Short communication

Isolation and identification of *Clostridium tetani* from tetanus suspected equine and their environment in selected sites of central Ethiopia

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Abstract

A cross-sectional study was carried out from November 2016 to May 2017 to isolate and identify *Clostridium tetani.* A total of 71 samples (equine deep wound swabs, feces, soil from the feces contaminated environment) were collected. Isolation of *Clostridium tetani* was carried out using an anaerobic *Viande et Foie* (VF) medium. Out of the 71 samples cultured on VF medium, 27 (38 %) of them were grown and all were confirmed to be *Clostridium tetani* using spore staining and biochemical tests. Study site and sample type had a statistically significant association (p<0.05) with *C. tetani* isolation in which higher occurrences were from the Bishoftu area, environmental, and feces samples. The present study showed the widespread occurrence of tetanus in the equine population inquiring about the need for designing feasible control strategies.

Keywords: *Anaerobic Culture; Central Ethiopia; Clostridium tetani; Equine; Tetanus.*

Introduction

Equines play a crucial role for the Ethiopian population in which 80 % of its people living in rural areas depend on agricultural activities. Equines are used for transportation, riding, and carting. The low level of development of road transport makes equines most valuable and affordable pack animals under smallholder farming system, but they are vulnerable to infectious diseases of various origins (Gebreab, 1998; Gebrewold *et al*., 2004).

More than 100 species of clostridia are known however, less than 20 species are pathogenic to human and domestic animals (Quinn *et al*., 2004). Clostridia are large (0.3-1.3 x 3-10 micrometer), Gram-positive, anaerobic, endospore-forming and the spores usually bulge the mother cell. Neurotoxic clostridia (*C. tetani* and *C. botulinum*) are among the pathogenic species of clostridia that secrete powerful exotoxins that are responsible for diseases such as tetanus, botulism, and gas gangrene when they are in their active form (Quinn *et al*., 2004). Tetanus is caused by *Clostridium tetani* associated with fatal wound infections affecting both humans and animals (Meshad *et al*., 2013).

The World Health Organization (WHO) global and regional immunization profile in 2014 reported tetanus cases in the European region to be 67 but it was 2900 in Africa. Tetanus is most commonly found in developing countries and it is rare in the developed world (Gibson *et al.,* 2009). The majority of tetanus cases in developing countries including Ethiopia occur due to the lack of effective immunization, poor treatment of injuries, and decline in protective antibodies. Thus, exposure to spores remains high. A retrospective cross-sectional study from medical records of patients admitted at Felege Hiwot Referral Hospital of Ethiopia reported that among 110 tetanus cases, 36 (32.7 %) patients were dead due to tetanus (Awoke *et al*., 2016). The mortality rate is much lower in developed countries due to the availability of facilities for intensive care (Anuradha, 2006; Chukwubike and God'spower, 2009; Tadesse and Gebre-Selassi, 2009; Khaskheli *et al*., 2013).

Equines are particularly susceptible to tetanus exotoxins. In developing countries where equines play a key role in the rural economy, tetanus is a major cause of death amongst horses, donkeys, and mules (Radostits *et al.,* 1994; Cullinane *et al.,* 1999). Retrospective analysis of the clinic database between 2003 and 2005 indicated that tetanus is one of the major causes of direct mortality in donkeys of Central Ethiopia (Bojia *et al*., 2006). There were different reports of dead and diseased animals due to tetanus cases in Welkayt woreda with a high rate of infected animals during the year 2007/8 - 2012/13 compared to the rest of the years (Gebru and Berihun, 2013).

There is a limited study conducted to isolate and identify *C. tetani* from equine tetanus cases and their environment. Hence, isolation and identification of the *C. tetani* from different sources including soil, deep wound from tetanus cases, and feces of equines would help to confirm the causative agent and provide evidence for further investigation on the transmission and potential risk factors for infection in Ethiopia.

Therefore, this study was conducted to investigate the occurrence of tetanus in equines and their environment in selected sites of central Ethiopia and to identify risk factors for *C. tetani* infection in equines.

Materials and methods

Study areas, animals, and design

The study was conducted in selected sites of central Ethiopia, which constitute Addis Ababa, Adama, Bishoftu, and Sebeta towns. These areas were selected purposively based on previous reports of tetanus in equine in central Ethiopia (Figure 1). Equine populations in Addis Ababa are 499 mules and 943 horses and 10,000 donkeys. In Adama town, there are an estimated 4621 donkeys, 1856 horses, and 1673 cart horses. The equine populations of Bishoftu include 1450 mules, 29045 donkeys, and 1298 horses. Of the horses in Bishoftu, 1,170 are estimated to be cart horses (SPANA, 2014).

The study animals were 46 tetanus suspected equines (43 donkeys and 3 horses) in the study area of which 27 and 19 were male and female respectively. Regarding age, 15 were in the age group of ≥2 and < 5 years, and 31 were ≥5 years. For the environmental study, we included soil samples from the area where tetanus suspected equines exist, i.e. barns, areas where the death occurred due to cases of tetanus, watering areas, and grazing lands.

