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The Ethiopian Veterinary Journal (*Ethiop. Vet. J.*) is a multidisciplinary peer-reviewed journal intended to promote animal health and production of national and regional/international importance. The journal publishes review articles, original research articles, short communication as well as technical notes in English. Under special circumstances, articles in Amharic may be considered for publication.

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Morphometrical study of the reproductive tract in Boran (*Bos indicus*) heifers in Ethiopia

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Abstract

Boran breed is the most suitable type of cattle breed for arid and semi-arid regions of Ethiopia due to their adaptive characteristics. Understanding their reproductive anatomy is important for successful reproductive management and has many implications for the application of assisted reproductive technology, and interpretation of physiological changes at different stages of their reproductive cycle. In this study, data of the anatomical structures of the reproductive organs of 20 Boran heifers were measured and characterized at different stages of reproduction. The mean (±SE) length (L) of the vagina, cervix, and body of uterus were 13.17±2.98 cm, 7.19±1.28 cm, and 6.70±1.08cm, respectively. The mean (±SE) length of the right uterine horn and oviduct was 18.47 ±2.54 and 18.17 ±1.27cm, respectively and that of the left were 18.05±2.73 and 17.45±1.80 cm, respectively. The mean ±SE weight (W) of right and left ovaries were 2.44±1.51 gm and 2.44±1.51 gm, respectively. The mean ±SE Length x Width x Thickness of the right ovary was 2.10±0.59 x 1.75±0.35 x 0.83±0.36 and that of the left was 1.97±0.41 x 1.58±0.29 x 0.67±0.33 cm. The mean (±SE) weight of the whole reproductive organ (without the vulva) was 301.35±66.34gm. The ovarian size was influenced by the presence of the corpus luteum(CL) usually larger with its presence.

Keywords: *Boran**;** Cervix; Fallopian tube; Heifer; Morphometry; Oary; Uterus.*

Introduction

Boran***"Indigenous African cattle with potential"*** are developed in Eastern Africa, more specifically the Borana plateau in Southern Ethiopia. Boran**,** are the most suitable type of breed for arid and semi-arid regions due to their

adaptive characteristics: tolerance to heat, ticks' infestation, feed, and water shortage (Solomon, 2001). The Boran cow has an excellent mothering ability. As with all zebus, the Boran has good heat tolerance. The sweat glands are more numerous and are larger than those of *Bos taurus* and the skin surface is increased by the presence of extra folds (Hailemariam *et al.,* 1998).

Animals with Boran genes have a relatively low maintenance requirement. Boran sired steers had a higher level of lipoprotein lipase activity in the subcutaneous fat depot than animals sired by other breeds (Sprinkle *et al.*, 1998). This is an indication that animals with Boran genes have low maintenance requirements, which enables them to survive the periodic feed shortages that frequently occur during drought in their homelands. The most outstanding characteristic of Boran cattle is that, while having low maintenance requirements, they are also able to produce efficiently when feeding conditions improve. Evidence from field data from the Borana Plateau has also demonstrated that young Boran animals can make dramatic recoveries after drought years when pasture conditions improve (Coppock, 1994).

Reproduction is a vital factor in determining the efficiency of animal production. At best, a cow is only likely to produce a single calf per year. The primary reproductive organ in female animals is the ovary, which performs dual roles of oogenesis and steroidogenesis by producing ova and ovarian steroids respectively (Arthur *et al.,* 1989). Other reproductive organs include the oviduct, uterus, and cervix with very important roles in female reproductive capacity and functioning. The semen deposited into the vagina or cervix during ejaculation must navigate the cervical folds and travel upward through the uterine body and the horn to get to the ampulla (Hafeez and Hafeez, 2000). The ovum shed during ovulation is collected by the fimbriae part of the infundibulum and conveyed in opposite directions to meet the spermatozoa usually in the isthmic-ampulla region of the oviduct where fertilization occurs and the formed zygote is transported back to the uterus. The main role of the uterus is to support an embryo during gestation (Ali *et al.,* 2006). The functions of all the aforementioned reproductive parts do overlap and sometimes, require stimulation/inhibition by some products of the other segments. Under normal anatomical and physiological conditions, all these parts work in collaboration and synergistically to achieve maximum reproductive capacity in female animals.
Morphometry deals with the shape, weight, and dimension (length, width, and thickness) variation of an organ. The knowledge of reproductive organs' morphometry of female animals (Boran heifers) is crucial and may determine the

extent to which these organs can perform their physiological roles (Memon, 1996). For instance, the number of cervical rings is related to the length of the cervix and determines the 'hurdles' the sperm has to cross before getting to the uterus. Animals in the luteal phase have slightly or completely curved or C-shaped. From our previous experiment on multiple ovulation and embryo transfer experience, we face difficulty to pass a flushing catheter through the cervix on the day of embryo collection even though the donor has a good response to the technologies. Therefore, for efficient assisted reproductive technology like artificial insemination (AI), embryo flushing, and embryo transfer (ET) information on the morphometry of the reproductive organs is important. The knowledge of such morphometry is also useful for pregnancy diagnosis and investigating cases of infertility in farm animals (Khaton *et al.,* 2015b). There are many reports about the morphometric values of the reproductive tracts of female animals in different countries of the world. However, presently there is a lack of information concerning the morphometry of the female reproductive organs in Boran breeds of cattle in Ethiopia. Therefore, the present study was undertaken to determine the morphometry of the internal reproductive system of Boran heifers in Ethiopia.

Materials and methods

Sources of the experimental animals

The study was carried out on 20 Boran heifers de-stocked from the Ethiopian Institute of Agricultural Research (EIAR), in Debre Zeit Agricultural Research Center (DZARC) Bishoftu. Age was estimated based on dentition as described earlier by Lawrence *et al.* (2001). Accordingly, the study animals were 3 - 4 years of age. The body condition score of the animal ranged from 2.5 - 3.5 on a 1-5 scale (1 poor, 5 fat) and was determined as described by Natumyana *et al*. (2008) Heifers were housed and provided with a feed of different mixes: *tef* (*Eragrostis tef)* straw and grass (*Andropogon abyssinicus*) hay as a basal diet and supplemented with commercially prepared concentrate, mineral salts, and alfalfa green fodder. Water was provided *ad-libitum*. Animals were regularly dewormed against a common parasitic disease and vaccinated for lumpy skin disease (LSD), foot and mouth disease (FMD), and other common infections.

Postmortem collection of reproductive organs

Reproductive organ samples were obtained from heifers de-stocked and slaughtered due to overstocking of the animal resulting in feed and barn shortage at the DZARC farm. Sampled organs were placed in a plastic bag and transported to the laboratory. Then as described by Bhajoni *et al.* (2018), the ovaries were allocated into two groups: ovary with corpus luteum (CL) and ovary without corpus luteum (CL). All findings were recorded on a predesigned data register.

Measurement of organs

All measurements length in cm were made using a ruler and Vernier's caliper (Mituoyo Stainless Steel) while weight [g] was measured using a sensitive electric balance (XY600BJ-1301146 Citizen, Delhi)). The whole reproductive organ was washed with lukewarm water, extra connective tissues were removed, and the weight of the entire tract (Figure 1) was measured and later on made flat on a table before separating individual organs. Each organ was individually measured: Ovaries (length, width, thickness, and weight), oviducts (length), uterine horns (length and diameter) body of the uterus (length and diameter), cervix (length, diameter, and ring), and vagina (length).

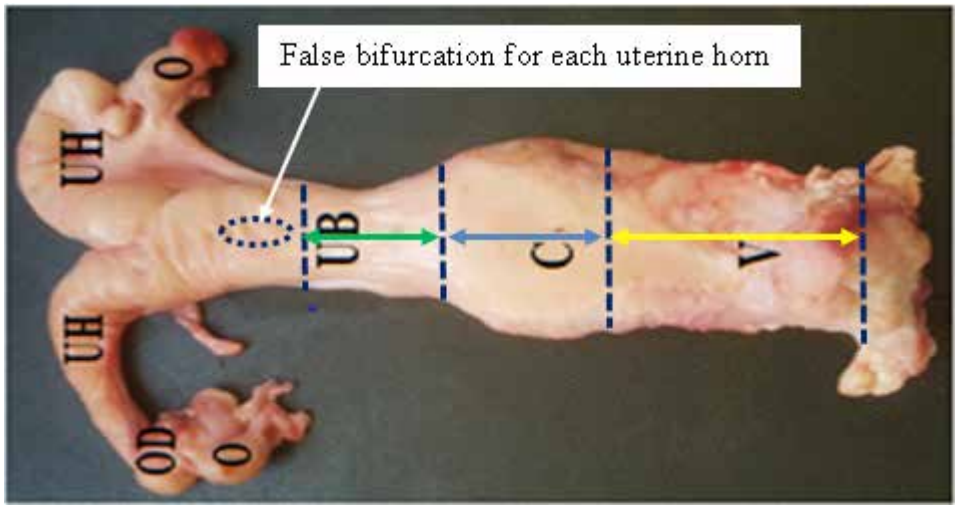


Figure 1: The reproductive system of Boran heifer: (V) =Vagina, (C) =Cervix, (UB) =Uterine body, (UH) = uterine horn, (OD) =Oviduct, (O) =Ovary

The length of the oviduct was taken from the top of the fimbria to the uterotubal junction. The length of the uterine horn was the length from the middle

of the bifurcation of the uterus to the utero-tubal junction (Figure 2). Diameters of the horn, body, and neck of the uterus were measured across. The length of the uterine body was measured from its bifurcation to the internal os of the cervix. The length of the cervix was the distance from the tip of the *internal os* to the tip of the *external os*. The length of the vagina was taken as the length from the external os of the cervix to the vestibule. Cervical rings/folds were considered as the number of rings/folds from *os-externum* to *os-internum* (Figure 4).

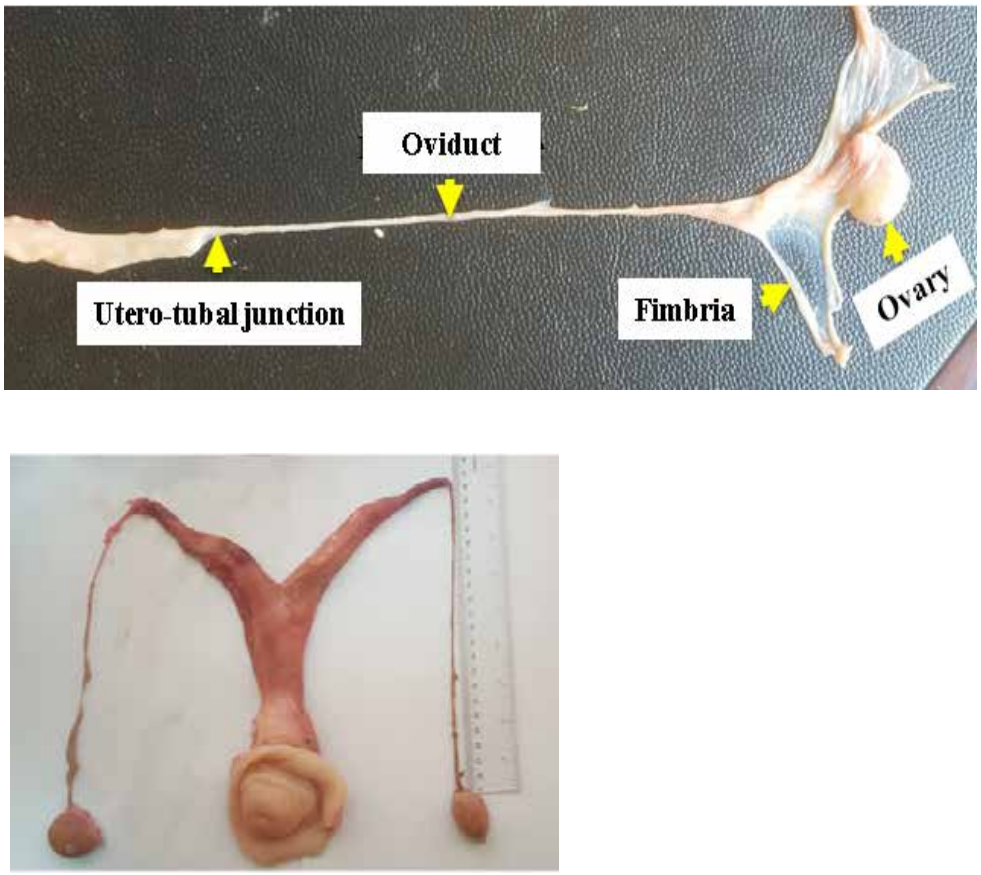


Figure 2: Ovary and oviduct (top), uterine horn and body, and the cervix (bottom) of Boran heifer showing measurement reference points

The ovaries, right and left were removed at their junction with the ovarian ligament as close to the ovarian tissue as possible after the weight of the whole reproductive organ was taken. Excessive tissues attached to the ovaries were carefully trimmed off and ovaries were weighed separately. The CL was identified when present and its color was classified (reddish=corpus hemorrhagicum (CH), yellowish or orange =mature (CL), and white= corpus albicans(CA); figure 3: The length of the ovary was measured from the anterior pole to the posterior pole along the length. The width of the ovary was measured from the medial to the lateral borders of the ovary. The thickness was measured at the thickest part of the ovary (Kouamo *et al.*,2017).

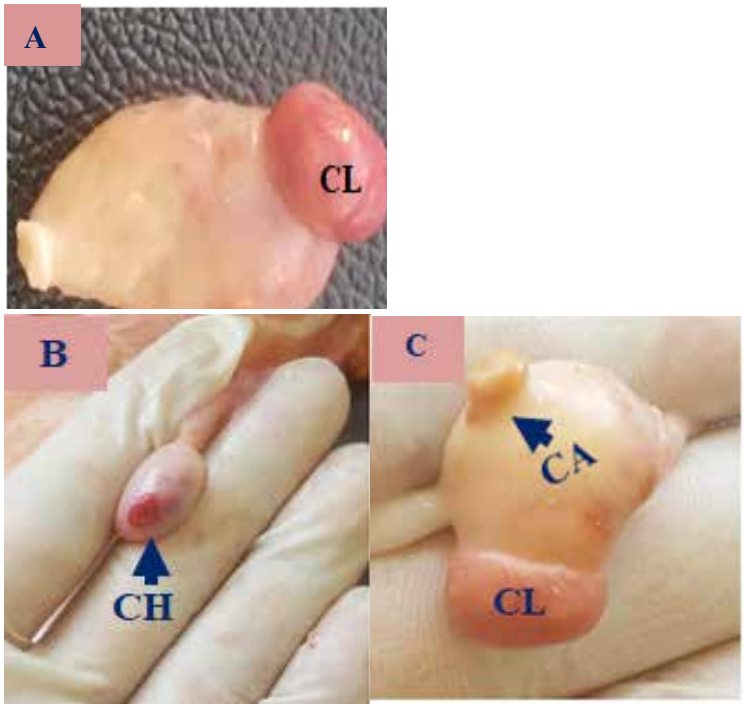


Figure 3: A) Corpus luteum (CL), B) Corpus hemorrhagicum (CH) and C) Corpus albican (CA) and D) Number of cervical rings (CR)

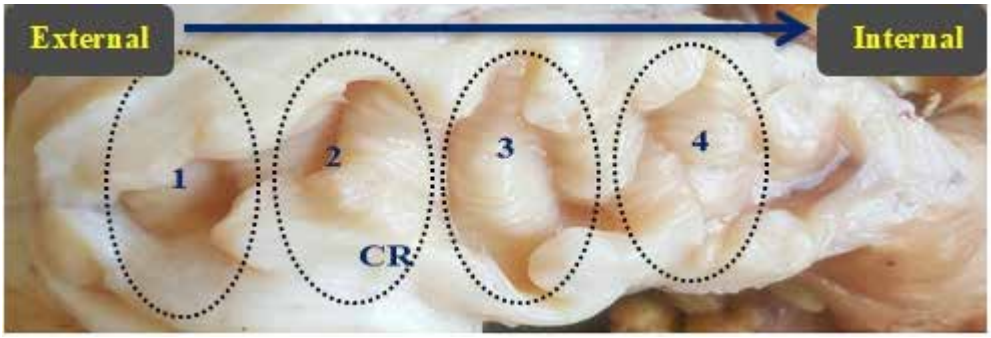


Figure 4: Number of cervical rings (CR), from *os-externum* **(end of vagina) to** *os-internum* **(beginning of uterus).**

Statistical analysis: In a parametric model, the data were presented as mean ± standard error. All analyses were performed using SPSS (Statistical Package for Social Sciences) (SPSS 20).

Results

In this study, ovarian morphometry of Boran heifers was evaluated in terms of length, width, thickness, and weight. *Generated data showed that the mean length, width, thickness, and weight right ovaries of(*2.10±0.59 cm*,* 1.75±0.35 *cm,* 0.83±0.36 *cm, and* 2.44±1.51 gm*) were not significantly* (p<0.05) *differed compared to that of left ovaries (*1.97±0.41 cm*,* 1.58±0.29 *cm,* 0.67±0.33 *cm, and* 2.06±0.93 gm, *respectively).* The mean (±SE) weight of the whole reproductive organ (without the vulva) was 301.35±66.34gm.

On the other hand, findings in ovarian morphometry revealed that the mean weight of ovaries with CL 2.95±1.60 was significantly (*P*<0.05) higher than the mean weight of ovaries without CL 1.67±0.83.as shown in Table 2.

Table 1: The mean length, width, thickness, and weight of ovaries in Boran heifers (Mean ± SE)

Organ	Measurements	Mean (±SE)
Right ovary	Length (cm)	2.10±0.59
	Width (cm)	1.75±0.35
	Thickness (cm)	0.83±0.36
	Weight (gm)	2.44±1.51
Left ovary	Length (cm)	1.97±0.41
	Width (cm)	1.58±0.29
	Thickness (cm)	0.67±0.33
	Weight (gm)	2.06±0.93

Table 2: Comparative dimensions of CL or non-CL bearing ovary (Mean± SEM).

Measurement [cm]	CL bearing ovary (15)	Non CL bearing ovary (25)
Width (cm)	1.87±0.35	1.52±0.29
Length (cm)	2.25±0.63	1.86±0.37
Thickness (cm)	0.95±0.36	0.65±0.30
Weight (gm)	2.95±1.60	1.67±0.83

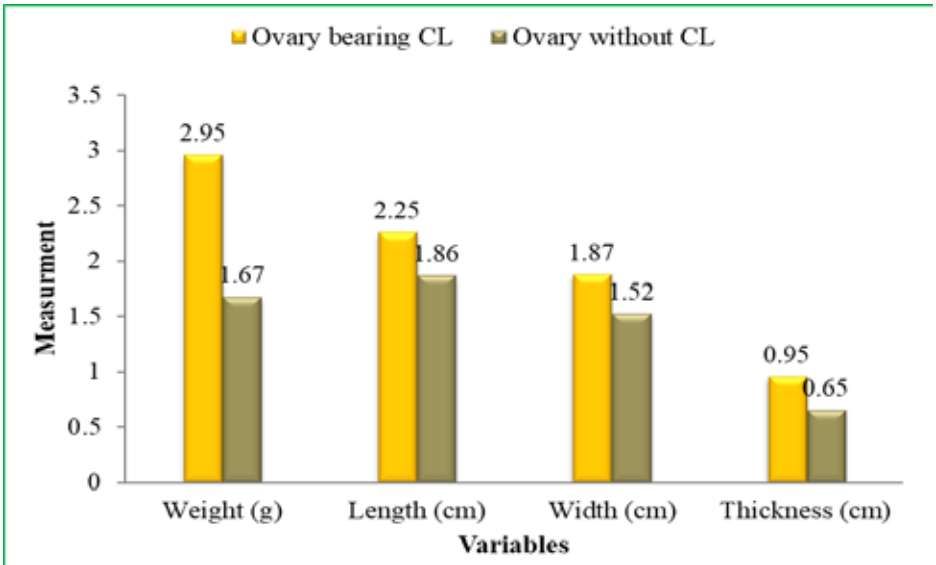


Figure 5: Morphometry of Boranovary with and without CL

Table 3: Comparative dimension of uterine morphometry

Measurement [cm]	Follicular phase [mean (±SE)]	Luteal phase [mean (±SE)]
Uterine body length	7.0±0.72	7.67±0.91
Uterine body diameter	3.56±0.93	3.7±0.21
Uterine horn length	17.5±0.57	18.5±0.44

Table 4: The mean length and diameter of the tubular part of the reproductive tract of Boran heifers

Organ measurements [cm]	Mean (±SE)
Length of right oviduct [cm]	18.17 ±1.27
Length of the left oviduct[cm]	17.45±1.80
Length of the right uterine horn [cm]	18.47 ±2.54
Length of the left uterine horn[cm]	18.05±2.73
Length of the body of uterus [cm]	7.86±1.08
Diameter of the body of uterus [cm]	3.65±0.51
Length of the cervix [cm]	7.19±1.28
Diameter of the cervix [cm]	3.40±0.50
Number of the cervical ring[number]	3.65±0.87
Length of vagina [cm]	13.17±2.98
Weight of whole organ [gm]	301.35±66.34

Discussion

All reproductive organs, the ovary, oviduct, uterus, and cervix play synergistic functioning in the reproductive system of females (Siddiqui *et al.,* 2005). The knowledge of morphometric parameters of these organs may determine the extent to which they can fulfill and perform their physiological roles towards the attainment of optimal reproductive capacity in female mammals.

Results obtained in the present study indicated that the average length (cm) of oviducts, uterine horns, and cervix commissure of Boran heifers were lower than those reported in another tropical zebu breed however higher in the body of uterus dimension. Accordingly, Moussa Garba *et al.* (2013) reported an average length (cm) of 19.1, 21.6, 8.2, and 18.9 for oviducts, uterine horns, cervix, and vagina commissure, respectively in Bos indicus breed of cattle. On the other hand, Riasat *et al.* (2006) reported an average length (cm) for the uterine horns and the cervix of 20.23 ±0.23 and 6.0 ± 0.22, respectively in Zebu cow. Further-

more, in dairy cows, Khaton *et al.* (2015 a) reported an average length (cm) for the oviducts, uterine horns, the body of the uterus, and vagina of 21.03, 25.56, 3.12, and 22.59, respectively for organs of dairy cows in Bangladesh In other findings reported by Carvalho *et al.* (2005) in the European breeds; an average length (cm) of 20.5-23.1, 35.3-39, and 4.1 for the oviducts, uterine horns, and uterine body, respectively. However, Kouamo *et al.* (2017), Khaton *et al.* (2015 a), and Moussa Garba *et al.*(2013) reported a lower vagina diameter compared to our finding. This difference could be possibly due to breed (genotype), age, environment, and management factors. Genotype and environmental factors play a crucial role in the productive and reproductive performance of cows/heifers (Kouamo *et al.,* 2017). The adaptation of the reproductive function in the animal's environment may justify the difference between local and European breeds (Kouamo *et al.,* 2017). Indeed, the physical development and maturity of the reproductive tract depend on the age of the animal.

In these findings, the weight and the size (length, width, and thickness) of ovaries obtained were lower than those obtained by Khaton *et al.* (2015, b) reported from the local breed of Bangladesh, and their cross with Holstein Friesian, and Jersey breed.

Our findings obtained in the present study indicated that the mean weight of right and left ovaries as well as mean length of right and left ovaries were lower compared to Raheem *et al.* (2016) report in Red Bororo cow and White Fulani cows. However, Kunbhar et al. (2003) reported mean width (cm) of the right and left ovaries in the Thari cow were 1.33 ± 0.08 cm and 1.30 ± 0.09 cm receptively which is lower compared to our study. On the other hand, results obtained in the present study indicated that morphometric measurements such as weight, length, width, and thickness were greater in the ovary bearing CL as compared to the non-CL bearing ovary, which is an incomplete argument with Bhajoniet al. (2018) findings.

In this study in most animals' examination of the cervix for an animal in the luteal phase (a functional CL in either ovary), indicate that their cervix is slightly or completely curved (C-shaped) which is difficult to path catheters through the cervix for embryo flushing or embryo transfer. This might implicate due consideration of developing certain procedures and materials that enable to pass through the cervix during the luteal phase or follow alternative techniques for further adoption and implementing the technologies.

Conclusions

From this study, it was concluded that the right ovary was wider in diameter, larger in length, and heavier in weight as compared to the left one but not statistically significant. This confirms that the fact of the right ovary being more active than the left one. On the other hand, ovary-bearing CL was significantly wider in diameter, larger in length, and heavier in weight as compared to the non-CL-bearing ovaries.

