Journal of Veterinary Medicine and Animal Health

Volume 9 Number 11 November 2017
ISSN 2141-2529

Academic Journals

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Journal of Veterinary Medicine and Animal Health

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academicJournals

Vol. 9(11), pp. 290-299, November 2017
DOI: 10.5897/JVMAH2017.0627
Article Number: 2B959F566284
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Effect of a low dose of BCG-Phipps vaccine on the development of reactivity to tuberculin skin test in neonatal calves and adult cows

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Received 21 July, 2017; Accepted 12 September, 2017

Bovine tuberculosis (bTB) has a direct impact in the productive and reproductive efficiency of dairy cattle. Nowadays, disease control programs based on tuberculin testing and removal of infected cattle are unaffordable for the developing countries, since there is no program of financial compensation, especially in high bTB-prevalence herds. Thus, control strategies based on vaccination are considered the best alternative. The aim of this study was to evaluate whether a low dose of BCG-Phipps vaccine induces reactivity to tuberculin skin test and its duration in neonatal calves and adult cows. For the analysis of the former, 69 TB-free Holstein-Friesian calves less than one-month old were used; of which, 54 calves were subcutaneously inoculated with 10^4 CFU of the BCG-Phipps vaccine, while the rest remained without vaccination. Under similar conditions of immunization, 133 single intradermal comparative cervical (SICCT) reactors and 133 non-reactors Holstein-Friesian cows of different age were also analyzed. In calves, the SICCT-reactivity was evaluated periodically in the first 14 months post-vaccination (mpv) while, in adult cows, the effect of vaccination on the test was evaluated at six months post-vaccination. A comparative ELISA was used by measuring the antibody levels in the groups. In calves, reactivity frequencies of 7.4 and 3.7% at 3 and 5 mpv, respectively were recorded. This reactivity disappeared at six months. None calves in the control group were reactor during the study. There were no variations in the degree of reactivity in the group adult reactor cows. However, in the non-reactor group, a conversion of 12.8% at six mpv was recorded. In addition, the conversion percentage was higher in older cows than in younger cows ($p<0.05$). The specific antibody levels did not increase in the vaccinated groups. Data indicate that the low dose of BCG-Phipps vaccine used had a reduced effect on the development of a delayed type hypersensitivity to tuberculin in neonatal calves, and heifers lesser than one-year-old.

Key words: *Bacillus* Calmette-Guérin, bovine tuberculosis, *M. bovis*, single intradermal comparative tuberculin test.

INTRODUCTION

Bovine tuberculosis (bTB) is one of the main diseases causing economic losses to dairy industry, and its incidence has been increasing in recent years. The severity of disease is more in immunosuppressed animals or in concurrent infections (Gupta et al., 2009). Therefore, it is required to develop effective control strategies. Hence, the use of Bacillus Calmette-Guérin (BCG) *Mycobacterium bovis* vaccine could be one of the most viable tools for this purpose. In this regard, it has been pointed out that vaccination with BCG in cattle at an early age diminishes pathological scores, so it could be an approach to decrease bacterial loads and reduce the transmission risk both from bovine to bovine and bovine to human (Lienhardt et al., 2012; Waters et al., 2012; Kaufmann et al., 2014).

However, field trials investigating protection have reported variable efficacies; furthermore, revaccination did not improve protective effect. Potential explanations of the variable levels of field protection includes the use of different BCG strains, administration of very high doses of BCG, different routes of inoculation, and previous exposure to *M. bovis* in maternal milk from infected cows, circumstances that diminish the effective protective immunity (González et al., 2007; Hope et al., 2011; Thom et al., 2012).

With respect to the optimum age of vaccination against tuberculosis, it has been determined that vaccine efficacy is higher in very young calves, lesser than one month old, compared to calves between five and six months old (Buddle et al., 2003; Hope et al., 2005). Why calves lesser than one month old develop higher levels of protection, may be that they have been lesser exposed to environmental mycobacteria which interferes with development of a protective immune response against bTB, compared to older calves (Siddiqui et al., 2012). It is therefore incontrovertible that eventual future application of vaccines will be used to improve the control of bTB. Thus, it is relevant to improve the knowledge of factors determining the development of a delayed type hypersensitivity (DTH) response, if it really develops when vaccine is given under a number of different modalities and stages (Cockle et al., 2002; Logan et al., 2005). Few studies talk about the development of a DTH response or conversion of tuberculin skin test (TST); as a result of the vaccine application, one of this studies demonstrated that DTH reaction is developed in the first months and lost in 90% of the vaccinated animals at nine months post-vaccination (Whelan et al., 2011; Buddle et al., 2013).

However, the theoretical framework of the BCG vaccine application indicates that it primes the animals to respond to the tuberculin test, and it is an impediment to use the BCG as a vaccine since this test is the primary tool for epidemiological surveillance used in many countries as a main strategy of bTB control, in México (Mexican Official Norm NOM-031-ZOO-1995; Good and Duignan, 2011; OIE, 2012). Today, the rate of conversion induced by the BCG, the correlation of DTH with protective immunity, and the factors determining the development of protection are unknown. These observations could have useful implications at some point in the future, for a solid improvement of BCG vaccines designed for the bovine cattle. The objective of this study was to determine whether the BCG-Phipps vaccine at low dose (10^4 CFU) induces reactivity to tuberculin skin test and its duration in neonatal calves as well as evaluate its effect on reactors and non-reactors adult dairy cows.

MATERIALS AND METHODS

Ethics statement

Animals used in this study belong to a commercial farm and have been submitted only to the standard clinical practices specifically regulated by the Mexican legislation on tuberculosis control program (Mexican Official Norm NOM-031-ZOO-1995), which were vaccination and blood sampling.

Experimental design for neonatal calves

Sixty-nine female calves between 6 to 30 days of age obtained from herds with no history of bTB were used. All animals were tested and confirmed negative for *M. bovis* by antigen-induced IFN-gamma produced by peripheral blood mononuclear cells (PBMCs), ELISA for detection of specific antibodies to a culture filtrate protein extract (CFPE), and PCR on nasal swabs were used. Fifty-four of these calves were inoculated subcutaneously on the neck with a dose of 10^4 CFU of BCG-Phipps strain in PBS. To avoid prime calves to bovine Purified Protein Derivative (PPD) by repeated tuberculin tests, they were divided in two groups for alternative testing; half of the animals were tested at 0, 3, 7 and 11 months post-vaccination, the other half, at 0, 5, 9 and 14 months, and results were analyzed altogether. Fifteen control animals were inoculated with PBS. All animals were isolated to eliminate any risk of infection from the main herd.

Experimental design for adult cows

The study was performed in a dairy herd with a bTB prevalence of 49.39% located in the State of Hidalgo, México, from which, 133 single intradermal comparative cervical tuberculin (SICCT) reactors and 133 non-reactors Holstein-Friesian adult cows were analyzed to determine the effect of vaccination on tuberculin skin test. Both study populations were immunized with 10^4 CFU/1.5 ml of BCG-Phipps subcutaneously injected on right side of the neck, like

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calves. The effect on SICCT test was evaluated at six months post-vaccination (mpv) in reactors and non-reactors cows. The latter were grouped by age to evaluate the influence of this variable on development of DTH response induced by vaccination, then forming the following groups: Group 1, ≤ 1 year old (n=36); Group 2, between 1 to 2 years old (n=37); Group 3, between 3 to 5 years old (n=34); and Group 4, between 6 to 9 years old (n=26). Cows pertaining to the Groups 2, 3 and 4 remain in production stable. The cows of Group 1 were isolated from the rest of animals to avoid exposure to new infections.

Production of BCG Phipps vaccine

For elaboration of BCG Phipps vaccine (Lot number 95 001-2013-7H9), seed was cultured on Middlebrook 7H10 OADC (Oleic Albumin Dextrose Catalase) solid medium for 15 days at 37°C. Next, bacteria were transferred to Middlebrook 7H9 ADC liquid medium and they were incubated in constant swirling at 37°C. Bacilli were subcultured once in liquid medium until they reached an early exponential growth phase (OD_{600nm} 0.3) and aliquots frozen in PBS at -70°C. For bacterial enumeration, serial dilutions were plated onto modified Middlebrook 7H10 OADC. Petri dishes were incubated for 3 to 4 weeks at 37°C, and CFU/ml were calculated by the formula UFC x DF x 10 = bacteria/ml; in which DF = dilution factor, and concentration was adjusted to 10^4 CFU/ml in sterile PBS.

Single intradermal comparative cervical tuberculin test

The development of a DTH response by vaccination in study populations was determined by SICCT test, which was applied according to NOM-031-1995, in the middle third of the neck. In two different shaved and cleaned spots, 0.1 ml (3,250 IU) of bovine PPD (PPD-B) strain AN5, and 0.1 ml (3,250 IU) of avian PPD (PPD-A) strain D4 (PRONAVIBE, México) were injected. After 72 h post-inoculation skin thicknesses were measured. A positive result was considered positive if the increase in skin thickness at the bovine PPD site of injection was at least 4 mm greater than the reaction at the avian PPD injection site. The reaction was suspicious, but inconclusive, when PPD-B reaction was between 2 to 3 mm greater than PPD-A reaction. A negative result was considered when PPD-B swelling was negative or, even being positive, for no more than 2 mm greater than PPD-A reaction.

Culture filtrate protein extract

To obtain mycobaterial CFPE from *M. bovis* AN5 and *M. avium* D4, these were cultured in Dorsett-Henley medium for six weeks at 37°C. At the end of the incubation period, the bacterial mass was eliminated by filtration, using cellulose filters, followed by 1.2, 0.45 and 0.22 µm Millipore filters (Bernardelli, 2007). The proteins present on the residuals liquids were precipitated with ammonium sulfate (Sigma Aldrich, St. Louis, MO, USA) at a final saturation of 80%, under constant whirling motion for 24 h at 4°C. The precipitates were centrifuged at 4°C for 60 min at 20 000 X *g*. The CFPE were recovered and finally dialyzed through a membrane with exclusion limits from 3000 kDa (Membrane Filtration Products, Inc. TX, USA) at 4°C for 48 h with PBS. The protein content of each CFPE was determined by the Bradford method and CFPE was stored at -70°C (Bradford, 1976).

IgG ELISA

A comparative ELISA was used for the evaluation of humoral immune response in the study populations. In this ELISA, the plates (Nunclon, Roskilde, Denmark) were coated with 1 µg/well of either *M. avium* or *M. bovis* CFPE dissolved in 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at room temperature at 4°C. Then, plates were washed three times with 0.1% Tween 20 in 0.01 M phosphate buffer at pH 7.4 (PBS-T). Free binding sites were blocked with 3% non-fat milk in PBS-T for 1 h at 37°C. Afterward, the plates were washed with PBS-T, next incubated with PBS-diluted (1:100) sera for 1 h at 37°C. After washing with PBS-T, 100 µl/well of Protein G-Peroxidase (1:10,000, in PBS) (P-8170 Sigma) were added and incubated for 1 h at 37°C. Next, the plates were washed and subsequently the chromogenic substrate solution was added; containing 0.04% O-phenylenediamine (Sigma P-3804) and 0.04% of hydrogen peroxide in citrate buffer at 37°C for 5 min. Reaction was stopped with 50 µl/well of 2 M sulfuric acid. Optical density was read at 492 nm (OD_{492nm}) in a microplate reader (Benchmark Plus, Bio-Rad, Hercules, CA, USA) (Estrada-Chavez et al., 2001).

The cut-off point was calculated as the media of the OD_{492nm} from all SICCT-negative animals before the vaccination plus two standard deviations, and animals having a value greater than this cut-off point was regarded as positives.

PCR

DNA was extracted from nasal exudate samples for detecting the presence of *M. bovis* by PCR. Briefly, the samples contained in 5 ml of sterile PBS (phosphate buffer saline 0.01 M, pH 7.4) were centrifuged at 12,000 X *g* for 5 min. The pellet was incubated with 400 µl of Tris-EDTA buffer (TE) (100 mM Tris-HCl, 10 mM EDTA, pH8.0) and 50 µl of lysozyme (100 mg/ml) 1 h at 37°C. 70 µl of 10% SDS and 5 µl of proteinase K (10 mg/ml) were added and incubated for 20 min at 65°C. Then, 100 µl of 5 M NaCl, and 100 µl of CTAB/NaCl (4.1 g of NaCl, 10 g of CTAB, 80 ml of distilled water) was added, and pre-warmed at 65°C for 10 min. After adding 750 µl of chloroform-isoamyl alcohol (24:1), mix was centrifuged at 12,000 x *g* for 5 min. After recovering the upper phase, this was mixed with 600 µl of absolute isopropyl alcohol for 30 min at -20°C. DNA was centrifuged at 12,000 x *g* for 5 min, supernatant discarded, and 1 ml of cold -20°C ethanol 70% was added to wash the DNA. Samples in microcentrifuge tubes were again centrifuged at 12,875 x *g* for 5 min; DNA was dissolved into 50 µl of sterile water, and stored at -20°C.

For PCR, primers TB1-F, 5'GAACAATCCGGAGTTGACAA3', and TB1-R, 5'AGCACGCTGTCAATCATGTA3', related to the *M. tuberculosis* Complex, were used; these primers amplify a 372 bp sequence from the gen MPB70 codifying the secreted protein MPB70 (Cousins et al., 1992).

PCR mix for a final volume of 25 µl was prepared with 0.5 µl of each primer (0.4 µM), 13.25 µl of sterile water, 2.5 µl of 10X buffer, 0.5 µl of dNTPs (250 µM), 2.5 µl of $MgCl_2$ (2.5 mM), 0.25 µl of Taq polimerase (0.5 U), and 5 µl of sample DNA (50 pg-250 ng). DNA obtained from *M. bovis* AN5, *M. tuberculosis* H36Rv, and from tuberculous lesions of diseased cows was used as positive controls. Sterile milli-Q water and DNA obtained from nasal swabs from IFN-γ and ELISA-negative calves were used as negative controls.

Cycling conditions were pre-warmed for 15 min at 96°C, and 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 60 s at 72°C; also a final step of extension for 5 min at 72°C (Gene Amp PCR System 9700, Applied Biosystems, Foster City, CA USA). 10 µl of each PCR product were analyzed in agarose 1.5% gels stained with ethidium bromide 1%, using 2.5 µl of molecular weight standards (Amresco K180-250 UL), and visualized with a Benchtop UV Transilluminator in a EpiChemi II Darkroom (BioImaging Systems, Upland California USA).

Table 1. Response to single intradermal comparative cervical tuberculin test (SICTT) in immunized heifers with a low dose BCG Phipp.

Group	Time post-vaccination					
	3 months (%)	5 months (%)	7 months (%)	9 months (%)	11 months (%)	14 months (%)
Group A, BCG (n=27)	2/27 (7.4)	-	0/27 (0)	-	0/27 (0)	-
Group B, BCG (n=27)	-	1/27 (3.7)	-	1+/-/27[a] (0)	-	0/27 (0)
Control (n=15)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	1+/-/15[a] (0)

Groups of 27 heifers each were tested by SICCT alternately. Group A, calves at 0, 3, 7 and 11 months post-vaccination, and Group B, at 0, 5, 9 and 14 months; results of both groups are displayed as a whole.[a] One calf showed a reaction around the cutoff value of the test considered as suspect.

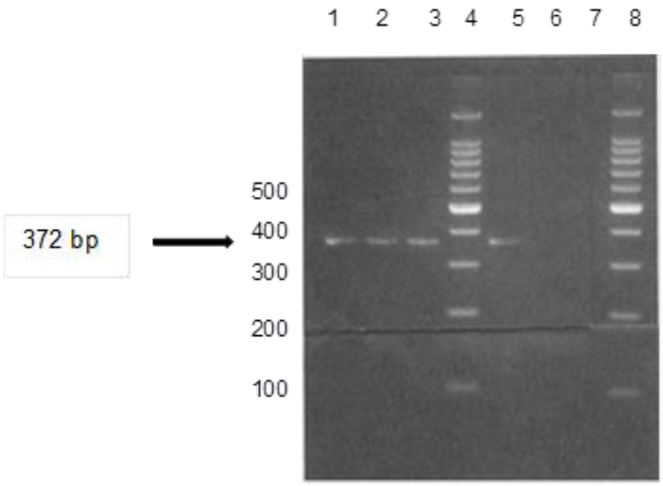

Figure 1. Products of PCR amplification from TB lesions and swabs of calves primers used were TB1-A and TB1-B which amplify a product with a length of 372 bp from the MPB70 gen of *M. bovis*. 1, *M. bovis* AN5; 2, *M. tuberculosis* H36Rv; 3, 5 DNA obtained from tuberculous lesions of diseased cows; 4, molecular weight markers (MWM); 6, DNA from swabs of a SITTC, IFN-γ and ELISA-negative calf; 7, water blank; 8, MWM. PCR showed that these calves concurrently remain negative throughout the study.

Statistical analysis

Data were analyzed with Student "*t*" test using the Statistical Analysis System software (SigmaStat Ver. 3.5) to determine significant differences between ELISA results before and after vaccination, and Xi^2 to analyze distribution of SICCT test data. A value of $p < 0.05$ was regarded as significant (Wayne, 2006).

RESULTS

SICCT test reactivity after BCG vaccination in calves

BCG vaccination in calves induced SICCT test reactivity in 2/27 (7.4%) at 3 months post-vaccination, and 1/27 (3.7%) at five months post-vaccination (Table 1); one animal alone was suspicious at 9 months; at 14 months post-vaccination, none of the animals showed reactivity. None of the control animals (n=15) was reactor at that time; however, one control animal showed a value around the cut-off point of the test. These data showed that at three months post-vaccination, the young female calves showed the greater frequency of reactivity, which diminished during the following months (Table 1). PCR analysis showed that both groups of calves concurrently remained negative all over the study (Figure 1).

