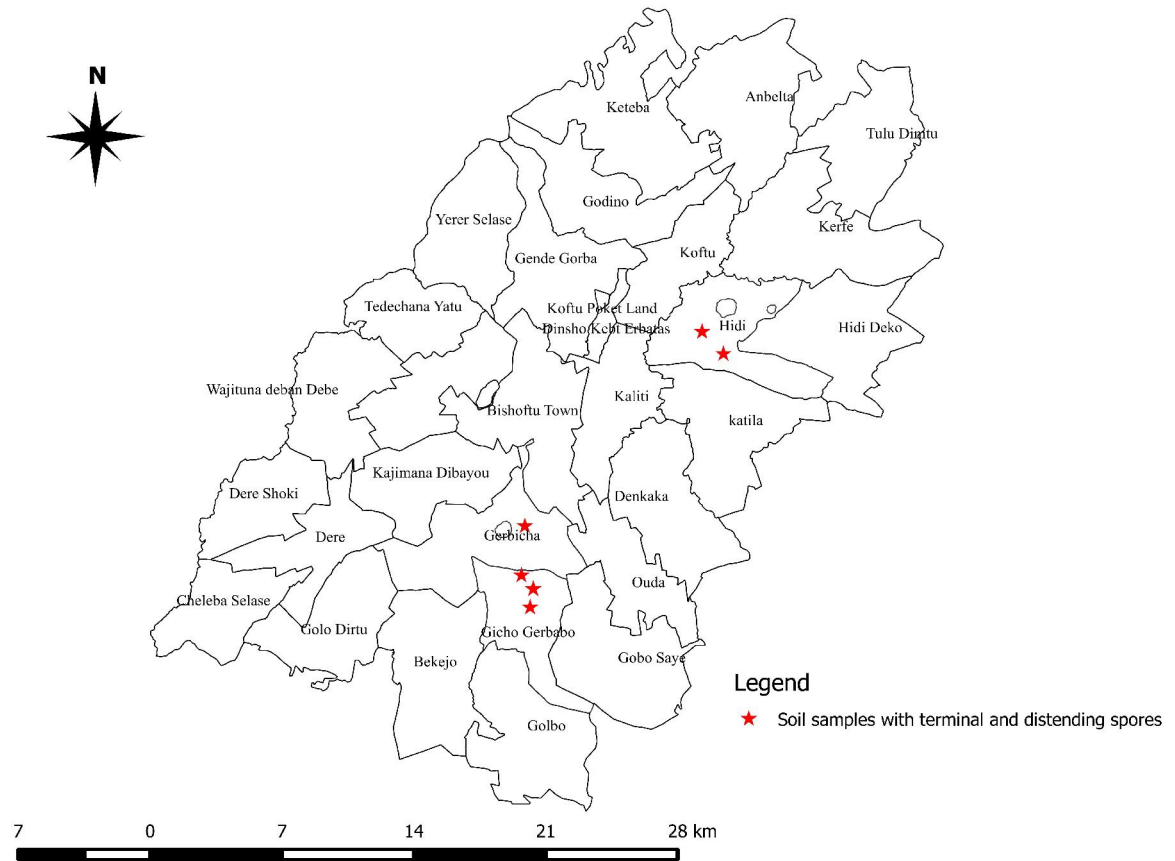


Figure 15: Peasant association's soil sample found with terminal spores and typical drumstick appearance microorganism by spore staining (red stars)

4.4. Results of experimental study

A total of 11 donkeys clinically diagnosed with tetanus between October 2016 and May 2017 were treated three treatment regimen. Of three donkeys treated with procaine Penicillin G one survived (3.33%) while two of them died. Four of the five donkeys (80%) treated with intravenous injection of metronidazole survived whereas one donkey was euthanized. Only one donkey died from three treated with combination of procaine penicillin and metronidazole while two of them (66.7%) survived (Table 6). All of affected donkeys had a visible wound on their different body parts predominantly in hoof and around fetlock. Out of visible wounds, a punctured wound has been observed frequently to the hoof (Table 6). From punctured hoof, different foreign materials were seen (Fig. 16). After treatment in all the three groups the mean rectal temperature was

found to be in the normal range (36.5-37.7°C). The group treated with metronidazole had slightly lower pulse and respiratory rates while the group treated with procaine penicillin G had higher pulse and respiratory rates. Interestingly the values of respiratory and pulse rates in donkeys treated with combination of procaine penicillin G and metronidazole remained constant throughout the study period (Table 7).

After the second day of treatments donkeys treated with metronidazole, showed improvements in terms of some clinical signs. These include resolution of stiffness and extension of head, dilation of nostrils, stiff gait, and locked jaw. Recovery was complete in this group in those donkeys which survived between 2 and 5 days. Donkeys began eating and drinking on day 3 of treatment. Donkeys treated with procaine penicillin G did not show improvement of clinical signs except one donkey, which showed resolution of erecting of ears on day 2 of treatment. The rest of the donkeys in this treatment group manifested clinical signs during the entire period of drug administration (Table 8). Complete recovery took more than a week in this group. Donkeys treated with combination of procaine penicillin and metronidazole showed less improvement in certain clinical signs over the period of treatment.