The difference in the size of the ovaries might be a reason for differences in ovarian activities potentially having implications for conducting other assisted reproductive technologies procedures such as ovum pick up (OPU). To the best of our knowledge, this is the first study to generate and document the morphometric parameters of reproductive organs in the Boran heifer in Ethiopia. These data for this breed may be useful as baseline data in further study related to the physiological function of individual structures measured in this study. Hence, further studies using different categories of age, parity of the Boran breed, and comparative study with other breeds are suggested to confirm the results in this study.

Conflict of interest

The authors declared no potential conflicts of interest relative to the research, authorship, and/or publication of this article.

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Quality of injectable oxytetracycline circulating in legal markets of Addis Ababa, Ethiopia using physio-chemical and sterility analysis

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Abstract

This study was conducted to evaluate the quality of different brands of injectable oxytetracycline solutions circulating in the legal markets of Addis Ababa with respect to physicochemical characteristics and sterility. Nine brands of oxytetracycline with thirteen different batches were randomly purchased from veterinary drug stores in the city. The physical assessment was performed by using a checklist that was prepared based on the World Health Organization guidelines. The qualitative and quantitative analysis of active pharmaceutical ingredients (API) was performed by High-Performance Liquid Chromatography. The sterility test was assessed by using the direct inoculation method. All samples passed the identity, the assay, and the sterility tests. However, there were statistically significant differences ($P<0.05$) among brands in the quantity of API. The highest percentage of the API was recorded in brand G (112.12%± 1.86) while the lowest was seen in brand H (92.61%± 1.5). This study revealed that all brands passed both physicochemical and sterility tests except for the differences in the API level among brands. Strict regulation, monitoring, and wider-scale surveillance are required to assure sustainable control of substandard, unsterile, and falsified pharmaceutical products in the country.

Keywords: *Batches; Brands; HPLC; Oxytetracycline; Physicochemical analysis; Sterility Test.*

Introduction

The quality of a drug is an integral part of a successful treatment regimen. To attain and sustain the required quality, there must be a system of quality assurance. Many developing countries do not have an effective means of monitoring the quality of generic drug products in the market. This results in the widespread distribution of substandard and counterfeit pharmaceutical products. Due to this, the World Health Organization (WHO) issued guidelines for global standards and requirements for the registration, assessment, marketing, authorization, and quality control of pharmaceutical products (Taylor *et al.*, 2001; Glass, 2014; Kelesidis and Falagas, 2015; WHO, 2015).

Oxytetracycline (OTC), which exhibits broad-spectrum antimicrobial activity against a variety of microbes, is the most frequently used antimicrobial drug, particularly in veterinary medicine. Some formulations, however, have been reported ineffective against disease-causing organisms (Michaloa *et al.*, 2004). This may be related to the quality of the drug or the development of resistance by the organisms. Reports showed that the counterfeit rate may reach up to 30% of drugs sold in Sub-Saharan Africa, of which antimicrobial agents have been the most popular ones (Glass, 2014; Kelesidis and Falagas, 2015; WHO, 2018). World Health Organization (2018) estimated that one in ten medical products in low and middle-income countries are substandard or falsified. Substandard products cause treatment failure, harm the patient, promote antimicrobial resistance, lead to loss of confidence, or trust in the healthcare system (Okeke *et al.*, 2007; WHO, 2018).

A drug like oxytetracycline is mainly imported to Ethiopia by private companies in which manufacturers are located outside the country. Quality tests are usually conducted during importing. The borders of the country are porous which may allow the entrance of substandard drugs. Hence, periodic assessment of drugs circulating in the market will make the quality control process complete and help to design systems or strategies to control the circulation of substandard drugs. The authors of this article conducted a quality test on albendazole, a quite commonly used drug to treat helminthic parasites, and found that 3 of the 10 batches contained a lower amount of albendazole per tablet than expected (Seifu *et al.*, 2019). This finding initiates us to test the quality of other drugs like oxytetracycline which is quite commonly sold and used drug for the treatment of widespread infectious diseases in animals (Tufa

et al., 2018). To the best of our knowledge, there was no information in the country concerning the quality and sterility of oxytetracycline (OTC) circulating in the market. Therefore, this study was conducted to evaluate the physicochemical quality and sterility of injectable OTC solutions circulating in the legal market of Addis Ababa, Ethiopia.

Methods

Study area

The study was conducted in Addis Ababa which is the capital city and administrative centre for the Federal Democratic Republic of Ethiopia. The city lies at 9.03° North latitude and 38.8° East longitudes (UN-Habitat, 2017). The city is the hub for the production, importation, and distribution of veterinary pharmaceuticals.

Study design and sampling

A cross-sectional study was conducted from November 2018 to April 2019. Thirteen brands of oxytetracycline injectable solution were included in the study and two vials were used for each brand for assessment. Drugs were purchased from randomly selected veterinary pharmacies in the city. During sampling, safety protocols were considered to avoid factors that reduce the quality of the drugs which means that purchased drugs samples were transported and stored as per the recommendation of the manufacturers. All laboratory activities were performed in Animal Products, Veterinary Drug, and Feed Quality Assessment Centre, which is located in Akaki Kality sub-city, Addis Ababa, Ethiopia.

Physicochemical analysis

High-Performance Liquid Chromatography (HPLC) (Agilent Technologies, Japan**)** was used for the identification and quantification of API in the samples. HPLC graded chemicals and reagents like methanol, orthophosphoric acid, acetonitrile, hydrochloric acid, oxytetracycline reference standard (USP, USA), triethylamine, and ultrapure deionized water were used for the analysis.

The mobile phase was prepared by mixing 4.075 ml of orthophosphoric acid in 1000 ml of ultrapure deionized distilled water and the pH of the solution was adjusted to 2.7 with triethylamine. Then, 650 ml of this solution was mixed

with 100 ml of methanol and 250 ml of acetonitrile, sonicated for 30 minutes, and passed through a filter with 0.5 µm pore size.

Oxytetracycline (OTC) (1 mg/ml) reference standard (USP) was prepared by taking two OTC reference standards (ref1 and ref2). For both, 0.025 g was accurately weighed and transferred to a 25 ml volumetric flask and dissolved in a diluting solution, and mixed by vortexing. Then, 10 ml of this stock solution was transferred into another 100 ml volumetric flask, and further diluted with distilled water to the required volume, and then transferred to HPLC vials after the syringe filter.

During the preparation of the sample, two vials of OTC of the same batch and brand were added into one beaker and mixed well. Then, an equivalent of 1 mg/ml concentration of reference standards was calculated depending on the percent concentration of API of the sample (10 and 20 %), and then the sample was diluted to half and sonicated for a few minutes. Then, 10 ml from the stock solution was taken and diluted to the volume by deionized distilled water and mixed well again. Afterward, the assay preparation was taken into a syringe, and passed through a 0.45 µm syringe filter, and filtered into 75 % of 1.5 ml HPLC vial, and it was ready for determination assay.

The HPLC system was equipped with a UV-vis detector (SPD-20A/20AV, Shimadzu Corporation, Japan), a 254 nm detection wavelength, and a 4.6 mm by 25 cm column that contains 5 µ particle size. The flow rate was about one ml per minute. The total chromatography run time was 12 minutes. Before running the samples, the suitability, and stability of the system were tested (USP, 2012).

An equal volume (about 10 µl) of the standard and the sample preparations were injected into the chromatograph separately. The chromatograph was recorded and measured for the response of peaks. For the identification, we compared the retention time of the OTC sample with that of the standard (Kupiec, 2004). The peak areas of the standard and the sample were used for quantification (Van Tonder *et al.*, 1996). All procedures were conducted three times to increase the precision.

According to the official compendia of USP, OTC for injection contains an amount of OTC Hydrochloride equivalent to not less than 90 % and not more than 120 % of the labelled amount of oxytetracycline (USP, 2015).

Sterility testing

Thioglycollate and soybean casein digest media were used for sterility testing. It was carried out by following basic microbiological aseptic techniques in the biosafety label II laboratory. The thioglycollate was used to test the growth of bacteria. Soya-bean casein digest was performed to test whether fungi or aerobic bacteria grew or not. Fluid thioglycolate was incubated at 35 °C whereas the Soybean casein digest was incubated at 25 °C.

The inoculation was conducted after making a serial dilution of Oxytetracycline up to 10^{-6} using a soybean digest medium. From each diluted tube, one ml of the fluid was transferred to the prepared media. Two-stage incubation was conducted to increase the accuracy and visibility. First, the sample was inoculated in the media and incubated for 14 days. After that one ml of the mixed fluid was taken and inoculated into the new media of the same type and incubated for an additional 4 days. In all cases, media without a drug was incubated and used as the negative control. As a positive control, thioglycolate was incubated with drug and bacteria (*Staphylococcus aureus*), and soybean casein digest was inoculated with diluted drug and fungi (*Candida albicans*). The interpretation was made based on the absence or presence of turbidity (Amrutha *et al.*, 2017).

Ethical consideration

An agreement was reached between the investigators and local veterinary pharmacists, after explaining the objective of the research. The owners agreed to sell the drugs with a full description as they are coming from the manufacturer or distributor. During sampling, the product manufacturer, brand name, and name of the pharmacy or owner were recorded and coded for anonymity. Hence, the brands were coded in the preparation of this manuscript.

Data analysis

The data were checked, coded, and entered into Microsoft Excel 2010. Descriptive statistics like percentage mean and relative standard deviation (RSD) were used to express and summarize the data. The content of API in each brand was compared by using a one-way analysis of variance (ANOVA). The Least Square Difference (LSD) was used to conduct pairwise comparisons. A *P-value* of less than 0.05 was considered statistically significant.

Results

Packaging information

All brands and batches fulfilled the criteria in terms of container character, strength (concentration), dosage statement, storage information, batch number, or lot number, manufactory, and expiry date. However, 2 (22.2%) brands and batches 3 (23.1%) had no leaflet or package insert (Table 1).

Table 1. Physical assessment of oxytetracycline

Product	Container and closer	API con centration (mg/mL)	Dosage statem ent	Storage informa tion	Leaflet or package insert	Batch Number	Manufa ctured and Exp ired Date
Brand A	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Brand B1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Brand B2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Brand C	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Brand D1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Brand D2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Brand E1	Yes	Yes	Yes	Yes	No*	Yes	Yes
Brand E2	Yes	Yes	Yes	Yes	No*	Yes	Yes
Brand F1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Brand F2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Brand G	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Brand H	Yes	Yes	Yes	Yes	No*	Yes	Yes
Brand I	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Brand B1 and B2 were in the same brand but containing different concentrations of oxytetracycline, the same is true for others (D1, D2, E1, E2, F1, and F2), Yes = Fulfil the criteria *No=do did not fulfill the criteria, API= active pharmaceutical Ingredient

Identification test

An identity test was conducted by comparing the retention time of the sample with that of the standard in the chromatographic response (Table 2 and Figure 1).

Table 2. Comparison of chromatographic retention time values of different brands of Oxytetracycline with a reference standard

Product	Concentration(mg/ml)	Retention time, minutes (sample),	Retention time minutes (standard)	Identity test
Brand A	200	3.33	3.29	Pass
Brand B1	100	3.27	3.29	Pass
Brand B2	200	3.26	3.29	Pass
Brand C	200	3.33	3.29	Pass
Brand D1	100	3.28	3.29	Pass
Brand D2	200	3.29	3.29	Pass
Brand E1	100	3.20	3.29	Pass
Brand E2	200	3.20	3.29	Pass
Brand F1	100	3.27	3.29	Pass
Brand F2	200	3.26	3.29	Pass
Brand G	200	3.29	3.29	Pass
Brand H	100	3.33	3.29	Pass
Brand I	100	3.29	3.29	Pass

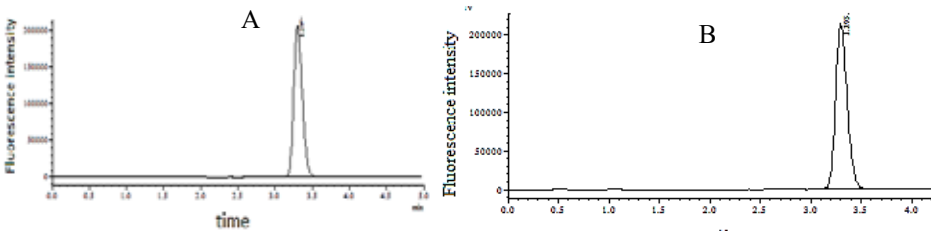


Figure 1. Representative image of the chromatograph of the standard (A) and one of the samples (B)

Assay of oxytetracycline

The results for the mean percentage label claim of the different brands of parenteral oxytetracycline preparation included in the study are depicted in Table 3.

Table 3. The assay of tested oxytetracycline brands

Product	Labelled concentration (mg/ml)	Assay (mean %± RSD)	Content (mg) obtained	Assay test decision
Brand A	200	102.15±0.03	204.3	Pass
Brand B1	100	96.71±1.03	96.71	Pass
Brand B2	200	98.82±0.23	197.64	Pass
Brand C	200	97.64±1.49	195.28	Pass
Brand D1	100	106.34±1.12	106.34	Pass
Brand D2	200	102.60±1.45	205.2	Pass
Brand E1	100	100.62±1.14	100.62	Pass
Brand E2	200	98.41±1.57	196.82	Pass
Brand F1	100	100.88±0.13	100.88	Pass
Brand F2	200	103.75±0.79	207.5	Pass
Brand G	200	112.12±1.86*	224.24	Pass
Brand H	100	92.61±1.50*	92.61	Pass
Brand I	100	96.45±0.79	96.45	Pass

*Acceptable range is between 90% and 120%, *RSD= relative standard deviation

Even though all brands and batches fulfilled both qualitative and quantitative criteria, there was a significant difference ($P<0.05$) among the brands. The highest mean content (112.12±1.86) and least mean (92.61±1.50) contents were seen in brands G and H, respectively (Table 3).

Sterility testing

All the brands passed the sterility testing which showed that they were free from aerobic bacteria and fungi (Table 4).

Table 4. The sterility test result of oxytetracycline

Product	Concentration (mg/ml)	Soya bean casein digest medium	Thioglycolate medium
Brand A	200	NT	NT
Brand B1	100	NT	NT
Brand B2	200	NT	NT
Brand C	200	NT	NT
Brand D1	100	NT	NT
Brand D2	200	NT	NT
Brand E1	100	NT	NT
Brand E2	200	NT	NT
Brand F1	100	NT	NT
Brand F2	200	NT	NT
Brand G	200	NT	NT
Brand H	100	NT	NT
Brand I	100	NT	NT

*NT= No turbidity (no bacterial or fungal growth)

Discussion

The use of drugs in food-producing animals is fundamental for their health and productivity. To be effective a drug should have the required physicochemical qualities and should be sterile. The quality of a drug might be affected by the storage conditions, humidity, packaging materials, transportation, formulation, the nature of the active ingredient, and the presence of microorganisms (Othman, 2017; WHO, 2019). The pharmaceutical products should constitute not only an adequate amount of API but also must be packed with proper packaging materials in the desired container, which protects environmental conditions that may affect the quality of the products. Besides, the drug should be labelled well and have the leaflet inserted for additional information. In this study, 22.2% of the brands or 23.1% of batches didn't have leaflets or package inserts. The absence of leaflet was also reported by Seifu *et al.* (2019) for albendazole in the same study area. This may lead to the inappropriate use of the drugs. Misuse or inappropriate use of drugs may have a direct impact on the recipient and an indirect effect on future treatment practices through the development of drug resistance (Clifford *et al.*, 2018).

In this study, all brands of oxytetracycline passed the quality assessment and the identification test. These indicated that oxytetracycline in the market in the study area had good quality during the study period which may be due to good manufacturing practices followed by the storage and transportation of the products in the proper conditions. The periodical assessment and effective control at the point of entry for imported drugs are needed to keep the quality and avoid the circulation of substandard or falsified drugs in the market. The result obtained in this study was also in line with the study conducted in Ghana (Owusuwaa, 2016), that reported all seven injectable oxytetracycline investigated brands passed the assay quantification test. Contrary to our finding, poor quality of tetracycline capsules was reported in Nigeria (Okeke and Lamikanra, 1995); the difference may be related to the control system and the practices in manufacturing processes and drug handling conditions.

In this study, the statistical analysis of the mean differences in drug content revealed that the mean API content of different brands was significant ($P<0.05$). The highest percentage content was obtained from brand G (112.12% ± 1.86) while the lowest drug content was obtained from brand H (92.61% ± 1.50). The reason for the relative difference in the mean OTC assay between the brands might have been related to the pharmaceutical manufacturer's formulation condition. Variations in the composition of ingredients may be related to the amount of excipients and API added to the products (Amrutha *et al.*, 2017). If the drug has higher API content than expected, the side effects may be intensified whereas if it contains a lower amount than expected, it will not be effective and may favor the development of drug resistance.

All forms of therapeutic products are expected to be sterile that is free of viable microorganisms. Principally, these include parenteral, ophthalmic, and irrigational preparations. Of these, parenteral products are unique dosage forms of drugs as they are injected subcutaneously, intramuscularly, or intravenously into the internal body compartments. As they have circumvented the highly efficient first line of body defense like the skin or mucous membranes, they must be free from microbial contamination (Amrutha *et al.*, 2017).

The sterility tests are intended for detecting the presence of viable microorganisms in a pharmaceutical preparation that is designed to be sterile (USP, 2012). The test was based on the principle that if microbial contaminants are available, they can grow in a suitable media and their presence will be indicat-

ed by the formation of turbidity in the medium. To produce therapeutic effects without predisposing the patients to other infections, the parental drug should be sterile. This is due to the possibility of the presence of drug-resistant bacteria that can survive in drugs and grow when conditions become suitable. The contamination may occur during the manufacturing process from the equipment, person, or dust (Arzai, 2008). All brands of oxytetracycline included in this study were free from aerobic bacteria and fungi. Contrary to our current finding, Arzai (2008) reported bacteria and fungi contamination of OTC ointments in Nigeria.

Conclusions

In summary, the physicochemical evaluation showed that all of the brands tested complied with the pharmacopeia specifications described in the USP. However, the statistical comparison for drug content indicated a significant difference among brands. Besides, sterility testing also showed the absence of both bacterial and fungal contaminants. Despite these favorable findings, strict regulations, monitoring, and surveillance are required to assure sustainable safety, quality, and efficacy of drugs in the country.

Abbreviations

API: Active Pharmaceutical Ingredient; HPLC: High-Performance Liquid Chromatography; OTC: Oxytetracycline, RSD: Relative Standard Deviation; SD: Standard Deviation; USP: United States Pharmacopeia; UVS: Ultraviolet-Visible Spectroscopy; WHO: World Health Organization

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Dairy cattle lameness prevalence, causes and risk factors in selected farms of southern Ethiopia

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Abstract

A cross-sectional study was conducted from October 2018 to December 2019 to estimate the prevalence of lameness in dairy cows, to assess the causes and associated risk factors in southern Ethiopia. A total of 2009 dairy cows were visually and clinically examined, and the overall prevalence of lameness was found to be 14. 1% (95% CI=12.7-15.7). The prevalence of lameness was significantly (P< 0.05) higher in Arsi Negelle, Yirgalem, and Wolaita Sodo than in Wondo Genet and Hawassa. The prevalence of lameness was also associated with the stages of pregnancy and parity (P < 0.05). Generally, the prevalence of lameness in dairy cows was increasing with the increase in parity and stage of pregnancy. The main abnormalities observed in this study were hooves overgrowth (6%), lesions between hooves (4%), lesions on legs (2.2%), trauma (0.6%), arthritis (0.5%), and a sole ulcer (0.3%). Most of these abnormalities were mainly due to faulty management. Hence, based on this finding it is recommended that hoof management and trimming are very essential components of lameness control. Furthermore, early detection and treatment of lame cows; training of the owners on hoof management; and improvement of the housing are helpful to keep lameness at a lower level.

Keywords: Dairy cows; Lameness; Risk factors; Southern Ethiopia.

Introduction

Various authors defined lameness differently; however, the sounder definition is ″an abnormal gait that is commonly caused by pain″ (Whay and Shrear, 2017). It is the most important condition that influences the productivity, health, and welfare of dairy cows. It has a negative effect on milk production (Specher *et al.*, 1997, Green *et al.*, 2002; Hernandez *et al.*, 2002; Bicalho *et al.*, 2007; Keyserlingk *et al.*, 2009; Huxley, 2013; Mandel *et al.*, 2018). Moreover, economic impact of lameness can be attributed to the cost of treatment and controls (Hernandez and Shearer, 2000), increased calving interval, and impaired reproductive performance (Hernandez *et al.*, 2001) and likely increase the risk of culling (Juarez *et al.*, 2003; Huxely, 2013; Cramer *et al.*, 2009). In addition to the economic impact, lameness also affects the behavioral expression of cows due to pain. Indeed, now a day lameness is one of the most important welfare issues in dairy cattle production (Tremetsberger and Winckler, 2015; Galindo and Broom, 2002).

The causes of lameness in dairy cows are often complex and multifactorial (Ranjbar *et al.*, 2016; Bell, 2015), but are generally recognized as poor quality floors in cattle housing, poor cow tracks, and cows being forced to stand for a too long time on hard surfaces, ineffective foot trimming, infectious diseases and poor nutrition.

In Ethiopia, there is a scarcity of information about the prevalence of lameness in dairy cows and the associated risk factors. Therefore, this study aimed to estimate the prevalence, identify the causes of lameness, and assess the potential risk factors involved in the occurrence of lameness in selected dairy farms in southern Ethiopia.

Materials and methods

Study area

The study was conducted between October 2018 and March 2019 in Hawassa, Wondo Genet, Yirgalem, Arsi Negelle, and Wolaita Sodo towns (Figure 1). Hawassa, Arsi-Negelle, Yirgalem, Wondo-Genet, and Wolaita Sodo are located at 7°3′43.4″N and 38°28′34.9″E, 7°2′59.9″N and 38°41′6″E, 6°45′N and 38°25′E, 7°5′3″N and 38°37′8″E, and 6°51′36″E and 37°45′41.7″, respectively. The study areas were located at the escarpment of the rift valley, and they are with good

dairy production potential. The altitudinal range of the study areas is from 1650 (at Hawassa) to 2500 (at Wolaita Sodo and Wondo Genet) meters above sea level (m.a.s.l). The range of annual rainfall was 900–1700 mm for Hawassa, 500–1100 mm for Arsi Negelle, 1314mm for Yirgalem, 1372mm for Wondo Genet, and 1446 mm Wolaita Sodo. The minimum and maximum temperature of Hawassa, Arsi-Negelle, Yirgalem, Wondo-Genet, and Wolaita Sodo were 13°C and 25°C, 12.6°C and 27.3°C, 14°C and 29°C, 9.9°C and 29.5°C, and 15°C and 28°C, respectively (NMA, 2019).



Figure 1. Map of Ethiopia with the regional states and showing the five study areas (1=Arsi Negelle, 2=Wondo Genet, 3=Hawassa, 4=Yirgalem, and 5=Wolaita Sodo)

Study animals and sample size

Information on dairy cows' lameness was collected from 157 small-scale dairy farms selected from the aforementioned five towns in southern Ethiopia. Farms were purposively selected based on the herd size of 5 or more cows. Based on this criterion, the total number of farms selected was 157. All dairy cows and heifers, at breeding age, found in the selected farms were included in the study. This constituted a total of 2,009 cows and heifers. The number of farms and animals selected from each town was shown in Table 1.

Table 1.Number of dairy farms and number of cows sampled for the study

Name of town	Number of farms selected	Number of animals selected
Hawassa	35	789
Yirgalem	34	280
Wondo Genet	43	360
Arsi Negelle	24	168
Wolaita Sodo	21	412
Total	157	2,009

Study design and methodology

A cross-sectional study was employed to assess the prevalence of dairy cows' lameness. All the animals in the selected farms were carefully observed and clinically examined for lameness. During the visit, the study animals were allowed to move and observed for any symptom of abnormal gait as described by Shearer *et al*. (2012) and visual locomotion. Those cows with apparent abnormal gait were clinically palpated for reaction and/or pain, observed for the presence of any lesions, shifting of weight on the limbs, and ticks. Potential risk factors for lameness such as herd size, parity, milking status, stage of pregnancy, hoof overgrowth, and study areas were recorded to assess their association with the disease.