A cross-sectional study design was conducted from November 2016 to May 2017 from clinically suspected cases of tetanus.

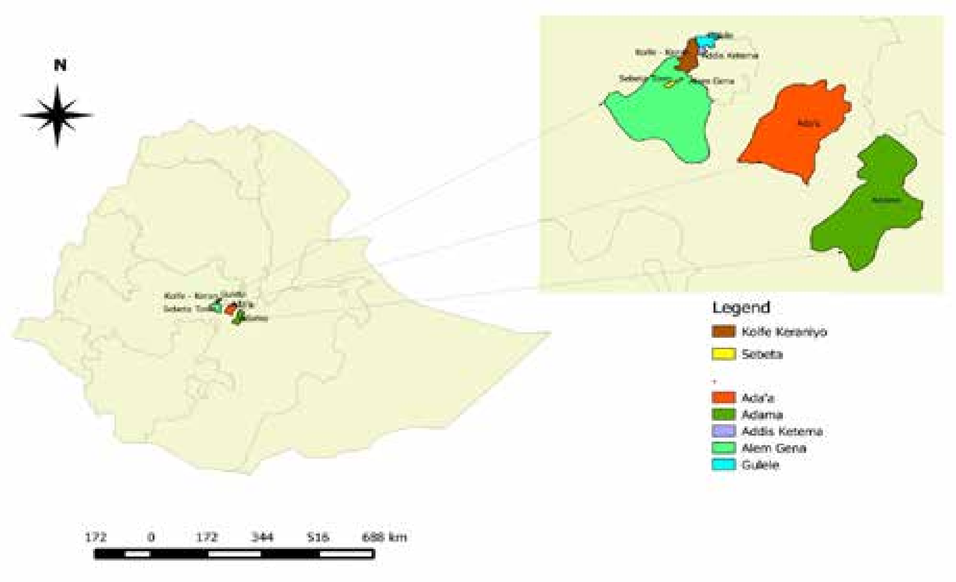

Figure 1. Map of Ethiopia that contains study area selected sites of central Ethiopia

Sampling methods, sample collection, and processing

Since the specimens were collected from those animals suspected to be infected by *C. tetani,* a purposive sampling technique was used to isolate the agent from the suspected cases and environmental samples. For this study, a total of 71 samples, 46 deep wound puncture swabs and fecal samples from suspected cases, and 25 soil samples from grazing land, watering point, and barns were collected. The samples were collected according to bacteriological standard safety conditions (CDC, 2009) in anaerobic transport media (Murray *et al*., 2007; WHO, 2007) and collected samples were transported to Addis Ababa University, College of Veterinary Medicine and Agriculture, Veterinary Microbiology Laboratory, Bishoftu, Ethiopia. Isolation of *C. tetani* from soil was carried out according to the modified method described by Sanada and Nishida (1965). Sample processing was carried out according to the methods of Smith and Hobbs (1974) and Lanitro and Muirhead (1975). Microbiology culture and identification were adapted from the methodology described in Murry *et al.* (2007).

Ethical considerations

Samples from horses and donkeys suspected case of tetanus were collected ethically. All protocols involving animals in the study were approved by the Research Ethical Review Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture, Bishoftu, Ethiopia.

Data management and analysis

All data collected from the study was transferred to a Microsoft Excel spreadsheet and coded for Statistical analysis using STATA Version 13.0 software and descriptive statistics were used. To consider a result to be statistically significant 95% confidence interval (CI) and $p < 0.05$ were considered.

Results

Isolation and identification *C. tetani*

In the current study, from a total of 71 samples collected (46 animal origin and 25 from environment origin), *Clostridium tetani* were isolated from 27 samples (38%) (Table 1)

Table 1. Proportion of C. tetani growth based on culture isolation on VF medium

Origin of sample	No of cultured	Growth on VF	Proportion (%)
Environment origin	25	18	72
Animal origin	46	9 (6 donkeys, 3 horses)	19.6
Overall samples	71	27	38

Spore staining was done on age of 8 days culture of VF medium, out of 27 grown samples, 26 (96%) showed, long slender rods with round ends spores (terminal spore), and spores were 2 to 3 times greater than the diameter of cells and it looks like "drum stick appearance" confirming the characteristics *C. tetani* spore (Figure 2).