ELISA in calves

The baseline levels of IgG antibodies to CFPE from *M. bovis* were similar between groups; these increased lightly in both groups without being significant at the first months post-vaccination; in the course of time, reaching a peak value of 0.34±0.24 *vs* 0.32±0.21 (OD_{492nm} ± standard error) for vaccinated group and control, respectively, at 3 months. In the subsequent two months,

Table 2. Tuberculin skin test conversion rate in adult non-reactor cows according to age, six months after having been immunized with a low dose.

Age	≤ 1 year old	1- 2 years old	3-5 years old	6-9 years old
Positive/total animals	0/36	4/37	6/34	7/26
Percentage (%)	0	10.8	17.6	26.9

levels remained without much variation for BCG vaccinated group. However, at 5 months post-vaccination the vaccinated group was higher than the control group ($P<0.05$); and at the end of the monitoring period of IgG antibodies, the values for the vaccinated group were even lower than from control group ($P<0.05$). For which, the evaluation of the humoral immune response was limited only to this period (Figure 2).

SICCT test reactivity after BCG vaccination in cows

We had the SICTT reactivities over the last three years for the dairy herd participating in this study. Figure 3 shows the bTB prevalence for 1 to 3 years in adult cows. The percentage of reactors at the beginning of the first year was 1.5% and at the end of the same year was 5.2% while for the second year, before vaccination of adult calves, it was 49.39%. As shown in Figure 3, bTB prevalence after vaccination increased from 0.9 to 50.5%, which is an insignificant increase. Because of an urgent requirement of alternative measures of bTB control, it was necessary to employ the BCG Phipps vaccine altogether with other biosafety measures to give response for the stated objectives.

Assessment of bTB prevalence in the studied dairy herd, by the SICCT test before BCG vaccination was needed with the aim to establish a reference point and from there to consider the impact of vaccination on the control of bTB prevalence. To determine the influence level of the BCG-Phipps vaccine on reactivity to the TST in adult animals, a high prevalence herd (49.39%) was chosen to analyze its effect in both reactors and non-reactors cows. Results indicate that the number of reactor cows increased slightly after BCG vaccination, and that the negative animals showed a reduction equivalent to the increase in positive ones. However, the increase in positive animals and the decrease in negative animals were not statistically significant according to the Chi-square test (Figure 4).

Related to the SICCT-non-reactor animals (n = 133), 17 animals converted to positive ones in a 12.7%. Further, a more detailed analysis showed that in older animals conversion rate was greater, as described in Table 2; conversion rates were: in animals ≤ 1 year old, 0%; between 1 to 2 years old, 10.8%; 3 to 5 years old, 17.6% and 6 to 9 years old, 26.9%. Thus, a greater conversion was observed in older cows, which could be related to a greater exposition to environmental mycobacteria considered a risk factor for bTB. These data complement those for calves, in which percentages of SICCT positive animals lesser than one month old after vaccination were low, and reactivity was diminishing during the studied period.

ELISA in cows

The prevalence of bTB was 49.39% before vaccination and antibody level was 3.15±1.01 (OD_{492nm} ± SD). Six months later, antibody level was 3.45±0.95. We found no significant differences by *t* Student test. Similarly, for isolated positive animals any differences were found.

DISCUSSION

Bovine tuberculosis is an important disease of dairy cattle, which has been difficult to control and eradicate. Incidence of this disease represents a significant problem to livestock production, because of a fast dissemination of the disease. Due to multiple factors, such as defective management, poor feeding, new infected animals in the herd, and mainly lack of adequate biosafety measures, bTB represents a risk of disease for humans (Thoen et al., 2006; Schiller et al., 2010; El-Sayed et al., 2016).

For many years, BCG vaccine effectiveness to reduce the bTB has been called into question, and a trouble that prevents its application is the interference of vaccination on tuberculin tests. However, the fundamentals of this interference and the involved factors are unknown. Considering that BCG vaccine represent an alternative for controlling bTB, and that it primes animals to give a DTH to tuberculin reaction, this results in an obstacle for using the BCG, since the TST is the primary tool in epidemiological surveys used in many countries as support for controlling bTB (OIE, 2012).

Today, conversions percentages to TST and protection effectiveness induced by BCG are unknown, as well as factors that intervene in both events. BCG strain, dose, age of vaccinated animals, role of environmental mycobacteria, and idiosyncrasy of each animal, among a number of factors, could influence in both events; conversion and protection. So our first objective was to determine the effect of BCG-Phipps vaccine in a reduced dose (10^4 CFU) on TST reactivity in bTB-free young calves of ≤ 1 month of age. The results showed a percentage of reactivity to the test in vaccinated calves

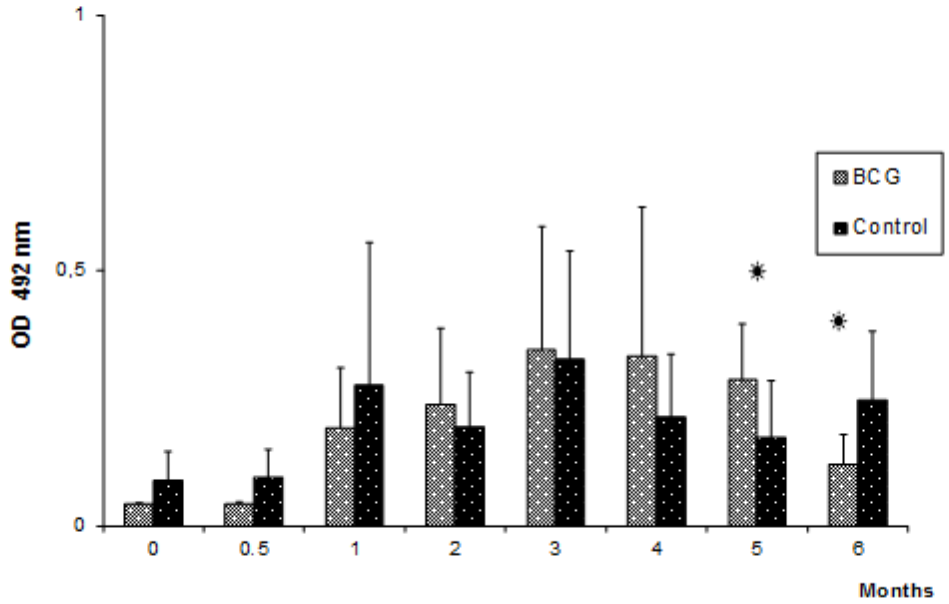

Figure 2. IgG antibody levels measured by ELISA in young female calves vaccinated with BCG Phipps compared to control non-vaccinated. Antibody levels were determined using a CFPE from *M. bovis* (see methods). Values are mean±SD; *p < 0.05; vaccinated vs non-vaccinated).

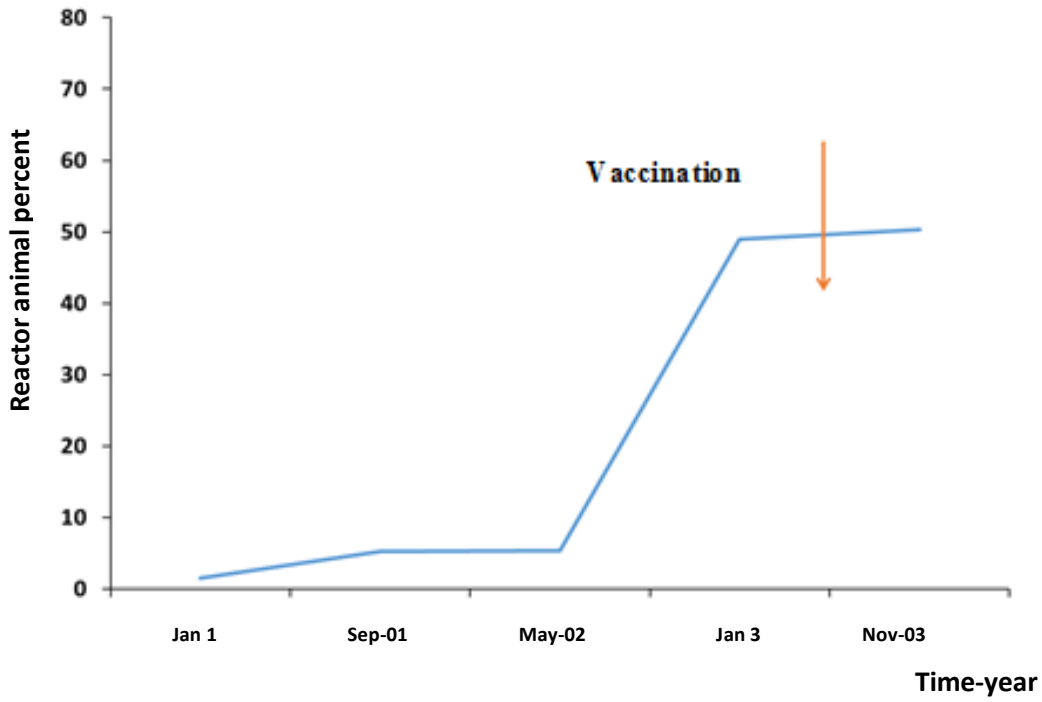

Figure 3. Historical records of bTB prevalence by SICCT test determined for the study herd three previous years vaccination, located in Hidalgo State, México. The arrow indicates the moment of vaccination of the groups of reactors and non-reactive cows to the tuberculin test

of 7.4% at three months post-vaccination, and 3.7% at five months, then wane at seven months post-vaccination. In non-vaccinated calves, there was no reactivity to TST in the first year, up to 14 months after

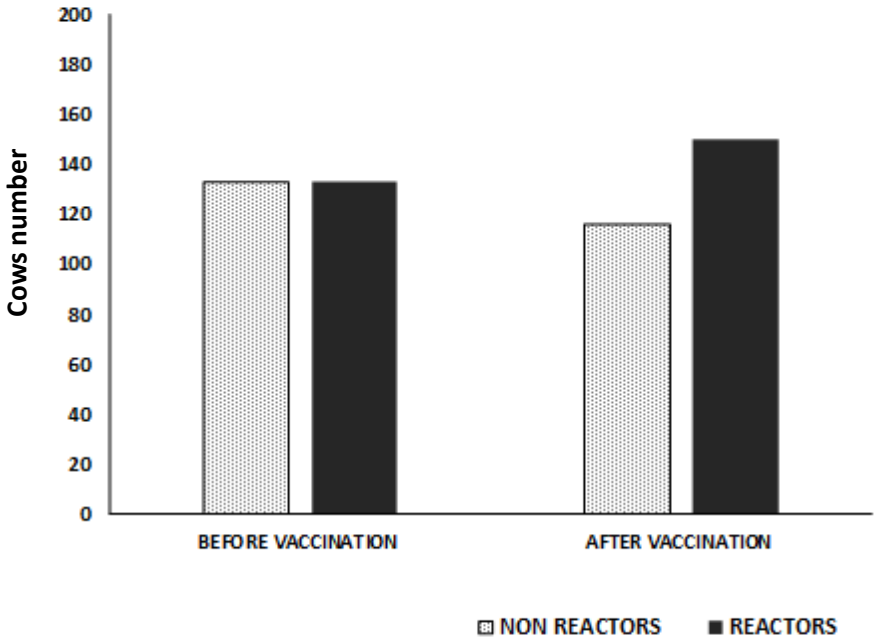

Figure 4. Number of cows that were positive or negative to the SICCT test before and after BCG-Phipps vaccination (six months post-vaccination). There were no significant differences between pre- and post-vaccination values ($p > 0.05$).

the study started in which one of them was suspicious. The percentages of reactivity observed in vaccinated calves in this study were much lower than that reported by Whelan et al. (2011). These authors, reported an 80% of TST conversion in calves vaccinated with BCG-Danish during the first six months post-vaccination, with a decay of 8% reactivity at nine months. This difference in reactivity percentages with our results could be related to the dose and strain used. Similar percentages of TST conversion have been reported in other studies using high vaccine doses between 10^6-10^8 CFU in humans, wildlife animals, and mouse model (Buddle et al., 2013; Tree et al., 2004). In addition, the protective efficacy reported in those studies was lower in vaccinated animals that showed a strong DTH response to bovine PPD than those animals that did not develop it (Skinner et al., 2001; Ritz et al., 2008). In follow-up of the possible establishment of *M. bovis* infections in vaccinated and control calves, PCR reactions performed from nasal exudates showed that both groups of calves were negative throughout this study. Even though we found no differences between vaccinated and unvaccinated groups in the detection of the DNA of *M. bovis* during time evaluated, there was concordance between TST and PCR tests after vaccination. Regarding the tuberculin conversion in vaccinated non-reactive cows, heifers less than one year old belonging to this group showed no reactivity. However, conversion percentages increased according to aging of cows, since the cows of 6 to 9 years showed a conversion percentage of 26.9% (7/26). This can be explained because cows during his long life were largely exposed to *M. bovis*, since they belonged to a herd with high prevalence of the disease. Consequently, the previous sensitizations in conjunction with the application of BCG vaccine favored the development of delayed type hypersensitivity in these animals while in the reactor group, there were no differences in the degree of reactivity or development of ulcerations and/or necrosis, at least with the dose of vaccine used. It has been mentioned that in infected cattle confirmed with at least two consecutive tests of tuberculin, as in humans, could developed an adverse reaction to vaccination with BCG (Koch phenomenon) in case of having a pathology advanced or generalized (Cardona, 2006). In this regard, Buddle et al. (2016) showed that vaccination of cattle with a high dose of BCG vaccine applied after an experimental infection with *M. bovis* increased the inflammatory response, but not tuberculosis pathology. However, all these observations should be taken with reserve and to carry out more studies, which show the benefit or prejudice resulting in the use of the BCG vaccine in infected cows. On the other hand, it has been suggested that the BCG vaccine is capable of preventing bacilli excretion. Thus, taking into account that airborne route is the most important transmission route for bovine tuberculosis, the BCG vaccine could reduce the risk of contagion among animals (López-Valencia et al., 2010).

In relation to the BCG vaccine, it is widely documented that because of the *in vitro* passage, the genome of the

original strain has been modified giving rise to a series of heterogeneous strains, which show clear variations in their genomes and protective immune properties (Castillo-Rodal et al., 2006; Abdallah et al., 2015). With regards to the BCG-Phipps the main regions of differentiation (RD) lost are RD1, RD2 and nRD18. RD1 is a DNA segment comprising 9.5 kb, which was deleted in all other BCG strains, that encodes T-lymphocyte epitopes such as ESAT-6, CFP-10, Rv3873, and PPE protein among others (Okkels et al., 2003). RD2 is a 10.7 kb DNA segment that encodes many proteins including Mpt-64 and CFP-21 (Joung and Ryoo, 2013) and nRD18 is a 1.5 kb segment containing genes encoding SigI, an alternative RNA polymerase sigma factor that was only lost in the strains BCG Pasteur, Phipps, Frappier, Connaught and Tice (Da Costa et al., 2014).

In a study realized by Zhang et al. (2016) assessing the virulence and efficacy of 13 BCG strains in SCID and BALB/c, showed that BCG strains of the DU2 group IV to which BCG-Phipps belongs, showed the highest levels of virulence among strains studied. They reported that these distinct levels of virulence could be explained by strain-specific duplications and deletions of genomic DNA. There appears to be a general trend that more virulent BCG strains are also more effective in protection against challenge. Similar results were reported by Castillo-Rodal et al. (2006) whom showed that BCG-Phipps immunized mice developed lesser pneumonia areas and had lesser bacterial loads than animals vaccinated with other strains, indicating resistance establishment against disease. In addition, reactivity to tuberculin was lower compared to animals vaccinated with other BCG strains and control groups. Considering the above, our results suggest that the BCG-Phipps vaccine can be applied to neonatal calves because we observed a low and transitory DTH reaction in these animals. Besides, the results obtained in a previous study showed that the use of low dose induced the development of good immunity against *M. bovis* in vaccinated calves under field conditions (González-González et al., 2012). Thus, BCG-Phipps vaccine constitutes a good candidate for use in disease control.

On the other hand, we have not observed significant difference in antibody levels to *M. bovis* for vaccinated calves and cows compared to respective controls at first months post-vaccination which may be related to the dose of vaccine used, such as previously reported by Lyashcehnko et al. (2004), whom with a similar dose of the BCG-Pasteur strain did not observe the development of antibodies to defined antigens of *M. bovis* in vaccinated animals.

In recent years, research has expanded on the development of new vaccines in livestock and wildlife, with encouraging results for potential in the control of the disease. However, so many questions remain without response. Biomarkers to predict a protective immunity against tuberculosis is required to be studied, in order to evaluate vaccine effectiveness and reduce challenge experiments, because of controversial issues related to bioethical principles. Additional studies are needed to put into test whether cattle vaccination extends protection in function of time, or affect the results of traditional diagnostic tests. Considering progress reached in the last decade, we optimistically think answers to these questions will be found, and TB vaccination will become a valuable control measure to control this disease in human beings, livestock and wildlife.

Conclusion

BCG-Phipps vaccination with a single reduced (UFC) dose, had a minimal transient effect on development of DTH to tuberculin in neonatal calves and heifers lesser than one year, and declined in the first six months post-vaccination. In contrast, under these conditions a greater number of older cows became reactors. In addition, PCR analysis used throughout the study did not show the establishment of *M. bovis* infections in vaccinated and control groups. In vaccinated non-reactive cows, the tuberculin conversion percentages increased according to aging of cows. For additional studies, age of vaccination should be considered since bTB is a chronic disease and the exposition risk increases as the age of animals increase, hence this parameter will be a critical factor in stables where bTB vaccines are considered as a control measure. Furthermore, it remains to be tested if protective immunity and long life is induced by low doses of BCG-Phipps vaccine.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Funding for this study came from the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) México (No. SIGI 14294534013). Thanks go also to the farmers that provided the animals and farms for the realization of this study.