Visual locomotion scoring

All cows in the selected farms were assessed visually for lameness and the gait was scored using a 1- 5 point rating system, where 1=normal, 2=mild lame, 3=moderately lame, 4=lame, and 5=severely lame (Flower and Weary, 2006; Chapinal *et al.*, 2009). The observed lameness was characterized as described by Sprecher *et al*. (1997), Thomsen *et al*. (2008), and Shearer *et al*. (2012). According to Sprecher *et al*. (1997) the definition and classification of cow lameness:

- Normal: Cow stands and walks with a level-back posture, the gait is normal.
- Mild lame: Cow stands with a level-back posture but develops an arched-back posture while walking; the gait remains normal.
- Moderately lame: Arched-back posture is evident, both while standing and walking. Cow's gait is affected and is best described as short striding with one or more limbs.

- Lame: Arched-back posture is always evident and gait is best described as one deliberate step at a time.
- Severely lame: Cow demonstrates an inability or extreme reluctance to bear weight on one or more of the limbs/feet.

Data analysis

All collected data were entered into Microsoft Excel Spread Sheet, edited and coded, and then, summarized using descriptive statistics like mean. Then, it was analyzed by univariable and multivariable logistic regression analysis to check the association of the aforementioned potential risk factors with the occurrence of lameness. The existences of collinearity among the variable were checked using gamma values (Dohoo *et al*., 2009). All the statistical analyses were performed on STATA software version 14.2 (STATA Corp, 4905 Lakeway Drive, College Station, Texas 77845 USA). The study considered a 95% confidence level and 5% of absolute desired precision.

Results

Prevalence of clinical lameness

From a total of 2,009 dairy cows and heifers examined 284 (14.1%) animals were found clinically positive for various degrees of lameness. The prevalence of lameness in each of the study towns is shown in Table 2.

Table 2. Dairy cattle lameness prevalence in the study areas

Study area	No examined	No positive (%)	Std. Er.	95% CI
Wondo Genet	360	35 (9.7%)	0.016	7.1-13.2
Hawassa	789	92 (11.7%)	0.011	9.6-14.1
Arsi Negelle	168	28 (16.7%)	0.029	11.7-23.1
Yirgalem	280	50 (17.9%)	0.023	13.8-22.8
Wolaita Sodo	412	79 (19.2%)	0.019	15.7-23.3
Overall	2009	284 (14.1%)	0.008	12.7-15.7

Lameness score

The locomotion of all the study animals' was recorded during the observation period. All dairy cows and heifers with locomotion scores 2 to 5 were found to

be lame. Among the animals observed to have lameness (284), 54.6% (n = 155), 32.7% (n = 93), 12% (n = 34) and 0.7% (n = 2) were mildly, moderately, lame and severely lame, respectively (Table 3).

Table 3. Proportion of locomotion score of dairy cows (N=2009)

Locomotion score	Description	No examined	Proportion (%)
1	Normal (Not lame)	1,725	85.9
2	Mildly lame	155	7.7
3	Moderately lame	93	4.6
4	Lame	34	1.7
5	Severely lame	2	0.1
Total		2009	100

Risk factors of lameness

The result of univariable logistic regression analysis of the prevalence of dairy cows' lameness with various risk factors has been shown in Table 4. Except for the herd size ($P > 0.25$), all the variables considered in the study, that means: study area, parity, milking status, and stage of pregnancy were found significant ($P<0.05$). All those independent variables, which were significant in the initial univariable analysis checked for co-linearity using Kruskal gamma statistics, and those variables whose gamma value ranged between −0.6 and +0.6 were considered in a multivariable logistic regression model. Accordingly, milking status was excluded from multivariable analysis due to collinearity with parity (gamma=0.8665). Thus, the variables included in the multivariable model were study area, parity, and pregnancy stage, and all of these variables were found significant ($P <0.05$) (Table 5). The Hosmer-Lemeshow goodness-of-fit test suggested that the model fit the data ($\chi^2= 10.96$; $P =0.1405$; ROC= 72.1%).

Table 4. Univariable logistic regression analysis of risk factors for the occurrence of lameness

Risk factors	Level of factors	No examined	No positive (%)	Std. Err.	Z	P-value
Study areas	Wondo Genet	360	35 (9.7%)	0.016	-	
	Hawassa	789	92 (11.7%)	0.011	0.97	0.332
	Arsi Negelle	168	28 (16.7%)	0.029	2.27	0.023
	Yirgalem	280	50 (17.9%)	0.023	2.97	0.003
	Wolaita Sodo	412	79 (19.2%)	0.019	3.67	0.000
Parity	* None	960	56 (5.8)	-	-	
	* 1-3	870	182 (20.9)	0.014	9.02	0.000
	* ≥ 4	179	46 (25.7)	0.033	7.83	0.000
Milking	No	1149	67 (5.8)	-	-	
	Yes	860	217 (25.2)	0.015	11.43	0.000
Pregnancy stage	None	1543	189 (12.2)	-	-	-
	1st trimester	276	46 (16.7)	0.022	2.01	0.045
	2nd trimester	105	27 (25.7)*	0.043	3.84	0.000
	3rd trimester	85	22 (25.9)*	0.048	3.53	0.000
Herd size	≤9	563	75 (13.3)	0.014		
	10 to 49	925	132 (14.3)	0.012	0.51	0.608
	≥ 50	521	77 (14.8)	0.016	0.69	0.490

Table 5. Multivariable logistic regression analysis of potential risk factors for lameness in dairy cattle, southern Ethiopia.

Risk factors	No examined	No positive (%)	OR	95% CI	P-value
Areas					
Wondo Genet	360	35 (9.7%)	-	-	
Hawassa	789	92 (11.7%)	2.71	1.19-2.29	0.007
Arsi Negelle	168	28 (16.7%)	1.98	1.01-3.01	0.048
Yirgalem	280	50 (17.9%)	3.27	1.40-3.88	0.001
Wolaita Sodo	412	79 (19.2%)	5.37	2.24-5.65	<0.001
Parity					
Heifers	960	56 (5.8)	-	-	
1-3	870	182 (20.9)	4.06	2.92-5.64	<0.001
≥ 4	179	46 (25.7)	6.11	3.80-9.82	<0.001
Pregnancy stage					
None	1543	189 (12.2)	-	-	
1st trimester	276	46 (16.7)	1.34	0.92-1.96	0.046
2nd trimester	105	27 (25.7)*	2.65	1.60-4.41	<0.001
3rd trimester	85	22 (25.9)*	2.59	1.49-4.51	<0.001

Abnormalities observed

During the study period, about eleven different types of abnormalities presumed to be the causes of lameness were observed. Clinically hoof overgrowth was the most commonly identified abnormality followed by lesion between hooves and on the legs (Table 6).

Table 6. Causes of lameness encountered during the study and their proportion

Abnormalities	Frequency	Proportion (%)
Hoof overgrowth	120	42.3
Lesions between hooves	80	28.2
Lesions on legs	44	15.5
Sole ulcer	6	3.3
Arthritis (Carpal and tarsal joint swelling)	10	5.5
Abscess on leg	2	1.1
Trauma and/or bruising on leg	13	7.2
Swelling on leg	4	2.2
Interdigital ticks infestation	9	3.2
Fracture of leg	6	2.1
Faulty injection	4	2.2
Two and more than two abnormalities	14	4.9

Discussion

The prevalence of lameness in dairy cows recorded in the present study, 14.1%, is comparable to the report from various parts of the world (Gitau *et al*., 1996; Haskell *et al*., 2006; Somers *et al*., 2-15; Abunna *et al*., 2017). However, it is lower than the reports from countries like the UK (Rutherford *et al*., 2009; Barker *et al*., 2010; Leach *et al*., 2010), Czech Republic (Šárová *et al*., 2011), Turkey(Yaylak *et al*., 2010), Canada and USA (Von Keyserlingk *et al*., 2012). In contrast, it is relatively higher than the reports from Sweden (Manske *et al*., 2002), New Zealand (Fabian *et al*. 2014), Denmark (Alban, 1995), the USA (Wells *et al*., 1993), and Ethiopia (Sulayeman and Fromsa, 2012). The variation in the prevalence of lameness between the different areas might be due to the differences in the management system, herd size, seasonal variation, breeds, level of productivity of the cows, and observer's subjectivity (Winckler and Willen, 2001; Channon *et al*., 2009; Huxley *et al*., 2012; Van Nuffel *et al*., 2015; Moreira *et al*., 2018). A dairy cow managed predominantly indoors will typically experience excessive net hoof growth because growth exceeds wear (Bell *et al*., 2009). Hoof overgrowth is one of the major causes of lameness as also observed during this study. It was noted that all cows with hoof overgrowth had lameness (Table 6). During the study period, the locomotion of all the cows was observed and scored as described by Sprecher *et al*. (1997) and Flower and Weary (2006) and the result was shown in Table 3. During this

study comparatively higher proportion of mild (7.7%) than moderate (4.6%) lameness was observed.

In the multivariable logistic regression analysis, the prevalence of lameness was significantly ($P< 0.05$) associated with the study area, parity, and stage of pregnancy. The odds of lameness in dairy cattle in Hawassa (OR = 2.71), Arsi Negelle (OR = 1.98), Yirgalem (OR = 3.27), and Wolaita Sodo (OR = 5.37) was significantly higher than in Wondo-Genet. During this study, the smallest and highest prevalence was recorded at Wondo Genet and Wolaita Sodo, respectively. These differences might be due to the variation in the management of cows between the two study areas. As observed during the study period in most of the dairy farms in Wolaita Sodo animals were managed indoor, while in Wondo-Genet animals had a certain period for outdoor exercises. The same holds true, i.e. indoor management, for Yirgalem and Arsi-Negelle. It was well documented that animals kept on pasture had a lower prevalence of lameness (Haaskell *et al.*, 2006; Olmos *et al.*, 2009; Adams *et al.*, 2017).

In the present study, the prevalence of lameness in dairy cows increased with an increasing parity number. This is also in agreement with several previous reports around the world (Groehn *et al.*, 1992; Wells *et al.*, 1993; Radostits, 2001; Mohamadnia *et al.*, 2008; Olmos *et al.*, 2009; Yaylak *et al.*, 2010; Solano *et al.*, 2015; Foditsch *et al.*, 2016; Daros *et al.*, 2019). It was noted that the likelihood of lameness was 6.1 and 4.1 times higher in cows with 4 or more parities and in their first to third parity compared to heifers, respectively. According to Solano *et al.* (2016), the problem of sole ulcer and white line diseases is increasing with parity and perhaps this may be responsible for the higher prevalence of lameness in multiparous cows.

The prevalence of lameness in dairy cows was significantly associated ($P<0.05$) with the stages of pregnancy. The odds of lameness was significantly higher in pregnant than non-pregnant cows, and there was an increase in the prevalence of lameness with the advancement of pregnancy. The OR was 1.5, 2.7, and 3.6times more in first, second and third pregnancy than non-pregnant, respectively. This is mainly due to the changes in hoof loading with pregnancy, which may predispose the cows' to lameness (Scott, 1988 ; Chapinal *et al.,* 2008).

In the present study, hoof overgrowth (6.2%) was the major cause of lameness followed by lesions between hooves (4%), lesions on legs (2.2%), trauma, and/or bruising (0.6%), and arthritis (0.5%). These abnormalities are reported to be

the cause for cattle lameness by other authors (Radostits, 2001; Huxley *et al*., 2012; Sulayeman and Fromsa, 2012; Bell, 2015). All cows found lame in this study had overgrown hooves (n=120, 6%). The association of hoof overgrowth with lameness had also been reported by different authors (Sulayeman and Fromsa, 2012; Solano *et al*., 2015; Sadiq *et al*., 2017; Oehm *et al*., 2019). The sole ulcer is a common report as a cause of lameness in dairy cows (Murray *et al*., 1996; Radostits, 2001; Barker *et al*., 2007; Huxley *et al*., 2012; Sulayeman and Fromsa, 2012; Bicalho and Oikonomou, 2013; Bell, 2015).

Conclusions

The overall prevalence of dairy cows' lameness in the southern part of Ethiopia was 14.1%. This study revealed that lameness in dairy cows is associated with increasing parity, stage of pregnancy, and management difference among the study sites. The most commonly observed abnormalities in the study animals were hoof overgrowth, arthritis, sole ulcer, interdigital ticks' infestation, and trauma and/or bruising.

Most of the abnormalities observed were due to faulty management, and hence, hoof management and proper trimming is a very essential component of lameness control. Furthermore, detection and treatment of lame cows and training of the farm owners are helpful to keep the prevalence of lameness at a lower level.

Conflict of interest

The authors declare that there is no conflict of interest

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Influence of breed on the quality of *in vivo* produced embryos from Boran and Holstein Frisian cross dairy breed in Ethiopia

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Abstract

The variation of the dairy breed can determine the success of bovine embryo transfer by influencing the quantity and quality of *in vivo* embryo production. In this experiment, output and quality of *in vivo* produced embryos using semen of progeny tested Holstein Frisian (HF) sire in Boran and HF*Boran F_1 cross cows, and semen from purebred Boran sire in HF*Boran F_1 cross and Boran cows were evaluated. Boran (n=18) and HF*Boran cross (n=18) breed donor dams were superovulated using a previously optimized follicular stimulating hormone (FSH) (Pluset®) dose regimen: 650 IU for HF*Boran cross and 250 IU for Boran breeds. Each cow was flushed on Day-7 post insemination and embryos were evaluated for their developmental stages and quality. Superovulatory response rates were 88.9% and 83.3%, respectively, for Boran and HF*Boran with no significant (P>0.05) breed differences. Total recovery rates were relatively lower (56.5%) in Boran compared to in HF*Boran (67.4%). The mean (±SE) embryo flush outputs were 6.5±0.8 for Boran and 6.9±0.7 for HF*Boran with no significant breed difference. Recovery of a transferrable embryo was significantly higher (68.0%; P<0.05) in HF*Boran dam inseminated with HF sire semen. Boran cows yielded a significantly higher (P<0.05) proportion of unfertilized ovum (57.6 %) irrespective of the sire breeds. Comparatively, a higher number of degenerated embryos were produced by HF*Boran cows. This study demonstrated that the presence of breed-related differences in both the quality and quantity of *in vivo* produced Bovine embryos.

Keywords: Bovine; dam; embryo collection; embryo transfer; sire; superovulation.

Introduction

In vivo embryo production is a reproductive technology generally used to improve the number of offspring from potentially elite cows using multiple ovulatory embryo transfer techniques (MOET) (Phillips and Jahnke, 2016). This technology has got more acceptances worldwide as the best and ethically acceptable method for getting quality embryos (Bó and Mapletoft, 2014; Bó *et al*., 2019). Quality embryos are used for transferring to the recipients and it is used mainly for genetic improvement to compliment increasing the numbers of a particular breed or phenotype (Junqueira *et al*., 2018). This can be done by applying the MOET technologies which require the meticulous selection of valuable donors in perfect gynaecological conditions, application of appropriate superovulation technique, the use of good quality semen, and reproductively sound recipients, all of which have a direct influence on the outcome of the embryo transfer program (Phillips and Jahnke, 2016; Bó *et al*., 2019).

Different studies (Peixoto *et al*., 2007; Perez *et al*., 2016; Perez *et al*., 2019; Vizoná *et al*., 2020) have been carried out elsewhere on *Bos indicus* and *Bos taurus* breeds showed that there are variations in response to the superovulation. This is due to individual variations where some females consistently produce large numbers of embryos in response to superovulation than other females of similar age and management but different breeds may also perform variably (Guerreiro *et al*., 2014; Batista *et al*., 2016). There are shreds of evidence for the variations originated from the genetic and physiological differences of the donors. As a physiological parameter of variations, differences in follicular dynamics of Ethiopian Boran breed and their HF crosses were characterized previously in Ethiopia (Degefa *et al*., 2016 and 2018). Concerning the sire effect, in a more recent study, Lemma and Shemsu (2015) reported that semen quality is highly influenced by age and breed of AI bulls which could influence *in vivo* fertilization. Besides the genetic factor, it has been indicated that production status, feeding, and insemination techniques are among other factors that can affect the quantity and quality of embryos both in single and multiple ovulations (Jemal and Lemma, 2015; Mikkola *et al*., 2019). Typically Sartori *et al*. (2010) have found that milk production, body condition score, the type and quantity of feed intake, and heat stress are the main potential risk factors that affect the quality of embryos. Still further researches are ongoing on how to improve the production of good quality embryos in cattle at a molecular level (Safari *et al*., 2018; Manzi *et al*., 2019; Vizoná *et al*., 2020).

In general embryo transfer program in Ethiopia has been initiated by the international livestock research institute (ILRI) in 1991 but was discontinued until subsequent works by Degefa *et al.* (2016) and Tadesse *et al.* (2016). The MOET techniques applied today in Ethiopia are those developed for *Bos taurus* with variable results calling the need to develop breed-specific modifications for better results. Currently, Boran and HF*Boran crosses are widely used in Ethiopia and East African countries' dairy sectors. Subsequently, evaluation of the efficiency and effectiveness of embryo transfer (ET) in these breeds is very economical.

Ovarian dynamics and superovulatory response are partially governed by breed-related physiological differences. In this study, we hypothesized that subsequent embryo quality and recovery rate could also be influenced by breed differences either of the dam or the sire considering indigenous Boran breeds and their HF F_1 crosses.

Materials and methods

Study area and study animals

The study was conducted at Debre Zeit Agricultural Research Centre (DZARC) and College of Veterinary Medicine and Agriculture (CVMA). The study site is located at 08° 44' N and 38° 58' E (Latitude/ Longitude) at 1900 meters above sea level (m.a.s.l) with the average annual temperature ranging from 9.8°C to 28.3°C. It receives an average annual rainfall of 851 mm in a bimodal season (EIAR, 2020). A two-stage screening was carried out to assign eligible donors**.** From 135 (pure and cross) HF and 60 Boran herd, a pool of 28 cross HF and 21 pure Boran donor cows were targeted based on the history (record book) for their blood level, reproductive stage (non-lactating and at least one parity) and body condition score (BCS >3.5 on 1 - 5 scale) at the time of screening (Klopčič *et al.*, 2011). Selected animals were further subjected to gynaecological examination using ultrasonography and only those with intact CL (cycling cows) and without reproductive health problems (uterine fluid and sign of endometritis) were submitted for the experiment. All animals were managed under the same condition, provided with good housing, and fed on forage and concentrate supplements. Water was provided *ad libitum*. The donors received vaccination against common infections and were regularly dewormed as they were also allowed to graze.

Study design

The two-level Dam-Sire combination method for embryo production was designed and experimental animals were randomly assigned for Pluset® treatment. Imported high-quality purebred Boran semen and progeny tested HF-Friesian (HF) Sire semen was used. Semen from either of the bulls were randomly assigned and inseminated to donor cows as per the design. The overall procedure was conducted according to the four combinations of treatments using a 2x2 design as described below in Table 1.

Table 1. An arrangement of 2x2 dam sire combination model

Combination	Boran donor dam	HF*Boran donor dam
Boran Sire	TR1= 9	TR3= 9
HF Sire	TR2= 9	TR4= 9
Total	18	18

TR-Treatment

Treatment protocol

A total of 36 (Boran breed n=18) and (HF*Boran cross n=18) were superovulated. The HF*Boran cows were treated with an IM injection of FSH (Pluset®, Barcelona, Spain) at a dose rate of 650 IU (5.75ml) while the pure Borans were given 250 IU (2.5ml) of the same Pluset® as previously described by Degefa *et al*. (2018). The treatment schedule was a twice-daily injection of dispensed Pluset® over four days starting Day 4 after the insertion of CIDR (Progesterone 1.38 gm, Hamilton, New Zealand) in a decreasing volume as 2.5ml, 1.5ml, 1.25ml, 0.5ml, for HF*Boran crosses and, 1ml, 0.75ml, 0.5ml, 0.25ml for Boran. On Day six, each cow received an IM injection of 2 ml prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (Cloprostenol Sodium, Germany) twice a day (b.i.d) while the CIDR was removed on Day 7. Animals were inseminated twice to attain the optimum concentration of semen for multiple ovulations upon the detection of standing estrus following the superovulation treatment. Besides, all flushed cows were injected 2 ml $PGF_2\alpha$ to avoid further conception.

Recovery of embryos and evaluation

Embryos were flushed using a two-way Foley catheter with 1 litre of lactated ringer solution mixed with 1-5% calf serum on the 7^{th}-day post-AI and consid-

ering 500ml for each uterine horn. Donors received 3-5ml of epidural anaesthesia (2% Lidocaine) during the procedure. Superovulatory response was evaluated based on an ultrasonic count of the corpus luteum (CL). The embryos were evaluated for their developmental stage (from stage 1 = 1-cell to stage 9 = expanded hatched blastocyst) and for their quality (from Grade 1 = excellent to Grade 4 = degenerate/ dead) according to the International Embryo Transfer Society guidelines described in Jahnke *et al.* (2015) and Baruselli *et al.* (2006). Embryos were categorized as transferable (Grades 1, 2, and 3), freezable (Grade 1&2 only), Degenerate (Grade 4), Poor quality embryos (Grade 3 and 4), Unfertilized ovum (UFO).

The quality of the embryo was determined based on the following 4 parameters:

- Excellent- An ideal embryo, spherical, symmetrical, and uniform sized blastomeres, with uniform color
- Good- Few extruded blastomeres, slightly irregular shape, few vesicles
- Poor- Definite but no severe problem, extruded blastomeres, vesiculation of few degenerated cells
- Degenerated- Severe problems, numerous extruded cells, degenerated cells of different sizes, large vesicles with dark color

Response rate (%) was computed as the number of cows with at least three CL (Vieira *et al*., 2014) and/or persistent follicles (PUF)/the Total number of treated cows*100.

Recovery rate (%) = Total recovery or flush output (UFO + embryos)/Total number of (CL+PUF) *100;

The proportion of UFO (%) = Number of UFO/Total recovery or flush output * 100;

Rate of transferable embryos = Total number of transferable embryos/ Total recovery or flush output* 100.

Endpoint definitions

- *Total recovery*- The recovery of the total substances (UFO and embryos) compared with the total ovulations in terms of the total CL and PUF counted
- *Total flush output*- The total average of recovered substances (UFO and embryos) per individual donor cow
- *Superovulatory response*- The extent of ovulation of preovulatory follicles in terms of CL and also the PUF

- *Persistent unovulatory follicles*- Those preovulatory follicles failed to ovulate and remaining persistent until days of embryo flush
- *The proportion of UFO*- The ratio of recovery of unfertilized ovum versus the *total flush output*

Statistical analysis

All data collected were summarized into a Microsoft Excel sheet. Skewed dependent variables of the superovulatory response (UFO, fertilized, transferable, freezable, degenerated) were transformed using the Zero-skewness log transform technique and tested for their significance effect and interaction terms using STATA® version 14.0. Poisson regression was run to test differences between variables (a breed of dam and sire), and embryo quality parameters. The effect of breed of the dam or sire was computed by Two-way factorial ANOVA and Bonferroni pairwise mean comparison test. The quality of embryos was evaluated by General Linear Model (GLM). The level of significance was held at $P<0.05$.

Results

Embryo production

Overall, 88.9% (n=16) of Boran and 83.3% (n=15) of HF*Boran donors responded to the superovulatory treatment. A total of 210 (3-18/ cow) and 191 (2-15/ cow) CL were observed in Boran and HF*Boran donors. The overall recovery rate was 56.7% and 67.4% in Boran and HF*Boran, respectively. 57.6% UFO collected over the total flush output (n= 117) from Boran donor dams while 17% (n=125) was from HF*Boran. Descriptive statistics of the superovulatory responses and embryo recovery outputs were summarized in both breeds (n=36) Table 2.

Table 2. Mean (± SE) of the superovulatory response and outputs of embryo flush in Boran and HF*Boran donor cows

Parameters	Boran (n=18)	HF*Bora (n=18)
CL count	11.6±1.0 (Range= 3 - 18)	10.6±0.9 (Range= 2 - 15)
Post unovulatory follicles	1.8 ± 0.4	1.4 ± 0.3
Total flush outputs (UFO + Embryo)	6.5 ± 0.8 (Range=1- 10)	6.9 ± 0.7 (Range=2- 10)
Unfertilized ovum (UFO)	3.6 ± 0.6	1.3 ± 0.3
Total embryos	2.8 ± 0.6	5.6 ± 0.6
Transferable embryos	2.3 ± 0.6	4.7 ± 0.6

Comparison of the Dam and/ or Sire effects

Comparison of donor and/ or sire effects on quality of recovered embryos shows significant effects of donors and to lesser extent sires and statistically no significant interaction effects. Individual effects were evaluated for the validation of their significance and have shown that dam significantly affect the quality of all recovery types than sire as described in Table 3.