Table 6: Outcomes of donkeys diagnosed with tetanus after experimental treatment

Case no	Age	sex	Wound	Wound Type	Wound location	Grade presentation	at Treatment group	Outcome	Recovery in days
1	7	F	Y	P	Hoof	2	PPF	SV	15
2	5	F	Y	NP	Limb+tail	3	MTN	SV	12
3	11	M	Y	P	Hoof	2	PPF+MTN	NSV	-
4	12	F	Y	NP	Ear	1	MTN	SV	6
5	8	M	Y	NP	Back	3	MTN	ET	-
6	4	F	Y	P	Fetlock	1	PPF+MTN	SV	21
7	8	M	Y	NP	Sacrum	3	PPF	NSV	-
8	3	M	Y	P	Hoof	2	PPF	ET	-
9	7	M	Y	P	Thoracic+fetlock	2	MTN	SV	14
10	8	M	Y	P	Ear+fetlock	2	MTN	SV	27
11	5	M	Y	P	Hoof	2	PPF+MTN	SV	29

PPF=Procaine penicillin, MTN=Metronidazole, PPF+MTN=combination of procaine penicillin and Metronidazole, Y= Yes, P = punctured, NP= Non- punctured, F= Female, M= Male, SV= Survived, NSV= Non-survived, ET= Euthanized

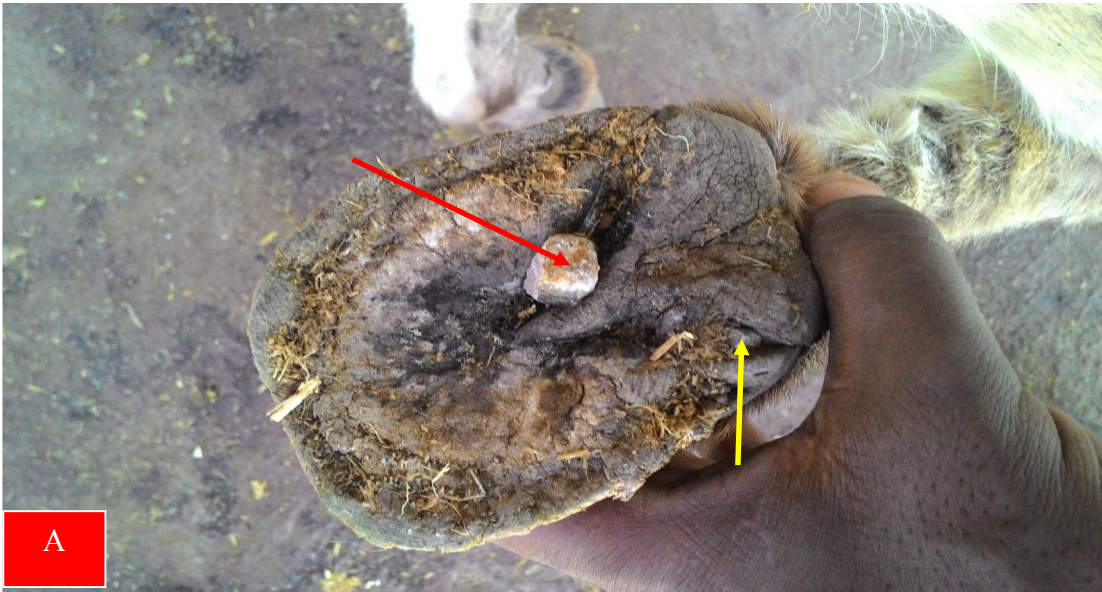


Figure 16: Punctured hooves cases: Case no 1. Hoof injured with sharp stone (A). Case no 3. Punctured hoof after hoof trimming with muddy and small stones (B)

Table 7: Mean and standard deviations of the respiratory rate (breaths/min), pulse rate (beats/min) and rectal temperature (°c) among treatment group

Vital signs	Treatment		Day 1	Day 2	Day 3	Day 4	Day 5
	Type	N					
PR (beats/min)	PPF	3	54.67± 6.11	60±10.58	60±4.00	61.33±2.31	60±4.00
	MT	5	62.40±5.36	53.60±6.70	50.40±5.36	50.40±6.07	53.60±6.07
	PPF+MT	3	52.00±10.58	49.33±10.06	52±4.00	60±4.00	58.67±2.31
RR(breaths/min)	PPF	3	46.67±12.22	60±20.78	58.67±16.16	57.33±15.14	61.33±16.65
	MT	5	40±10.20	36.20±9.28	40.00±11.31	37.60±9.63	38.40±8.76
	PPF+MT	3	37.33±16.16	40±17.43	34.67±12.22	38.67±11.55	36±13.85
RT (°c)	PPF	3	38.06±0.32	37.87±0.25	38.06±0.38	37.80±0.60	37.80±0.43
	MT	5	37.44±0.41	36.60±1.46	36.02±1.13	36.78±1.36	36.96±1.34
	PPF+MT	3	37.46±0.47	37.36±0.72	37.83±0.55	37.88±0.55	38.03±0.25