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academicJournals

Vol. 9(11), pp. 300-312, November 2017
DOI: 10.5897/JVMAH2017.0617
Article Number: E78E99C66286
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Health and welfare status of donkeys in and around Hawassa Town, Southern Ethiopia

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Received 30 June, 2017; Accepted 26 July, 2017

A cross-sectional study was conducted from November 2016 to April 2017 with the objective of assessing the welfare situation and identifying major health problem of donkeys in association with risk factors identification using physical examination and questionnaire survey in and around Hawassa town, Southern Ethiopia. Three hundred and eighty four donkeys were physically examined for health related and management problems and three hundred and eighty four donkey owners and cart drivers were interviewed for awareness and welfare assessment in the study area. Out of the total 384 donkeys, the prevalence of wound, lameness, skin problem, other illness signs, eye problem, dental problem and change on visible mucous membrane were found in 47.7, 38.8 36.2, 14.3, 9.4, 7.6 and 6.3%, respectively. Statistically significant ($p<0.05$) association was observed between the occurrence of wound and age, type of work, duration of work and body condition of the study animals. The prevalence of wound in old age groups, poor body conditioned animals, donkeys that transport construction material and those working for more than 10 h were 76.6, 74.6, 71.2 and 93.8%, respectively. Furthermore, the welfare problems association with donkey users' ownership status, age, and educational status were assessed and 51.3% of cart drivers' owners, 47.1% young drivers and 62.2% had educational status under grade four and have little experience on handling working donkeys. Out of 384 respondents, 63.2% were not aware of common animal welfare freedoms. The health and welfare problems of cart pulling donkeys in the study area were created and complicated with multiple influential factors. The whole community should participate on awareness creation, introduction of improved design of harnesses and carts, also training on animal welfare to owners should be given to reduce health and welfare problems on donkeys.

Key words: Donkeys, health problems, risk factors, welfare, wound.

INTRODUCTION

The donkey (*Equus asinus*) is believed to be the first member of the Equidae family that was domesticated (Rossel et al., 2008). The world donkey population is estimated to about 44 million; half is found in Asia, just

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over one quarter in Africa and the rest mainly in Latin America (Fielding and Starkey, 2004). Ethiopia has the largest population of donkeys in Africa and the second largest donkey population in the world next to China (Kumar et al., 2014).

Equines are important animals to the resource poor communities in rural and urban areas of Ethiopia, providing traction power and transport services at low cost. The use of equines in door-to-door transport service also provides urban dwellers with the opportunity of income generation. The majority of the income generation product of equines mainly comes from donkeys (Biffa and Woldemeskel, 2006; Tesfaye et al., 2016; Abdela et al., 2017; Genetu et al., 2017). Donkeys are one of the most important domestic animals most intimately associated with humans. They contribute a lot through their involvement in different social and economic sectors (Ayele et al., 2006). The donkey is harder than the horse, survives with much less attention, drives sustenance from poor quality food and can tolerate considerable heat and dehydration. This makes it a suitable animal for harsh environments and difficult working conditions. Its main role is that of a beast of burden, typically transporting materials such as grains, fuel wood, water, crop and building materials (Kiros et al., 2016).

Since these animals are working animal, they are always expected to undergo suffering in their day to day life due to stress, strain due to overwork, fatigue due to working with poor health, feed, nutrition and drinking water. Pains are due to unscientific ill-fitting equipments, harness devices, poorly designed agricultural equipments and carts. Non availability of proper veterinary care, working under hot and dusty environment, lack of proper shelter, care and management, crude castration, restraining and tethering devices, walking long distances and overloading, poor handling during loading and unloading and inhumane slaughter is common. Most of the animal owners are not even aware of animal welfare practices and as a result, animals have to undergo significant suffering due to improper handling, transport and husbandry practices (Biswas et al., 2013).

The animal welfare is being compromised due to several constraints such as poverty and lack of knowledge. Research conducted in Ethiopia demonstrated that improvements in the welfare of donkeys had significantly improved their work output which in turn improved livelihood situations of the poorest communities in the rural and peri-urban areas (Smith, 2003). The welfare of working donkeys in developing countries is therefore crucially important, not only for the health and survival of those animals, but also for the livelihoods of those people dependent on them (Wilson, 2002; Pearson and Krecek, 2006).

The causes of poor welfare outcomes frequently identified for working animals include poor nutrition, poor harness design and use, overwork and inappropriate management practices, such as beating and working animals at too young an age. Additional related problems include wounds, lameness, colic in equids and preventable infectious disease. Initiating factors are usually multi-layered. They include traditional or cultural beliefs and economic constraints. Cultural factors can include traditional beliefs in harmful practices such as nostril-slitting and firing. Economic factors commonly constrain marginalized communities from accessing resources such as feed and water, good harnessing and carts, quality shoes, shelter, and health care for their working animals, which all have a direct impact on animal welfare (Rahman and Reed, 2014).

As any other animals, donkeys are vulnerable to a variety of disease of biological origin, nutritional diseases and other miscellaneous cause that leads them to ill health, suffering, considerable loss of work output and reduced longitivity (Gebreab and Fanta, 2007). In countries like Ethiopia they are subjected to a variety of health disorder including multi-parasitism, back sore and other wounds due to different causes, hoof problems, colic, various infectious diseases such as strangles, tetanus and others. Feed shortage and disease are the major constraints to productivity and work performance of equines. The major and common clinical manifestations of disease which affect organs of support are lameness, failure of support, insufficiency of movement and deformity (Amene et al., 2015).

They are brutally treated, made to work overtime without adequate feed or healthcare. They suffer from lack of shelter from sun, rain or biting insects at markets or working sites. These have a potential to negatively affect their welfare and quality of life. This was justified by low number of donkeys presented annually to the clinic compared to other domestic animals; 270 donkeys vs. 20,000 head of other domestic animals such as cattle, between 1987 and 1988 (Yilma et al., 1991).

According to recent central statistical agency (CSA, 2013), there are about 2.03 million horses, 7.43 million donkeys, 0.4 million mules, and about 1.16 million camels in the sedentary areas of the country. In Ethiopia the use of donkeys as pack animal or for pulling cart has enabled small scale farmers to participate in the market economy. Donkeys are used for fetching water, for household shifting, for carrying the sick to hospital, for carrying sick calves, for transportation, and for pulling materials needed for construction. Probably one of the most important limitations is the general lack of information on the proper management and welfare problems of donkeys, which leads them to receive minimum care. Donkey has spent hundreds of years being used by man but, despite that only little attempt has been made to study any aspect of this animal until recently and particularly in countries where they are most important (Amene et al., 2015).

Even though donkeys are used as the source of income generation and provides invaluable and cheap energy for the whole communities in the study area, their health and welfare situation are compromised through poor husbandry practices, lack of animal welfare knowledge, problem of the daily income of drivers, absence of legislation policy for working animal and poor design harness material. In Hawassa and its surrounding, large population of cart pulling donkeys are found, but their health and welfare situation show that they suffer from multi-factor life threatening problems. Therefore this study was planned with the following objectives: to assess welfare situation of cart pulling donkeys in Hawassa town and also to identify major health problems and associated risk factors of the Donkeys in the study area.

MATERIALS AND METHODS

Study area

The study was carried out in and around Hawassa Town, Southern Nations, Nationalities and Peoples of Southern Ethiopia. Hawassa is situated at 275 km South of Addis Ababa (the capital of Ethiopia) at latitude between 6°83' to 7°17' N and longitude 38°24' to 38°72' E on the escarpment of the Great Rift Valley along the Addis Ababa Moyale highway. The altitude ranges from 1650 to 1700 m above sea level. The annual average rainfall and temperature are 955 mm and 20.3°C, respectively. Hawassa is the capital of South nation, nationalities and people region (SNNPR) and has eight sub cities, namely Addis Ketema, Mehal, Menariya, Misrak, Hayk Dar, Behal Adarash, Tabor and Hawela Tula sub cities. The population of donkey (Equus asinus), mule (Equus hemonious) and horse (Equus cabalis) of Hawassa city are 13961, 369, and 5161, respectively (CSA, 2008). A total of five areas of Hawassa were included in the study (Aroge Gebeya, Adis Gebeya, Atena Tera, Alamura Sefer and Hawela Tula Gebeya).

Study animals and sampling procedure

Working donkeys (384) which were used for cart pulling to transport different material were sampled. Working donkeys of all age groups from different localities in and around Hawassa city were randomly selected for the study. The breed of the donkeys were local breed called Abyssinia type with the characteristic feature of short, compact, lower feed requirement and easily manageable. The assessment of welfare and health was done with the corresponding risk factors such as age, behavior, body condition, lameness, harnessing condition, wound distribution and types of wound. Animals were physically examined for their health and problems associated to welfare and owners or cart drivers were interviewed with structured/semi-structured questionnaires.

Sample size and sampling method

A total of 384 donkeys were randomly selected for physical examination and questionnaire survey. The sample size has been determined according to the formula given by Thrusfield (2007) based on expected prevalence of 50%. Based on simple random sampling methods and 95% confidence interval with required 5% precision, the sample size was determined as:

$$N = Z^2 \times Pexp\ [1\text{-}Pexp] / d^2$$

Where, N= required sample size, Z= the Z-value at 95% confidence level, Pexp=expected prevalence=50%, and d= required precision.

Study design and methodology

A cross sectional study was carried out on cart pulling donkeys found in five selected areas of in and around Hawassa city. The sites were selected purposively based on their accessibility, easy of logistic and number of donkey populations in the area. The sites were Aroge gebeya, Adis gebeya, Atena tera, Alamura sefer and Hawela Tula gebeya. Donkeys were randomly selected, all are indigenous breeds irrespective of age, sex and body condition score to investigate the health and welfare situation and associated risk factors.

Data collection

Direct assessment

A structured direct assessment format was developed and data was collected by direct physical examination of the animals. Prior to the assessment, consent was obtained from animal's owners or users after explaining the objective of the study. If the animal owner is not willing, then opportunity was given to the next willing animal owner. The same procedure was continued steadily until the sample size was attained throughout the study period. All sampled animals were physically restrained by animal owner and causal worker. Mouth was thoroughly examined for the presence of any feed pack on teeth. If there is feed pack, the tooth is removed so as not to interfere with age estimation and also cause abnormal teeth identification.

Information regarding general health parameters such as: type of wound, anatomical distribution of wound/physical injuries, lameness, skin problems, problems of visible mucus membrane, and eye conditions and other signs of illness were properly recorded on data collection format. Assessment was carried out at field level, market and around homestead on the day time. Animals were allowed to stand for maximum 10 min after being held by head collar and lead rope assessment began, without causing major disturbance to donkey routine work.

Age determination

Age of the animal was estimated based on the observation of the animal's front teeth (Incisors) (Crane, 1997). Accordingly, the study animals were categorized into three age groups as less than 5 years, 5 to 10 years, and above 10 years. Dental abnormalities were also observed and recorded. But for the ease of study simplification and absence of donkeys that are too old, this study took three age categories as <5, 5-10 and >10 that are considered as young, adult and old, respectively.

Wound assessment

Body lesions were then recorded with regard to type of wound and anatomical location as wither sore, back sore, tail and tail base

Table 1. Age, body condition score and behavior of study animal (n=384).

Variable		Frequency	Percentage
Age	Less than 5 years	51	13.3
	Between 5 to 10 years	218	56.8
	Above 10 years	115	29.9
BCS	Poor	63	16.4
	Medium	186	48.4
	Good	135	35.2
Behavior	Anxious	7	1.8
	Friendly approach	120	31.3
	Friendly not approach	13	3.4
	Depressed	87	22.7
	No response	142	37
	Tail tuck	9	2.3
	Biting	6	1.6

BCS: body condition score.

sore, girth sore, neck sore, chest sore, mouth-commissure sore, head sore, and other sore in study animal.

Body condition of animal

Body condition score was done according to the criteria described by Pritchard et al. (2005) and animals were examined from all sides. The donkey body condition was scored as 0 to 5 (0 = very thin, 1 = thin, 2 = fair, 3 = good, 4 = fat and 5 = very fat). However, for the purpose of data analysis, body conditions 0 to 5 were categorized into three distinct groups: categories 0, 1 and 2 were grouped as “poor", category 3 was defined as "medium" and body condition scores 4 and 5 were categorized as "Good".

Demeanor of the animal

The behaviors of all animal sampled were assessed as anxious, friendly approach, not friendly approach, depressed, no response, tail tuck and biting which involve an observation of general alertness versus unresponsiveness to the environment to correlate these behaviors with physical problem and diseases (Morka et al., 2014).

Indirect welfare assessment

Semi-structured questionnaire was developed to collect data on the major constraints health and welfare considering use of donkeys, veterinary service, disease management system, management practice (feeding, watering, health care, shelter and resting time), working nature (duration on work, weight carried, length of journey covered, nature of working environment), educational status, and age of donkey cart drivers and other people working on animal. Accordingly, 384 people were interviewed to generate information which was missed during direct assessments of the animal.

Data management and analysis

The data collected from the 384 donkeys and interviews made with 384 drivers were entered into Microsoft excel spread sheet and analyzed using SPSS version 20 statistical software. Descriptive statistics were used to quantify the problems and Chi-square (χ^2) was used to determine the association of the problem with the risk factors. In all calculations, the confidence interval was set at 95% and statistical significant differences were considered as $p<0.05$.

RESULTS

Direct assessment results

The direct assessment data was collected by assessing age, work type, body condition, different wound types, distribution of wound, behavior, causes of lameness, origin of lameness, degree of lameness, dental problem, skin problem, mucous membrane problem, and other illness signs observed from the examined donkeys. For the 384 studied animals, results for the distribution of age, body condition and behavior are illustrated in Table 1.

During the study period, the donkeys were thoroughly examined for type of wound and distribution of wound. Out of 384 donkeys sampled, 183 of them were found to be wounded; the overall prevalence of wound was 47.7% (Table 2).

Out of 183 wound exposed donkeys, 56.3% were found with abrasion, 24% were positive with laceration, 13.7% had puncture, and 6% were affected with incision wound (Figure 1).

From the examined donkeys, many health problems were identified with thorough examination of the sampled animal. Out of 384 examined for other illness signs,

Table 2. Wounds identified during the study period and their anatomical distribution.

Wound distribution	Frequency	Percentage	Overall prevalence (%)
Wither sore	48	8.7	47.7
Back sore	62	11.2	
Tail/Tail base sore	17	3.1	
Girth/Belly sore	23	4.2	
Chest sore	16	3	
Ribs/Flank sore	32	5.8	
Loin sore/croup sore	7	2.26	
Knee sore	4	0.72	
Hock sore	4	0.72	
Mouth-commissure sore	28	5.07	
Foreleg other than knee	5	1	
Hindquarter other than hock	3	0.5	
Lip lesion	6	1.1	
Head sore	4	0.72	

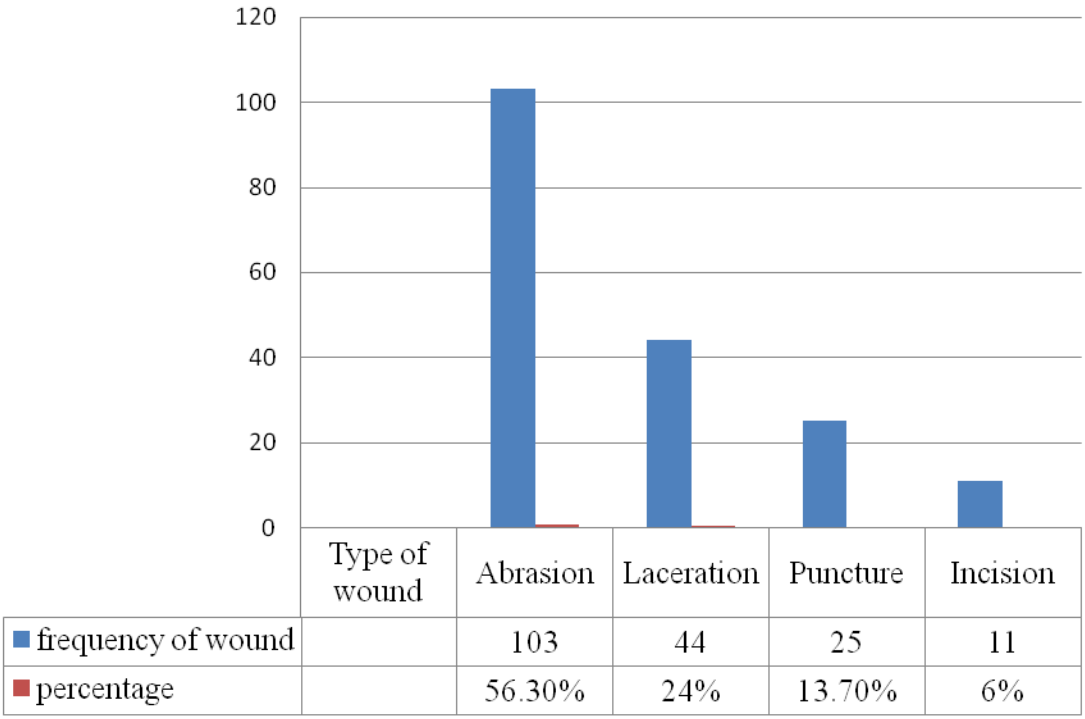

Figure 1. Wound type identified during study period.

14.3% (55) donkeys were positive for other illness signs. For the dental problem examined, 7.6% (29) donkeys had dental problem. The abnormality on visible mucous membrane was found in 6.3% (24) donkeys.