Table 3. Two-way ANOVA of dam and sire effect on embryo quality

Embryo quality	Dam*			Sire			Dam^sire interaction		
	MS	F	*P* value	MS	F	*P* value	MS	F	*P* value
UFO	2.98	11.15	0.002	0.64	2.42	0.129	1.00	3.75	0.061
Fertilized	0.55	13.20	0.001	0.25	6.12	0.018	0.02	0.65	0.413
Transferable	0.82	9.98	0.003	0.37	4.48	0.042	0.08	1.06	0.310
Freezable	0.97	4.42	0.043	0.81	3.70	0.063	0.17	0.81	0.375
Degenerated	0.44	19.86	0.000	0.00	0.24	0.625	0.01	0.69	0.425

^ refers interactions; df=1; MS= Mean square; * significant association of dam to all outcomes than sire

Status of embryo qualities

All embryos were sorted into grades while freezable (Grades 1 & 2, and Stages 4-8) and poor-quality embryos (Grades 3 & 4) were computed for the dam-sire combination effects. Unlike the lower fertilized embryo counts in Boran cows, there was a higher count of G1 and G2 embryos from the overall recovered embryos regardless of the sire effect. Similarly, HF*Boran cows produce higher count G2 embryos than other quality grades in the breed contest (Figure 1).

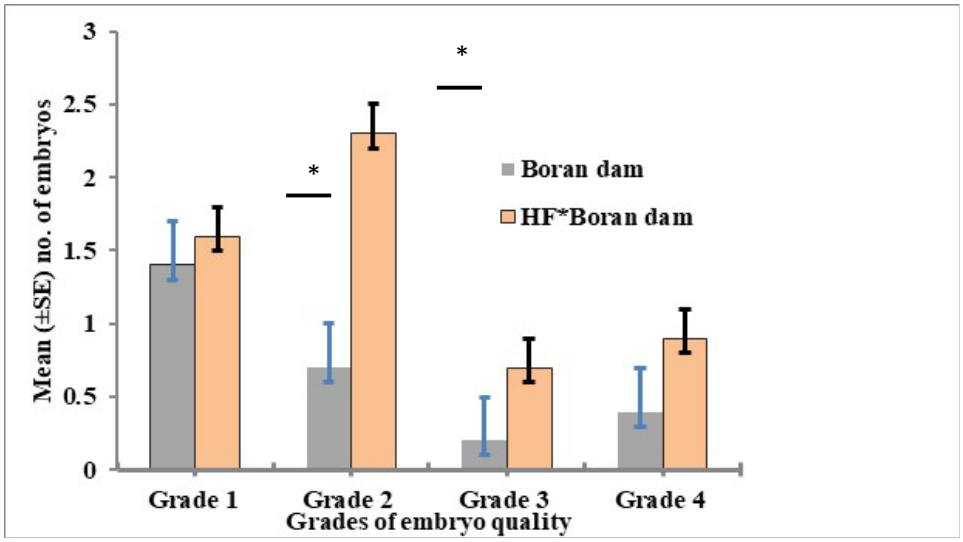


Figure 1. The overall quality of recovered embryos in both bred

On the other hand, when comparing each grade of embryo quality, HF*Boran dams inseminated with HF sire yield a higher number of excellent and good embryos (Grade 1 & 2) and also had yield embryos of poor-quality grades (Grade 3 & 4) than Boran cows inseminated with Boran sire (Figure 2).

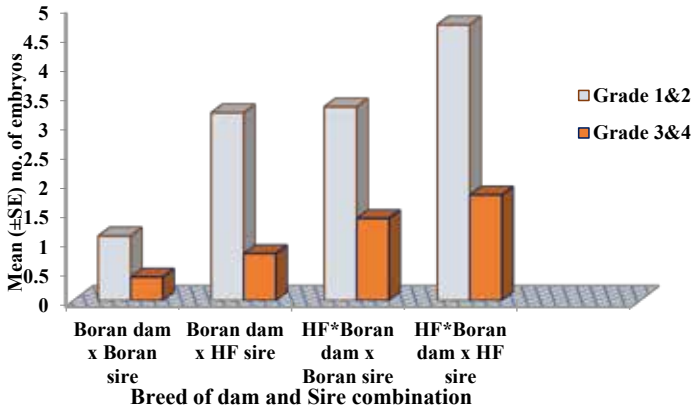


Figure 2. Classification of embryo qualities based on their dam-sire breed

Further analysis of different qualities of embryos recovered during alternate use of dam and sire breed showed a higher number of fertilized and transferable embryos when HF*Boran dams inseminated with either of the sires. Whereas a larger number of degenerated and poor quality embryos were found when HF*Boran donor dams were inseminated with HF sires, while a higher number of UFOs were recovered when Boran dams were inseminated with Boran sire (Table 4).

Table 4. The average number of different categories of embryo qualities resulting from the four dam-sire combinations of Boran and HF*Boran crossbreeds

Embryo category	Mean (±SE) number of embryos for dam sire combinations (x)			
	Boran dam x Boran sire	Boran dam x HF sire	HF*Boran dam x Boran sire	HF*Boran dam x HF sire
Fertilized	1.8 ± 0.8	3.8 ± 0.8	4.8 ± 0.8[a]	6.4 ± 0.8[b]
Transferable	1.2 ± 0.8	3.2 ± 0.8	4.1 ± 0.8	5.3 ± 0.8[a]
Freezable	1.2 ± 0.8	3.1 ± 0.8	3.3 ± 0.8	4.7 ± 0.8[a]
Degenerated	0.6 ± 0.8	0.7 ± 0.8	1.9 ± 0.8[a]	1.8 ± 0.8[a]
Poor quality	0.4 ± 0.3	0.8 ± 0.3	1.4 ± 0.3	1.8 ± 0.3[a]
UFO	3.9 ± 0.7[a]	3.3 ± 0.7	2.1 ± 0.7	0.6 ± 0.7

Superscripts significantly different at P<0.05 and 0.001, for the values "a" and "b", respectively

Discussion

In this study, the superovulatory response was not significantly different between donor breeds which agrees to previous reports by Batista *et al.* (2016), Degefa *et al.* (2016), and Degefa *et al*. (2018). However, other authors (Purohit *et al*., 2013; Guerreiro *et al*., 2014) showed that *Bos indicus* have an inherently low ovarian follicular population and respond less than the *Taurus* breeds. On the other hand, *Bos indicus* animals are said to be more sensitive to exogenous gonadotropins and respond better to superovulation (Ferreira *et al*., 2014). This variation might indicate the presence of a more important individual variation rather than breed influence when it comes to superovulatory treatment in bovine (Curtis, 2015). The average CL detection was comparable to previous studies for the HF crossbreeds (Ferreira *et al*., 2014; Vieira *et al*., 2014; Tadesse *et al*., 2016) though fewer numbers have also been reported (Hussein *et al*., 2014). Several other studies (Peixoto *et al*., 2007; Vieira *et al*., 2014; Mapletoft *et al*., 2015; Perez *et al*., 2016; Perez *et al*., 2019; Vizoná

et al., 2020), confirmed that the variations in response to superovulation and recovery rate are not only due to breed differences but also the status of donors at the time of superovulation. In line with a report by Degefa *et al*. (2018) on similar animals, the Boran breed showed a comparable ovarian response as the HF*Boran, however, recovery of UFO was much higher in Boran cows compared to the HF*Boran crosses. A likely cause is fertilization failures were reported by several authors (Sartori *et al*., 2010; Rasolomboahanginjatovo *et al*., 2014; Dorice *et al*., 2019) where a higher proportion of non-fertilized oocytes and degenerated embryos have been frequently recovered. Fertilization failure in superovulated cattle is generally more pronounced, averaging approximately 45%, much lower than the present finding. It is mainly associated with poor gamete transport due to hormonal imbalances or suboptimal oocyte quality which is not uncommon in zebu breeds (Dorice *et al*., 2019). Hasler (2012) in his study in Red Angus beef breeds reported a closely similar proportion of UFO and a higher proportion of degenerated embryos in superovulated cows.

The mean number of transferable embryos found in the present study is much higher compared to a previous report by Tadesse *et al*. (2016) from a similar HF*Boran crossbreeds. However, it was smaller than those reported for Nelore breeds of donor cows where local variability was confirmed to be due to farm management and donor age as the main factors (Silva *et al*., 2009). A closer analysis of the effect of the dam-sire combinations in this study confirms the insemination of Boran dam with Boran sire to be the source of the largest proportion of UFOs recovered. This finding is in agreement with Degefa *et al*. (2018) where a higher yield of UFO was recorded in Boran cows inseminated with semen from Boran sire. Probable causes for the high proportion of UFO in Boran cows might be related to the breed itself or the condition of the dam including nutritional status at the time of insemination (Ferreira *et al*., 2014). Sire effects are also related to fertilization ability, semen quality and dose, immunogenetics, and other related factors which might contribute to the variation (Hasler, 2012). However, the use of high-grade imported Boran semen in our study has not significantly altered the incidence of UFOs from Degefa *et al*. (2018) who used both AI and the natural service of indigenous Boran bulls. It is imperative to assume then the likely source of higher incidence of UFO is probably the oocyte quality than the semen. On the other hand, a significantly larger proportion of degenerated embryos were harvested from HF*Boran donor cows in the current study. Hasler (2012) and Trigal *et al*. (2012) reported that the male effect is more evident on the variation of su-

perovulatory outcomes in beef breeds and from using sex-sorted semen. Contrary to this, the crossing of HF*Boran dam with semen from pure HF bulls produced a significantly higher proportion of transferable embryos relative to the Boran dams in all possible combinations. This probably shows the role of the donor dam breed which is also confirmed previously by Degefa *et al*. (2018) though the overall result was comparatively smaller in our study. More yield of freezable embryos was collected than is reported by Vieira *et al*. (2014) while the average number of fertilized embryos from HF*Boran cows is comparable. The HF*Boran donor cows produced a significantly higher number of Grade 1-3 quality embryos compared with the Boran breed in this study. Different kinds of literature (Alarcón *et al*., 2010; Trigal *et al*., 2012; Vieira *et al*., 2014; Tadesse *et al*., 2016; Chinchilla-Vargas *et al*., 2018) suggest the source of these variations to be related to breed, reproductive behaviour, environmental factors or interaction of all.

Conclusions

It can be concluded that the recovery rate of the embryo might be comparable for the two breeds in any combination of the dam and sire we compared. However, a higher incidence of UFO was evident in Boran cows irrespective of the sire breeds while a higher number of degenerated embryos were produced by HF*Boran cows. Further, both the quality of the embryo and the proportion of transferable embryos are associated to breed differences. Interestingly, comparatively more transferable embryos were recovered when semen from pure HF sire was used in both breeds of the dams.

Conflict of interest

The authors declare that they have no conflict of interest

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Bovine trypanosomosis and its vectors: prevalence and control operations in Kellem Wollega, Western Ethiopia

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Abstract

A cross-sectional study was conducted to estimate the prevalence of bovine trypanosomosis and to assess farmers' perception of the disease and its control operations. From October to April 2012, a total of 586 cattle were sampled for the prevalence study. Buffy coat procedure and haematocrit value determination were performed. To capture the fly that was involved in the transmission dynamics, one hundred monopyramidal traps were deployed for 72 hours. A semi-structured questionnaire was conducted to study farmers' perceptions of the diseases and their control operations. Trypanosomal infections were diagnosed in only 8.7 % (51) of animals. The overall prevalence of trypanosome infection in cattle was significantly varied between study districts (33.1% Dale Sadi and 66.9% Dale Wabera). Most infections were due to *Trypanosoma congolense* (81.8%) followed by *T. vivax* (15.6%) and mixed infections (2.6%). The association of hematological value changes and trypanosome infections was profound. The overall Packed Cell Volume (PCV) values of sampled cattle were 25.8%. A significant ($P< 0.05$) variation in PCV values was recorded in infected (20.8%) and non-infected (26.5%) cattle. In the study period, a total of 2055 flies were captured and of which 92% belong to the genus *Glossina* followed by *Stomoxys* and *Tabanids*. Four types of tsetse species (*G. pallidpes, G. m. morsitans*, *G. tachinoides,* and *G. f. fuscipes*) were identified. The questionnaire survey revealed that trypanosomosis is the most important problem for agricultural activity and animal production in the study areas. Farmers are well aware of the problem, means of transmission, and the different control

methods. Integrating tsetse control program with other trypanosomosis control options is recommended.

Keywords: Bovine; Farmer's perception; Prevalence; Trypanosomosis; Vector.

Introduction

Tsetse transmitted trypanosomosis is one of the most devastating diseases of protozoan origin caused by different species of unicellular parasites found in the blood and other tissues of vertebrate hosts. The disease remains the main constraint to livestock production and agricultural development in humid and sub-humid zones of Africa (Swallow, 2000; Abebe, 2005). In Ethiopia, the disease affects a total of 14.8 million heads of cattle, an equivalent number of shoats, 7 million equines, and quite a considerable number of other domestic animals are at risk (Swallow, 2000; Abebe, 2005).

There are considerable variations on reports of the land cover of tsetse infested areas of Ethiopia since the first report appeared by an author (Langridge, 1976) nearly four decades ago where a total area was estimated as 98,000 km^2. The Ministry of Agriculture had estimated the potential area of tsetse infestation to be about 220,000 km^2 (MoARD, 2004). These areas are mainly found in the Southern, Southwestern, and Western parts of the country (Abebe and Jobre, 1996; Abebe, 2005). Many of the areas are the most suitable for livestock and crop production. However, they are virtually devoid of cattle and other domestic livestock (MOA, 1995; PATTEC, 2001; MoARD, 2004; Abebe, 2005). To date, five species of *Glossina* (*G. m. submorsitans, G. pallidipes, G. tachinoides, G. f. fuscipes, and G. longipennis)* have been recorded from Ethiopia. The tsetse flies transmit four species of trypanosomes: *Trypanosoma congolense*, *T. vivax*, *T. brucei brucei,* and *T. rhodesiense* (Abebe, 2005; Tadesse and Tsegaye, 2010).

Due to the presence of large fertile lands, the Dale Sadi and Dale Wabara districts of Kellem Wollega Zone in western Ethiopia comprise settlement areas for people from drought-stricken areas of the country. Most of the settlement areas are tsetse-infested which poses major constraints to livestock keepers to sustain in their new home areas. In the beginning, the settlement schemes have a history of heavy loss of cattle as a result of trypanosomosis mainly due to inadequate veterinary services and lack of tsetse control operations. As a

result of the threat of tsetse flies, a large proportion of the livestock population of the settlement areas was exposed to trypanosomosis.

Although the impact of livestock trypanosomosis is a well-known constraint to livestock production in several tsetse infested areas of Ethiopia, little attention has been paid to the trypanosomosis situation in settlement areas of Birbir valley in western parts of the Oromia region. Furthermore, sufficient information was lacking on the status of the disease prevalence, tsetse fly challenge, and the attitude of farmers on tsetse and trypanosomosis intervention programs being practiced in the study districts. Understanding the epidemiology of the disease will facilitate the choice of suitable control methods and help in planning for development programs in the areas. Therefore, the study helps to estimate the prevalence of trypanosomosis situation, estimate the species composition and distribution of tsetse flies, the perception and knowledge of farmers on the diseases, management of the disease problems, tsetse fly challenge, and the impact of intervention practices in the study areas.

Materials and methods

The study area

The study was conducted in Dale Sadi and Dale Wabera districts of Kellem Wollega zone in Oromia regional state located in Birbir valley (Baro Akobo river system), western Ethiopia (Figure 1). The two districts are located 600-700 km West of Addis Ababa. The agro-climate of the areas alternates with long summer rainfall (June to September) and winter dry season (December to March), with annual rainfall ranging from 1300 to 1600mm. The mean minimum and maximum temperatures are 11.0-15.5°C and 26.1-34°C respectively. The altitude range for Dale Wabera is between 1100 and 1800 m.a.s.l. and that is 1400-2000 meter above sea level (m.a.s.l.) for Dale Sadi. The livelihood of the society largely depends on mixed livestock and crop production. The crops that are commonly cultivated include maize, sorghum, pepper, pulse, and finger millet. Coffee is the dominant one and is the main source of income for society. The dominant livestock population in both Dale Sadi and Dale Wabara districts in the Birbir valleys is cattle followed by goats and sheep where all of them are raised under the traditional management system. Other animals such as equines, poultry, and bees are also bred. In the livestock-crop mixed farming livelihood of the settlement schemes, cattle are used for drought power traction and milk production while sheep and goats, bees, and poultry are

raised both for household consumption and income generation (KWZRADO, 2010).

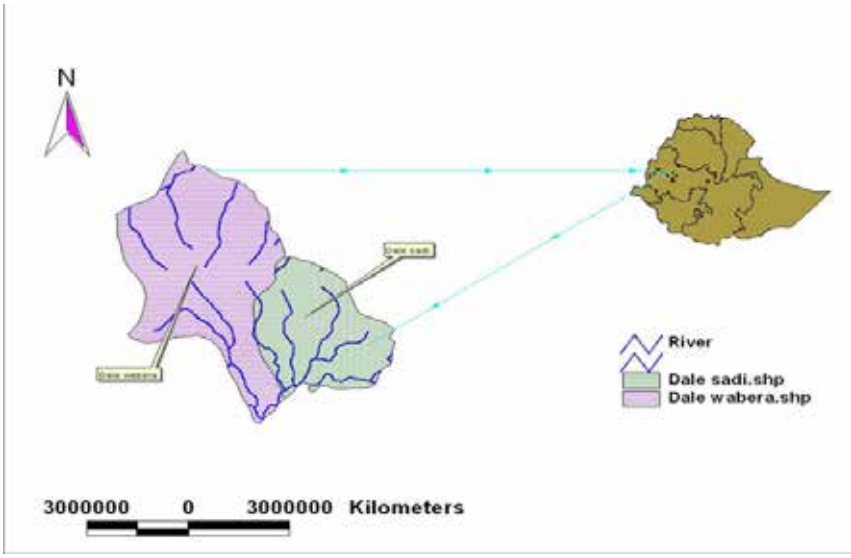


Figure 1: Map of Dale Wabera and Dale Sadi Districts

Study animals

From October to April 2012, a total of 586 cattle were sampled for the prevalence study Indigenous East African zebu cattle kept under traditional extensive husbandry systems with communal herding were used for blood sampling. Animals of all sex, age, and body condition groups were included. The animals were categorized into three age categories, calves (< 1 year) young (1-3 years), and animals that were able to move far distance away from homestead to graze were considered as adults (> 3 years).

Sampling and sample size determination

The sampling was based on combinations of multistage and purposive sampling methods applied according to Thrusfield (2005) and Toma *et al*. (1999). The two districts representing tsetse-infested settlement areas were selected from the Kellem Wollega zone in the Oromia region. Then within each district sampling frame was listed for every peasant association (PA) obtained from district agricultural offices and the PA were selected for sampling pur-

posively based on accessibility and tsetse infestation. Announcements were made for farmers by local agricultural or animal health extension workers and community leaders to bring their cattle for examination. This was followed by systematic random selection of households that have brought their cattle for examination at specified locations among those registered on each sampling day. Similarly, among the cattle presented to the site for examination, animals were selected randomly. To determine sample size an expected prevalence of 50% (to increase the sample size) was taken into consideration and the desired sample size for the study was calculated using the formula given by Thrusfield (2005) at a 95% precision level. Accordingly, the computed sample size was 384 animals. However, 589 animals were sampled to maximize the precision of the sample.

Study-design

A cross-sectional study design was employed to estimate the prevalence, vector density and assess farmers' perception of the disease and its management.

Parasitological and haematological examinations

Blood samples were collected from marginal ear veins using microhematocrit capillary tubes and sealed on one side with cristaseal (Hawskely Ltd.). The capillary tubes were then transferred to a hematocrit centrifuge and spun for 5 min at 1200 revolutions per minute. Before processing the tubes for parasitological examination, measurement of packed cell volume (PCV) was made using a hematocrit reader (Murray *et al.*, 1983). Then, the capillary tube was cut using a diamond-tipped pen 1 millimeter (mm) below the Buffy coat to include the uppermost layers of the red blood cells and 3mm above to include the plasma. The content of the capillary tube was expressed on the slide, homogenized onto a clean glass slide, and covered with a cover slide. The slide was examined under ×40 objective using phase-contrast (Murray *et al.*, 1977) or dark field microscopy (OMAX 40× 200× Binocular, Compound) to examine for the presence/absence of motile trypanosomes. For species identification of trypanosomes, Giemsa-stained thin blood film readings were made according to an established standard procedure to identify the different species of trypanosomes.

Entomological survey

To assess the apparent densities, distributions, and species of tsetse flies and other biting flies, entomological data were collected along with the suitable tsetse habitats. These include livestock grazing areas, watering points, wild game reserve areas, rivers and riparian, savanna grassland, and sub-savanna area of dense riverside forests in the Districts. For the present entomological survey, standard monopyramidal traps developed for tsetse fly catch were used (Drees and Jackman, 1998). The traps were deployed at an interval of about 100-200 meters to assess the fly density. The coordinates of each trap position were recorded with a Global Positioning System (GPS). All the traps were baited uniformly with acetone and cow urine. The poles of traps were greased to prevent fly predators mainly ants. A total of 100 traps were deployed early in the morning and maintained in position for 48 hrs in trapping sites. The cages from these traps were emptied and caught flies were counted, identified morphologically, and sexed for the tsetse fly (Walle and Shearer, 1997).

Questionnaire survey

A semi-structured questionnaire survey was conducted on 105 selected farmers (56 farmers from Dale Wabera and 49 from Dale Sadi district). Community elders and key informants like local PA administrators and agricultural extension workers were involved in the study. They helped in the identification of households within the area that kept livestock. Those farmers that possess cattle are only interviewed. The interviews were conducted in Oromifa and Amharic languages. Mostly the questions are the open-ended type with a limited number of questions having a list of choices. Before the interview, the objective of the research was explained to each participant and full consent of the interviewers was obtained. The questionnaire focused mainly on general livestock production, perception of the presence and management of trypanosomosis in cattle, and impact of tsetse and trypanosomosis control efforts in the area.

Data management and analysis

Data collected were entered into MS Excel to create a database. The edited data were then transferred to the statistical software program called SPSS versions 20 for windows. Descriptive statistics, univariable and multivariable logistic regression, confidence intervals, and analysis of variance were applied in the analysis of the data. The prevalence of bovine trypanosomosis was expressed as the number of parasitaemic animals through Buffy coat microscopy

to the total number of animals examined (Murray *et al*., 1983). Hematological findings were expressed as a percentage of the RBC to the total blood content in percentage as measured by hematocrit reader. To compare trypanosomosis prevalence and the pooled data of mean PCV between aparasitaemic and parasitaemic animals a combination of frequency distribution and independent t-test were used. A logistic regression model was used to test the association and the odds for the degree of severity between the prevalence of trypanosome and the assumed risk factors. The apparent fly density was calculated as the mean number of tsetse and biting flies caught per trap per day. In all cases, a 95% CI was employed to extrapolate sample results to the target population in the study area to assess the dynamics of trypanosomosis and the vectors.

Results

Prevalence of trypanosomosis

A total of 589 blood samples were examined from cattle in settlement schemes of the study districts (Table1).

Table 1: The geographical locations of sampling sites (PA's) within the two districts used for sample collection and the respective number of samples collected

Zone	Districts	Sampling PA	Number of samples	Altitude (m.a.s.l.)	Latitude	Longitude
Kellem Wollega	Dale Sadi	Bikilabirbir	101	1409	N84^{0}75'23	E35^{0}111'20
		Village 14	94	1477	N84^{0}84'46	E35^{0}112'08
Subtotal			195			
	Dale Wabara	Midega	71	1175	N84^{0}14'11	E35^{0}07'18
		Village 8	145	1172	N85^{0}417'93	E35^{0}051'90
		Kombo	100	1537	N85^{0}07'79	E35^{0}066'53
		Chirecha	78	1528	N85^{0}17'78	E35^{0}072'89
Subtotal			394			

Trypanosomal infections were only diagnosed in 51 (8.7%) animals. The difference in the overall prevalence of trypanosomosis between the two districts was

significant (P<0.05). Most infections were due to *T. congolense* followed by *T. vivax* and mixed infections (2.6%) (Table2).