N= number of cases, PPF=Procaine Penicillin G, MT=Metronidazole, PPF+MTN=combination of Procaine Penicillin G and Metronidazole, data are presented as mean±s.d

Table 8: Changes in clinical signs among different treatment groups for five days of experimental period

Clinical signs	Treatment Type	Day 1	Day 2	Day 3	Day 4	Day 5
Ears pulled back	PPF	3(100)	3(100)	2(66.67)	2(66.67)	2(66.67)
	MT	2(40)	1(20)	1(20)	1(20)	1(20)
	PPF+MT	1(33.33)	1(33.33)	0	0	0
Sow horse stance	PPF	2(66.67)	2(66.67)	2(66.67)	2(66.67)	2(66.67)
	MT	3(60)	3(60)	2(40)	2(40)	2(40)
	PPF+MT	1(33.33)	2(66.67)	2(66.67)	2(66.67)	2(66.67)
Lock jaw	PPF	1(33.33)	1(33.33)	1(33.33)	1(33.33)	1(33.33)
	MT	2(40)	2(40)	1(20)	1(20)	1(20)
	PPF+MT	0	0	0	0	0
Stiff gait	PPF	3(100)	3(100)	3(100)	3(100)	3(100)
	MT	5(100)	5(100)	3(60)	2(40)	2(40)
	PPF+MT	3(100)	3(100)	3(100)	3(100)	3(100)
Extended head	PPF	2(66.67)	2(66.67)	2(66.67)	2(66.67)	2(66.67)
	MT	4(80)	4(80)	4(80)	2(40)	2(40)
	PPF+MT	3(100)	3(100)	3(100)	3(100)	3(100)
Dilation of nostrils	PPF	3(100)	3(100)	3(100)	3(100)	3(100)
	MT	5(100)	5(100)	3(60)	2(40)	1(20)
	PPF+MT	3(100)	3(100)	3(100)	3(100)	3(100)
Tail raised	PPF	2(66.67)	2(66.67)	2(66.67)	2(66.67)	2(66.67)
	MT	4(80)	4(80)	4(80)	3(60)	3(60)
	PPF+MT	3(100)	3(100)	3(100)	3(100)	3(100)

PPF=Procaine Penicillin G, MT=Metronidazole, PPF+MTN=Combination of Procaine Penicillin G and Metronidazole, N (%) indicates number of animals (%)

5. DISCUSSION

This study showed that tetanus is one the standing health problem of equines in Bishoftu and Addis Ababa. Taking into account the role of equines particularly donkeys, this finding suggests significant losses can occur due to the disease. Cases of tetanus were found to aggregate in Gerbicha and Gicho Gerbabo peasant associations of Ada'a district. The authors suggest that the variation could be associated with land topography and nature of work as all are transporting thorny firewood to the towns on regular basis. Gerbicha and Gicho Gerbabo are plains situated between two small mountains (Appendix VII). Spores can be easily washed down from uplands by floods during rainy season and lodged in the plain. The grazing areas in the plains are featured by their widespread thorny bushes, gravels and sharp stones that can cause wounds to the animals which serve as portal of entry for the spores. Donkeys examined in the area have prevalent injuries on their feet with thorns and gravels as depicted in (Appendix VI). Culture of soil samples collected from few areas with high organic matter yielded *Cl. tetani*. It has been reported that spores of *Cl. tetani* are mostly found in warm climates where the soil is rich in organic matter and in the soil contaminated by animal feces (Andrew and Jack, 2006; Peter *et al.*, 2017). This implies that these two areas have high source of tetanus cases compared to other peasant associations. These peasant associations also have high population of donkeys that provide multiple services to the community. That is, tetanus is hampering livelihood of equine owning community in the areas. Tetanus cases admitted to Merkato clinic is higher from Addis Ketema sub city as compared to other sub cities due to clinic location. Otherwise, the variations would not be as such wide.

The case fatality observed in this study, which is 40.61%, is significant for the community in which equines provided multitude of functions. Comparable case fatality was reported in Croatia (Damjan *et al.*, 2015). However, the finding of this study is lower than the case fatality reported in USA 75 % (Green *et al.*, 1994), Belgium 68% (Van Galen *et al.*, 2008) and Morocco 54% (Kay and Knottenbelt, 2007). This variation in case fatality could be attributed to variation in the treatment protocol used, the grade of tetanus at presentation, durations of hospitalization, onset of the disease, secondary

complications and presence of nursing cares consisted of maintaining an equines in slings, offering feed and water regularly using stomach tube.