Eye problems were found in 9.4% (36) donkeys. The skin problem was one of the major finding constraints. Out of 384 examined donkeys, 139 (36.2%) were affected. In the study area, donkeys have crucial importance to the transportation of goods. However, lameness is the main problem affecting transport services of the donkeys in the study area. From the total examined donkeys, 38.8% (149) were found with lameness problem. Table 3 illustrates the other illness signs, dental problem, skin problem, eye problem, change on visible

Table 3. General health problem encountered.

Encountered health problem	Number affected	Prevalence (%)	Overall prevalence (%)
Other illness signs			
Coughing	12	3.1	
Discharges from nostril	11	2.9	
Fever	3	0.8	
Listlessness	2	0.5	
Constipation	7	1.8	14.3
Diarrhea	6	1.6	
Refuse to move	8	2.1	
Refuse to eat	4	1	
Swollen belly	2	0.5	
Dental problem			
Overbite problem	9	2.3	
Periodental disease	13	3.4	7.6
Diastema	7	1.8	
Visible mucous membrane			
Pale	11	2.9	6.3
Congested	13	3.4	
Eye problem			
Lacrimination	17	4.4	
Inflammation	9	2.3	9.4
Loss of vision	2	0.5	
Swelling	8	2.1	
Skin problem			
Rough hair coat	73	19	
Alopecia	16	4.2	
Sarcoid	4	1.0	36.2
Habronemiasis	7	1.8	
Ectoparasites	21	5.5	
Loss of elasticity	18	4.7	
Degree of lameness			38.8
Low grade	67	17.4	
Moderate grade	58	15.1	
High grade	24	6.3	
Causes of lameness			
Hoof problem	77	51.7	
Wound	32	21.5	
Fracture	37	24.8	
Arthritis	3	2	
Origin of lameness			
Above knee	55	37	
Below knee	56	37.5	
Above hock	16	10.7	
Below hock	22	14.8	

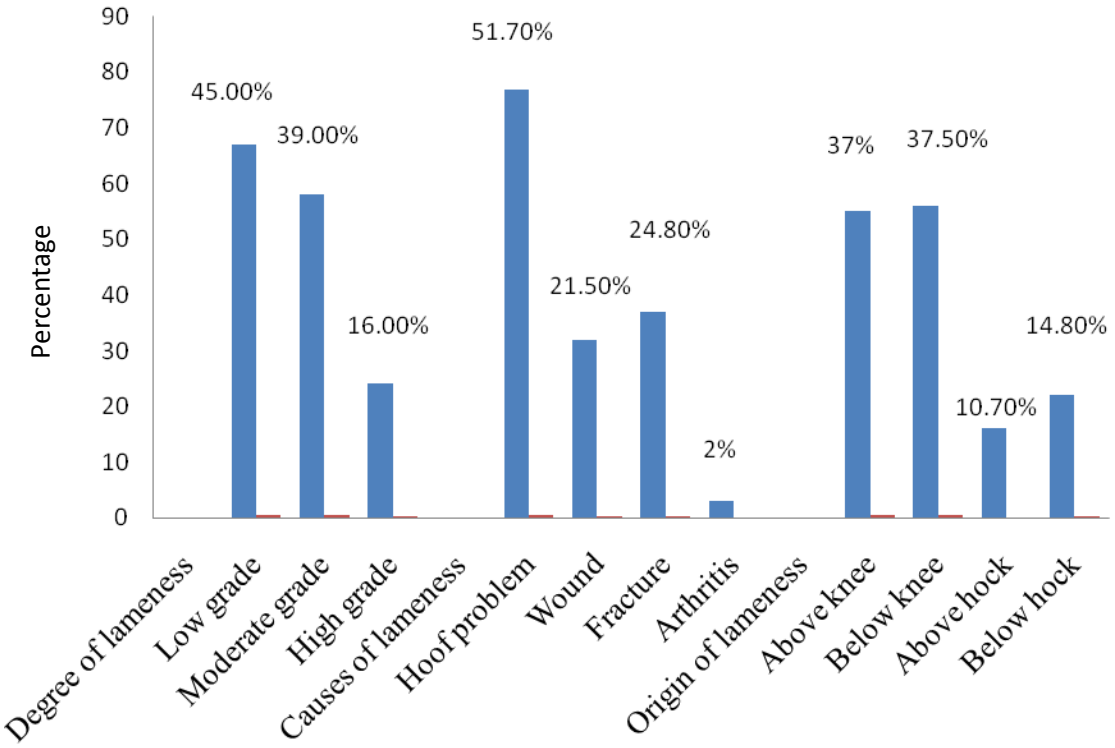

Figure 2. Grade of lameness, factors that cause lameness and origin of lameness.

mucous membrane problem, degree of lameness and origin of lameness, and causes of lameness. In Figure 2, the grade, causes of lameness and origin of lameness are shown.

The prevalence of wound associated with different age groups were examined and the statistical association reflects that the donkeys between five and up to 10 years had high prevalence of wound than the age groups of below five and above 10 year (χ^2 =10.745, P-value=0.005). The prevalence of wound with body condition of the donkeys are highly significant in poor body conditioned donkeys (χ^2=21.971, P=0.00). Also, there was a statistically significant association observed (χ^2=63.883, P-value=0.000) for prevalence of wound with the type of work animal used. The prevalence of wound is strongly associated (χ^2=89.817, P-value=0.000) with duration on work and the donkeys worked above 10 h (Table 4).

Result of questioner survey

Out of a total of 384 interviewed, 51.3% (197) of the respondents are owners of donkeys and 48.7% (187) respondents were not the owners. Age distribution of drivers was 47.1% (181) young, 44.8% (172) adult, and 8.1% (31) old. The drivers educational status were 62.2% (239) below grade four, 32.8% (126) between grade 5 and up to 8, and 5% (19) above grade eight. The survey of experience of working year using this animal were 29.7% below one year, 35.9% one to two years and 34.4% above two years (Figure 3).

Out of the total respondents, 63.2% (243) were not aware of animal welfare, 27.9% were not aware of freedom from thirst and hungry, and 8.9% were not aware of freedom from injury and disease. The animal welfare knowledge source was veterinarian (16.7%), radio programme (9.6%), their friends (3.4%) and world animal day (7%). The drivers' responses about care for sick animal were 43.2% (166) taken to Government Veterinary Clinic or private clinic, 34.4% (132) given traditional medication and 22.4% (86) left to self-healing. Out of the total respondents, 90.4% (347) owners were forced to use their donkeys that had wound and only 9.6% (37) owners use their donkeys after wound healed. In the study area, 81.6% (313) did not get any training or consultation about animal welfare from Veterinarian and only 18.4% (71) cart drivers were trained on animal welfare and management. The opinion of respondents about responsibility for donkey health and welfare assessed and their response were 42.4% veterinarian, 32% owner, and 25.6% government. Also, respondents reflects different standing on severe diseased or old

Table 4. Prevalence of wound based on the age group, BCS groups, type of work, and duration on work and harnessing condition.

Category	Number of examined	Number of affected	Prevalence (%)	χ^2	p-value
Age of animal					
below 5 years	51	29	7.6		
Between 5 and 10 years	218	88	22.9	10.745	0.005
Above 10 years	115	66	17.2		
BCS					
Poor	63	47	74.6		
Medium	186	78	41.9	21.971	0.000
Good	135	58	43		
Type of work					
Multipurpose	140	78	55.7		
Construction material	104	74	71.2		
Wood and charcoal	48	9	8.8	63.883	0.000
Flour from mill house and farm products	64	14	21.9		
Garbage	28	8	28.6		
Duration on work					
Less than 6 h	45	10	22.2		
6 up to 10 h	259	98	37.8	89.817	0.000
Above 10 h	80	75	93.8		
Harness condition					
Tire rope	166	80	48.2	0.034	0.854
Mill rope	218	103	47.2		

donkey not used for work purpose: out of 384 respondents, 62.5% (240) owners showed their response to leave out of home and 37.5% (144) owners keep their severed diseased or old aged donkeys not used for work purpose at their home (Table 5).

The questionnaire survey result of 384 donkeys drivers and owners interviewed from five selected areas indicated that all animal owners do provide water and feed to donkeys at home and working places, out of 384; only 70.3% (270) provide shelter to donkeys at their home and 29.7% (114) of the owners of cart donkeys in and around Hawassa city reflected that they could not provide shelter at home specially at night and they keep their donkeys in fenced compound without any shade paying off fee; this is due to the fact that the owners do not have their own house and live in rented homes as a result of this, donkeys were exposed to different welfare problems. 41.4% fed their donkeys three times per day and 58.6% fed their donkeys twice in day. The owners fed their donkeys depending on the income they got daily and availability of feeding material (Table 6).

DISCUSSION

In Hawassa, all donkeys are kept to transport different materials in order to insure their owners' daily income. This study is in agreement with reports by Usman et al. (2015), Solomon et al. (2013) and Pritchard et al. (2005) describing that equids are mainly kept for transport purposes and only rarely as source of meat or milk. The current study revealed that 100% of the owners were using their animals for transportation or carrying load for more than 6 h a day in average which is in agreement with Biswas et al. (2013) and Panwar et al. (2008).

In this study area, 47.7% donkeys population had overall prevalence of wound, 56.3% were found with abrasion, 24% were positive with laceration, 13.7% had puncture and 6% were affected with incision wound. Similarly, the finding of Fikru et al. (2015) reported that 50.6% of abrasion wound was examined from the same study area. During the study period, the wound was classified depending on the anatomical distribution and result indicated that the high frequency of back, wither, ribs/flank, mouth-commissure, tail/tail base and chest wound were examined. The current study agreed with those of Helen (2001) who reported similar situation in the Northern Ethiopia and this higher prevalence of wound at the back region could be due to improper harnessing that cause injuries in working donkeys. Similarly, the present result also agrees with the previous report of Mandefro (2008), in which, those ill-fitting and

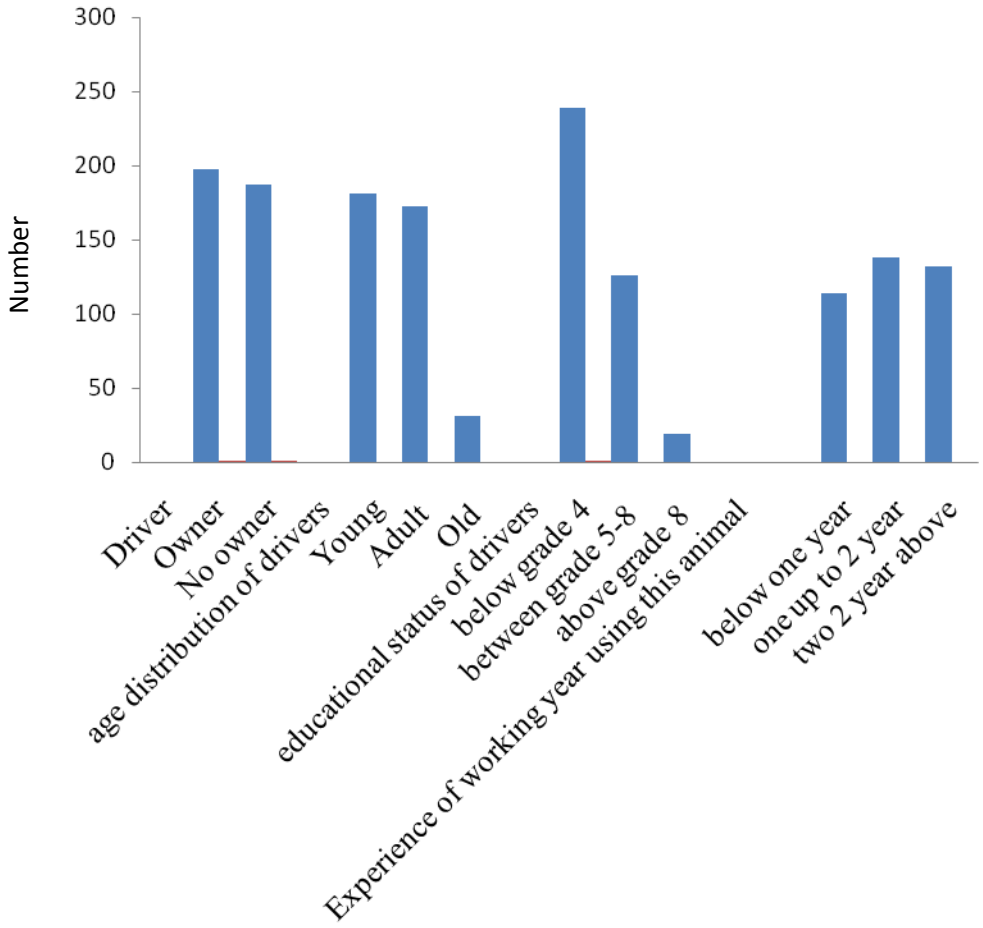

Figure 3. General status of cart drivers.

improperly made tail straps that usually has sharp edge, causes lesions on the underneath of the base of tail of working donkeys.

In current study, wound had higher prevalence than the reports of Morka et al. (2014) in and around Nekemte Town, East Wollega Zone (38.4%) and lower prevalence of donkey wound than the result of Fikru et al. (2015) and Biffa and Woldemeskel (2006) who revealed 63.4 and 79.4%, respectively. These differences might be due to variation in care for animal health, management practices, the weight of load and the work type of the donkeys in the region. All the donkeys examined for the present study were used for cart pulling purpose in the study area.

The current study revealed that higher prevalence of wound was examined in above 10 year old donkeys (48.1%) than other age groups. This finding is in line with those of Tesfaye et al. (2016) who reported 69.2% wound in older age groups than other age groups. This study agreed with reports of Biffa and Weldemeskel (2006) in Hawassa who reported that older equines had greater wound risk than other age group. This might be due to more exposure to work and carrying heavy load over a long distance, less owners' attention to wound management and also the immune defense mechanism also reduce with age advancement.

There was significant association between prevalence of wound and body condition (P=0.000) with 74.6% of poor body conditioned donkeys affected with wound. This result was found to be in agreement with the reports of Abdela et al. (2017), Tesfaye et al. (2016), Tsega et al. (2016) and Henneke et al. (1983) who reported strong association between prevalence of wound body condition. These might be due to dehydration which decreases the elasticity of the skin in poor body condition animals and the prominence of bones leading to easy skin injury.

In this study, the prevalence of wound and type of work donkeys regularly involved has significant association (p=0.000). The donkeys commonly used for transporting construction materials were found to be wounded (71.2%) than donkeys transporting other materials. This

Table 5. Responses of welfare knowledge of cart drivers (n=384).

Welfare issues	Frequency	Percentage
Owner's welfare knowledge		
Not aware	243	63.2
Freedom from thirst and hungry	107	27.9
Freedom from injury and disease	34	8.9
Freedom from pain and discomfort	0	0
Freedom to express normal behavior and enough space to move	0	0
Freedom from fear and distress	0	0
Awareness source		
Veterinarian	64	16.7
Radio programme	37	9.6
Their friends	13	3.4
World animal day	27	7
Care for sick animal		
Taking to vet clinic or private clinic	166	43.2
Traditional medication	132	34.4
Leave to self-healing	86	22.4
The owners use their animal while wounded		
No	37	9.6
Yes	347	90.4
Trained or consulted by veterinarian		
Not trained	313	81.6
Trained	71	18.4
Responsible for donkey		
Veterinarian	163	42.4
Owner	123	32
Government	98	25.6
Decision on severe diseased or old donkey not used for work purpose		
Leave out of home	240	62.5
Keep at home	144	37.5

is due to the transportation of highly loaded metals, sand, cement, stones and woods from far distance for long time without rest. This study is not in agreement with those of Tesfaye et al. (2016) in Mirab Abaya, Gamo Gofa and Kumar et al. (2014) in Mekele who reported that the highest prevalence was recorded in charcoal transporting donkeys (52.1%). This may be due to burning characters of charcoal and wider surface area of sack that lay on the back of the donkeys in which the whole surface not covered by proper harness leads to at least injury in one area of the anatomical location.

In the present study, the prevalence of skin and skin associated problems were found with overall prevalence (36.2%). These findings are higher than Mulisa et al. (2015) in Wolaita zuria (12.6%), Kumar et al. (2014) in Mekelle city (23.7%) and Tesfaye et al. (2016).

In the current study, eye problem and dental problem were identified as the health problem of the studied animals in the study area with overall prevalence, eye problem (9.4%) and dental problem (7.7%). This result disagrees with those of Tesfaye et al. (2016) in Mirab Abaya, Gamo Gofa zone with prevalence with unilateral eye problem (5.4%), bilateral eye problem (11.4%) and dental problems (11.5%). But these outcomes are lower

Table 6. Response of the respondents to the way of management; (n=384)

Respondent knowledge	Frequency	Proportion (%)
Feeding time		
Before loading	83	21.6
After loading	104	27.1
Both before and after loading	197	51.3
Feeding material		
Concentrates	283	73.7
Concentrates and sugar cane	55	14.3
Concentrates, grass and sugar cane	46	12
Feeding condition		
Separately feeding	271	70.6
Feeding with other animal	113	29.4
Feeding frequency		
Twice per day	225	58.6
Three times per day	159	41.4
Watering frequency		
Once per day	40	10.4
Twice per day	325	84.6
Three times per day	19	5
Presence of rest in week		
One day in week	273	71.1
Two day in week	77	20
No rest in week	34	8.9
Shelter		
Absent	114	29.7
Present	270	70.3

than that in the report of Kumar et al. (2014) in Mekelle city; 19.3% eye problem and 16.2% dental problem and higher than the report of Abutarbush et al. (2014) with 4% problem in Jordan. These differences might arise due to difference in topographical nature and misuse, low level of donkey health care keeping characteristics, feeding characteristics and age of working donkey.