Table 2: The prevalence of trypanosomes against the risk factors assumed

Risk factors	No. examined	Positive	negative	% Positive	%Tc	%Tv	% Mixed
Districts							
Dale Sadi	195	13	182	6.7	84.6	15.4	0
Dale Wabara	394	29	365	8.7	78.9	15.8	5.3
PA's							
Bikilabirbir	101	12	79	28.6	19.6	5.8	0
Village14	94	1	93	2.4	2	0	0
Midega	71	8	61	19.1	29.4	0	0
Village8	145	17	138	40.5	25.5	7.4	0
Kombo	100	3	97	7.1	2	2	3.9
Chirecha	78	1	77	2.4	2	0	0
Age							
≤3	173	16	157	9.25	62.5	37.5	0
>3	416	35	381	8.4	91.4	5.7	2.9
Sex							
male	338	39	299	11.54	84.6	15.4	0
female	251	12	239	4.78	75	16.7	8.3
BCS							
good	143	9	134	6.3	100	0	0
medium	366	30	336	8.2	80	16.7	3.3
poor	80	12	68	15	75	25	0

PA= Peasant Associations

The risk of infection in the higher altitude was relatively lower than that of lower altitude where *T. congolense* had shown a statistically significant difference (P<0.05). The prevalence in males (11.54%) was relatively higher than that of the female animals (4.78%) and the difference was significant (P< 0.05). The poor (15%) body conditioned animals were shown significant (P< 0.05) variation in trypanosome prevalence with reference to good (6.3%) and medium body conditioned cattle (8.2%) (Table3).

Table 3: The logistic regression output of the overall prevalence of bovine trypanosomosis in different factors considered

Factors	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]
districts	0.11	0.12	-2.07	0.038	[0.01, 0.89]
PA's					
Village14	0.07	.077412	2.47	0.014	[0.01 0.58]
Midega	6.37	6.934121	1.70	0.089	[0.75 53.8]
Village8	7.30	7.670551	1.89	0.059	[0.93 57.2]
Kombo	1.72	2.013836	0.46	0.644	[0.17 17.1]
Chirecha	(omitted)				
Sex	0.39	0.1559274	-2.36	0.018	[0.18 .85]
Age	1.04	.4153521	0.11	0.915	[0.48 2.28]
Body condition					
Medium	1.97	.9018469	1.48	0.139	[0.80 4.83]
Poor	3.92	2.094172	2.55	0.011	[1.37 11.17]

NB. Bikilabirbir PA and Good body conditions were used as a reference

Hematological findings

The study revealed that the PCV values of cattle were generally low in both districts (Fig1). However, there was a significant difference in the mean PCV of the two districts (Dale Sadi, 26.5% CI=26-27.1%) Dale Wabara (20.0% CI= 18.0- 22.5%). The PCV of animals within the normal range (PCV≥24) was significantly higher (27.6%, 95% CI= 27.4-28.0%) than those with lower PCV (PCV<24) (19.8%, CI= 19.4-20.2%) (Table4).

The PCV of cattle was 20.8% (95% CI= 19.5- 22.1%) for infected animals and 26.0% (CI=25.6- 26.3%) for non infected animals. The difference between the means of the two groups was 5.2% and it was statistically significant (Table4).

Table 4: The result for PCV in infected and noninfected animals and districts

PCV		Obs	Mean (%)	SE	SD	[95% CI]	*P* value
Noninfected		538	26.0	0.18	4.2	[25.6 26.3]	≤0.0001
infected		51	20.8	0.64	4.6	[19.5 22.1]	
PCV status	PCV≥24	430	27.7	0.14	2.9	[27.4 27.9]	≤0.0001
	PCV<24	159	19.8	0.20	2.5	[19.4 20.2]	
District	Dale Sadi	195	26.5	0.29	4.0	[26.0 27.1]	0.0001
	Dale Wabara	394	25.0	0.23	4.6	[24.6 25.5]	

Obs=Observation SE= Standard Error SD=Standard Deviation CI=Confidence Interval

Entomological survey

A total of 2055 flies were caught using 100 monopyramidal traps deployed at different sites of the study areas. Out of which 1890 (92 %) belongs to tsetse flies of the genus Glossina (tsetse fly) followed by Stomoxs 159 (7.7 %) and Tabanids 6 (0.3 %). Four tsetse species were identified (*G. pallidpes, G. m. morsitans*, *G. tachinoides,* and *G. f. fuscipes).* The overall apparent density of tsetse flies was 18.9 F/D/T. Of which 9.1 F/D/T was from Dale Wabera and 9.8 F/D/T was from Dale Sadi.

The apparent density of tsetse flies in Dale Wabera was: *G. pallidpes* 1.8 fly/ trap/day, *G. m. morsitans* 0.9 fly/trap/day, *G. tachinoides* 0.9, and *G. f. fuscipes* 1.6 fly/trap/day whereas the apparent density of tsetse flies in Dale Sadi district was *G. pallidipes* 5.9 fly/trap/day/ *G. m. morsitans* 6.2 fly/trap/day, *G. tachinoides* 0.8 fly/trap/day and *G. f. fuscipes* 1.1 fly/trap/day. Generally, Savanna species appear to be dominant in both districts. Altitude had a significant effect on the apparent density of tsetse in the Dale Wabera district which is found relatively in higher altitudes. The survey revealed that 93.3% of the total fly catch was from a low altitude whereas, 6.7% of the total catch is from a higher altitude. There was a significant difference for *T. congolense* prevalence between the low altitude (1172-1190 masl) and higher altitude areas (1431-1488 masl) (P<0.05). However, this was not the case for *T. vivax* (Table5).

Table 5: The apparent density and tribes of flies caught during the study period

Trap type	Altitude (m.a.s.l)	Site	Tsetse flies								Sto moxys	Taba nus	haem atop ota	FTD
			G.m. submor sitans		G. palli dipes		G. tachin oides		G. fusc ipes					
			M	F	M	F	M	F	M	F				
Monopyr amidal	1293	D. sadi	190	155	145	185	22	23	34	26	53	0	0	28
Monopyr amidal	1393	D. Wab ara	61	68	68	195	58	74	95	130	239	6	2	9

Questionnaire survey

Livestock use and management

Cattle, sheep, goats, and equines are common livestock species kept by farmers in the study districts. All of the interviewed people were well responded to the prepared questionnaire format. The majority of the resident communities of the two districts have lived in the area for more than five years (Fig.2) and were able to describe the animal disease situation of the districts effectively.

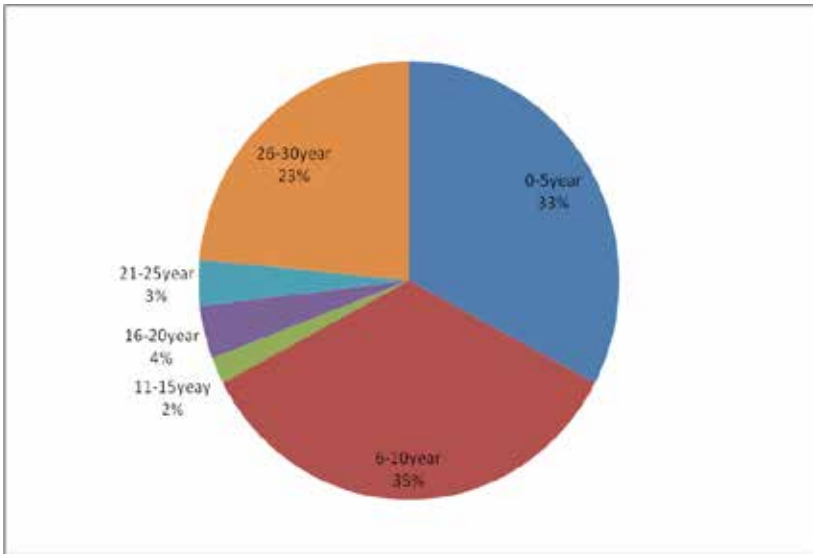


Figure 2: The percentage of farmers describing the time of starting farm activities in the study area

The farmers in the area ascertained that they keep livestock mainly for agricultural activities. Accordingly, above 99% of the respondents' livelihood depends on a mixed crop-livestock production system. The most common livestock species kept in the area based on interview response are cattle (53.3%) followed by cattle and small ruminants (34.3%) and others (12.4%). Most farmers kept the animals mainly for drought power and milk (Table 6). According to the respondents, livestock feed shortage would have been observed during the dry season when almost the grazing land is overgrazed. Better feed is available during the late and early rainy season (July-September) when the pasture is green and the feed biomass is relatively high. The main constraint of livestock in the area as mentioned by 100% of the respondents were animal diseases but the shortage of grazing land and less availability of veterinary drugs were also mentioned.

Table 6: The purpose of animal breeding in the study area

Purpose of breeding	frequency	%
Drought power	38	36.2
Milk	3	2.9
Milk and drought power	62	59.1
Fattening	1	0.95
Fattening and drought power	1	0.95

Constraints of livestock production in the study areas

Based on the interview result, the main livestock diseases in the order of importance are trypanosomosis, pasteurellosis, ectoparasites, blackleg, Lumpy skin disease, and Anthrax as shown in Figure 3 below.

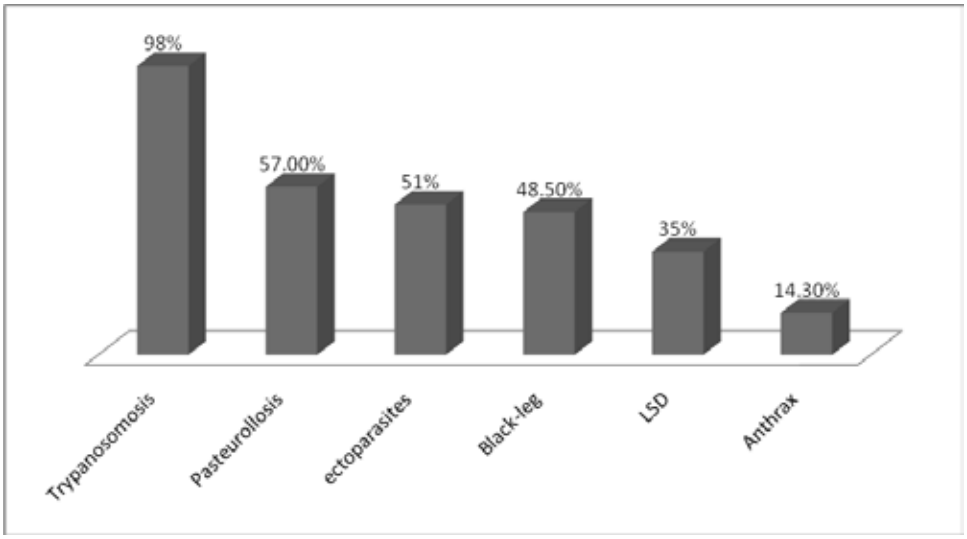


Figure 3: Diseases in the area in the order of their importance

All the respondents think that trypanosomosis is a major constraint and serious threat to livestock production and utilization of the fertile arable land. It is known by all locally as "Ghendi" and mostly affects cattle (100% responders). Clinical signs observed by the farmers: emaciation, ruffle hair coat, constipation, change in color coat, lack of appetite, necrosis and cut-off tail, weakness, eating soil, dry feces, grinding of teeth, and finally death. Most farmers (91.4%) said that the incidence of disease (trypanosomosis) is high in the rainy season but infection occurs throughout the year. Out of the 105 interviewed farmers, 14.4%, 48.6 and 29.5% of respondents know the disease for the last 5 years, last 10 years, and last 15-20 years respectively. Almost all (99%) of them also affirmed that the situation of the disease is now improving as described by the reduction of its impacts on livestock.

Means of transmission

Out of the 105 respondents, 97% of them knew flies as transmitters of trypanosomosis to their cattle (Table 2). When asked to specify the fly type, they call it "ghendi zinb/Tisisa ghandi) and described it as having long legs, large size, brown color biting their animals when the animals move to the forest and savanna vegetation types their equivalent might be tsetse fly other flies bigger in size, cause bleeding at the point of bite locally call as "*Warana*" their equiva-

lent are tabanids and stomoxyes flies. Most believe that the fly population is higher in the rainy (58.1%) and following after the rainy (41.9%) season. All respondents believe that fly density was high around water bodies and valleys.

Table 7: Respondent's perception of the source of trypanosomosis

Source	No of respondents	%
Tsetse flies	102	97.14
Infected grass and water	1	0.95
Others	2	1.91

Trypanosomosis and vector control

All respondents acknowledged that tsetse and trypanosomosis control operations have been there for a long time. They (88.6%) also added that control of trypanosomosis is carried out mainly through a combination of treatment of affected animals with trypanocidal drugs and tsetse flies population control in the area**.** Only 7.6% and 3.8% of them believe that fly control alone and chemotherapy alone was the control option available respectively.

About 88 % of respondents get trypanocidal drugs from government veterinary clinics, while others obtain the drugs from private clinics (8.6%) and illegal sources (3.8%). Most of the farmers (88.6%) send their animals to veterinary clinics and treat them by animal health personnel while 11.4% of them responded to treat by themselves/family members at home. Diminazene aceturate (Berlin) and Isometamidium chloride (Samorin) are the commonly used drugs to treat trypanosomosis (Figure 4). Information gathered from the respondent farmers and animal health personnel emphasized it is a common practice to monthly send animals to animal health posts for trypanocidal treatment irrespective of the presence or absence of disease and this trend is locally called "*Worawi*' (monthly) in the community.

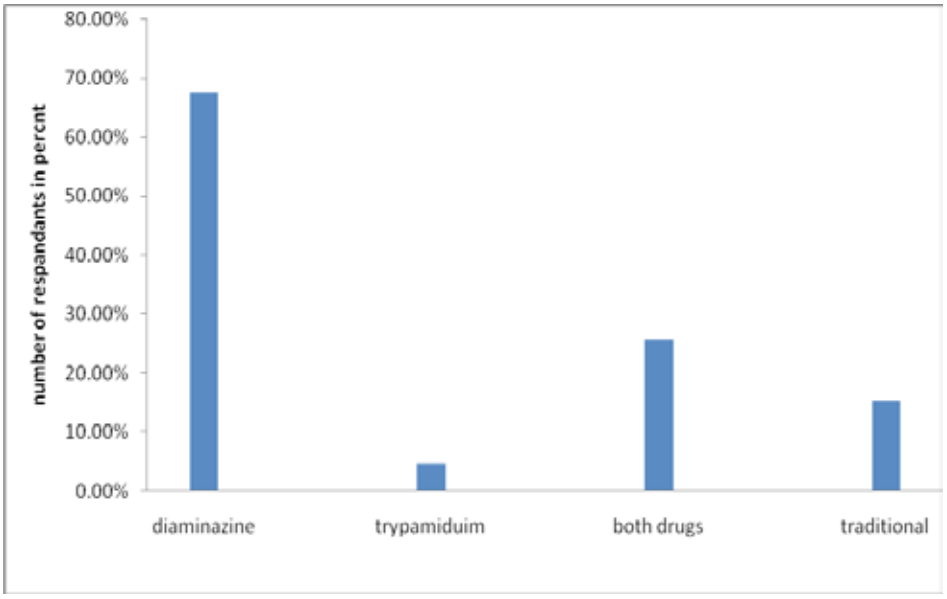


Figure 4: Most common trypanocidal drugs farmers used to treat their animals.

As the respondents mentioned, the National Tsetse and Trypanosomosis Investigation and Control Center (NTTICC, 1996) has been conducting surveillance, updating distribution maps, undertaking trypanocidal drugs sensitivity tests, and implementing control schemes through the integration of pour-on with odor baited and insecticide-impregnated target technologies for the last few years in the study area. All the interviewed farmers also indicated that this control program is the most essential and it brings good results in their area in terms of fly population reduction and the activity is participatory (training of community on target deployment technique, about the impact of drug resistance and the vectors of the disease, etc.). As information revealed by farmers, all techniques employed for the control program are requiring their participation so that, about 99% of respondents said that they have been actively participating in the deploying target in site, extensively training and giving information to others farmers and supporting the technicians accordingly. After the launching of the control program, 99% of the farmers said that the incidence of the disease is getting better compared with previous periods. They describe the changes in terms of an increasing number of their animals, increasing working efficiency, increasing milk yield, reduction in the cost of treatment, increasing

selling prices, improvement of animal body condition, and decreasing mortality rate. The changes in the livestock health were so significant that all respondent farmers of the two woredas they want to continue and enhance their active participation in the control of tsetse and trypanosomosis by volunteering money and labor and protection of properties (traps, targets, etc. in the field against theft and fire) to ensure the sustainability of the program.

Discussion

The study revealed the presence of bovine trypanosomosis in the settlement schemes of the Biribir valley. This has shown a 7.1% overall prevalence of trypanosomosis and dominance of *T. congolense* (81.8%) over *T. vivax* (15.6%). The result was relatively lower than the previous report (8.5%) of Senbata (2010) in Southwestern Ethiopia but higher than the reports (2.4%) of Tadesse and Tsegaye (2010) in Western Ethiopia. However, it was lower in contrast to the reports of other authors elsewhere in tsetse infested areas of the country (Rowlands *et al.*, 1995; Abebe and Jobre, 1996; Yohannes *et al.*, 2000; Tefera, 2008; Bekele *et al.*, 2010; Biyazen *et al.*, 2014). Accordingly, Terefa (2008) and Bekele *et al.* (2010) reported a prevalence of 15.1% and 21% in tsetse-infested Western and Southern Rift valley areas, respectively. Similarly, a higher prevalence of 17.2% and 17.5% were reported in tsetse-infested Metekel and upper Didessa areas (Yohannes *et al.*, 2000).

This study has clearly shown the relatively lower prevalence of trypanosomosis in the settlement schemes in contrast to the findings of other tsetse-infested areas. This disparity emanates from many factors that explain the lower prevalence of trypanosomosis observed in this study area. The study was conducted during the early dry period and this might have affected the vector population and subsequently the trypanosome prevalence (Leak *et al.*, 1993). Furthermore, the expansion of veterinary services up to peasant association, deforestation for crop cultivation (settlement), frequent usage of trypanocidal drugs and the husbandry practices on animals, and presence of trypanosomosis and fly control program in the area might have contributed positively to the observed lower prevalence.

This finding of the trypanosome species is in accordance with the reports of other authors (Rowlands *et al.*, 1995; Abebe and Jobre, 1996; Yohannes *et al.,*

2000; Tewelde, 2001; Tefera, 2008; Bekele *et al.*, 2010; Tadese and Tsegaye, 2010). This is in general agreement about *T. congolense* as the dominant trypanosome species in tsetse-infested regions of Ethiopia. Abebayehu and Biniam (2010) from Southwestern Ethiopia and Bekele *et al*. (2010) from the Southern rift valley reported *T. congolense* as dominant species. They reported 36.4% and 70% *T. congolense* and 18.2% and 20% *T. vivax* infection, respectively. In contrary to the prevalence of *T. congolense* in tsetse-infested areas of Ethiopia, *T. vivax* is the only species with a major health problem to cattle in non-tsetse-infested areas of Ethiopia (Cherinet *et al.,* 2006; Misganaw, 2006; Sinshaw *et al.*, 2006).

The predominance of *T. congolense* infection in cattle of tsetse-infested areas may be due to the high tendency of the parasite to develop new strains capable of escaping the host immune response. The host has the potential to the development of better immune response against *T. vivax* compared to *T. congolense* infections (Leak, 1999). In the present study mixed infection is not significant in contrast to the reports from other areas but that does not mean to rule out its importance.

The overall prevalence of trypanosomosis between the districts was significant ($P<0.05$) where the disease is 11% more likely to occur in Dale Wabara than in Dale Sadi. The study involved four PAs from Dale Wabara and two from Dale Sadi with relatively higher prevalence being reported in Village 8 (40.8%) and the lowest being in Village 14 (2.4%) and Chirecha (2.4%). Village 8 is the lowland area with an altitude of 1172 m.a.s.l while Village 14 and Chirecha PAs are found relatively at higher altitudes with 1477 and 1528 m.a.s.l. respectively. Altitude difference has been identified as one of the main factors for variation in trypanosome prevalence. This variation between districts and villages in the study could be attributed to the presence of suitable habitat for the vectors which results in high fly density. Variation in tsetse density appeared to be the main factor for variation in the prevalence of trypanosomosis. The tsetse and other biting fly populations and types present in each locality, which are dependent on microclimate, animal herd density, the distance between herds, and other various factors (Leak *et al.*, 1993).

Although the relatively lower prevalence was recorded in adults (8.4%) than the young animals (9.3%), the infection with trypanosomes seems unrelated to the age of the animals (P>0.05). On the contrary, other reports show that trypanosomosis is a chronic disease increase with the age of animals and its infection is usually higher in adult than in young animals (Rowlands *et al.,* 1993; Rowlands *et al.,* 1995; McDermott *et al.*, 2003;). Similarly, the attraction of tsetse flies more by the odor of older animals has been reported (Torr, 1995; Torr *et al.*, 2006; Torr and Mangwiro, 2000).

The prevalence in males (11.54%) was higher than the females (4.78%) and the difference was significant (P>0.05). Different authors were also reported a higher infection rate in male animals than females (Muturi, 1999; Yohannes *et al*., 2000; Tewelde, 2001) and the possible suggestion could be the use of males more for drought purposes traveling long distances to an area of tsetse challenge for grazing and its use in plough as draft power which causes stress so the animal becomes susceptible to trypanosomosis.

Trypanosomosis is a disease that is usually associated with poor body condition and emaciation. In the present study, animals with poor body conditions were more associated with the disease as compared with animals with medium and good body conditions and the difference was significant (P<0.05). However, it does not mean that trypanosomosis is necessarily associated with poor body condition or emaciation. Other factors such as helminthiasis and feed shortage may be implicated as the cause of the deterioration of the condition (Parkins and Holmes, 1989).

The trypanosome-infected cattle were shown profound changes in hematological values of infected animals. The overall PCV value of cattle was 25.8%. Of which 20.8% was due to the presence of trypanosomes infection. The difference in PCV of infected and noninfected animals was significant (P<0.05). However, a considerable number of animals (76.7 %) were anemic (PCV<24%) without having trypanosomes infection. Some animals (2.5%) were positive to trypanosomosis but their PCV was normal (PCV≥24%). This may suggest the presence of other anemia-causing factors and on the other hand individual variability in the maintenance of normal PCV in infected animals. Trypanosomosis is also known to causes a reduction in weight gain and productivity (Rowlands *et al*., 2001). However, the difference in mean PCV value between infected and non-

infected animals indicates that trypanosomosis reduces the PCV values in infected animals (Bekele *et al*., 2010).

The damage of tissue due to trypanosomosis is probably multifactorial in etiology, but the underlying feature is progressive anemia throughout the course of the disease. The case of anemia is due to hemolysis caused primarily by erythrophagocytosis due to simulation and expansion of the mononuclear phagocytosis system (Seifert, 1996). Taylor (1998) indicated that anemia persists during the chronic stages of infection when parasitaemia is generally quite low, probably because different mechanisms are involved in its genesis during the acute and chronic stages of infection. This suggests that control of parasitaemia and control of anemia is unrelated in the chronic phase when immune infections are depressed and anemia is sustained through dyserythropoiesis.

Monopyramidal trap was the only trap type used from a variety of traps that have been designed to catch different species of tsetse and other biting flies because of its availability. The apparent density of tsetse flies was about 18.9 flies per trap per day. A total of 2055 flies were caught of which 92 % belong to the genus *Glossina* (tsetse flies) the followed by Stomoxs and Tabanids. Four types of tsetse species (*G. pallidpes, G. m. morsitans*, *G. tachinoides,* and *G. f. fuscipes*) have been identified. The apparent density of tsetse flies recorded significantly differed between the two districts. Similarly, other researches support the present finding from the Gawo District of West Oromia (Terefa, 2008). However, Senbata (2010) has reported higher tsetse density from the same study area and Hawa Gelan Districts. In contrast to the present finding, Tadesse and Tsegaye (2010) reported lower apparent densities of tsetse flies in Southwestern Ethiopia.