Retrospective analysis of medical records of equines with tetanus showed that the incidence of the disease is higher during the summer season (August and July). During summer the feet get wet can easily be macerated and become liable to penetration by gravels, stones and thorns or other foreign materials becoming portal of entry for the bacilli spores. Higher incidences of tetanus cases were reported during the rainy season (Ayele *et al.*, 2015). This highlights the need for intensive feet care during the rainy season. Inspection and cleaning of donkey's feet with a hoof pick at least once a day has been recommended practice (Svendson, 1997). It has been shown that when owners of donkeys become reluctant to practice daily foot care the incidence of tetanus raises (Kumar, 2007).

Although the difference was not significant equines that receive TAT have a higher chance of survival than those which did not receive TAT. Tetanus antitoxin is theoretically indicated to neutralize unbound toxin whereas toxins which travel within axons are not accessible to TAT (Linnenbrink and McMichael, 2006). In addition, immunoglobulin molecules in TAT preparation are enough and do not readily cross the blood-brain barrier to neutralize unbound toxins already found in the central nervous system (Miranda-Filho *et al.*, 2004). In consistent to our observation, Kay (2006) reported lack of association between the use of TAT and survival of affected equines. This implies that further experimental studies involving large number of animals is need to explore if the use of TAT is beneficial to the affected animals or economically justifiable.

Survival of equines diagnosed with tetanus was found to be dependent on clinical signs such as constipation and retention of urine, saw horse stance, locked jaw, flared nostrils, prolapse of 3rd eyelid, retracted lips, dysphagia, recumbency and opisthotonus. This could be due to the manifestation of these signs when large concentrations of toxins are accumulated in the CNS and bind to their receptors. This causes stiffness of muscles and

impairment of functions of several tissues. Hence, these signs can be used to monitor the progress and prognosis of the disease. Previous study revealed that dyspnoea and recumbency were indicators of poor prognosis in tetanus affected equines (Van Galen *et al.*, 2008). Prognosis of tetanus is associated with grades of tetanus. This suggests careful examination is needed to predict prognosis and provide due nursing care. This observation is in agreement with the reports of Kay and Knottenbelt (2007) and that of Ayele *et al.* (2015).

This study revealed significant association between duration of illness before hospitalization and survival status of equines affected with tetanus. This is due to the fact that if longer time is lapsed before hospitalization and commencement of therapy, large concentration of toxins is bound to its receptors and exerts its effect. These situations can be difficult to treat. Similar observations were reported previous in Ethiopia (Ayele *et al.*, 2015) and elsewhere in the world (Reichmann *et al.*, 2008; Van Galen *et al.*, 2008). Duration of hospitalization was also observed to affect the survival of affected equines. That is, the longer the equines are kept under hospital management, the better chance of survival. This is due to provision of supportive treatment and continual monitoring of the progress of therapy and recession of clinical signs until the animals fully recover. Early release of animals from hospital can expose them to certain stimuli, which can aggravate the clinical situations. This observation is in agreement with previous reports made elsewhere (Ansari & Matros, 1982; Green *et al.*, 1994; Van Galen *et al.*, 2008).

Analysis of matched case control study revealed that presence of the visible wound on the body of animals and the body condition scores of the animals are important risk factors for the occurrence of tetanus in donkeys. Equines with wounds have higher odds of acquiring tetanus than those which do not have wounds. This is as a result of contamination of the wounds by spores of the *Clostridium tetani* that are present in the environment such as soil and animal dung. Furthermore, it is assumed that contamination could be initiated by rolling habits of the donkeys on the dust or soil that causes soiled wound. Elsewhere it was shown that human hands contaminated with soil has been observed to contaminate wounds and lead to development of tetanus (Raza *et al.*, 2004).

The wound is assumed to be caused by lack of proper padding, harnesses and management. Equines with good and moderate body conditions are prone to tetanus than Equines with poor body conditions. This is due to maintenance of anaerobic conditions in the deep and massive tissue once the spores of *Cl. tetani* got access to the tissues of equines. Animals with good and moderate body conditions have higher tissue mass than animals with poor body conditions, which favors reduced oxygen and stimulate germination and growth of the bacterium (Francisco *et al.*, 2016).