The present study revealed that 77.6% of respondent provide care for their donkeys (43.2% taken to government veterinary clinic or private clinic and 34.4% traditional medication) and 22.4% left to self-healing. Also, most of the owners are forced to use their donkeys having wound (90.4%). The result is closely related with those of Tesfaye et al. (2016) in Mirab Abaya that showed 84.2% of the respondents provide care for their sick animal out of which 48.3% took donkey to nearby veterinary clinic.

In this study, the owners fed their donkeys depending on the income they got daily and availability of feed. The responses for supply water to animal were 10.4% once in day, 84.6% twice in day and 5% three times in day. The result of the study shows that 51.3% fed their donkeys both before and after loading at home and working places. However, in the report of Tesfaye et al. (2016), 48.3% provided feed before loading only.

The current study indicated that 70.3% provide shelter to donkeys at their home and 29.7% of the owners keep their donkeys in fenced compound without any shade paying off fee. This result agree with those of Morka et al. (2014) with 76.6% providing shelter to equine at home and 23.6% of the owners of cart horses in Nekemte town reflected that they could not provide shelter at home especially at night and they release them to the street or forest after work. This is due to the fact that to cover a wide range of role of equine, the owners do not have their own house and live in rented homes as a result of this,

animals were exposed to predators', environmental factors, car accident and easily stolen by thieves.

Conclusion

The present study revealed that the cart pulling donkeys in and around Hawassa were faced the multi-factorial health and welfare problems. Poor design wood worked carts, no standard in harnessing equipment, absence of approved policy in working animals, poor animal welfare knowledge, dependence of daily income source on cart work, overloading, overworking and work types were major factors causing health and welfare problems. In the current study, abrasion, laceration, puncture and incision were the identified wound types. Furthermore, the distribution of wound on different body parts were assessed and back sore, wither sore, mouth-commissure sore, tail/tail base sore, ribs/flank sore, chest sore and girth/belly sore were the major wounds examined on working donkeys. Lameness problem, skin problem, other illness signs, and dental problems frequently occurred in donkey's health situation in this study area. Lack of awareness of animal welfare knowledge and less attention to husbandry practices to feeding management, housing condition, watering the donkeys, and heath care of animals were indicators of the minimum understanding of the health and welfare of this study population. These situations strongly call for awareness creation on animal welfare by government and charity organizations interested in animal welfare. The country should also have policy on working animal handling and management. The standardized harnessing equipment should be available in market. Moreover, the donkey sanctuary model carts should be used for all cart pulling donkeys.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 9(11), pp. 313-319, November 2017
DOI: 10.5897/JVMAH2017.0597
Article Number: 5F085EF66309
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Anthelmintic resistance in gastrointestinal nematodes of goats in southern Mozambique

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Received 5 June, 2017: Accepted 25 July, 2017

Small ruminant production is significantly constrained by gastrointestinal parasites once they cause serious production and economic losses for both small-scale and large-scale farmers in the developing world. The control of helminth parasites is most exclusively based on the use of anthelmintics for the economic production of ruminants. However, the resistance of nematodes to the commonly used groups of anthelmintics represents a threat to the production, particularly for small ruminants. The objective of this study was to assess the efficacy of albendazole, the most frequently used anthelmintic in Gaza and Maputo provinces in the southern region of Mozambique, in gastrointestinal nematodes of goats between November and December, 2016. Eleven goat farms in Gaza (n = 5) and Maputo (n = 6) were surveyed. The faecal egg count reduction test was used to assess the efficacy of the drug. The flocks were considered resistant when the reduction in eggs per gram of faeces was less than 95% and the lower limit of the confidence interval was less than 90%. Resistance to albendazole was detected in 60% (3/5) of the farms in Gaza province and 83.3% (5/6) of the farms in Maputo province. The percentage of faecal egg count reduction varied from 51 to 97% in Maputo and from 0 to 100% in Gaza in the farms surveyed. In pre-treatment coprocultures, *Haemonchus* spp., *Oesophagostomum* spp. and *Trichostrongylus* spp. were the predominant nematode species. Post-treatment larval cultures indicated that *Haemonchus* spp. and, to a lesser extent, *Oesophagostomum* spp. and *Trichostrongylus* spp., were resistant to albendazole. This study provided further evidence that anthelmintic resistance of gastrointestinal nematode parasites in goats is currently a problem of great significance in this region of the country and that appropriate measures must be taken to reverse the situation.

Key words: Albendazole, efficacy, gastrointestinal parasites, small ruminants, Mozambique.

INTRODUCTION

Small ruminants make important contributions to human livelihoods in developing countries. In 2012, about 30% of the approximately 1 billion populations of goats in the world were located in Africa (FAO, 2015; reviewed by

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Zvinorova et al., 2016). Gastrointestinal nematode infections of ruminant livestock cause major problems for both small-scale and large-scale farmers in the developing world, with a major impact in sub-Saharan Africa (Sissay et al., 2007; Hussain, et al., 2014; Preston et al., 2014; Pawar et al., 2017; Shija et al., 2014; Singh et al, 2017a; Wondimu and Gutu, 2017), including Mozambique with its tropical and sub-tropical areas where favourable climates for the survival and development of gastrointestinal nematodes prevail (Akkari et al., 2013; Shija et al., 2014). *Haemonchus* spp. are highly pathogenic and economically important gastrointestinal parasites affecting ruminants across the world (Chaudhry et al., 2015; Zvinorova et al., 2016). The control of helminth parasites is often essential for the economic production of animals, especially small ruminants. Current control measures rely heavily upon the use of anthelmintic drugs (Gaba et al., 2012; McArthur and Reinemeyer, 2014). However, the ability of parasites to develop quickly resistance to these drugs is spreading globally (Preston et al., 2014). Anthelmintic resistance is a globally threatening problem to sustainable livestock industry and production (Kenyon et al., 2013; Shalaby, 2013; Chaudhry et al., 2015; Singh et al., 2017b). A strain of *Haemonchus contortus* resistant to all classes of anthelmintics has been reported to have developed in South Africa, a neighbouring country of Mozambique, probably because of frequent use of anthelmintics and inappropriate dosage (Van Wyk et al., 1997). Shalaby (2013) stated also that the factors considered most significant in the development of anthelmintic resistance have been the excessive frequency of treatments and administration of inadequate anthelmintic doses.

Given that the use of anthelmintics is required for an efficient and sustainable control of helminth parasites, the knowledge of the levels of resistance to the drugs commonly used will lead to the implementation of adequate zoo technical management practices to mitigate the spread of anthelmintic resistance. The objective of this study, therefore, was to assess the efficacy of albendazole, the most frequently used anthelmintic, through the faecal egg count reduction test, in goats in Maputo and Gaza provinces, two of the 11 provinces of Mozambique.

MATERIALS AND METHODS

Study locations and animals

The study was carried out between November and December 2016, in seven districts of the southern region of Mozambique, namely, Xai-Xai (farms G1 and G2), Chibuto (farms G3 and G4) and Chókwe (farm G5) in Gaza province, and in Magude (farm G6), Namaacha (farm G7), Boane (farms G8 and G9) and Moamba (farms G10 and G11) in Maputo province. Mozambique is located on the eastern coast of Africa, between Latitudes 10° 20' S and 26° 50' S. The study areas were located between Latitudes 24° 38' S and 26° 02' S, and between Longitudes 33° 39' E and 32° 07' E (Figure 1). The study areas were characterized by a tropical dry climate influenced by the motions of the Indian Ocean, with a hot rainy season from October to March and a cool dry season from April to September. The average maximum temperature is 30°C in January/February, with a maximum of 43°C in January, and the minimum average temperature is 15°C in June/July. The mean maximum precipitation is 152 mm in January, and the minimum average precipitation is 10 mm in June/August (Diniz et al., 2012).

Eleven farms, five in Gaza province and six in Maputo province comprising 1,040 goats, were selected. The number of goats in each of the flocks varied from 26 to 180 animals, and three flocks, two in Gaza (G1 and G3) and one in Maputo (G8), were kept extensively on communal grazing without supplementation throughout the year. The rest of the flocks were kept semi-intensively on private delimited pasture areas with supplementation during the dry season. For the study purpose, only 26 to 30 goats were randomly allocated to two groups of 13 to 15 animals in each flock; thus, a total of 355 goats were surveyed. In order to assess the profile of the zoo technical management systems, a questionnaire was administered to the owners or managers of the farms.

Faecal examination

Faecal collection was done directly from the animals' rectal bulb in order to avoid contamination, and the samples were kept cooled in Coleman thermoelectric cooler until the determination of the eggs per gram of faeces (EPG) was performed at the Central Veterinary Laboratory, Animal Sciences Directorate of the Agricultural Research Institute of Mozambique (IIAM) in Maputo province, and at the Provincial Veterinary Laboratory in Gaza province, respectively.

Reinecke's (1961) modification of the McMaster technique was used for the quantitative determination of nematode eggs in the faecal samples. The number of nematode EPG was calculated using the formula: $EPG = Te_1 + Te_2 \times 100$, where Te_1 is the number of eggs in chamber 1 and Te_2 is the number of eggs in chamber 2 of the McMaster slide (Ueno and Gonçalves, 1998).

One hundred larvae per culture were identified (Gupta and Singla 2012), unless there were fewer than 100 in which case all were identified, using the descriptions of Georgi et al. (1985) and Ueno and Gonçalves (1998).

Determining anthelmintic resistance

The efficacy of oral suspensions of albendazole (5 mg/kg body weight; Albenol-100®, Interchemie, Holland), which is frequently used in Mozambique, was assessed using the faecal egg count reduction test (FECRT). The latter was performed according to the methods recommended by the World Association for the Advancement of Veterinary Parasitology (Coles et al., 1992), described and updated by Coles et al.(2006) and interpreted using RESO® to determine anthelmintic resistance.

Once a flock was selected, 26 to 30 goats were randomly allocated to two groups of 13 to 15 animals each, identified with coded ear tags, and weighed. On each of the 11 farms, an untreated control group was formed to monitor the changes in the nematode egg counts during the test period and a treated group that received oral administration of albendazole (5 mg/kg body weight). Individual faecal egg counts and pooled larval cultures were performed from samples collected on the day of treatment and 14 days after the anthelmintic treatment.

Goats with less than 100 nematode EPG in their pre-treatment samples and those with missing values for either the pre- or post-treatment EPG were excluded from the analyses.

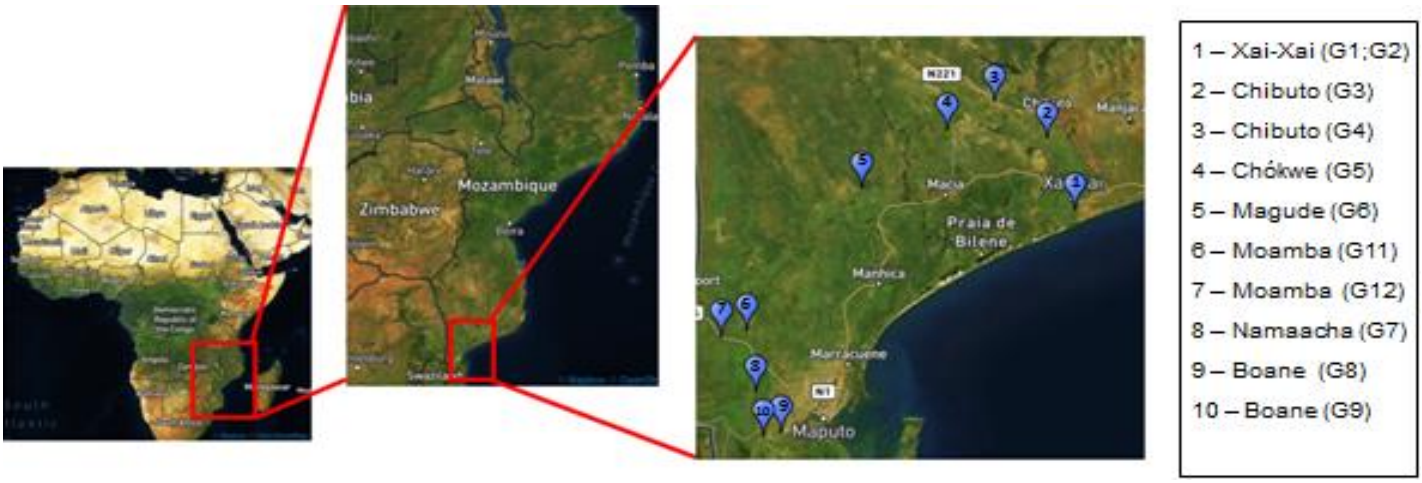

Figure 1. Map of Mozambique indicating the study locations, namely Xai-Xia (G1;G2) and Chokwe (G5) districts in Gaza province and Magude (G6), Namaacha (G7), Boane (G8;G9) and Moamba (G10;G11) districts in Maputo province.

The percentage faecal egg count reduction (%FECR) was calculated, as *100 × (1 - T2/C2)*, where *T2* was the mean egg count of the treated group at day 14, and *C2* was the mean EPG count of the untreated control group 14 days after treatment. The flocks were considered resistant when the EPG reduction percentage was less than 95% and the CI's lower limit was less than 90%. They were considered suspicious if the flock only complied with some of the aforementioned criteria. Flocks that did not comply with any of the required criteria were considered susceptible (Coles et al., 1992, 2006; Canul-Ku et al., 2012).

Statistical analysis

The Pearson's Chi-square test was performed to check for possible differences between the animals surveyed with reference to an association between EPG and factors such as age, sex, and goat breeds. The comparison of susceptibility and resistance of gastrointestinal nematodes to albendazole between farms relating to the frequency of anthelmintic use, grazing system, location at provincial, district, and locality levels was also performed using the Pearson's Chi-square test with 95% confidence interval (CI). All statistical analyses were performed using SPSS version 21, and $p < 0.05$ was considered significant.

RESULTS

Questionnaire

The analysis of the zoo technical management information as per the questionnaire administered indicated that the main objective in all of the flocks (100%) was to produce goat meat. The animals were kept either in communal or private pastures during day hours and shared the pastures with cattle and/or sheep. The animals were reared under extensive systems in communal pastures during the day and kept in corrals at night in 27.3% of the farms (flocks G1 and G3 in Gaza and flock G7 in Maputo). In the rest of the flocks (72.7%), goats were reared under semi-intensive systems in private pastures and kept in cement or metal slabs at night.

Drenching of animals with anthelmintics was based on visual estimative weight in 90.9% (10/11) of the farms except in flock G7 in Maputo, where animals were weighed to determine the adequate anthelmintic dosage. In 81.8% (9/11) of the farms, all animals were drenched with anthelmintics on each occasion, and no EPG tests were performed to decide which animals should be drenched in all farms. Anthelmintics were chosen without any criteria, no drug combination was used, and no quarantine was undertaken in case of acquiring animals from other countries or from other locations within the country in all the studied farms. The data from the questionnaire indicated also that most of the farms had poor management practices, including inadequate disease control measures, inadequate nutrition during the dry season, and face disease challenges, mainly gastrointestinal parasitism.

Faecal egg counts, percentage faecal egg count reduction, statistical analysis, and larval cultures

The arithmetic means of the faecal egg counts before and after treatment for each of the eleven flocks surveyed in Gaza and Maputo provinces and the %FECR are presented in Tables 1 and 2, respectively. According to Coles et al. (1992), flocks are considered resistant when %FECR is less than 95. Resistance to albendazole was detected in 60% (3/5) of the farms in Gaza and 83.3% (5/6) of the farms in Maputo. The %FECR varied from 0 to 100% in Gaza and from 51 to 97% in Maputo.

Possible differences between the animals surveyed with reference to the EPG and factors such as age, sex and goat breeds were assessed using the Pearson's Chi-square test. No statistical difference was found between

Table 1. The arithmetic means of faecal nematode egg counts before and after treatment and %FECR of five farms (G1 to G5) in Gaza province.

Farm no.	Control			Albendazole			% FECR
	No. of goats	Before treatment	After treatment	No. of goats	Before treatment	After treatment	
G1	12	425	567	10	560	20	96
G2	13	846	1885	14	707	593	0[R]
G3	12	658	1092	11	786	0	100
G4	11	355	500	12	708	375	34[R]
G5	11	1345	1218	13	1138	577	0[R]

R, Resistant.

Table 2. The arithmetic means of faecal nematode egg counts before and after treatment and %FECR of six farms (G6 to G11) in Maputo province.

Farm no.	Control			Albendazole			% FECR
	No. of goats	Before treatment	After treatment	No. of goats	Before treatment	After treatment	
G6	12	417	500	12	492	183	68 [R]
G7	11	391	245	13	400	108	81[R]
G8	11	245	245	11	209	18	97
G9	12	908	2025	14	886	279	51[R]
G10	10	250	360	11	218	182	68[R]
G11	10	240	290	10	580	160	72 [R]

R, Resistant.

Table 3. The percentages of gastrointestinal nematode species found in pre-treatment and post-treatment larval cultures on five flocks tested in Gaza province.

Farm no.	*Haemonchus* spp. (%)		*Oesophagostomum* spp. (%)		*Trichostrongylus* spp. (%)		*Strongyloides* spp. (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
G1	37	0	4	0	59	0	0	0
G2	40	84	26	1	31	6	3	9
G3	65	0	24	0	6	0	5	0
G4	95	100	1	0	2	0	2	0
G5	88	100	10	0	0	0	2	0

the animals surveyed in terms of an association between EPG and all analysed factors ($p>0.05$).

In the pre-treatment larval cultures, *Haemonchus* spp. (37 to 95% in Gaza and 19 to 100% in Maputo) and *Oesophagostomum* spp. (up to 26%) were the predominant nematode species, while *Trichostrongylus* spp. And *Strongyloides* spp. (except flock G9) were present in small numbers, except in two flocks (G1 and G2) in Gaza province where *Trichostrongylus* spp. appeared in large numbers (31 and 59%) as indicated in Tables 3 and 4. Post-treatment faecal cultures indicated that *Haemonchus* spp., and to a lesser extent *Oesophagostomum* spp. and *Trichostrongylus* spp., were resistant to albendazole. However, *Strongyloides* spp. appeared in large numbers in the post-treatment larval cultures of three flocks, one in Gaza (G2) and two in Maputo (G7 and G9).