The density of tsetse flies within and between districts might be affected by the number of traps deployed, the site of trap deployment, and the months of the study period. Moreover, the density is affected by the favorable environment such as enough moisture, vegetation growth, and suitable habitat. The fly species identified also agrees with the reports of Terefa (2008) and Senbata (2010). *G. pallidipes* covered a vast area with high apparent densities. This could be because this species inhabits a wide range of vegetation types that are available in the area. While *G. m. morsitans* was found to be almost restricted to savannah vegetation and thus its distribution is highly dependent on the season and vegetation coverage (IAEA, 2004).

G. f. fuscipes and *G. tachnoides* were distributed along the large river of Birbir and its tributaries like Keto, Chabal, Hindina, Kile, and others, which cross the study areas and thus provide a conducive environment. The sex ratio of flies was biased towards females. This is in agreement with the report of other authors (Mohamed-Ahmed *et al*., 1989; Msangi 1999; Leak 1999) where female flies comprise 70-80% of the mean tsetse fly population.

The result of the questionnaire survey revealed that trypanosomosis is the most important problem for agricultural activity and animal production in the study areas. Above 99% of farmers' livelihood depends on a mixed crop-livestock farming system. The majority of livestock reared in the area were cattle followed by small ruminants and equines. The livestock disease, shortage of grazing land, and scarcity of modern veterinary services were the challenges for the development of the area. Several other works demonstrated the same constraints elsewhere in Ethiopia (Dangnachew *et al*., 2005).

The disease trypanosomosis was reported to be the most and the first important livestock constraint limiting the overall agricultural activity and livestock productivity by almost 100% of interviewed people. Similar findings were reported by Tewelde (2001) and Yohannes *et al.* (2000). The incidence of the disease reaches its peak during and after rainy seasons while the infection is persistent throughout the year in the current study area. However, Yohannes *et al*. (2000) in Metekel and Tewelde (2001) in west Ethiopia reported that the incidence of trypanosomosis was high throughout the year.

Farmers acknowledged that tsetse and trypanosomosis control program is present in their area and the activity is participatory. They also added that the situation is improving since the introduction of the intervention program through fly control by pour-on insecticide spray and fly trapping. Observations in other areas indicated that tsetse fly control schemes around Arbaminch Zuria have significantly reduced the prevalence of trypanosomosis and fly density followed by a reduction in calf mortality (Gechere *et al*., 2012). However, due to the long history of the disease in the area, they developed the habit of frequently treating their animals with chemotherapeutics such as Diminazine aceturate and Isometamidium chloride. A similar finding was reported about the drugs used by previous researchers in many parts of the country (Yohannes *et al*., 2000; Seyoum *et al*., 2013). According to Van den Bossche *et al. (*2000), the majority of farmers prefer to use Diminazine aceturate than Iso-

metamidium chloride. The study also supported this conclusion in that majority of the respondents affirmed that they use Diminazine may be due to its fast curative response. While the observation that the majority of the respondents use veterinary services for their source of trypanocidal drugs and injections is encouraging, a proportion of them are still using drugs from illegal sources and administering them on their own. This casts suspicion on the development of drug resistance in the area. Similar findings were reported by Seyoum *et al.* (2013) at Gojeb and Baro-Akobo River Basins.

Conclusions

The results from the study demonstrated the presence of trypanosomosis as the major health problem of livestock in the settlement schemes. The most widely distributed and dominant species of trypanosome was *T. congolense.* Four more common and important tsetse flies species were identified. A significant reduction in the level of PCV was observed associated with the disease. Farmers are well aware of the problem, means of transmission, and the different control methods. The problem can be solved by integrating tsetse control with other trypanosomosis control options like the use of effective drugs for the treatment of the diseases. Continuous community awareness creation should be done about drug resistance and the use of veterinary services as appropriate sources and administration of the drugs.

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Enzymatic and fecundity Evaluation of *Fasciola hepatica* exposed to different doses of ɣ- irradiation in Ethiopian sheep

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Abstract

The upshot of ɣ-irradiated *Fasciola hepatica* infection on the activity of plasma glutamate dehydrogenase (GLDH) and ɣ-glutamyl transpeptidase (GGT) was evaluated in 36 sheep infected with a single dose of 30, 60, 120, and 240 grays and those kept as negative and positive control over 17 weeks. During this period, serum and faecal samples, as well as body weight gains, were taken at weekly intervals. Furthermore, the effects of the irradiation dose (500) for oral vaccination and on the recovery of adult flukes were assessed following primary infection. Eggs were first detected in the faeces of infected sheep on week 8 post-infection. The parasite viability was severely affected by doses of ɣ-irradiation of 120 Gy or 240 Gy. In the aforementioned doses, relatively low numbers of mature flukes of about 60 (17.1%) and 38 (10.8%) were recovered than the control group, respectively. The sensitized lambs also showed less hepatic damage compared with the controls as indicated by lower levels of the serum enzyme glutamate dehydrogenase and ɣ- glutamyl transferase significant body weight loss was observed between weeks 6 and 8 post-infection followed by a steady increase of the mean weight of infected animals across time. In conclusion, vaccination of sheep with ɣ irradiated metacercariae of *F. hepatica* appeared to affect the number and development of the fluke population resulting in reduced hepatic damage during migration, reduced fecundity after patency, as measured by worm and egg counts, levels of serum glutamate dehydrogenase and ɣ- glutamyl transferase.

Keywords: Gamma irradiation; *F. hepatica*; immune response; liver damage; worm and egg recovery; body weight changes.

Introduction

Fasciola hepatica is a trematode parasite with a global distribution, which is responsible for considerable disease and production losses. The commonly known species of *Fasciola* in tropical and subtropical countries is *Fasciola gigantica* whereas *Fasciola hepatica is* found in countries with cold and temperate climates where both species overlapping in subtropical areas (Urquhart *et al*., 1996; Yilma and Malone, 1998; Mas-coma *et al.*, 1999; 2005; Tadesse *et al.*, 2019a). In Ethiopia, *F. hepatica* is mostly associated with disease in sheep, cattle, and goats (Yilma and Malone, 1998; Biffa *et al.*, 2006; Tadesse *et al.*, 2019a, b).

The findings of the abattoir study are inadequate to reveal information regarding the development of the disease in live animals. However, data gathered is useful to disclose the cross-sectional studies (prevalence's, seasonal variations), liver damages, fluke examinations and measurements, assessment of liver fluke burden, and economic importance of the disease (Yilma and Malone, 1998; Biffa *et al.*, 2006; Tadesse *et al.,* 2019a, b).

The presence of eggs in feces is the best way to know if a host is harboring flukes in the live animal. False positives may occur due to the retention of eggs in the gall bladder for at least 2 weeks after successful treatment. Furthermore, it has shown daily variation in egg counts and a maximum rate of egg recovery of only 45-60% (Flanagan *et al.*, 2011; Rojo-Vázquez *et al.*, 2012; Sargison *et al.*, 2016).

Irradiation has been successful in the attenuation of the infective stage of the parasites for use as vaccines against a number of parasites including *Fasciola spp*. Some studies had revealed that the optimal protection was found to be dependent on the dose of irradiation, the number of immunizing cercariae, and the number and time course of immunizations (Minard *et al*., 1978). The evolution of hepatic enzymes during the infection is shown to be important in assessing the level of liver damage by infective flukes. Most work on methods using blood parameters has focused mainly on the determination of enzymes

particularly aspartate aminotransferase (AST) and gamma- glutamyltranspeptidase (GGT) (Simesen *et al*., 1973; Vyckoff and Bradley, 1985; Urquhart *et al.,* 1996; Taylor *et al.*, 2007).

Recovered liver fluke length and width, the total mass of recovered flukes, and liver damage are the major factors often used to indicate the protection against the severity of parasite infection (Valero *et al*., 2002; Valero *et al*., 2006). A detailed study to generate data on all aspects of the host-parasite relationship is still required to better understand the disease dynamics and identify ideal irradiation doses for experimental vaccination trials. Therefore, this study aims to evaluate the enzyme profiles (GLDH and GGT), the enumeration and fecundity assessment with egg counts of recovered flukes as well as assessment of liver and body weight changes in sheep ɣ- irradiated by different doses of *Fasciola hepatica* metacercariae for its vaccination potential.

Materials and methods

Study area: The infective metacercariae of *Fasciola hepatica* used in the experimental sheep have been obtained from artificially infected *Galba truncatula* snails collected from water at Angolela river and villages situated in fasciola endemic areas around Debre Berhan city. The city is located in the Semien Shewa Zone of the Amhara Region, about 120 kilometers northeast of Addis Ababa on the paved highway to Dessie. It has a latitude of 9°41′N and longitude of 39°32′E and an elevation of 2,840 meters. The experiment was conducted at Aklilu lemma Institute of Pathobiology.

Fasciola hepatica infective metacercariae

F. hepatica metacercariae were produced in laboratory reared snails of *Galba truncatula* from river Angolela. The viability of the metacercariae of *Fasciola hepatica* was checked by stimulation of the metacercariae by artificial digestion before dosing of sheep (Wikerhause, 1960). The experiment consists of 36 six-month-old local breed sheep divided into six groups of animals each. The first four groups were vaccinated with 500 *F. hepatica* metacercariae per os with an increasing level of irradiation dose. The fifth and sixth groups were served as infected control and negative control, respectively. The irradiation doses used were 30, 60, 120, and 240 grays for groups I, II, III, and IV, respectively (see table 1). The gamma irradiation process uses Cobalt 60 to attenuate

the metacercariae of *F. hepatica*, which has been widely used by SIT (Sterile Insect Technique) project located in Kaliti, Addis Ababa. The 500 metacercariae were separately counted under a dissecting microscope. Administration of the doses was done by giving in a gelatin capsule as described in (Boray, 1983) and (Wikerhause, 1960). At the time of infection, metacercariae were 2 months old and had 82% viability. It was assessed by using the criteria of works done that depict the motility of the worms and clear observation of the excretory granules in some cysts under dissecting microscope.

Table 1. Details of experimental groups exposed to various levels of irradiation doses

Group	Number of sheep	Number of parasites (Metacercarae)	Irradiation dose (Killo radiance or Gray)
I	6	500	3 (30)
II	6	500	6 (60)
III	6	500	12 (120)
IV	6	500	24 (240)
Infected Control (PC)	6	500	-
Non-Vaccinated	6	-	-

Study animals

The animals used in this experiment were the local breed of sheep. They were purchased from the market in the Gojo district in central Ethiopia. Gojo is situated in the West Shewa zone in the Oromiya region of Ethiopia with geographical coordinates of 9° 16' 0" North, 38° 5' 0" East, and with an altitude of 2905 meters above sea level. The sheep were brought to the market from the surrounding areas of Gojo town. The area was supposed to be free or pose a very low risk for fasciola species. The sheep breed was the Arsi Bale type which has a wider distribution in the central and southern parts of the country and is susceptible to fasciola species (Alemu and Merkel, 2009).

Study design and sampling

The study was a randomized controlled experiment where sheep were selected based on approximate similarity in estimated age, body weight, and sex (all male). The sheep were matched and randomly allocated into groups consist-

ing of six animals each to begin the experiment. Upon arrival, all the sheep have been treated with Triclabendazole (Fasinex, 10-12 mg/kg) to preclude any fluke infection and Oxytetracycline (10mg/kg) to control opportunistic bacterial diseases. All the animals were kept for about a month before the commencement of the actual experiment. Animals were tested for the presence of internal parasites and fasciola species before the beginning of the experiment using simple floatation and sedimentation methods, respectively. These tests have been done throughout the adaptation period. The sheep were maintained with concentrate and well-dried hay free of parasites. Each sheep were provided with 25gms of concentrate (75% wheat bran and 25% Nug cake) and with hay and water provided ad libitum. Sheep were weighed twice before and every other week after the commencement of the experiment. The body and liver weight of each animal was measured using a standard weighing scale at a weekly interval. Each liver was placed in a large basin after evisceration for subsequent weighing. The changes in body weight gain and loss recorded in different groups were compared to determine the effect of vaccinations. Blood samples for biochemical analysis were collected from the external jugular vein into marked vacuum tubes and transported at +4 °C to the laboratories every week starting at week one till the end of the experiment. The serum was separated from blood samples and collected into Eppendorf tubes to be stored at -20°C before analysis. The blood tubes were centrifuged at 3000 rpm for 10 minutes for serum separation.

The faecal sample was examined at the weekly interval after 8 weeks of the experiment. For the estimation of prevalence fresh faeces were randomly collected regularly, directly from the rectum of all animals, and transported to the laboratory in an airtight condition. The specimens were then subjected to qualitative and quantitative coproscopic examination for the presence of characteristic *Fasciola* eggs and egg counts by direct sedimentation technique employing a standard procedure (Urquhart *et al*., 1996). At the end of the experiment, a postmortem examination was carried out on all animals. The bile ducts and liver parenchyma was examined and the number and size of adult flukes inside were observed and counted.

Spectrophotometric analytical methods

Due to the poor stability of enzymes, the serum samples were analyzed within 6 hours after collection. The activity of liver enzymes γ-glutamyl transferase (GGT) and glutamate dehydrogenase (GLDH) were conducted spectrophotometrically to assess liver damage using commercial kits (Chemical analyser) and adjusted to 37ºC. The levels were expressed as international units per liter (IU/L). The enzymes used are a product of Bio vision Inc.

Statistical analysis

The data collected from each group (number and size of parasites, enzyme parameters) were compared by two-way analysis of variance (ANOVA), followed by Tukey post hoc test for differences between the means of control and experimental samples at each time interval.

Results

Dynamics of hepatic enzymes

The dynamics of hepatic enzymes during the experimental period on sheep γ-irradiated by metacercariae of *F. hepatica* was shown in Figures 1 and 2 below. Animals exposed were responded well to the infection as shown by the evolution of hepatic and gall bladder enzymes. The enzyme level varies with the dose of γ-irradiation and non-sensitized groups of animals. Generally, irrespective of immunization, all the infected animals exhibited increasing trends in the GLDH and GGT levels from week 2 onward post-infection. In all cases, the uninfected control sheep (NC) maintained a low profile of enzymes that were well within the normal ranges of each enzyme (GLDH= 1-12; GGT= 34-100 IU) in sheep.

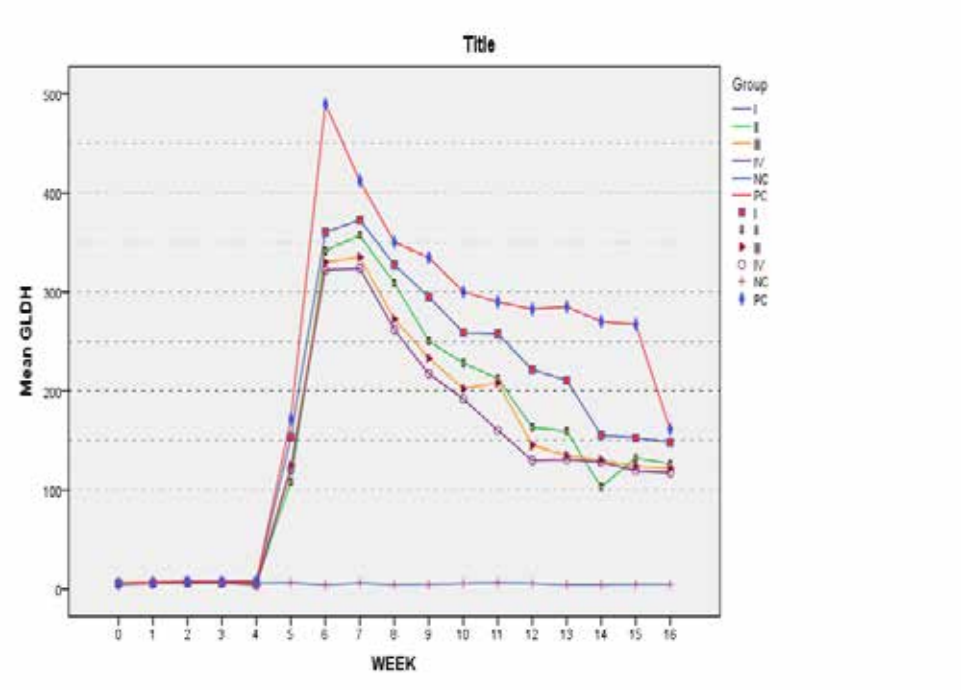


Fig 1. The weekly dynamics of GLDH during the experimental period in sheep

The mean GLDH level of positive control sheep (489.67±16 IU) was significantly (P <0.05) higher compared to the other vaccinated groups at GI (360±9 IU), GII (356.6±8 IU), GIII (335.3±8 IU), and GIV (324.4±12). The comparative progressive decline in the mean levels of serum GLDH of the infected control sheep observed between weeks 6 and 16 was in line with the decline observed in all other treatments.

All vaccinated groups had maintained the serum GLDH level greater than 100 IU at week 16 post-infection. However, sheep that were dosed with 30 Gy (minimum γ-irradiation dose used in the present experiment) had shown significantly higher (P<0.05) mean serum GLDH level between weeks 5 and 16 compared to the relatively lower levels of other treatment groups during the same period.

In the group of sheep dosed with 60, 120, and 240 Gy the mean serum GLDH level lies in a range of 100 and 150 IU after week 6 post infections. However, these values were relatively stable between weeks 12 and 16 post-infection, regardless of a statistically insignificant (P>0.05) difference between the groups. On the other hand, animals vaccinated with a low dose of 30 Gy kept the stable serum GLDH level in the range of 150 - 200 IU between weeks 14 and 16 post-infection. The serum GLDH profile of non-sensitized positive control sheep reached a minimum level of 200 IU only at week 16 following a sharp decline from 267± at week 15 to 200 at week 16 post-infection. The more pronounced changes in levels of the enzyme at positive control and lower dosed groups indicated a relatively higher level of damage to the liver.

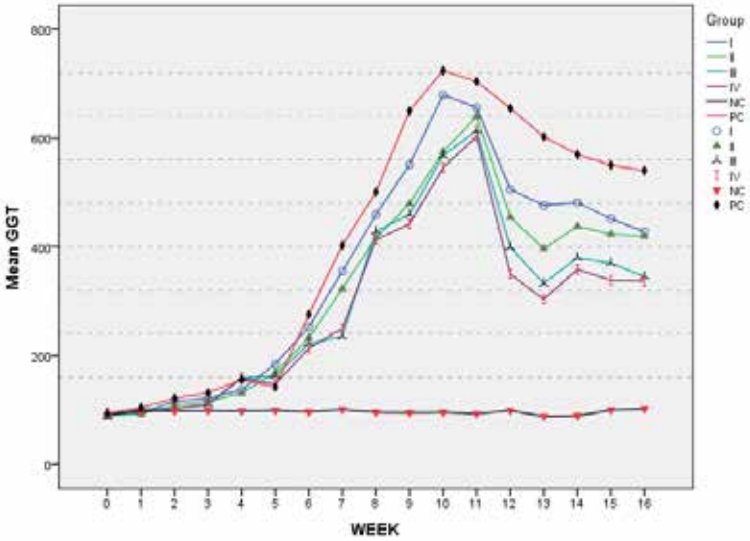


Fig 2. weekly dynamics of GGT during the experimental period in sheep

The mean serum γ-glutamyl transpeptidase (GGT) concentrations remained altered in all infected sheep except the negative control. In this regard, the mean levels of the enzymes persistently increased from week 5 post-infection onward until the end of the experiment. After week 5 there was no much fluctuation of the enzyme profile among the treatment groups except that levels were raised till week 11. The difference in GGT levels of all infected sheep with respect to the positive control was significant (P>0.05), but these were more pronounced between weeks 10 and 11. In all groups, infected animals had ex-

hibited peak levels of GGT concentrations between weeks 10 and 11 across time. However, after week 12 to the end of the experiment, all infected sheep had invariably maintained relatively raised constant GGT levels. The difference in the mean peak values of GGT between positive control and other treatment groups as well as between group I and groups II, III, and IV at weeks 10 and 11 were significant ($P< 0.05$).

The discharge of fluke eggs in the faeces and the EPG

All the vaccinated and infected control groups were found to shade eggs except the uninfected negative controls. Eggs were seen to shade starting from the eighth-week post-infection. These were first observed in infected control groups (PC) and those vaccinated with a low dose of the parasite (GI dosed with 30 gray and GII sheep with 60 grays). The animals in high-dose vaccinated groups (GIII received 120 grays and GIV dosed with 240 grays) began to shade eggs only after week 9 post-infection. The uninfected control animals (NC) were never appeared to excrete eggs. The eggs discharged by all infected and vaccinated animals continued to be recovered until the end of the experiment 17 weeks post-vaccination. Higher EPG output was observed in infected control (PC) followed by those animals vaccinated with low doses of the gamma irradiation (GI and GII). The EPG of all groups steadily increased with time (Figure 3).

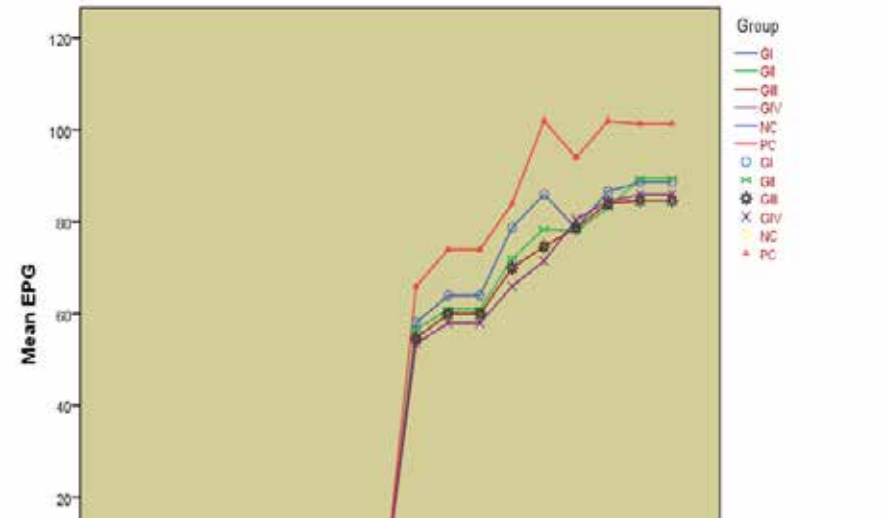


Fig 3. The eggs per gram of faeces in different experimental groups across time

The EPG of all groups steadily increased with time. The pattern of EPG raise was similar in all sheep that had received the irradiated infective metacercariae and those that were infected with the same number of a non-sensitized normal fluke. The clear differences in EPG output were noted between week 12 and 15 post-infection. Infected control sheep had shown significantly (P<0.05) higher EPG level reaching peak 13 post-infection compared to the group of sheep that received different levels of gamma sensitized metacercariae.

The higher EPG of eggs (108) were recorded for positive control animals at the end of the experiment, 17 weeks post-vaccination than the other groups. However, the EPG of higher-dose animals (GIII and IV) appeared lower than those vaccinated with lower doses (GI and II). In all cases, a slight variation of EPG was observed between those higher dosed groups of animals in GIII and GIV. Similarly, the EPG of GI animals was relatively higher than that of GII from the lower dosed animals (Figure 3). Generally, with the exception of infected control animals, a relatively nonsignificant EPG output was induced following vaccination with 500 metacercariae attenuated with 240Gy of γ-irradiation (GIV), or attenuated with 120Gy (GIII) of γ -irradiation compared to metacercariae γ-irradiated at 30Gy and 60Gys. These findings disclosed the effect of the degree of parasite attenuation (dose levels) on starting time of egg shedding and the level of EPG output in sheep irradiated with gamma cells provided, the levels of irradiation-attenuated infective stages were kept constant (500 metacercariae).

Fluke recovery and measurements

Out of the 500 flukes administered per os to each group, the mean (M± SE) number of flukes recovered varies significantly (P<0.05). The present finding indicated that the higher the irradiation dose, the lowest was the recovery of flukes in necropsy examinations (Table 2). That means a strong dose-response was evident in the number of parasites recovered in the groups dosed with incremental doses of γ-irradiation of *F. hepatica* metacercariae. The recovered parasites were morphologically normal, patent, immature, and adult liver flukes. The percentage of the flukes recovered (percentage reduction in fluke burden) unirradiated from the liver of the sheep were highest in infected control groups (22.5%; 112.3±4.3) followed by GI animals dosed with the lowest irradiation dose (18.6%; 93± 3.4). The percentage reduction in the burden of flukes in group II and III sheep was 13.4% (67.8 ± 2.8) and 11.2% (56.5± 7.18)

respectively. Similarly, those animals in the highest irradiation dose (GIV) yield the lowest recovery (7.7%, 38.3± 3.3). Accordingly, the higher the irradiation dose, the lowest was the recovery of flukes in necropsy examinations.