Although procaine penicillin is commonly used to treat tetanus in humans and animals, systematic investigation on the efficacy of therapeutic approaches has not been carried out in equines. In this study we examined the effects of the metronidazole, procaine penicillin G and combination of procaine penicillin G and metronidazole on the fate of tetanus in donkeys. The result revealed that donkeys treated with metronidazole had better improvement. This bears important implication in reducing mortality in affected equines. To the best of our knowledge, this the first reports on the effect of metronidazole on tetanus in equines. Previously trials carried out on humans elsewhere showed that metronidazole has superior effect in treating tetanus than procaine penicillin (Ahmadsyah and Salim, 1985; Sanford, 1995; Ganesh *et al.*, 2004). This is due to better bactericidal activity of metronidazole against *Cl. tetani* and its rapid bioavailability in almost all body fluids and tissues. It has been shown that metronidazole has good effect against anaerobes and accumulates in tissues faster (Ahmadsyah and Salim, 1985; Ganesh *et al.*, 2004; Alhaji *et al.*, 2011). Moreover, donkeys treated with combination of procaine penicillin G and metronidazole showed better survival and improvement in clinical signs than those treated with procaine penicillin G. This lower in survival rate and increment in vital values procaine administered group could be associated GABA antagonist nature of procaine penicillin G which may potentiate the action of tetanospasmin that could worsen muscular spasm and increased risk of convulsions that has been observed with high doses of penicillin in animal models (Farrar *et al.*, 1997; Sahal *et al.*, 2011). Furthermore, inadequate tissue perfusion at the wound site may reduce delivery of penicillin to the site of local infection (Alhaji *et al.*, 2011). Fluctuation of pulse rate and heart observed in entire treatment group may be associated to disturbance of the animals with human

intervention or veterinary visits for medication and measurements of vital values. An exaggeration of all signs with stimuli has previously been described especially in response to a handclap and tapping the head (Pearce 1994; Peter *et al.*, 2017).

6. CONCLUSION AND RECOMMENDATIONS

The present study showed that tetanus remains an important disease with high case fatality. It proved welfare and economic significance of tetanus in the study area. If it were not for the intervention of a much equipped charity clinic in the college, the death toll would invariably be high. The study is limited to only cases that could access the college; we would assume that the disease problem and associated economic losses and social disruption are expected to be much higher. Tetanus is spatially aggregated in areas with physical factors favoring survival of *Cl. tetani* in the soil and predisposing animals to infection with the spore of the bacterium. Survival of equines affected with tetanus is dependent on prognostic signs and grade of the disease.

The risk factors identified were wound and body condition score with wound cases having higher odds of tetanus while with poor body condition protective compared to better conditions. The results of the experimental study showed that metronidazole has better activity in resolving clinical signs associated with muscular spasm and reducing mortality. Taking into consideration the results of this study, the following recommendations are suggested:

- Wound prevention using proper packsaddles and harnesses made of natural materials should be a priority to educate communities and mainstreamed into the districts livestock extension package
- Further large scale controlled trials needs to be carried out to elucidate the efficacy of metronidazole in the management of tetanus cases
- Training of farmers is important to reduce the effects of predisposing factors on the occurrence of tetanus
- Further characterization of *Cl. tetani* isolates from soils in the study area is needed
- Although it is beyond the mandate of this research, I would like to bring the need to access tetanus toxoid by any means by the veterinary and livestock authorities

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8. APPENDICES

Appendix I: Tetanus case recording format

Tetanus Case Recording Sheet						
Owner's Detail: Name _____ Sex _____			Animal Detail			
Case no: _____ Date: _____ District: _____ Peasant Association: _____ Tel: _____			Sex: _____ Age: _____ BCS: _____ Species: _____ Breed: _____			
History			Predisposing Factor			
Duration: _____ Reluctant to graze/chewing _____ Difficulty of urination _____ History of puncture wound: _____ History of uterus contamination: _____ History of recent parturition: _____			Use of _____ saddling		Rural	
			Padding			
			Hobbling		Dry season	
			Urban		Wet season	
			Pregnancy		Retained placenta	
			Sore on (tick)			
Sign Observed			Head		Cervical	
Constipation and urine retention		Extended head	Lumbar		Sacral	Thoracic
Ears pulled back		Flicking of 3 rd eye	Chest		Carpal	Coccygeal
Saw horse stance		Flared nostrils	Umbilical cord (young)			Hoof

Table (Continued)

Ears erect and Stiff		Lips retracted	Laboratory exams (tick)			
Lock jaw		Elevated tail head	Bacterial isolation			
Stiff gait		Sweating	CSF analysis			
Pyrexia		Dysnea	Grade and Prognosis (tick)			
Tachycardia		Peripheral vasoconstriction	Grade I		Grade III	
Dysphagia		Drizzling saliva	Grade II		Grade IV	
Recumbency + Opisthotonus		Convulsion	Prognosis Good		Prognosis-guarded	
Treatment Control			Stomach tube feeding			
Kept under – environment	Quiet	Cool	Dark	Intravenous fluid therapy		
					Parenteral	local
Plug ears w/cotton				Penicillin		
Blindfolding the head				Tetanus antitoxin		
Place soft feed and water at level of muzzle				Intrathecal TAT injection		
Manual evacuation of rectum				Neuromuscular symptom control , ACP		
Bladder catheterization				Time to recovery		
Debride, flush wound with oxidizing agents; hydrogen peroxide				Vet in Charge		
				Signature		
Comment:						