DISCUSSION

The present study demonstrated that goats from all the flocks surveyed in seven districts of Gaza and Maputo provinces were infected with gastrointestinal nematodes,

Table 4. The percentages of gastrointestinal nematode species found in pre-treatment and post-treatment larval cultures on four of the six flocks tested in Maputo province.

Farm No.	*Haemonchus* spp. (%)		*Oesophagostomum* spp. (%)		*Trichostrongylus* spp. (%)		*Strongyloides* spp. (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
G6	58	50	26	50	0	0	16	0
G7	88	100	8	0	4	0	0	9
G8	*	*	*	*	*	*	*	*
G9	19	7.5	2	0	2	0	79	92.5
G10	100	100	0	0	0	0	0	0
G11	*	*	*	*	*	*	*	*

*The larvae collected from coprocultures of the two flocks were reserved for molecular diagnostics since the numbers of larvae were too small.

and eight of the flocks were considered resistant to albendazole.

Knowledge about the species of the gastrointestinal nematodes present and the anthelmintic resistance status of the farms is important for performing control and treatment strategies, and to identify where alternative control measures should be used (Burgess et al., 2012). Laboratory analyses should include knowledge on the reality in the field to make the application of intervention strategies feasible for the farmers (Salgado and Santos, 2016). The faecal egg counts are the most used *in vivo* techniques for detecting infections by nematode parasites, and they help farmers decide when and whether or not to drench animals (Lira, 2005). The chief method for the detection of resistance remains the FECRT, which can be used for all anthelmintic groups. The FECRT estimates anthelmintic efficacy of one or more drugs by comparing the faecal egg counts of animals at the time of treatment and at defined times after treatment, depending on the anthelmintic group used (Coles et al., 1992; Pena-Espinoza et al., 2014). Although the test is considered reliable only if more than 25% of the worms are resistant (Martin et al., 1989; Coles et al., 2006), the FECRT is still the most widely used and most feasible test and is considered the gold standard for detecting anthelmintic resistance *in vivo*.

On all the farms where resistance of the worms to albendazole was detected in both provinces, albendazole had been used over long durations, and the owners had been complaining of the failure of this drug to control nematode infections. Therefore, some of them have dismissed albendazole and used ivermectin or moxidectin to drench animals. According to Van Wyk (2001) and Pena-Espinoza et al. (2014), frequent anthelmintic treatments, use of anthelmintics with similar mode of action for several years, and underdosing are some of the factors that contribute to the development of resistance. Thus, inappropriate use of anthelmintic drugs in small ruminants has led to failures in their effectiveness, leading to a global constraint of anthelmintic resistance (Salgado and Santos, 2016).

On the three farms (G2, G4 and G5) in Gaza province, where resistance to albendazole was detected, most of the goats (mainly Boer breed) were imported from Zimbabwe and South Africa, while the goats (Kalahari red breed) were imported from South Africa in farm G11 in Maputo province. An important feature to note in the referred farms is that animals were neither kept in quarantine nor dewormed after they had been imported before introducing them to their new farms. Shalaby (2013) indicated that introducing newly acquired animals to a farm without prior quarantine and deworming constitutes a faster way to spread resistance of gastrointestinal nematodes.

Indiscriminate use of anthelmintics to treat the entire flocks was observed in the surveyed farms, and this may have probably contributed to the higher levels of anthelmintic resistance in the present study. Das Neves et al. (2014) suggested that the low cost and practicability of anthelmintic administration encourage farmers to treat entire flocks irrespective of the individual's needs, thus resulting in the emergence and rapid spread anthelmintic resistance of gastrointestinal nematodes. Geary et al. (2012) also suggested that, although unsustainable with regard to selection for anthelmintic resistance, routine treatment of the entire flock rather than selective treatment of individuals has become a common practice. Therefore, refugia-based drenching regimes have been widely recommended to slow down the development of anthelmintic resistance (Van Wyk, 2001; Kenyon et al., 2013). Based on the levels of resistance to albendazole detected in the farms studied, "refugia" should be explored to prevent and delay the development of anthelmintic resistance, and even to reverse the situation in flocks where resistance levels are already high.

The occurrence of different levels of resistance to benzimidazoles in gastrointestinal nematode parasites, as evidenced by the FECRT results, is in agreement with other studies undertaken elsewhere, including Denmark (Pena-Espinoza et al., 2014), India (Chaudhry et al. (2015), Mexico (Herrera-Manzanilla et al., 2017), North Ireland (McMahon et al., 2013) and Scotland (Kenyon et

al., 2013), where resistance of *H. contortus*, *Trichostrongylus* spp. and *Oesophagostomum* spp. to benzimidazoles was registered. Kumsa and Abebe (2009) reported anthelmintic resistance in *Haemonchus* spp. to albendazole and tetramisole in goats in Southern Ethiopia. Chagas et al. (2013) reported the presence of a multiple-resistant strain of *H. contortus* in Southeast Brazil. Borges et al. (2015) reported the occurrence of resistance of *Haemonchus* spp. and *Trichostrongylus* spp. to albendazole in the northeast Bahia State in Brazil.

In the present study, resistance to albendazole was detected in eight (72.7%) of the 11 farms surveyed. The results indicated high levels of resistance to the tested anthelmintic, and demonstrated that there was an alarming increase in the resistance rate compared to the results reported by Atanásio et al. (2002).

Any viable eggs in post-treatment coprocultures indicate that some resistant worms may have been present in the animals at the time of treatment. Evidence of small percentage of survivors may indicate a resistance problem that could develop with further rounds of treatments, and should be monitored (Pena-Espinoza et al., 2014). Since large numbers of infective larvae of *Strongyloides* spp. in the post-treatment larval cultures compared with the pre-treatment larval cultures were observed only in three of the flocks (G2, G7, and G9), it was not possible to determine whether the resistance of this species to albendazole has occurred.

Conclusion

This study provided evidence that anthelmintic resistance of gastrointestinal nematode parasites in goats is currently a problem of great significance and imposes severe constraints on the production of small ruminants in this region of the country. Our findings were in agreement with previous studies that indicated the resistance to benzimidazoles as an emerging problem in southern Mozambique. Different control strategies or appropriate measures including sustainable control strategies using integrated approaches and an alternation strategy of drug groups in order to minimize the pressure for parasite adaptation must be taken to reverse the situation. Refugia-based anthelmintic treatments are recommended to slow down the development of resistance to drugs, and target selective treatments to extend the efficiency of anthelmintics mainly in the farms where the levels of resistance are low or anthelmintic drugs are still effective must be applied. Adoption of strict quarantine procedures should be instituted for all newly acquired animals to prevent the importation of resistant nematodes to the farms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

This study was financed by the Ministry of Science and Technology, High Education and Technical Vocational Training (MCTESTP) through HEST Project-World Bank. The author gratefully acknowledges the technical assistance provided by Dr Aida Cala, Mr Carlos Sitoe, and Mrs Ana Paula from the Parasitology Laboratory at Animal Sciences Directorate (DCA) of the Agricultural Research Institute of Mozambique (IIAM) and Mr Gedeão Macanze from the Provincial Veterinary Laboratory in Gaza. Sincere appreciation is extended to the IIAM General Director, Dr Olga Fafetine and the DCA Director, Dr Zacarias Massango, for logistical support during the field work, and to Dr Muller Ribeiro for generating the study locations map. The authors would like to thank reviewers for proof reading the manuscript. The authors express special acknowledgements to Editage for professional editing and English review of the manuscript.

Ethical approval

The clearance by the Ethical Commission on the Use of Animals at the School of Veterinary Medicine-Federal University of Bahia (UFBA), Brazil, has been registered under EMZV-UFBA No. 09/2017.

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academicJournals

Vol. 9(11), pp. 320-326, November 2017
DOI: 10.5897/JVMAH2017.0604
Article Number: 411AB1766312
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Sero-prevalence and associated risk factors for *Brucella* sero-positivity among small ruminants in Tselemti districts, Northern Ethiopia

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Received 9 June, 2017; Accepted 20 July, 2017

A cross sectional study design was employed with the aim to determine sero-prevalence of brucellosis among sheep and goats and identify factors associated with sero-positivity to *Brucella*. A total of 558 sera were collected randomly and aseptically from small ruminants from November, 2015 till October, 2016 in Tselemti district, Northern Ethiopia, following proper restraining. All the sera were primarily screened for the presence of *Brucella* antibodies using Rose Bengal Plate test (RBPT) and then confirmed by Complement Fixation Test (CFT). The overall sero-prevalence of disease in the study area was 1.79% (n=10). Most of the risk factors including peasant association, species, sex, age, parity, herd size, lactation, and pregnancy status had no significant effect on the sero-positivity to *Brucella* (P>0.05), whereas animals with previous history of abortion and retained fetal membrane had significant effect (P<0.05). Hence, the odds of being sero-positive to *Brucella* was found to be 5.68 (COR=5.68; 95% CI: 1.13, 28.53) and 4.05 (AOR=4.05; 95% CI: 1.01, 16.22) times higher in animals with previous history of retained fetal membrane and abortion when compared with animal with no history of retained fetal membrane and abortion, respectively (P<0.05). The results of the current study demonstrated that brucellosis is endemic and the cause for reproductive loss and failure. Hence, the finding suggests that there is a need for implementation of better management practice such as culling of positive animals from the flock, burning/burial of aborted or retained fetal membrane, and also community awareness about zoonotic importance of the disease should be raised.

Key words: Risk factors, small ruminants, sero-prevalence, Tselemti districts.

INTRODUCTION

Ethiopia owns a huge resource of small ruminant population with an estimated number of 27.34 and 28.16 million heads of sheep and goats, respectively (CSA, 2014). Small ruminants provide various benefits particularly to smallholder farmers. They may be used as a source of immediate cash income, meat, milk, skin,

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manure, risk spreading, and various social functions (Berhanu et al., 2006). Besides this, small ruminants are also considered as means of investments and insurance for the small holder farmers in order to provide income for the purchase of food during the seasons of crop failure because sheep and goats have high fertility rates, short generation interval, and small feed requirement and adaptability to harsh environmental conditions as compared to large ruminants which make them best suited for smallholder farming practice in the country (Berhanu et al., 2006; Tsedeke, 2007).

The current levels of contributions of the livestock sector in Ethiopia, at either the macro or micro level are below the country's potential. The levels of foreign exchange earnings from livestock and livestock products are also much lower than would be expected, given the size of the livestock population (Berhanu et al., 2007). This is due to the prevailing animal disease, feed shortage both in quality and quantity, low genetic potential and management problems. Of these, infectious diseases are the major constraints for enhancing small ruminant production all around the globe including Ethiopia (Singla, 1995; Getahun, 2008; Gizaw, 2010; Kaur et al., 2013).

Brucellosis is a zoonotic infectious disease affecting a wide range of species of animals and humans with an estimated half a million human cases reported annually. It is caused by different *Brucella* species of the genus *Brucella*. It is facultative intracellular Gram negative bacteria. The disease is one of the most widespread zoonoses and is endemic in many developing countries (Corbel, 2006; Pal et al., 2013).

Brucella melitensis is the most important cause of brucellosis which primarily affects sheep and goats and also very pathogenic for human beings. The disease is also caused by *Brucella ovis* which severely affects sheep. Although the disease has preferred hosts, the bacteria have an ability to cross infect other domestic animals. Hence, sporadic infections in small ruminants could also be caused by *Brucella abortus* or *Brucella suis*, but such cases are rare (Corbel, 2006; OIE, 2015).

Small ruminant brucellosis mainly affects the reproductive tract of animals which is manifested by late term abortions, retention of placenta in the case of female animals, epididymitis and orchitis in males. Additionally, the disease also poses major constraint to international trading of animal and animal products (Benkirane, 2006; Radostits et al., 2007; Seleem et al., 2010).

As the disease often goes undetected, identification of infected herd and animals is of prime importance for control of the disease. Having huge livestock resource at hand coupled with intermingling of livestock species may cause uninfected animals to easily get exposed to the disease from multiple sources such as abortion discharges and direct contact with infected animals. Mixed farming especially raising goats and sheep along with cattle was also reported by many researchers to be a risk factor for *Brucella* transmission between different animal species (Godfroid et al., 2013; Padilla et al., 2010).

In Ethiopia, the existence of small ruminant brucellosis has been reported from different parts of the country. Most of the authors used serological surveys to determine the prevalence and associated risk factors. Such kind of information on the status of small ruminant brucellosis in Tselemti district, Northwestern Zone of Tigray Regional State is absent, although there are cases of abortion and retained placenta among small ruminants according to oral information given by farmers, local authorities, and experts. This problem is probably because of brucellosis. So far, the existence of the disease in small ruminants is little known in the study area and so is the circulating *Brucella* spp. in sheep and goats. As there are major risk factors for the occurrence of the disease, a detailed study on small ruminant brucellosis is necessary to establish disease effective control program.

Therefore, the aims of this study were to estimate the sero-prevalence of brucellosis in small ruminants in Tselemti district and to determine risk factors associated with *Brucella* sero positivity.

MATERIALS AND METHODS

Study area

The study was conducted from November, 2015 till October, 2016 in Tselemti district of Northwestern Zone of Tigray Regional State, Northern part of Ethiopia. The study area is situated at 38°15' E and 13°48' N and 1178 km away from the capital city of Addis Ababa. The study area is among the six districts of the Northwestern zone of Tigray and border with districts of Asgede Tsimbla on the North, Welkayit on the West, Tanqua Abergelle on the East and Amhara region on the South.

In Tselemti districts, six Peasant Associations (PA) were used for the current study. Geographically, the Medinealem which is located at longitude 13°35′21″ N and 38°8′48″ E with an altitude of 1361 m, Wihdet at 13°33′0″ N and 38°5′48″ with 1156 m, Mayteklit at 13°37′42″ N and 38°3′4″E with 1227 m, Mayayni at 13°40′38″N and 38°9′51″ E with 1413 m, Maytsebri at 13°58′04″ N and 38°14′17″E with 1370, Mayambesa at 13°37′29″N and 38°13′2″E with 1405 m and Mayayni located at 13°40′38″N and 38°9′51″E with altitude of 1413 m. The area coverage of the district is approximately 2702.5 km^2 with an altitude ranging from 800 to 2870 m above sea level. The mean annual temperature of the area ranges from 16 to 38°C. The annual rainfall also ranged from 758 to 1100 mm and has a mono-modal pattern.

In addition to this, the livestock production system is predominated by extensive production system. The dominant ruminant species in the study area are cattle and goats and followed by sheep with an estimated number of 268, 647, 264, 429, and 13,276 thousands of cattle, goats, and sheep, respectively (OoARDT, 2014).

Study design and study animals

A cross-sectional study design was employed to estimate the sero-prevalence of brucellosis in small ruminants in Tselemti districts.

The current study was conducted on small ruminants kept under extensive production system. Sheep and goats within the age of 6 months and above and both sexes in the selected flock with no previous history of vaccination against brucellosis were included in the study. Individual animals belonging to the study household flock were selected randomly for blood sampling to examine for brucellosis using serological tests. All data related to potential risk factors were also collected.

Sampling techniques

A combination of purposive and two stage random sampling were used to select district, PA, and individual animals. A two stage cluster sampling was employed to determine the sero-prevalence of brucellosis considering districts as primary clustering units and PA as the secondary clustering units. There are six districts, namely, Medebay Zana, Tahtay Koraro, Asgede Tsimbela, Tselemti, Lalay, and Tahtay Adiyabo in the Northwestern zone of Tigray. From the six districts, Tselemti was selected purposively based on high livestock population and ease of transportation service in order to synchronize the present study with research activities of the Shire-Maitsebri Agricultural Research Center. In Tselemti districts, there were 25 PA, of which 6 were selected purposively for the study based on proximity to the main roads and ease of transportation. Finally, individual households having sheep or goats or both species were selected randomly for blood sampling from the selected PA.

Sample size determination

The sample size for the study was determined according to the formula given by Thrusfield (2005) for random sampling method. A 5% absolute precision and 95% confidence interval was used to determine the sample size. An expected prevalence of 50% was taken to determine the maximum sample size. Accordingly, 384 animals were used during the study period. In order to increase the accuracy, the sample size was increased to 558 animals.

$n= 1.96^2 \times Pexp (1 - Pexp) / d^2$

Where, n=total sample size; d=absolute precision; and Pexp=expected prevalence.

Accordingly,

$n= 1.96^2 \times 0.5 (1 - 0.5) / (0.05)^2$

Sample collection

About 10 ml of blood sample was collected aseptically from the external jugular vein of each animal using plain vacutainer tubes after the animal was restrained properly. All samples were serially identified and labeled properly using permanent marker. The blood sample was allowed to clot in slant position at room temperature and transported using an ice box to the Maitsebri Veterinary Clinic. After 24 h of collection, serum was then separated gently by decanting into 2 ml cryo vials tubes following centrifugation at 3000 rpm for 3 min and stored at -20°C in Maitsebri Veterinary Clinic until tested. The sera were then transported to Mekelle University College of Veterinary Medicine and National Animal Health Diagnostic and Investigation Center (NAHDIC) for serological diagnosis.

Serological diagnosis

The Rose Bengal Plate Test (RBPT) and Complement Fixation Test (CFT) were used as screening and confirmatory tests for brucellosis (OIE, 2009).

Rose Bengal Plate Test (RBPT)

Initially, the entire serum sample was tested using Rose Bengal Plate Test (RBPT) by adding an equal volume of antigen (30 µl) and serum onto glass slides. The antigen and test serum were then mixed thoroughly by plastic applicator, shaken for 4 min and the degree of agglutination was observed visually and recorded immediately as positive for the presence of agglutination and negative for its absence of agglutination (OIE, 2009).