A total of 350 (82%) recovered fluke lengths were measured from sheep in all infected (PC) and vaccinated groups. The overall number of flukes counted from the positive control group was significantly higher (P<0.05) than other vaccinated groups of the experimental animals.

Table 2. The number of metacercariae administered per os and the flukes recovered in different vaccination groups at necropsy

Group	Number of animals	Number of parasites (met)	Irradiation dose (gray)	Fluke recovery				
				Mean ±SE	%	Max	Min	Range
I	6	500	30	93± 3.4	18.6	103	80	23
II	6	500	60	67± 2.8	13.4	75	60	15
III	6	500	120	56.5± 7.18	11.2	75	30	45
IV	6	500	240	38.3± 3.3	7.7	50	30	20
Infected Control (PC)	6	500	-	112.3± 4.3	22.5	123	100	23
Non-Vaccinated	6	-	-	-	-	-	-	-

A relatively large number of flukes were recovered and measured (98; 28%) from sheep in the positive control than the vaccinated groups. The number of flukes measured in groups GI, GII, GIII, and GIV was 77 (22%), 77 (22%), 60 (17.1%), and 38 (10.8%) respectively. The length of flukes recovered ranged from 0.30cm to 2.3cm. The mean length of *Fasciola hepatica* recovered in the positive control group was 2.2cm. The corresponding length in GI, GII, GIII, and GIV was 2.1, 1.8, 1.6, and 1.5cm respectively. However, in all the infected control and vaccinated groups the majority (Mean + SEM) of flukes recovered (69 ± 5.1) had lengths between 1.3cm and 1.5cm. Similarly, a significant number of flukes had a length greater than 1.6cm and less than 1.9cm (66 ± 4.74). In all treatment groups, the mean number of those flukes with lengths less than 0.5cm (43 ± 8.3) and more than 2cm (57) was relatively small. The mean number of flukes measuring less than or equal to 1cm was significantly lower in the group infected with high irradiation dose in GIV (15 ± 1.3) and GIII (27

± 3.1) respectively in that order. However, the reverse is true for animals that received parasites exposed to lower irradiation dose in GI (37 ± 2.1) and GII (37 ± 3.4). The overall number of flukes counted from the positive control group was significantly higher (P<0.05) than other vaccinated groups of the experimental animals (Figure 3).

Similar trends were recorded on the width of flukes recovered corresponding to their lengths where the widths of the flukes had seen to rise with the increasing length of liver flukes. The width of flukes recovered ranged from 0.30cm to 1.2cm. However, in all the infected control and vaccinated groups the majority (Mean + SEM) of flukes recovered (68± 5.1) had widths between 0.7cm and 0.9cm. Similarly, a significant number of flukes had a width greater than 0.5cm and less than 0.6cm (66 ± 4.74). In all treatment groups, the mean number of those flukes with lengths less than 0.3cm (43 ± 8.3) and more than 1.2cm (53) was relatively small. The mean number of flukes measuring less than or equal to 0.5cm was significantly lower in the group infected with high irradiation dose in GIV (10 ± 2.3) and GIII (16 ± 2.1) respectively in that order. However, the reverse is true for animals that received parasites exposed to lower irradiation dose in GI (23 ± 3.1) and GII (23 ± 3.4). The overall number of flukes counted from the positive control group (29 ± 5.2) was significantly higher (P<0.05) than other vaccinated groups of the experimental animals (Figure 4). These findings are an indication for strong host immunological responses at higher gamma-irradiated groups of animals (GIII and GIV) than those who received parasites exposed to low irradiation and uninfected controls (PC).

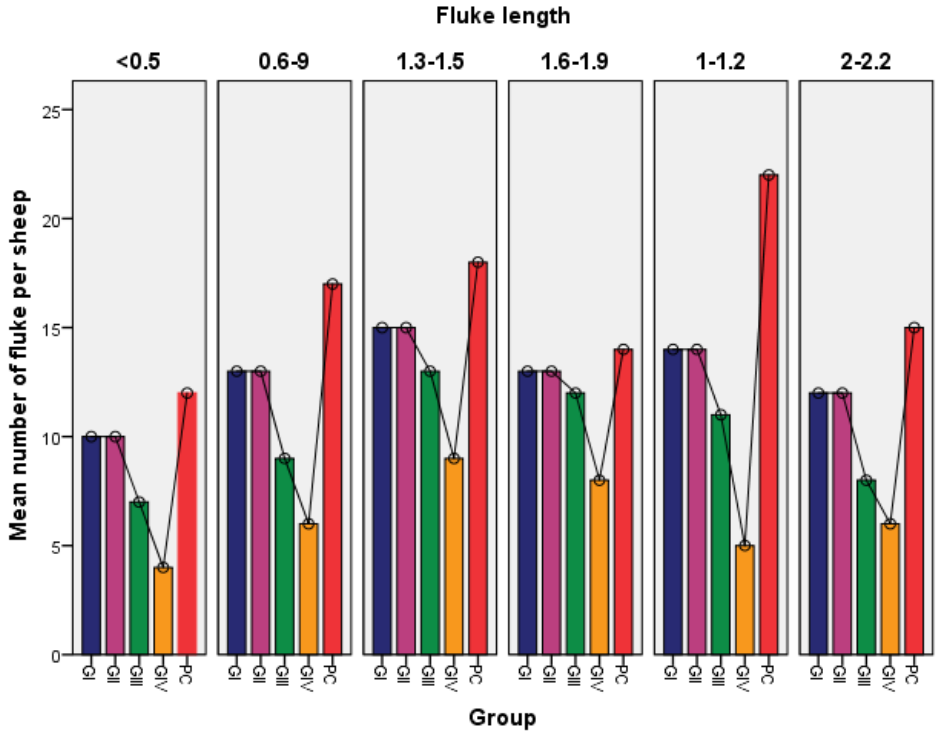


Fig 4. The number of flukes recovered and the measurement of fluke length (cm) in different treatment groups

Similar trends were recorded on the width of flukes recovered corresponding to their lengths where the widths of the flukes had seen to rise with the increasing length of liver flukes. The width of flukes recovered ranged from 0.30cm to 1.2cm (Figure 4).

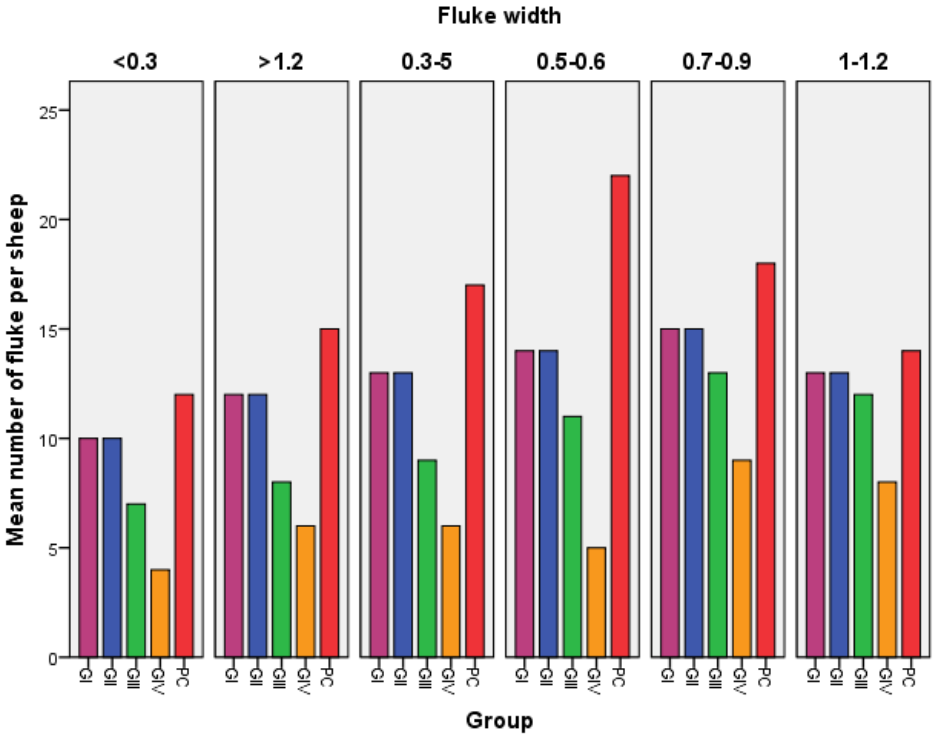


Fig 5. The number of flukes recovered and the measurement of fluke width (cm) in different treatment groups.

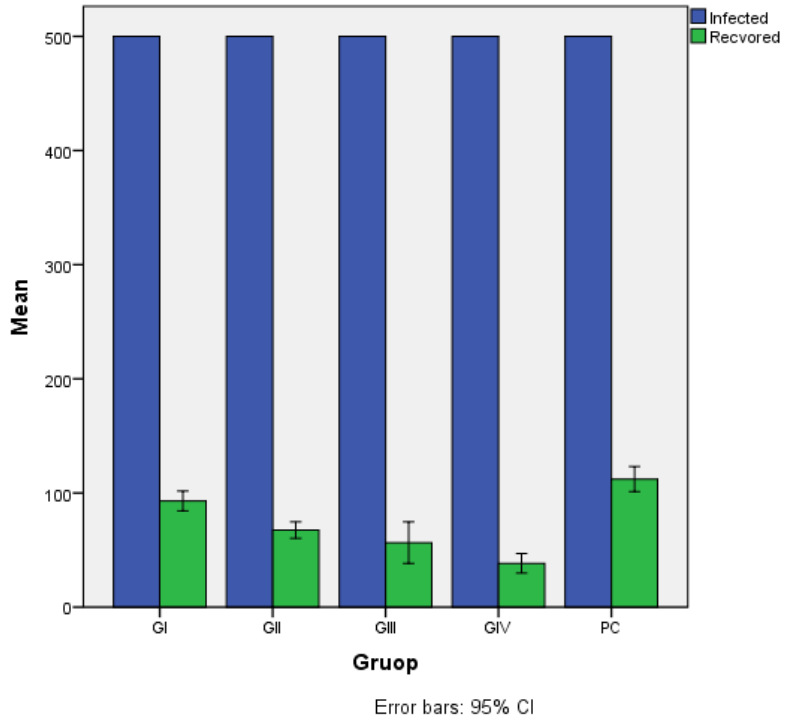


Fig 6. The number of flukes recovered from the different treatment groups at end of the experiment

Table 3. The mean Fluke length, width, and mean liver weight of infected and control groups

Group	Mean number of fluke ± SEM	Mean Length ± SEM (cm)	Mean width ± SEM (cm)	Mean liver weight ± SEM (gm)	Mean differences of liver weight (PC-other groups) ± SEM (gm)
PC	112.3± 4.3	2.3 ± 0.2	1.75 ± 0.1	396	Reference
GI	93± 3.4	2.1 ± 0.3	1.5 ± 0.2	378	18 ± 2.3
GII	67± 2.8	1.8 ± 0.1	1.3 ± 0.1	368	28 ± 3.4
GIII	56.5± 7.18	1.6 ± 0.1	1.2 ± 0.1	356	40 ± 2.3
GIV	38.3± 3.3	1.5 ± 0.15	1.1 ± 0.1	348	48± 3.3
NC	-	-	-	325	71 ± 2.5

The liver weight and the irradiation dose

The results disclosed the difference in the mean change of the liver weight between the infected and control groups at necropsy (Table 3). The highest mean liver weight changes were observed between positive and negative control groups with a difference of 71± 2.5 g and this was significant (P<0.5%). Comparison between highly dosed groups at GIV with that of positive control had shown a mean difference of 48± 3.3 g. This was followed by the mean difference of 40± 2.3 g with that of group III animals. The relative comparative mean live weight changes recorded for GI and GII were 18 ± 2.3 and 28 ± 3.4gm. Generally, the liver weight relatively tends to decline with the progressive increase in irradiation dose and subsequent decrease in the recovery of the fluke and reduction in the size of *F. hepatica* at necropsy examinations.

Bodyweight changes

Comparison of the relative mean weight gain (M± SD) of highly dosed GIV (20.8±3.5kg) sheep and non-infected controls (22.5±4.5 kg) had detected non-significant (4kg) differences (P<0.05). Similarly, relatively lower but statistically insignificant (3.7kg) difference was recorded between uninfected control (22.5±4.5 kg) and group GIII (20.8±3.5kg) animals. The variation detected in mean weight gain between negative controls (22.5±4.5kg) and that of lower dosed group GI (18.8±2.3kg) and GII (18.6± 2.1kg) sheep were significant with a difference of 7.1and 6.9kg respectively. However, the relative difference (8.1kg) was more pronounced between negative control (22.5± 4.5) and infected control (18.4± 0.96) groups. Generally, the mean weight gain of all groups tends to increase with time despite the differences in weight gain of the treatment groups used for the vaccine trials. Despite the nearly linear increase in the mean weight gain of positive controls and the groups received the lower irradiation doses of 30 and 60 gray, a significant body weight loss was observed between weeks 6 and 8 post-infection followed by a steady increase of the mean weight of infected animals across time (Figure 7).

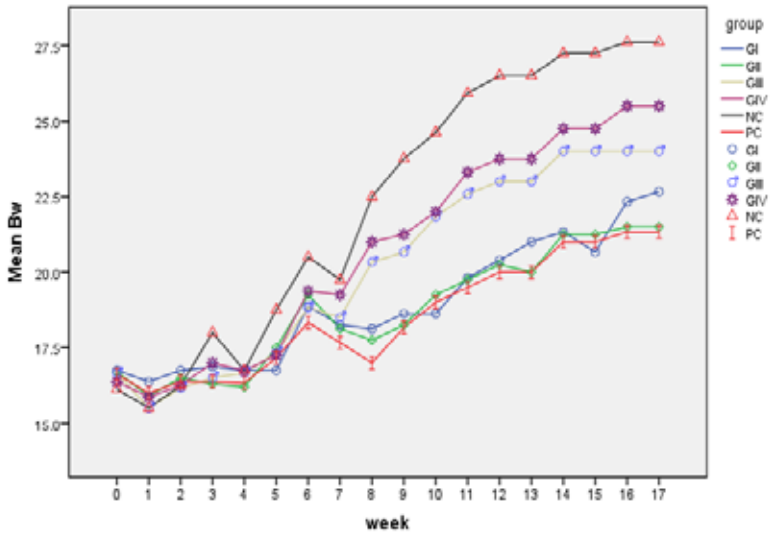


Fig 7. Bodyweight changes of sheep vaccinated with different doses of *Fasciola hepatica* **across time**

Discussion

To deter the detrimental effects of pathogens to the host, huge successes have been made in vaccine development against viruses and bacteria over the past several years. Except for the live attenuated Huskvac vaccine for lungworm, there are no commercially viable vaccines for animal helminth parasitic pathogens (McKeand, 2000). Recent advances in vaccination with recombinant helminth antigens have been successful against cestode infections of livestock and new vaccines are being tested against nematode parasites of animals (Hewitson and Maizels, 2014). More recently, a new vaccine for *H. contortus*, Barbervax, consisting of native gut-derived antigen complex has been launched in Australia www.barbervax.com.au.

Attenuated antiparasite vaccines enable the host to mount a protective immune response against the organism without the development of the pathological symptoms of infection. Despite the practical nature of this strategy, several irradiation-attenuated parasite species have been used experimentally to induce protection in various host species (Smith *et al*., 1993), including cattle

against infection with the liver fluke, *Fasciola hepatica,* and sheep and cattle against infection with *F. gigantica* (Haroun and Hillyer, 1986).

Several authors previously depicted that plasma activities of liver enzymes are sensitive indicators of liver damage in sheep and cattle (Rowlands and Clampitt, 1979; Bulgin *et al*., 1984; Ferre *et al*., 1994; Fere *et al*., 1995a; Fere *et al*., 1996) and with this regard, our findings were not different. In ruminants (bovine and ovine) fasciolosis young flukes may be found in the hepatic parenchyma for approximately 6-8 weeks, migrating through the tissue and growing. The flukes then enter the biliary system, mature, and lay eggs which can be found in faeces many months after initial infection (Taylor *et al*., 2007).

In the present study, the parasitological findings disclosed that sheep appear to show reduced liver damage against *F. hepatica* infection when dosed with 120 and 240 gray γ-irradiated metacercariae, the size of the inocula being always 500 metacercariae in a single experimental infection. This is evidenced by the significant reduction of worm egg outputs and the recovery of mature flukes in groups γ-irradiated with 120 and 240 grays compared to the positive control and groups kept at 30 and 60 gray of γ-irradiated metacercariae.

In this study, primary vaccination of sheep with 500 γ-irradiated (30 and 60 grays) metacercariae of *Fasciola hepatica* did not generate a significant reduction of liver damage compared with 500 γ-irradiated (120 and 240 grays) metacercariae and the positive control as measured by the reduction of faecal egg output and recovery of flukes from liver and bile ducts. All the vaccinated and infected control groups were found to shade eggs except the uninfected negative controls. Higher EPG output were observed in infected control (PC) followed by those animals vaccinated with low doses of the gamma irradiation (GI and GII). However, a study (Campbell *et al*., 1978) stated that vaccination of sheep with either 100 or 1000 γ-irradiated (25 grays) metacercariae of *Fasciola hepatica*, on two occasions six weeks apart, did not generate significant protection against intraruminal challenge with *F. hepatica* six weeks after the second vaccinating dose as measured by the recovery of flukes from liver and bile ducts, twenty weeks after challenge.

The present results were in agreement with most of the previous studies in that vaccination of sheep with irradiated metacercariae of *F. hepatica* yielded matured and immature parasites with varying levels of reduction across treatment groups. In contrary to some authors (Rickard and Howell, 1982; Haroun and Hillyer, 1986; Boyce *et al.*, 1987) stated that sheep do not acquire resistance against *Fasciola hepatica* as indicated by the yields of mature parasites from primary and secondary infections, the result of the present study revealed differences in the response of sheep to yield matured parasites in vaccinated and control groups from primary infection. In coincident with this, the mean number of flukes recovered from infected groups varies significantly ($P<0.05$). That means a strong dose-response was evident in the number of parasites recovered in the groups dosed with incremental doses of γ-irradiation with *F. hepatica* metacercariae. Accordingly, the yield of matured parasites from Ethiopian sheep ranged from 7.7% to 22.5%. In the studies with European fleece sheep, yields of *F. hepatica* ranged from 16 to 38% after primary infection and from13 to 31% after secondary infection (Rickard and Howell, 1982). A significant reduction in parasite numbers (from 17% in control animals to 3.4%) was reported in Sudanese desert sheep vaccinated with irradiated metacercariae of *F. gigantica* following a secondary challenge (A'Gadira, *et al*., 1987).

Presently, the percentage of the flukes recovered (percentage reduction in fluke burden 81.5%) unirradiated from the liver of the sheep were highest in infected control groups (22.5%; 112.3±4.3) followed by GI animals dosed with the lowest irradiation dose (18.6%; 93± 3.4 recovered / 83.4% reduction). The percentage reduction in the burden of flukes in group II and III sheep was 86.7% (13.4%; 67.8 ± 2.8 recovered) and 88.8% (11.2%; 56.5± 7.18 recovered) respectively. Similarly, those animals in the highest irradiation dose (GIV) yield the lowest recovery 92.3% (7.7%, 38.3± 3.3 recovered). Accordingly, the higher the irradiation dose, the lowest was the recovery of flukes in necropsy examinations. There was, however, a significant increase in the proportion of flukes retarded in the parenchyma of vaccinated groups with the significant effect observed in those vaccinated with 120 and 240 grays of γ-irradiated (30 and 60 grays) metacercariae. The percentage of retarded flukes was positively correlated ($r = 0.72$) with the degree of liver damage. According to the works of some authors (Creaney *et al*., 1995), no significant reduction of fluke burdens was observed in any group, although a non-significant 20% reduction was observed in sheep vaccinated with 2000 metacercariae irradiated with 100 grays.

The present study used parasitological parameters such as recovered liver fluke length and width, the total mass of recovered flukes, and liver damage (enzyme profile) which are major factors often used to indicate the protection against the severity of parasite infection. This method of assessing has been used in evaluating vaccination against fasciolosis by different studies including a multivalent vaccine of recombinant stage-specific antigens (Boray, 1983; Valero *et al*., 2002; Valero *et al*., 2006; Jayaraj *et al*., 2009).

Cysteine proteases are common virulence mediators of parasites and are produced by all stages of the fluke lifecycle. They mediate biological functions including excystment, tissue invasion, and immune evasion (Berasain *et al*., 2000). Adult fluke cathepsin L and Newly Excysted Juvenile (NEJ) cathepsin B are the prominent proteolytic enzymes of their respective ES (excrete tory secretory) materials. *F. hepatica* cathepsin L5 (fifth stage larvae) (Creaney et al., 1995; Smooker *et al*., 2000; Irving *et al*., 2003; Law *et al*., 2003; Meemon *et al*., 2004; Beckham *et al*., 2006; Kennedy *et al*., 2006) and *F. gigantica* cathepsin L1 (Grams *et al*., 2001) are promising targets for vaccines against *Fasciola* infection.

Similar to the current finding, other authors (Jayaraj *et al*., 2009) depicted that, in single and multivalent recombinant protein vaccinations of adult stage *F. hepatica* cathepsin L5, metacercarial stage *F. gigantica* cathepsin L1g, and juvenile stage *F. hepatica* cathepsin B against *F. hepatica* challenge infection, the rats vaccinated with recombinant proteins were shown to have significantly fewer and smaller flukes than the control rats. That means maximum protection of 83% was seen in the group vaccinated with a combination of cathepsin B and cathepsin L5 (Jayaraj *et al*., 2009). Although there was a variation in the level of protection, the percentage reduction of the fluke population of gamma-irradiated *Fasciola hepatica* metacercarial infection in Ethiopian sheep ranged from 81.5% (22.5%, 112.3$\pm$4.3 recovered) to 92.3% (7.7%, 38.3$\pm$ 3.3 recovered).

In agreement with the present study, vaccination with the multivalent cathepsin B/L5 leads to less liver damage, lower fluke numbers, and the lowest mean wet weight compared to control rats. This result indicates liver fluke development was retarded in the vaccinated groups. It may be that a fluke vaccine does not need to induce sterile immunity, but reduce the pathology to a level that is tolerated by the animal. A reduction in fluke burdens will also reduce the number of eggs passed by infected animals, reducing pasture contamination. A study demonstrated (Jayaraj *et al*., 2009) that juvenile stage-specific

recombinant proteins (B, L1g) can mediate immunity to liver fluke infection, but that protection is greatest when a juvenile stage protease (cathepsin B) is used in concert with an adult stage protease, L5. A work reported (Jayaraj *et al*., 2009), the protective immunity elicited by recombinant protein vaccination appears to evoke effector and memory responses against infection. Although such vaccination induces strong humoral responses (Jayaraj *et al*., 2009), the precise effecter mechanisms leading to fluke control have not been elucidated. That a cocktail of juvenile and adult stage *Fasciola* recombinant proteins induced better protective immunity than individual protein alone indicates that stage-specific, multivalent recombinant vaccines against parasites may be feasible (Jayaraj *et al*., 2009).

The number and size of fluke's recovered had a significant effect on the observed weight changes of the liver in different groups. The results disclosed the difference in the mean change of the liver weight between the infected and control groups at necropsy. The highest mean live weight changes were observed between positive and negative control groups with a difference of 71$\pm$ 2.5 g and this was significant (P<0.5%). The presence of flukes in positive control was defiantly responsible for the increased weight of the liver than the uninfected negative control sheep. Comparison between highly dosed groups at GIV with that of positive control had shown a mean difference of 48$\pm$ 3.3 g. This was followed by the mean difference of 40$\pm$ 2.3 g with that of group III animals. The relative comparative mean live weight changes recorded for GI and GII were 18 $\pm$ 2.3 and 28 $\pm$ 3.4gm. Generally, the liver weight relatively tends to decline with the progressive increase in irradiation dose and subsequent decrease in the recovery of the fluke and reduction in the size of *F. hepatica* at necropsy examinations.