Appendix II: Treatment follow-up format

Treatment group _____

Wound: Yes No Type of wound: Punctured Not Punctured

Wound location _____

Wound debridement: Yes No Type of chemical _____

Vital values and clinical signs	Day 1	Day 2	Day 3	Day 4	Day 5	Condition & date
Constipation and urine retention						
Ears pulled back						
Sow Horse stance						
Ears erect and stiff						
Lock Jaw						
Stiff gait						
Extended head						
Prolapse 3rd eyelid						
Dsyphagia						
Erected tail						
Dilation of nostrils						
RT(°c)						
PR (beats/min)						
RR (breaths/min)						
Others (specify)						
Grade/severity of disease						

✓ Mark for presence or note yes

Appendix III: BCS determining methods

Grade 1: poor: emaciated, ribs, spine and tuber coxae very prominent, dull coat

Grade 2: Below average: spine prominent, coat dull

Grade 3: Good: Spinous process palpable but not prominent, coat and skin supple, shiny hair and in good condition

Grade 4: Fat: Spinous process not easily palpated, shiny coat and intact skin.

Grade 5: Obese: Body well rounded with generous muscle and fat cover. Spinous process is not palpable, shiny coat and intact skin.

Source:-Svendsen, 1997

Appendix IV: Geographical coordinates of Soil sampled sites

Name of area	Longitude	Latitude
Gerbicha near to kebele	038°59'7.38"	08°41'43.92"
Giche 02	038°59'1.44"	08°40'17.16"
Giche birka	038°59'22.08"	08°39'53.88"
Gerbicha river	038°59'16.56"	08°39'21.54"
Kajima Dibayou warka1	038°55'745"	08°43'178"
Kajima Dibayou warka2	038°55'44.58"	08°43'9.54"
Hidi near to primary school 1	039°04'861"	08°47'403"
Hidi near to primary school 2	039°04'869"	08°47'358"
Ude near to veterinary clinic	039°02'10.14"	08°40'26.46"
Ude near to gubaseye	039°02'18.84"	08°39'44.94"

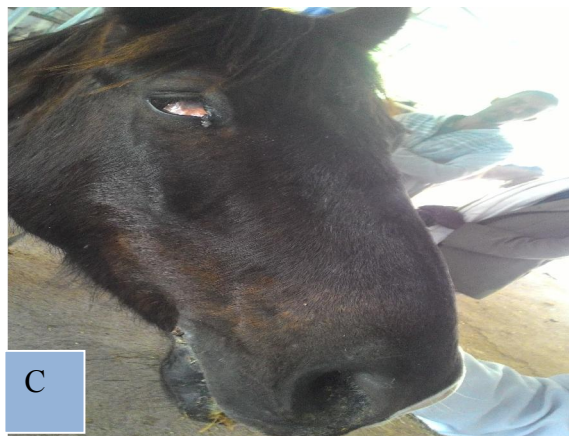
Appendix V: Selected pictures on tetanus clinical signs (photo by DHWP)



A: Note the stiff posture of the donkey with neck extended and saw horse stance



B: Donkey with tetanus exhibited unable to eat, erected tail and ear



C: Protrusion of third (nictitating) eyelid is pathognomonic for tetanus

Appendix VI: Some selected photos of donkeys with different foreign materials in hoof



A

A: Nails from hoof of donkeys that usually causes puncture wound (Photo DHWP)



B

B: Small sands recovered from punctured hoof of donkey with tetanus (Photo DHWP)

Appendix VII: Selected pictures taken during field visit to describe peasant associations



Photo1: Irregular shape of the land and sharp stones at boundary between Gerbicha and Giche Gerbabo peasant association



Photo 2: The two mountains surrounded Gerbicha and Giche Gerbabo peasant association.

A. Mountain on southeast of Gerbicha and Giche Gerbabo



B. Mountain on north part of Gerbicha



Photo 3: Plant with sharp-pointed tip resembling a spike on a stem (thorns) at Gerbicha PA



Photo 4: A sharp thorn from hoof of donkeys affected tetanus (black arrow)



Photo 5: Thorny plants on the way to Giche Gerbabo

Appendix VIII: VL and VF media preparation for isolation of *Cl.tetani* from soil

A. Procedure of VL (VIAND ET LOUVRE) semi solid media

Preparation Composition

➤ Tryptose	10gm
➤ Sodium chloride	5gm
➤ Glucose	2 gm
➤ L-cystine	0.25gm
➤ Yeast extract	5 gm
➤ Beef extract	2gm
➤ Bacto agar	6gm
➤ distilled water	1000ml
➤ pH	7.48

Procedure

1. Mixed all ingredients in distilled water.
2. Boiling the ingredients in water bath for 70 °C to dissolve completely.
3. Placed the boiled ingredients to test tube
4. Sterilize by autoclaving at 120 °C for 30 minutes.
5. Cool the semi solid media and placed at + 4 °C until inoculation time.
6. Boiling if solid appear, the semi solid media before inoculation of samples, at water bath in 100 °C for expel absorbed oxygen found in media following by rapid cooling to 37 °C.