Agglutination was then recorded as 0, +, ++, +++ according to the degree of agglutination where 0 indicates absence of agglutination, + indicates barely visible agglutination, ++ indicates fine agglutination, and +++ indicates coarse clumping. The samples identified with no agglutination (0) were recorded as negative, while those with +, ++, and +++ were regarded and recorded as positive.

Complement fixation test (CFT)

All the sera tested positive to RBPT were confirmed using Complement Fixation Test (CFT). A known antigen was incubated at 37°C with test and control sera to form immune complexes. A defined amount of complement was added to reaction mixtures. An immune complex was then produced in positive antigen and antibody reaction which was suggestive of the complement was fixed or consumed. In negative sera, an immune complex was not produced.

An animal is considered positive if tested for sero-positive on both RBPT and CFT in serial interpretation. The use of RBPT/CFT combinations, the most widely used serial scheme, is generally recommended (Dohoo et al., 2003).

Data collection and statistical analysis

Data obtained on serological test was entered and stored on Microsoft excel sheet. Statistical analysis was performed using STATA version 11.1 statistical software. Chi-square and univariate logistic regression analysis were used to check the association between the outcome and explanatory variables and the degree of association was then expressed as odds ration and 95% confidence interval. Those independent variables that were statistically significant were again subjected to multivariate logistic regression. For all analysis, a cut-off point of $P<0.05$ was used for significance difference. The final model was developed using a step wise reaction. In the final model, all variables with a P value < 0.05 were considered statistically significant and retained in the model.

RESULTS

A total of 558 animals sera comprising 145 sheep and 413 goats were examined for the presence of *Brucella* antibodies. A total of 13 and 10 sera were found positive for RBPT and CFT tests, respectively. Accordingly, the overall sero-prevalence of brucellosis in the study area was 1.79% (10/558). The higher prevalence was recorded in goats (2.18%, 9/413) as compared to sheep (0.69%, 1/145). This observed difference was found to be statistically insignificant (P>0.05) (Table 1).

In the present study, explanatory variables such as PA,

Table 1. Sero-prevalence of small ruminant brucellosis in Tselemti district.

Test variable	Total sera examined	Serological tests	
		RBPT positive N (%)	CFT positive N (%)
Sheep	145	2 (1.38)	1 (0.69)
Goats	413	11 (2.66)	9 (2.18)
Total	558	13 (2.33)	10 (1.79)
P-value	-	0.37	0.24

sex, age, species, pregnancy status, lactation status, parity and herd size had no effect on being sero-positive to *Brucella* (P>0.05). However, animals with previous history of abortion and retained fetal membrane were found to be statistically associated with small ruminant brucellosis (P<0.05) (Table 2).

More importantly, the sero-positivity to brucellosis was higher in small ruminants with previous history of abortion (5.17%) as compared to animals with no previous history of abortion (1.08%). The odds ratio indicates that animals with previous history of abortion were found to be 4.05 (AOR=4.05; 95% CI: 1.01, 16.22) times more likely prone to the infection when compared with animals with no previous history of abortion. The difference observed was found to be statistically significant (P<0.05).

The prevalence of the disease was also found to be higher in those animals with previous history of retained fetal membrane (9.09%) as compared to animals with no previous history of retained fetal membrane (1.73%). Accordingly, the odds of being sero-positive to *Brucella* were found to be 5.68 (COR=5.68; 95% CI: 1.13, 28.53) times higher in sheep and goats with previous history of retained fetal membrane as compared to animal with no history of retained fetal membrane (P<0.05).

DISCUSSION

The present study indicates that the overall prevalence of small ruminant brucellosis was 1.79%. Several authors in Ethiopia have reported different sero-prevalence values of the infection in different parts of the country. Prevalence of 1.76% in Debrezeit and Modjo export abattoirs (Tsegay et al., 2015) and 1.9% in Somali (Teshale et al., 2006) was reported which is comparable to the current finding. However, higher prevalence of the disease was also recorded with 16% in Afar (Teshale et al., 2006), 13.6% in Afar (Adugna et al., 2013), and 3.5% in Southern part of Tigray (Teklue et al., 2013). Conversely, relatively low prevalence of 0.7% was also recorded in Kombolcha (Tewodros and Dawit, 2015). The difference in the prevalence rates in the current study and other studies might be due to differences in management practice and agro ecology.

Statistically, species of the animals had no significant effect on the sero-positivity in the current study. Sheep and goats were found equally susceptible to the infection but the magnitude of the disease was higher in goats (2.18%) as compared to sheep (0.69%). This finding is in agreement with those of Teklue et al. (2013) and Bekele et al. (2011). As opposed to the current study, there are significant differences in species susceptibility to the infection in which goats were found at higher risk of getting the infection than sheep as reported by Teshale et al. (2006), Ashenafi et al. (2007), Adugna et al. (2013), and Tegegn et al. (2016). This observed difference could be due to the fact that cattle and goats are the principal livestock species in the study area, while sheep is domesticated and raised in small pocket areas of the district which might not be the same in other study areas.

Additionally, statistically insignificant difference was also noted between sex of the animals and sero-positivity to *Brucella* in which higher prevalence was recorded in females as compared to males. Similar findings were also reported by Teshale et al. (2006), Ashenafi et al. (2007), Bekele et al. (2011), Debassa et al. (2013), Teklue et al. (2013), and Tsehay et al. (2014). Conversely, sex had an effect on the prevalence of the disease as reported by Tegegn et al. (2016). Lack of difference between the female and male animals on the prevalence of the disease observed in the current study and other study might be due to smaller samples of male animals used. Small ruminants especially male goats are considered as first candidates for marketing and serve as immediate source of income to satisfy house hold demands and purchase of agricultural inputs. Another reason for such variation in the present study and others might be small number of male animals kept as sires for the purpose of breeding in the study area.

Similarly, higher sero-positivity to *Brucella* was detected in adult animals as compared to young animals. The difference observed was found to be statistically insignificant (P>0.05). The present study was consistent with the work of Teklue et al. (2013), Tewodros and Dawit, (2015), and Tsehay et al. (2014). Contrary to the present study, there was significant association between age of the animal and sero-positivity to *Brucella* spp. as reported by Adugna et al. (2013), Ashenafi et al. (2007), and Bekele et al. (2011). The variation observed might be related to small sample size used in the present study. Additionally, there is high market and consumer preference for small ruminants especially when the age

Table 2. Logistic regression analysis of the effect of risk factors on prevalence of small ruminant brucellosis.

Variable	Total examined	Positive [N (%)]	COR (95% CI)	P-value	AOR (95% CI)
Peasant Association					
Maytsebri	98	1 (1.02)	-	0.53	-
Medhinealem	188	4 (2.13)	-	-	-
Wihdet	160	5 (3.13)	-	-	-
Mayteklit	59	0	-	-	-
May Ambesa	17	0	-	-	-
May Ayni	36	0	-	-	-
Sex					
Male	73	0	-	0.21	-
Female	485	10 (2.06)	-	-	-
Age					
Young	73	0	-	-	-
Adult	485	10 (2.06)	-	-	-
Species					
Sheep	145	1 (0.69)	-	0.24	-
Goats	413	9 (2.18)	-	-	
Lactation status					
Lactating	259	4 (1.54)	-	0.39	-
Non lactating	226	6 (2.65)	-	-	-
Pregnancy status					
Non pregnant	329	8 (2.43)	-	0.40	-
Pregnant	156	2 (1.28)	-	-	-
History of abortion					
No	369	4 (1.08)	1	-	1
Yes	116	6 (5.17)	4.97 (1.37, 17.95)	0.01	4.05 (1.01, 16.22)
History of retained fetal membrane					
No	463	8 (1.73)	1	-	1
Yes	22	2 (9.09)	5.68 (1.13, 28.53)	0.03	2.44 (0.42, 14.03)
Herd size					
1-20	111	1 (0.90)	-	0.42	-
>20	447	4 (2.01)	-	-	-
Parity					
No	56	0	-	0.29	-
1-3	269	7 (2.60)	-	-	-
>3	160	3 (1.88)	-	-	-

of the animals reaches 6 to 12 months in the districts.

Higher sero-positivity to brucellosis was found in animals with previous history of abortion (5.17%) than animals with no history of abortion (1.08%). Accordingly, the odds ratio (OR) indicates that sheep and goats with previous history of abortion were found to be 4.05

(AOR=4.05; 95% CI: 1.01, 16.22) times more likely prone to brucellosis as compared to animals with no previous history of abortion (P<0.05). The present finding was in agreement with reports of Teklu et al. (2013) and Tadeg et al. (2015). This is due to the fact that there exist tropism/preference of *Brucella* spp. to the key target cells called trophoblasts. Growth of *Brucella* inside trophoblasts is apparently enhanced synergistically in the presence of high concentration of steroid hormones and erythritol during the final gestation of ruminants. The capacity to replicate rapidly and extensively in trophoblasts can compromise the integrity of the placenta and infection of the fetus, resulting in abortion or birth of weak offspring (OIE, 2012; Xavier et al., 2009).

Likewise, the odds of being sero-positive to *Brucella* were 5.68 times higher in (COR=5.68; 95% CI: 1.13, 28.53) sheep and goats with previous history of retained fetal membrane as compared to goats and sheep with no history of retained fetal membrane. The observed difference was statistically found to be significant (P<0.05). The current study was found to be consistent with the work of Tadeg et al. (2015).

Conclusions

The current study revealed that the prevalence of small ruminant brucellosis is low in the study area as compared to previous works. Risk factor analysis also revealed that factors such as species, age, lactation and pregnancy status, parity, and herd size had no significant effect on the sero-positivity of *Brucella* spp. However, small ruminants with previous history of abortion and retained placenta had significant effect on sero-positivity to *Brucella* spp. More importantly, animals with history of abortion were identified as a risk factor in the final model. The existence of positive animals in the flock can serve as foci of infection to in contact animals and humans and responsible for the spread of the infection. Hence, implementation of better management practices like introducing brucellosis free animals, the use of maternity pen of separation for animals during parturition, use of personal protective equipments, proper disposal of fetal membranes and/or aborted fetus, culling of positive animals, and proper cleaning and disinfection activity. Moreover, extensive extension service including health education must be launched to make animal owners, animal attendants and the consumers aware of the public health significance of the disease.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are very much indebted to many individuals and organizations for their support and encouragement. Their utmost gratitude goes to farmers, Bureau of Agricultural and Rural Development of Tselemti District, Tigray Bureau of Agricultural and Rural Development, Tigray Agricultural Research Institute, Mekelle University and National Animal Health Diagnostic and Investigation Center for their invaluable, persistent and unlimited encouragement and support given.

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Vol. 9(11), pp. 327-333, November 2017
DOI: 10.5897/JVMAH2017.0570
Article Number: 0CA2A8E66315
ISSN 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Growth performance, feed conversion efficiency and blood characteristics of growing pigs fed on different levels of *Moringa oleifera* leaf meal

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Received 27 February, 2017: Accepted 24 May, 2017

To determine the effects of inclusion, at different levels of *Moringa oleifera* leaf meal (MOLM) in growing pig diets on pig's daily feed intakes (DFI), growth performance, feed conversion efficiency (FCE), haematology and plasma lipid indices, a total 24 pigs aged 2.5 months old were selected and assigned to 4 treatment diets (T) containing: 0% (T1), 3% (T2), 6% (T3) and 12% (T4) MOLM concentrations, each with 2 replications of 3 pigs. The DFI and weekly pig weights were monitored for 7 weeks, after which 2 sets of blood samples were drawn from 2 pigs per replication for haematology and serum lipid determination. The DFI for the T4 (3.16 kg) was significantly higher than T1 (2.90 kg), T2 (2.61 kg) and T3 (2.54 kg). Pigs in T2 had significantly higher daily weight gains (0.836 kg) compared to T1 (0.807 kg), T3 (0.810 kg) and T4 (0.810 kg) groups. Furthermore, pigs in T2 and T3 had significantly higher FCE (31.57 and 31.23% respectively) compared to T4 (28.05%) and T1 (30.31%). Inclusion of MOLM in the diet significantly increased haemoglobin concentration only to the level of T3 (14.70 g/dL) after which there was a reduction in T4 (12.70 g/dL). Higher mean corpuscular volume was also observed for T1 (60.0 fL) compared to T3 (52.30 fL). MOLM diet also improved the white blood cell counts; 16.70×10^9/L in T2 compared to 14.50×10^9/L from T1. Total cholesterol in T2 (2.80 mg/mL) were significantly reduced compared to T1 (3.90 mg/mL). This implies, MOLM at lower levels (<6%) in the diet improves haemoglobin concentration, white blood cell counts and exhibits hypocholesterolemic effects, thereby improving growth performance of the animals.

Key words: Growth, haematology, *Moringa oleifera,* total cell count, total cholesterol, pigs.

INTRODUCTION

Pig production is gaining importance in societies that currently are undergoing a shift from ruminant to non-ruminant livestock production in Kenya. However, increasing feed costs, especially the protein sources, have limited the expansion and profitability of the pig enterprise (FAO, 2012). As a result, farmers have adopted a variety of feed ingredients perceived to be cheaper without taking cognizance of their influences on

the animals' body systems (Etim et al., 2014).

MO is a plant in the family Moringaceae introduced in East Africa in the 20th century from India and Pakistan (Foidl et al., 2001). Due to its rich nutritional value, the plant has been used for numerous purposes such as human food, animal feeds as an alternative growth promoter and medicinal purposes (Richter et al., 2003; Sanchez et al., 2006; Nkukwana et al., 2014; Babiker et al., 2017; Caturao et al., 2017). Nutritionally, MO leaves contain between 19.3 and 28.0% crude protein, 2.2% ether extracts, 19.2% crude fibre, 7.1% ash, 42.0% nitrogen free extractives, 0.3% phosphorus and 8.6% calcium (Foidl et al., 2001; Aregheore, 2002; Ferreira et al., 2008; Mustapha and Babura, 2009; Nuhu, 2010; Madukwe et al., 2013; Gakuya et al., 2014).

Haematological values can serve as baseline information for comparing conditions of nutrient efficiency, physiology and health status of farm animals (Ameen et al., 2007; Togun et al., 2007; Isaac et al., 2013; Etim et al., 2014). The phytochemicals from MO seeds, roots and leaves have been shown to have some effects on the haematological and plasma lipid profiles in humans and animals. For instance, El Tazi and Tibin (2014) recorded improved red blood cell indices in broiler chickens fed on MOLM diets. MOLM seed extracts also exerted blood hypocholesterolemic effects in chicken, mice and dogs (Fahey, 2005; Ghebreselassie et al., 2011; Garcia et al., 2015). However, Gakuya et al. (2014) reported that the plant leaves did not have any effects on total cholesterol and total triglycerides in chicken.

Despite the increased use of the plant as a nutritional supplement in humans and animals, there have been varied results on the effects of its inclusion at different concentrations in animal diets on growth performance, haematology and plasma lipids and thus, the need for the study. This study was therefore designed to determine the effects of inclusion of MOLM at varying levels in pig's diet on growth performance, feed conversion efficiency, haematological parameters and plasma lipid profiles in growing pigs.

MATERIALS AND METHODS

This study was conducted at the University of Nairobi, College of Agriculture and Veterinary Sciences, located in Nairobi County. The area receives an average of 869 mm annual rainfall with average daily temperature of 19°C. Growing pig diets were formulated using the NRC (2012) guidelines using maize meal, wheat pollard and vegetable oil as energy sources while MOLM, cotton seed cake, sunflower cake, fish meal and soybean meal served as protein sources. Vitamin mineral premix, Di calcium phosphate and limestone were also included as vitamin and mineral sources (Table 1). The nutritional compositions of the treatment diets are shown in Table 2.

This study was conducted in accordance with the University of Nairobi Faculty of Veterinary Medicine Research Animal Use and Ethics guidelines. Twenty four (24) large white growing pigs (2.5 months old) were selected and assigned to four treatment diets (T): 0% (T1) as the control, 3% (T2), 6% (T3) and 12% (T4) MOLM, each with 2 replicates of 3 pigs in a concrete floor housing system using the design of Reese et al. (2010).

Feeds were weighed each morning and fed in 3 portions to minimize wastage. At the end of the day, feed left in the troughs were weighed and subtracted from the total weight of feed provided for the day to get the daily feed intake. Average daily feed intake for each of the treatment groups was calculated for the entire experimental period. Water was provided *ad libitum*.

At the start of the experiment, each pig was weighed followed by weekly weighing for a total of 7 weeks. At the end of the experiment, pigs were starved for 12 h, with provision of drinking water only. Four pigs from each treatment were randomly selected and 2 sets of blood (5 ml each) drawn from jugular vein using 9 ml vacutainers; one treated with anticoagulant (EDTA) and the other with serum clot activator. Red blood cells, total white blood cell counts, granulocyte, lymphocyte and mid-range absolute count (MID) and differential counts were determined in the laboratory using an automated haematology analyser. Serum lipid profiling was undertaken after centrifuging blood for 15 min at 3,000 rpm (Li and Kim, 2014). Serum triglycerides, total cholesterol, HDL and LDL were analysed using the serum lipid profiling Kits.

Data on pigs' voluntary daily feed intake, weekly weights, haematological and lipid profiles were entered into Ms excel and exported to Statistical Analysis Software version 9 (SAS Inc, 2002) for descriptive statistics and analysis of variance (ANOVA). Tukey's test was used to determine whether there were significant differences between the means of the treatment groups.

RESULTS

MOLM used in formulating pig diets had 27.37% crude protein (CP), 8.90% crude fibre (CF), 46.01% nitrogen free extractives (NFE), 5.73% ether extract (EE) and 11.91% ash on dry matter basis.