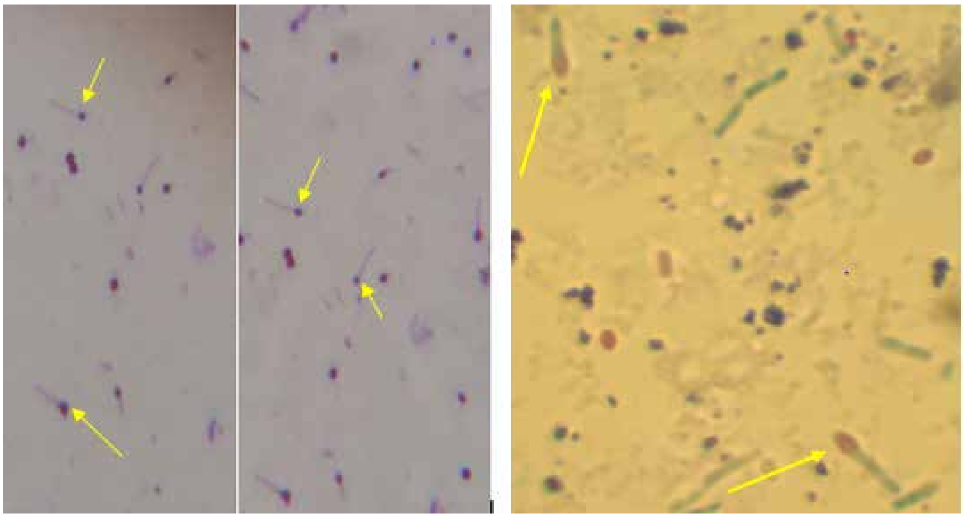

Figure 2. Drum's stick appearance of *C. tetani* spores (arrow) of positive samples after spore staining

Association of different risk factors with the occurrence of clinical tetanus

Association of environmental and animal factors like the selected site, sample type, and sample origin with the proportion of *C. tetani* isolation revealed that site of isolation and sample type had a statistically significant ($p>0.05$) difference among the group considered. Accordingly, the Bishoftu area and soil sample showed a higher proportion of *C. tetani* isolation (Table 2).

Table 2. Association of different risk factors with the occurrence of clinical tetanus in sampled equine and environmental samples in central Ethiopia

Variables	No Examined	Proportion (%)	95% CI	χ^2	p-value
Sex				0.002	0.959
Male	27	6 (22)	(8.0 - 42.0)		
Female	19	3 (15.8)	(33.0 - 39.0)		
Age				0.002	0.959
≥2 and < 5 years	15	4 (26)	(8.0 - 55.0)		
≥5 years	31	5 (16)	(5.0 – 33.0)		
Selected Sites				19.6	≤0.001
Adama	6	0 (0)	-		
Bishoftu	36	20 (55.6)	(38.0– 72.0)		
Addis Ababa	13	7 (53)	(25.0-8.0)		
Sebeta	16	0 (0)	-		
Sample type				21.7	≤0.001
Soil	25	18 (72)	(50.0-88.0)		
Wound swab and feces	46	9 (19.6)	(9.0-34.0)		
Sample origin				18.9	≤0.001
Animal	46	9 (19.56)	(9.0-34.0)		
Environment	25	18 (72)	(50.0-88.0)		

Discussion

The isolation of *C. tetani* from the deep wound puncture and feces in 9 (19.6%) of cases in the current study was comparable with Hajra *et al*. (2015) who isolated *C. tetani* from 16% of the cases. However, it is lower than the reports of James *et al.* (2009), who isolated *C. tetani* from 53.57% of samples from deep wounds. In Louisiana (USA), in 2015 the Department of Health and Hospitals reported the isolation of *C. tetani* in 30% of cases (Louisiana Dept. of Health & Hospitals, 2015). The difference in the results might be related to the difficulty of identifying wounds caused by anaerobic bacteria in which growth occurs after the healing of minor wounds. From soil samples, *C. tetani* were isolated in 18 (72%) of the cases. This result was higher than the reports of Bukar (2008) who reported 60%. The high proportion of isolation from soil samples in this study may be due to the spore formation of bacteria which might allow the organisms to persist in the environment for years in the most fertile and virgin soils that contain organic matter (Quinn *et al*., 2004).

The rate of isolation of *C. tetani* from soil samples (72%) was higher than the rate of isolation from wound samples (17%). This finding is in line with the findings of Carol and Tracy (1996) who indicated that *C. tetani* is commonly found in the soil and can be isolated from the wound in only about one-third of the cases.

The present finding was different from Green *et al.* (1994) who suggested age as a predisposition for younger horses, but did not report an association between age and survival, while Van *et al.* (2008) in a retrospective study of 31 cases suggested that young horses are particularly vulnerable to tetanus and their prognosis is poorer than that of older horses. This variation in age susceptibility might be due to older horses being more likely to be immune through natural exposure to the disease (Galen *et al.,* 2008) and equines that are appropriately vaccinated are solidly immune to the condition of tetanus disease. Estimates of the occurrence of *C. tetani* between animal sex showed no significant difference ($p>0.05$). This was supported by previous reports (Malikides *et al.,* 2002; Gracner *et al.,* 2015; Dajman *et al.,* 2015).

Conclusions

The current study showed for the first time that tetanus is widespread in equines and their environment in central Ethiopia. A high proportion of *C. tetani* was detected and isolated from clinical tetanus cases and the environment by using various bacteriological diagnostic techniques from the different samples collected during the study period. It is clear from the present findings that those animals exposed to *C. tetani* can be easily affected unless they take tetanus antitoxin as a preventive measure inquiring the need for future development of tetanus toxoid vaccine in Ethiopia.

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