B. Procedure of VF (VIANDE ET FOIE) broth media

Preparation Composition

➤ Glucose	5 gm
➤ L-cystine	0.5gm
➤ Yeast extract	3 gm
➤ Meat and liver extract broth	>1000ml

- Paraffin 200ml
- pH 7.6

Procedure

7. Mixed all ingredients in distilled water (de mineralized) except paraffin oil.
 8. Boiling the ingredients in water bath for 70 °C to dissolve completely.
 9. Placed the boiled ingredients to test tube and add 200 ml paraffin at top on each test tube
 10. Sterilize by autoclaving at 120 °C for 30 minutes.
 11. Cool the broth media and placed at + 4 °C until inoculation time.
 12. Finally boiling the broth media before inoculation of samples, at water bath in 100 °C for expel absorbed oxygen found in media following by rapid cooling to 37 °C
- Note: equal amount of cooked ground meat and liver pellets are put on the bottom of each test tube and paraffin added at top and sterilization by autoclaving at 120 °C for 30 minutes

Appendix IX: Isolation of *Clostridium tetani* from soil

- 2 grams of soil is added to 10ml of distilled water in a test tube. This is mixed thoroughly by centrifuging at 1,600 rpm for 10min and then heated at temperature of 60° C for an hour using water bath.
- The VF media is heated at 100 °C for 1 hour to remove oxygen. 2ml of the processed soil sample is inoculated in VF broth media at bottom of the test tube that is incubated for 24 hours at 37°C.
- In case of *clostridial spp* growth uniform turbidity and gas formation are observed. Gram staining is done to see the morphology of anaerobic bacteria from cultured sample. After seven days, spore staining is done to see morphology of the spores.

Appendix X: Gram staining and spore staining procedures

A. Gram staining

- Prepare a heat fixed smear of each of the cultures
- Flood smear with 1 % crystal violet stain for 1 min.
- Rinse off excess stain with water.
- Flood smear with Gram's iodine for 1 min.
- Pour off the iodine. Rinse with water.
- Flood the smear with 95% ethyl alcohol for 10 to 30 seconds and quickly rinse with water.
- Flood the smear with 1% safranin for 20 seconds to 1min.
- Rinse with water.
- Blot with absorbent/filter paper and air dry for a few minutes.
- Examine the stained smear with low-power, high-power and oil-immersion objectives.

Interpretation

- Gram positive bacteria appear blue/ violet or purple
- Gram negative bacteria appear red/ pink

B. Spore staining (Haksen's method)

- Prepare the film
- Stain with steaming (heat the stain so that it steams) carbol fuchsin for 5 minutes. (Do not let the stain dry while heating it). Then, wash with water.
- Decolorize with 5% acetic acid till the film is a light pinkish and wash with water
- Counter stain with methylene blue for 3minutes
- Examine the stained smear with low-power, high-power and oil-immersion objectives.
- Interpretation: spores appears bright red and vegetative part seems blue

Appendix XI: Procedures of biochemical test

- ✓ Boil VL media until it melts
- ✓ All VL media containing test tubes are labelled according to sample code and sugar type (Maltose,Sucrose,Fructose and Lactose)
- ✓ 0.5ml from all sugars are added to control test tubes that contain *C. chauvoei*
- ✓ 0.5ml from each sugar is added to labelled VL test tubes
- ✓ 0.5ml of sample is taken from VF media and added to VL media that contains sugar using Pasteur pipette
- ✓ Add 2ml of phenol red to each VL media including the controls
- ✓ Incubate at 37°C for about 24 hours

Interpretation: no color (fermentation) change if it is *C. tetani*

Biochemical reactions of *Clostridial* species are indicated as follows

Spp	Glucose	Maltose	Lactose	Salicin	H ₂ S	Gel liq.	indole	Lit.milk
<i>Cl.chauvoei</i>	+	+	+	-	+	+	-	acid
<i>Cl.septicum</i>	+	+	+	+	+	+	-	acid
<i>Cl.tetani</i>	-	-	-	-	+	±	+	No change
<i>Cl.novyi</i>	+	+	-	-	+	+	-	acid
<i>Cl.perfringens</i>	+	+	+	-	+	+	-	Stormy
<i>Cl.hemolyticum</i>	+	-	-	-	+	+	+	acid
<i>Cl.botulinum</i>	+	+	-	-	+	+	-	acid
<i>Cl.sordelli</i>	+	+	-	±	+	+	-	digested

Source: (Merchant and packer, 1967)

Appendix XII: Selected photos of laboratory results of *Cl. tetani* isolation from soil