In the feed trials, pigs from T4 registered a higher ($P < 0.05$) daily voluntary feed intakes than those in T1, T2 and T3 groups. T1 group on the other hand had higher ($P < 0.05$) daily feed intakes compared to T2 and T3 groups. The T2 group had a higher ($P < 0.05$) average daily weight gains compared to T1, T3 and T4.

Furthermore, feed conversion efficiency (FCE) was higher in T2 and T3 compared to T1 and T4 (Table 3). All the blood parameters measured in this study (haematological and serum lipid profiles) were within the normal range for T1, T2, T3 and T4.

However, there were variations in haematological parameters and serum lipid profiles with variations of MOLM levels in the diet. From the study, the red blood cell (RBC) and haemoglobin concentration (Hb) was

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Table 1. Percentage (%) Compositions of the growing pig diets.

Ingredients	Dietary treatments (#MOLM inclusion levels, %)			
	T1 (0%)	T2 (3%)	T3 (6%)	T4 (12%)
Maize meal CP%	40.0	40.0	40.0	40.0
Moringa oleifera (27% CP)	0.0	3.0	6.0	12.0
Wheat pollard	30.0	26.0	23.0	16.8
Vegetable oil	1.0	2.5	3.3	3.5
Cotton seed cake (43% CP)	3.9	3.5	3.1	2.8
Sunflower meal (32% CP)	1.4	1.3	1.2	1.3
Fishmeal (50% CP)	10.0	10.0	10.0	10.0
Soybean meal (46% CP)	10.0	10.0	10.0	10.0
Di calcium phosphate	0.0	0.0	0.0	3.3
Limestone	3.4	3.4	3.1	0.0
Vitamin mineral premix	0.3	0.3	0.3	0.3
Total (%)	**100.0**	**100.0**	**100.0**	**100.0**

#MOLM = *Moringa oleifera.*

Table 2. Calculated nutritional compositions of the pig diets.

Nutrient	Dietary treatments (#MOLM inclusion levels, %)			
	T1 (0%)	T2 (3%)	T3 (6%)	T4 (12%)
Amino acid tabulations				
Lysine (%)	0.9	1	1.1	1.3
Threonine (%)	0.6	0.7	0.8	1
Methionine (%)	0.3	0.4	0.4	0.4
Methionine+Cysteine (%)	0.6	0.5	0.5	0.5
Tryptophan (%)	0.2	0.3	0.3	0.4
Isoleucine (%)	0.7	0.8	0.8	1.2
Leucine (%)	1.3	1.5	1.7	1.1
Valine (%)	0.8	0.9	1	1.3
Proximate tabulations				
Crude fibre (%)	5.2	5.2	4.2	5.6
Ash (%)	5.5	5.7	6.6	5.9
Ether extracts (%)	5.7	5.7	4.8	5.8
Digestible energy Mcal/kg	3.4	3.5	3.2	3.3

higher (P <0.05) in T3 group compared to T1, T2 and T4 (Table 4). Mean cell volume (MCV) was lower (P <0.05) in T3 compared to T1, T2 and T4; mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) on the other hand did not vary (P >0.05) with the diet (Table 4).

The T2 group had a higher (P = 0.05) concentration of white blood cells compared to T1, T4 and T3 treatment groups. There was also an increase in lymphocytic concentration with increase in MOLM in diet Table 5. Granulocyte cell concentration was, on the other hand, lower (P <0.05) in the MOLM treatment groups compared to the control (T1) groups. Differential cell counts showed that granulocyte proportions declined with increased MOLM in diet, but started to rise again with increased MOLM in T4 (12% MOLM). The mid-range absolute counts (MID) cell proportions further increased (P <0.05) with increase in MOLM in the diet and similarly for the lymphocytes (LMP).

Total cholesterol reduced (P <0.05) with the increase in MOLM in the diet but increased again at the higher levels of dietary MOLM (T3 and T4). T1 group also had the highest level of LDL (2.89 mg/ml) compared to T2 (2.27 mg/ml), T3 (2.26 mg/ml) and T4 (2.5 mg/ml), though not

Table 3. Mean voluntary daily feed intake, weight gains and feed conversion efficiency (FCE) of pigs fed on MOLM diets (± standard error of the mean).

Variable	Dietary treatments (#MOLM inclusion levels, %)			
	T1 (0%)	T2 (3%)	T3 (6%)	T4 (12%)
Starting weight (kg)	26.35 ± 0.10[a]	25.95 ± 0.11 [a]	26.10 ± 0.11[a]	26.31 ± 0.10[a]
Final weight (kg)	66.46 ± 0.54[a]	65.24 ± 0.55[a]	66.55 ± 0.57[a]	65.16 ± 0.53[a]
Daily feed intake (kg/day)	2.90 ± 0.09[a]	2.61 ± 0.09[b]	2.54 ± 0.09[b]	3.153 ± 0.09[c]
Daily gains (kg/day)	0.807 ± 0.04[a]	0.836 ± 0.05[b]	0.810 ± 0.05[a]	0.810 ± 0.05[a]
Feed conversion efficiency (%)	28.05 ± 0.49[c]	31.57 ± 0.48[a]	31.23 ± 0.48[a]	30.31 ± 0.48[b]

T = treatment, #MOLM = *M. oleifera* leaf meal. The treatment means denoted by the same superscripts ([a, b and c]) in the same row did not have significant differences at P <0.05.

Table 4. Mean red blood cell parameters from pigs fed on diets with different levels of MOLM (± standard error of the mean).

Red blood cell parameters	Dietary treatments (#MOLM inclusion levels, %)				
	T1 (0%) (n=4)	T2 (3%) (n=4)	T3 (6%) (n=4)	T4 (12%) (n=4)	Normal references
RBC (x10^6/mm^3)	7.50[a] ± 0.20	8.00 [a] ± 0.42	8.70 [b] ± 0.25	7.10 [a] ± 0.09	5.00-8.00
Hb (g/dL)	13.50 [a] ± 0.20	13.70[a] ± 0.25	14.70[b]± 0.23	12.30[c] ± 0.26	10.00-16.00
HCT (%)	45.00 [a] ± 0.40	44.30[a] ± 0.89	45.00[a] ± 0.42	43.40[a] ± 0.70	32.00-50.00
MCV (fL)	60.00 [a] ± 0.01	56.70[a] ± 0.62	52.30[b] ± 0.25	58.00 [a] ± 1.14	50.00-68.00
MCH (pg)	18.00 [a] ± 0.01	17.30 [a] ± 0.47	17.30[a] ± 0.25	17.00 [a] ± 0.42	17.00-21.00
MCHC(g/dL)	30.50 [a] ± 0.20	31.00 [a] ± 0.42	32.30 [a] ± 0.25	30.30 [a] ± 1.73	30.00-34.00

#MOLM = *M. oleifera* leaf meal, T = treatment, RBC = Red blood cell counts, Hb = Haemoglobin concentration, HCT = Haematocrit concentration; MCV = Mean cell volume, MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration. The treatment means denoted by the same superscripts ([a, b and c]) in the same row did not have significant differences at P <0.05.

Table 5. Mean white blood cell parameters of growing pigs fed on the different levels of MOLM in diet (± standard error of the mean).

White blood cell parameters	Dietary treatments (#MOLM inclusion levels, %)				
	T1 (0%) (n=4)	T2 (3%) (n=4)	T3 (6%) (n=4)	T4 (12%) (n=4)	Normal reference
WBC (10^9/L)	14.50 ± 0.20[a]	16.62 ±0.23[b]	15.63 ± 0.23[a]	14.87 ± 0.42[a]	11.00-22.00
LYMPC(10^9/L)	7.50 ± 0.20[a]	8.70 ± 0.23[b]	9.30 ± 0.25[b]	8.70 ± 0.23[b]	3.80-16.50
MIDC(10^9/L)	1.00 ± 0.17[a]	1.00 ± 0.17[a]	1.00 ± 0.17[a]	1.30 ± 0.25[a]	0.10-5.00
GRANC(10^9/L)	6.50 ± 0.20[b]	7.00 ± 0.23[b]	5.00 ± 0.23[a]	5.30 ± 0.25[a]	5.00-13.90
LYMP (%)	50.00 ± 1.42[a]	52.09 ± 1.7 [a]	60.70 ± 2.1[b]	56.80 ± 1.30[c]	39.00-62.00
GRA (%)	43.50 ± 1.40[a]	41.90 ± 0.25[a]	32.60 ± 0.42[b]	34.64 ± 0.65[b]	28.00-50.00
MID (%)	6.50 ± 0.54[a]	6.00 ± 0.65[a]	6.53 ± 0.23[a]	8.40 ± 0.33[b]	4.5.00-13.00

#MOLM = *M. oleifera* leaf meal, T = treatment, WBC = White blood cell counts, LYMPC = Lymphocyte cell counts, MIDC = Mid-range absolute counts, GRANC = Granulocyte cell counts, LYMP = Lymphocyte proportions, GRA = Granulocytic proportions, MID = Mid-range absolute count proportions. The treatment means denoted by the same superscripts ([a, b and c]) in the same row did not have significant differences at P <0.05

statistically significant. TGS and HDL however did not vary significantly with the diet(Figure 1).

DISCUSSION

The crude protein levels of MOLM in this study were close to 27.51% reported by Oduro et al. (2008), and 29.55% recorded by Nuhu (2010) in Ghana but higher than 23.30% reported by Gakuya et al. (2014), in Kenya. This could be attributed to differences in ecological zones and the physiological stage of harvesting where younger fresh materials could have had higher protein levels, NFE and lower crude fibre contents (Samkol et al., 2005; Gakuya et al., 2014).

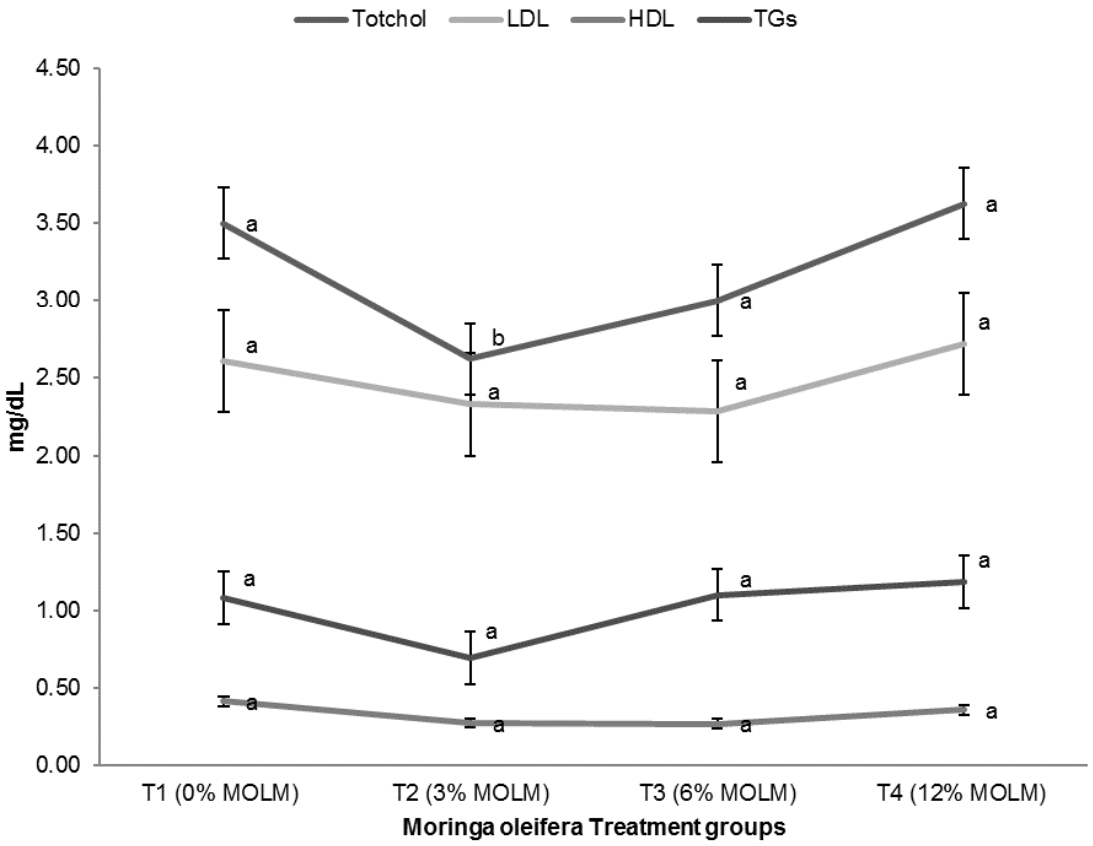

Figure 1. Effects of *M. oleifera* leaf meal diets on the mean serum lipid levels of growing pigs fed on MOLM leaf meal diets (n=16). Key: Totchol = Total cholesterol, LDL = Low density lipoproteins, TGs = Total triglycerides, HDL= High density lipoproteins, MOLM = *M. oleifera* leaf meal. The treatment means denoted by the same superscripts ($^{a, b \text{ and } c}$) in the same series did not have significant differences at P <0.05

The MOLM diets in all treatment groups were well tolerated by the pigs and no mortalities were recorded. MO has shown a high safety margins both in human and animal research (Stohs and Hartman, 2015). Tolerance of MOLM has also been reported by Gakuya et al. (2014) in chicken, Nuhu (2010) in rabbits and Adedapo et al. (2009) in rats. The higher pig feed intake recorded in T1 and T4 groups may be attributed to limited nutrient availability in the diets as well as higher fibre contents which might have increased the rate of passage in the gut (Afuang et al., 2003). The highest average daily weight gain recorded in T2 was close to that of Mukumbo et al. (2014) who recorded the highest pig weight gain at 5% MOLM, attributed to high protein content from MOLM and higher digestibility. Nkukwana et al. (2014) reported the highest weight gains among broiler chicken fed on MO based diets attributing this to enhanced nutrient utilization. However the average daily gain weight in this study was different from that of Acda et al. (2010) who reported that MOLM up to 10% could substitute commercial pig pre-starter diets and Caturao et al. (2017) Who reported enhanced growth of Oreochromis niloticus by inclusion of 10% dried MO in the diet.

Diet has been found to influence haematological parameters (Etim et al., 2014). MOLM improved the red blood cell counts and haemoglobin concentration in blood to a level of 6% after which the levels declined significantly. These results were similar to those of El Tazi and Tibin (2014) who recorded higher levels of Hb in broiler chickens fed on MOLM diets. This has been attributed to higher levels of protein and minerals, mostly iron, which are responsible for the formation of haemoglobin in the MO plant (Madukwe et al., 2013; El Tazi and Tibin, 2014). The higher the haemoglobin concentration the better the oxygen circulation in the body, hence, better performance of the animal (Olugbemi et al., 2010). At higher levels (greater than 6% MOLM) however, haemoglobin concentration declined and could possibly be due to the potential toxicities by high levels of flavanoids and tannins in the plant leaves (El Tazi and Tibin, 2014). Increased MOLM (12% MOLM) led to increased MCV implying that there might have been

increased release of immature RBC or increased iron or folic acid levels that enhanced red blood cell formation (Fahey, 2005). This therefore implies that MOLM should be used in moderation since high levels may lead to toxicity and reduced efficiency in oxygen transportation in the body, hence, reduced performance. Higher levels of MCV could further imply existence of chronic liver diseases hence inefficiency of liver detoxification. This also could be as a result of increased levels of flavonoids which might have led to impairment of liver function at the highest MOLM in the diet (Fahey, 2005).

The T2 group had the highest white blood cell counts followed by T3 and T4 while T1 had the least counts. These results were similar to those of Gupta et al. (2012) which implied that higher vitamin and protein concentrations in MOLM may have led to improved immune system in animals; indicated by higher body defence cell levels. This is important since the treatment groups would be able to fight diseases compared to controls, hence, minimizing drug usage and thereby reducing the cost of production and subsequently, increasing the safety of pork (Pascoal et al., 2012). MID cells increased with increased MOLM in the diet, implying that the white blood cell precursors had increased therefore enabling the animal to readily counter any infections that may arise. These findings support those of Gaikwad et al. (2011) and Stohs and Hartman (2015) who documented that MO stimulate both cellular and humoral immune systems. This Immunomodulatory potential of *M. oleifera* leaves could be attributed to the presence of flavonoids, polyphenols and terpenoids which may modulate immune-mechanisms. Granulocytes in most instances are responsible for the immune defense against bacterial infections. In this case, MOLM antimicrobial properties may have led to suppression of the pathogenic microbes hence resulting decline in granulocyte levels.

Fahey (2005) and Ghebreselassie et al. (2011) reported that, MOLM exerts hopocholesterolemic effects when taken in the diet. This has also been supported by this study. Increased MOLM reduced cholesterol levels significantly, perhaps by lowering the serum concentrations of LDL by β-sitosterol; the bioactive phytoconstituents isolated from *M. oleifera* (Ghasi et al., 2000). However, pigs on the highest concentration of MOLM showed increased cholesterol levels therefore necessitating further studies to establish reasons for the increased cholesterol levels.

CONCLUSION AND RECOMMENDATIONS

This study therefore concludes that low levels of MOLM in the pig's diet could enhance haemoglobin and WBC formation which, could increase efficiency in oxygen circulation in the body and boost animal's immunity and enhance better performance. However, higher levels beyond 6% could interfere with the normal haematological parameters and subsequently affect negatively the pig's performance. MOLM also at lower levels in diet has hypocholesterolemic effect which could reduce predisposition to cardiovascular diseases associated with higher levels of LDL and total cholesterol. Further studies however, ought to focus on the actual immune response in relation to specific infectious agents in pigs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors acknowledge Carnegie Foundation through the Rise AFNETT for funding the project, The University of Nairobi for provision of the research facilities.

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