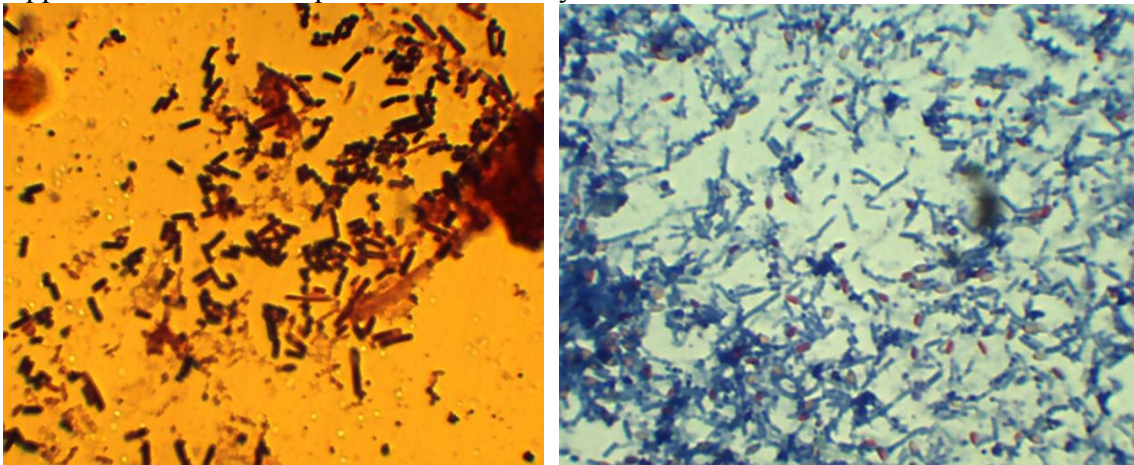


Photo A: Gram-positive rods appeared in single or in clusters (Gram staining)

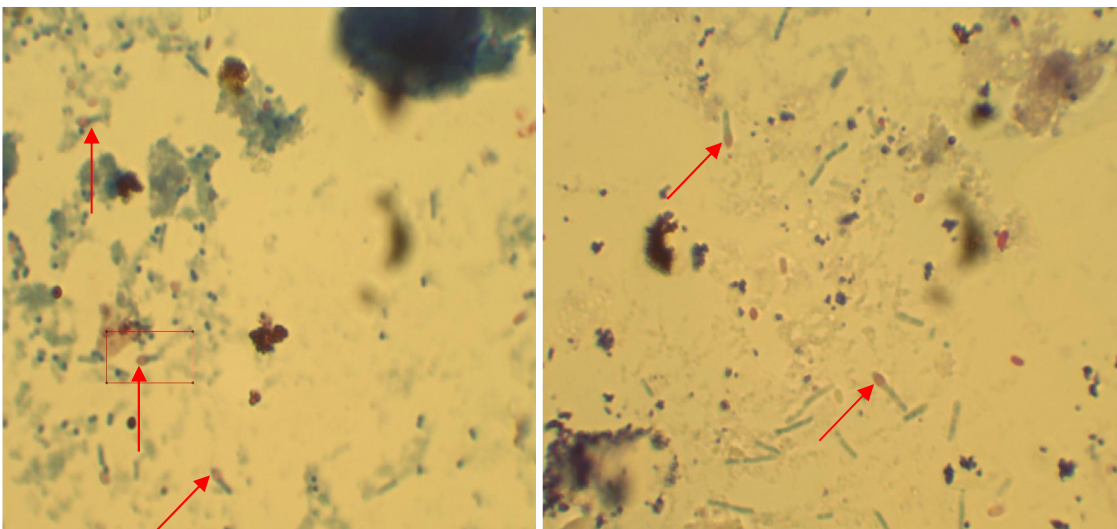


Photo B: *Clostridium tetani*. Terminal spores are observed in several rods, giving the typical drumstick appearance to the organism (red arrows)



Photo C: No fermentation of sugars (Glucose, Maltose, Fructose and Lactose) which is a biochemical property of *Cl. tetani*

Back of the rack (yellow color); fermentation of sugars by control microorganism: *Cl. chauvoei*

Appendix XIII: Ethical clearance certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/11/06/09/2017

Name of Applicant: Zerihun Asefa (DVM, MSc fellow)

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: Epidemiological investigation of tetanus: retrospective, matched case control study and evaluation of treatment options in equines in Central Oromia, Ethiopia

Date of application: 02/05//2017
 Nature of the project: Non-invasive
 Target animal species: Donkeys
 Number of animals involved: depends on available case
 Study area: Central Oromia, Ethiopia

Minutes No. and date of review: VM/ERC/06/09/017, 06/06/2017

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is allowed to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

Dr Getachew Terefe
Chairman

Dr. Dinka Ayana
Dean
College of Veterinary Medicine
and Agriculture

Signature

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Please quote Our Ref. No. When replying

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