The experiment was only resumed after three weeks of acclimatization and all animals began to gain weight after the second week post-infection and this gain was continued till the end of the experimental period. Generally, the mean weight gain of all groups tends to increase with time despite the differences in weight gain of the treatment groups used for the vaccine trials. Despite the nearly linear increase in the mean weight gain of positive controls and the groups received the lower irradiation doses of 30 and 60 gray, a significant body weight loss was observed between weeks 6 and 8 post-infection followed by a steady increase of the mean weight of infected animals across time. Given

the young growing age of sheep used in this experiment, the initial live weight gain has been expected. However, the effect of fasciola on body weight loss of treatment groups has been described between weeks 6 and 8. This might be due to damage caused by the migrating flukes to the liver parenchyma. Rates of growth were significantly reduced by 14.7% and 14.1% in steers receiving a superimposed artificial infection rate of 1200 metacercariae and grazed at 3.54 beasts/hectare and 4.39 beasts/hectare respectively. Similarly, group body weights were depressed 3% and 20% in steers receiving 600 metacercariae and grazed at 3.54 beasts/hectare and 4.39 beasts/hectare respectively (Chick *et al*., 2008).

Along with parasitological tests (faecal examination for fluke eggs and the EPG count), laboratory analysis of the hepatic enzymes has been a useful indicator for damages caused by liver flukes. In this regard, the first is the estimation of plasma levels of enzymes released by damaged liver cells. Two enzymes are usually measured. Glutamate dehydrogenase (GLDH) is released when parenchymal cells are damaged and levels become elevated within the first few weeks of infection. The other, gamma-glutamyl transpeptidase (GGT) indicates damage to the epithelial cells lining the bile ducts; elevation of this enzyme takes place mainly after the flukes reach the bile ducts and raised levels are maintained for a longer period (Taylor *et al*., 2007; Hoffmann and Solter 2008; Washington and Hoosier, 2012).

Serum GGT and GLDH determinations for diagnosis of hepatic disease in domestic animals have received less attention in Ethiopia than in some other countries. The determination of GLDH activity is best done in conjunction with the determination of other hepatic enzymes (GGT) and other indicators of hepatic injury or disease.

Many of the early studies on *F. gigantica* and *F. hepatica* assumed that infection with the former was essentially similar to that of *F. hepatica*. However, based on fluke biomass, average fluke size, degree of liver damage, plasma glutamate dehydrogenase (GLDH) levels, and gamma-glutamyl transpeptidase (GGT) responses during the first 10 weeks of infection, *F. hepatica* develops more rapidly than *F. gigantica*, resulting in increased plasma levels of GLDH, indicating greater damage to the liver parenchyma(Boyd, 1962; Meeusen *et al*., 1995) and an increase in the GGT levels at 10 weeks post-infection, indicating

epithelial damage in the bile duct (Chauvin *et al*., 1995; Martínez-Valladares, 2010b).

The results of the present study indicated that a peak in GGT activity was associated with the onset of patency. Tissue damage, which is shown by fluctuations in GLDH and GGT levels after adult flukes have become established in the bile ducts, is considered to be due to the feeding activity of adult flukes and the deposition of immune complexes in the liver parenchyma. In this sense, the serum was successfully assayed for the presence of the enzymes glutamate dehydrogenase (GLDH) and gamma-glutamyl transferase (GGT), as indicators of liver and bile duct damage respectively.

In the present study, animals that have been exposed to infection of the metacercariae of *F. hepatica* were responded well to sensitization and infection as shown by the evolution of hepatic (GLDH), bile duct, and gall bladder (GGT) enzymes. The degree of visual hepatic damage and burden of *F. hepatica* were significantly positively related to levels of GGT and GLDH. The enzyme level varies with the dose of γ-irradiation and non-sensitized groups of animals. In all cases, the uninfected control sheep (NC) maintained a low profile of enzymes that were well within the normal ranges of each enzyme (GLDH= 1-12; GGT= 34-100 IU).

Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme that catalyzes the removal of hydrogen from L-glutamate to form the corresponding ketimine acid that then undergoes spontaneous hydrolysis to 2-oxoglutarate. The liver has by far the highest concentration of GLDH activity. Lesser amounts are found in the kidney and small intestine, where the GLDH activity is located in the proximal and distal tubular epithelial cells and the mucosal epithelial cells, respectively. The GLDH activity of non-hepatic tissues is relatively small compared to that found in the liver, where GLDH is concentrated in the central areas of the lobule. In all species, increases in serum GLDH activity are considered liver-specific (Boyd, 1962; Keller, 1981).

Serum GLDH activity is used most commonly in food animals. Because of its location within mitochondria, GLDH should be released only with irreversible cell injury. The sensitivity of GLDH activity varies depending on the nature of the disease. For example, in a study of calves with hepatic disease, GLDH

activity increased in only 60% of the animals (Pearson *et al*., 1995). Similarly, in cattle, the sensitivity of GLDH activity for the detection of hepatic lipidosis, hepatic abscessation, leptospirosis, and fascioliasis was only 28%, 53%, 71%, and 72%, respectively (West, 1991). The determination of GLDH activity is best done in conjunction with the determination of other hepatic enzymes and other indicators of hepatic injury or disease (Hoffmann and Solter, 2008; Washington and Hoosier, 2012).

The elevation of the enzymes and the damage inflicted on bile ducts have shown that they have positively correlated (r = 0.68). Irrespective of the infectious groups, similar trends in the elevation GGT profile (an indicator of epithelial damage in the bile duct) were observed for all sheep infected with *F. hepatica* metacercariae until the end of the experimental period. All animals kept in the sensitized and infected groups had maintained the steady rise in the level of GGT until week 4 post-infection, then after the GGT profile had shown marked elevation before reaching peak levels between weeks 9 and 11 post-infection where the majority of flukes reach patency (epithelial damage to the bile ducts and gall bladder). The peak in GGT activity could be associated with the onset of patency. Then after the level of enzymes progressively decline before it had shown relatively stable enzyme levels between weeks 14-16 post-infection. However, sheep infected with non irradiated normal *F. hepatica* metacercariae (PC) and Group I animals that received the infective parasite exposed to low levels γ-irradiation of 30Gy had shown significantly higher GGT levels compared to the animals in other infected groups that received 120Gy and 60Gy of irradiated *F. hepatica* metacercariae.

A study in goats immunized with Sm 14 antigen of *F. hepatica* disclosed that plasma levels of GLDH and GGT were within the normal range before challenge (week 10). Three weeks after challenge infection (week 13), GLDH levels were elevated until week 18, GGT increased in week 16. No significant difference was noted between groups during the study (Mendes *et al*., 2010).

Although sheep that received the 30 Gy irradiated metacercariae (500) had a relatively higher GGT profile compared to sheep that were treated with the same number of metacercariae and kept at 60 and 120 Gy, the difference was not statistically significant ($P>0.05$). A statistically significant difference ($P<0.05$) in serum GGT levels was only observed between infected control sheep (PC) and the other sensitized groups (GI, GII, GIII, and GIV) after week

8 post-infection. However, between weeks 9 and 11 a statistically significant (P<0.05) rise in serum GGT profile was observed in GI (γ-irradiated with 30Gy) sheep compared to other sheep that had received relatively higher doses of γ-irradiation (60 and 120 Gy). The difference in mean (M±SD) GGT profile of group GII and GIII and also GIII and GIV were not significant (P >0.05). Particularly the steady rise of GGT levels of groups GII, GIII, and GIV were indistinguishable until the values had shown clear relative comparative difference after week 12 post-infection.

Conclusions

This trial was the first attempt on experimental vaccination of sheep against γ- irradiated *F. hepatica* metacercariae in Ethiopia. In the present settings, which include faecal examinations for *Fasciola hepatica* eggs and the EPG, recovery of flukes and measurement of the sizes and enzyme analysis (GLDH and GGT) became useful indicators for primary vaccination trial of sheep in determining the irradiation attenuating dose. The determination of GLDH activity was best done in conjunction with the determination of GGT as indicators of hepatic injury. The degree of visual hepatic damage and burden of *F. hepatica* were significantly positively related to levels of GGT and GLDH. The enzyme level varies with the dose of γ-irradiation and non-sensitized groups of animals. The findings disclosed that sheep appear to shows significantly reduced liver damage against *F. hepatica* infection when dosed with 120 and 240 grays of γ- irradiation while the size of the inocula is 500 metacercariae in a single infection. A strong dose-response was evident in the number of parasites recovered in the groups dosed with incremental doses of γ-irradiation with *F. hepatica* metacercariae.

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Conflict of interest

None of the authors have any financial or personal interests that could inappropriately influence or bias the contents of this paper.

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Estrus characteristics, ovarian response to synchronization hormones, and fertility of crossbred dairy heifers managed under a semi-intensive system

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Abstract

This study aimed to characterize estrus sign/s which best predict the time of ovulation and determine ovarian responses to different synchronization protocols and conception rates to fixed-time artificial insemination in Boran*Holstein crossbred heifers. In the first part of the study, twenty-seven heifers were observed for estrus signs (from induced and natural) and the relationship of various estrus signs with ovulation time was evaluated by using ultrasonography. In the second part, 91 Boran*Holstein crossbred heifers were randomly grouped into three groups. Group one heifers (n=28) received 100µg gonadotropin-releasing hormone (GnRH) on day zero (D0), 500µg prostaglandin F2α (PGF2α) on day 7 and 100µg of GnRH on day 9. Group 2 heifers (n=32) were treated as group one but additionally received progesterone as controlled internal drug release (CIDR). Group three heifers (n=31) were treated as those in group 2 but without injection with GnRH on day 0. In all the 3 groups' insemination was made at 19h of the second GnRH. The results showed that irrespective of estrus source type (induced or natural), score for standing to be mounted, mounting other heifers, and non-receptive mount by other heifers showed a strong positive correlation with ovulation time (r=0.67, $P<0.05$). Standing estrus duration, and time elapsed from standing estrus to ovulation were shorter ($P<0.05$) in induced estrus. The conception rate was 39.3% in synchronized ovulation in the absence of CIDR. The conception rate in timed insemination was 56.3% when ovulation was synchronized by combining CIDR, PGF2α, and GnRH. In conclusion injection of GnRH on day zero

together with treatment with CIDR improved ovulation rate, the number of new corpus luteum, and conception rate. Heifers that ovulated within the first 24h after timed insemination had a higher (P<0.05) conception rate than heifers ovulated after 24h of timed insemination. Cycling heifers at day zero had a greater (P<0.05) conception rate than non-cycling.

Keywords: Conception rate; crossbred dairy heifers; estrus sign; ovarian response; synchronization.

Introduction

Estrus induction by PGF2α and subsequent insemination is used to speed up cattle genetic improvement in Ethiopia. The average conception rates after estrus induction and insemination were reported to be lower than 40% (Gizaw *et al*., 2016). Many factors influence the efficacy of prostaglandin-based estrus induction; among which are the stage of the estrus cycle at prostaglandin administration, estrus sign detection, and submission of animals in estrus for artificial insemination (AI). Previous studies (Bainesagne, 2015; Destalem, 2015) indicate that 24.2% to 50.9% of cows had milk progesterone concentration higher than 3ng/ml at insemination, which strongly indicated that insemination was done at the wrong time.

Protocols that allow for AI without the need for estrus detection are a better alternative to increase the number of animals inseminated at an appropriate time. These protocols need manipulation of follicular waves and ovulation before insemination. These protocols have been successfully applied in *Bos taurus* herds. However, where the environment is predominantly subtropical to tropical, and when *Bos taurus* breed was crossed to *Bos indicus*, a decrease in conception rates to ovulation synchronization and timed AI has been reported (Hiers *et al*., 2003). When dairy heifers of *Bos taurus* breed are subjected to Ovsynch protocol, a greater incidence of estrus before timed artificial insemination (TAI) was frequently observed (Rivera *et al*., 2004) and that leads to a low conception rate to TAI for dairy heifers. Suppression of premature estrus expression using Controlled Internal Drug Release (CIDR) improves conception rate to TAI in taurine dairy heifers.

Most of the data on ovulation synchronization and conception rate to timed AI was from sub-tropical countries like Brazil, Argentina, and the Southern Unit-

ed States. Similarly, the zebu cattle used for crossing to *Bos taurus* in these countries were commonly the Nelore breed. Little is known on the subject in tropical African and non-Nelore zebu cattle crossed to *Bos taurus* cattle. The objectives of the present study were, therefore, to characterize estrus sign/s which best predict the time of ovulation, and determine ovarian responses to different synchronization protocols and conception rates to fix time of artificial insemination in crosses of *Bos indicus* X *Bos taurus*.

Materials and methods

Study area

This study was conducted in six dairy farms, three farms from Bishoftu, and three from Modjo towns. Both towns are located in the Eastern Shewa Zone of Oromia Regional State. Bishoftu is located at 47km southeast of Addis Ababa at 8°45′N latitude, 38°59′E longitude, and an elevation of 1885 meters above sea level. The mean annual rainfall of the town is 866 mm with a bimodal pattern. The mean annual minimum and maximum temperatures are 14^0C and 26^0C, respectively and relative humidity was 61.3%. Modjo town is located at 66Km southeast of Addis Ababa at 8°35'N latitude and 39°7'E longitude. The altitude is 1790 meters above sea level. The average annual rainfall, temperature, and mean relative humidity are 776mm, 19.4 ^{o}C, and 59.9%, respectively (National Meteorology, 2010, unpublished data).

Experimental design and treatments protocols

This study has two experiments. Experiment one (Exp 1) involves 27 crossbred dairy heifers aged between 18 and 26 months and with a mean body condition score of 3.7± 0.2 (on 1-5 scale, Ferguson *et al*., 1994). The heifers were given two doses of 500µg prostaglandin F2 alpha (PGF2α) (Synchromate®, cloprostenol sodium, Warburg, Germany) at 11 days intervals. After gathering data on estrus, the heifers were rested for one estrus cycle and crossed over for gathering data on natural estrus. In natural estrus, an observation started from day 16 of the previous estrus. Experiment 2 (Exp2) involved 91 crossbred dairy heifers randomly assigned to three groups. In group 1, 28 heifers received100µg gonadotrophin-releasing hormone (GnRH) through IM injection (Gonadorelin diacetate tetrahydrate, Merial limited Duluth, USA) on day 0 (D0), 500µg PGF2α on day 7 and the second dose of GnRH (100µg) on day 9. The group was assigned as GnRH-PGF2α-GnRH (GPG). Group 2 (CIDR-GPG) heifers (n=32) received all treatments of Group 1 and were additionally treated

with CIDR (CIDR 1380, EAZI BREED™, New Zealand) from day 0 to 7 and at CIDR removal (D7) PGF2α was given. Group 3 (CIDR-P-G) heifers (n=31) had the same treatment as those in Group 2 but without the initial (day 0) GnRH treatment. In all groups, heifers were inseminated using frozen-thawed semen at 19h of the last GnRH injection.

Estrus detection

After 24h of the second PGF2α injection in experiment 1, heifers were visually observed every 3h for 30 minutes (6 am, 9 am, 12 am, 3 pm, 6 pm, 9 pm, 12 pm, and 3 am) for estrus sign for 120h in estrus induced group or starting from day 16 of the former estrus in spontaneous estrus. Observed estrus signs were scored using a method recommended by van Eerdenburg *et al*. (1996) with slight modifications in relation to signs associated with vaginal anatomy, which were not considered in the present study. Each time a symptom was observed, the assigned number of points was recorded (Table 1). If the sum of the points exceeded 50 during two consecutive observation periods, a heifer was considered to be in estrus. A heifer was said to be in standing estrus when it stood receptive on mounting by another heifer, with or without the other symptoms of estrus. Cessation of estrus was when heifers are no more receptive to mounting after previously being in standing estrus

Table 1. Estrus scoring criteria. A heifer was considered to be in heat when the cumulative score was 50 at two observations (Modified from van Eerdenburg *et al*., 1996).

Estrus sign	Points assigned
Flemen	3
Restlessness	5
Bellowing (repeated)	5
Sniffing vagina of another cow	10
Being mounted but not standing	10
Resting with chin on another cow	15
Mounting (or attempting) other cows	35
The mounting head side of another cow	45
Standing heat	100

Ovarian ultrasonography

Mindray ultrasound (DP.50vet, China) with a 7.5 MHz linear array rectal probe was used. In Experiment 1, starting from 24h of the second PGF2α in estrus induced group or day 16 of previously induced estrus in natural estrus group, ovarian structures were scanned twice a day (8:00 am; 8:00 pm) until heifers were in standing estrus. Beginning from first standing estrus, ovarian examinations were made at every 6h interval until heifers were off estrus. From off estrus to ovulation, ovarian examinations were made every 3h. In Experiment 2, ovaries were monitored on days 0, 2, 7, 8, and 9, and then at 24h, 36h, and 48h after day 9 to assess ovulatory outcomes and size of the ovulatory follicle. On ultrasonographic examination, the size of follicles, the location of the dominant follicle, and the corpus luteum were recorded. Ultrasonographic examinations were also performed on day 10 after AI to determine CL diameter. Ovulation was confirmed on the disappearance of a previously identified dominant follicle (≥8mm) and the presence of CL on the same site (modified from Ginther *et al.,* 1989).

Pregnancy determination

Conception was checked on day 32 of AI. On ultrasound, the presence of a fluid-filled uterine horn and a conceptus were used as positive indicators of conception (Fricke *et al*., 1998).

Statistical analysis

Duration of estrus was calculated by subtracting the time initial estrus was detected from the time the last standing mount was detected. The interval from PGF2α injection to the onset of estrus was calculated as the interval from PGF2α to the first estrus detected. The estrous response was defined as the number of heifers displaying estrus after PGF2α injection divided by the total number of heifers treated. To evaluate the length of time intervals of each behavioral estrous sign to an ovulation time, analysis of variance (ANOVA) in STATA software was used. All heifers that ovulated between 10 pm and 6 am were not included in the ovulation time interval as determining ovulation every 3h within this time gap was interrupted in some heifers. Pearson correlation was used to evaluate the correlation between estrus signs and ovulation time, follicle size at standing estrus and ovulation, follicle size and ovulation time, ovulation from the same ovary at different estrus. $P < 0.05$ was considered to be significant. Conception rate was defined as the number of heifers that became pregnant, divided by the number of heifers that were inseminat-

ed. All count measurements were indicated as mean ± SE (standard error of the mean). Five heifers that did not respond to PGF2α were excluded from estrus induced group.

Result

Estrus behavior characteristics and frequencies

The detailed descriptions of estrus signs manifested are listed in Table 2. There was no statistically significant difference among induced and natural estrus (P>0.05) on the frequency of various estrus signs. However, there was a tendency that standing to be mounted, attempt to mount other heifers, and non-receptive mount were frequently seen in heifers from the induced group. Irrespective of estrus type (induced or natural), sniffing ano-vaginal region and resting chin on other heifers were the most frequently observed estrus signs. Estrus signs were more frequently observed when more than two heifers were in estrus at the same time.

Table 2. The frequency and percentages of estrus sign observed (mean ±SE) in relation to estrus type and number of heifers in estrus

Estrus sign	Mean frequency (±SE) and (percent) of estrus signs		
	Induced estrus	**Natural estrus**	
	≥ two heifers were in estrus (synchronized)	*One heifer in estrus*	*≥ two heifers in estrus*
Sniffing	20.2±8.3, (92)[a]	15.6±3.6, (90)	16.3±2.7,(94)
Chin resting	22.6±10.5, (90)	18.5±5.2, (96)	21.2±10.6, (94)
Mounting others	9.9±4.2, (82)	6.7±2.6, (71)	7.9±3.2, (74)
Mount not received	8.2±1.5, (64)[a]	2.6±1.2, (63)	4.2±1.8, (69)
Standing estrus (mounts received)	7.5±2.8, (63.6)	4.7±1.5, (54)[a]	7.2±2.3, (59.1)
Bellowing	9.4±3.7, (58.9)	7.9±2.5, (50.9)	8.8±3.3, (54.9)

a= Cells with superscript **a** across the column were significantly different (P<0.05) from others

Estrus duration and time of ovulation

The details of durations of different estrus behaviors and intervals from different estrus signs to ovulation are listed in Table 3. The mean (± SE) estrus length was not affected by estrus type (induced and natural). The interval from estrus onset to ovulation was slightly shorter in induced estrus (30.7±0.6h) than natural estrus (32.1±0.8h) although the difference was not statistically

significant (P>0.05). Heifers that showed less than 24h estrus length in the induced group were also likely to have less than 24h estrus length when crossed over to the natural estrus manifestation group (r = 0.85; P<0.05). Standing estrus was started at an average of 7.1 ± 0.4h of onset of estrus in induced estrus and 8.6 ± 2.0h in natural estrus (Table 3). In all heifers that ovulated, ovulation occurred within the first 24h after the end of estrus. From the total of 32 ovulations observed, the large majority (60.4%) ovulated from the right ovaries (P<0.05). Heifers that had ovulations from right ovaries in estrus induced group were more likely to successively ovulate from the right ovaries (r= 0.6, P<0.05) when crossed over to ovulate from natural estrus. In the present study, it was found that standing to be mounted and mounting other heifers have a 100% correlation with ovulation rate (r=1) and interestingly the time of ovulation was quite consistent (range 16 -21h) and was with minimum variation between individual heifers with standing to be mounted.

Table 3. Duration of estrus, estrus onset to standing heat, and estrus signs to ovulation (hrs)

Estrus sign	Estrus type & interval to ovulation			
	Induced		Natural	
	Mean± SE	Range	Mean ±SE	Range
Duration of estrus	18.4±4.2	11.0-29.0	20.2 ±3.9	10.0 -30.0
Interval from onset to standing estrus	8.1 ±0 .6	6.0 -20.0	10.4±1.5	8.0-23.0
Standing estrus duration	7.1 ± 0.4[a]	5.0 – 12.0	10.6 ± 2.0[b]	3.0-16.0
Estrus onset to ovulation	30.7 ± 0.6	18.0-38.0	32.1±0.8	17.0-39.4
Estrus end to ovulation	12.3± 1.1	6.0-20.0	15.4±1.2	6.4-20.3
Standing estrus to ovulation	19.0±1.9[a]	12.0-21.0	25.0 ±4.0[b]	15.0-26.0
Mounting other heifers to ovulation	26.8±4.3[a]	16.0-35.4	30.3±2.1[b]	21.2-43.5
Non-receptive mount to ovulation	24.7±5.4	10.5-45.0	21.5±1.9	16.4-39.6
Sniffing (start) to ovulation	29.4±1.2	20.0-38.5	31.4±5.3	22.0-36.5
Chin resting (start) to ovulation	30.5±5.2	17.0-40.5	30.5±4.7	20.0-37.0

a, b cells with superscripts a,b within the row were different (P<0.05) from others

Follicle and corpus luteum size by types of estruses

The mean (±SE) diameter of the larger follicle at first standing estrus did not differ significantly (P>0.05) due to estrus type (8.64±1.32 in induced; 9.22± 0.73 in natural estrus). Similarly, the mean (±SE) diameter of the ovulatory follicle didn't show a significant difference (P>0.05) between induced (16.15±0.53mm)

and natural (17.43±1.53) ovulations. There was a strong positive correlation between follicle size at standing estrus and size at ovulation in which heifers with large follicles at standing estrus had larger ovulatory follicles at ovulation (r = 0.72, $P<0.05$). There was also a positive correlation between follicle size and ovulation time in which, heifers with larger follicles at standing estrus ovulated earlier than heifers with smaller follicles (r = 0.15, $P>0.05$). The diameter of CL did not significantly differ ($P>0.05$) between induced (17.5±1.6 mm) and natural estrus groups (17.7± 2.2mm).

Ovulation rate and ovulation time

The details of the ovulation rate to GnRH and time of ovulation were described in Table 4. Ovulation to day 0 GnRH injection was significantly higher ($P<0.05$) in heifers of the GPG group (28.6%) than the CIDR-GPG group (12.5%). Ovulation to day 9 GnRH injection was significantly higher ($P<0.05$) in the CIDR-GPG group than CIDR-P-G and GPG group. In the CIDR-GPG group, all heifers ovulated to day 0 GnRH injection ovulated also to day 9 GnRH injection. However, in the GPG group, 21.1% of heifers that ovulated to day 0 GnRH injection did not ovulate to day 9 GnRH injection.

The mean time (h) from PGF2 treatment to ovulation and from GnRH injection on day 9 to ovulation was not affected by treatment type ($P>0.05$) except in the CIDR-GPG group which had a significantly longer mean time (h) from PGF2α treatment to ovulation ($P<0.05$) than the other groups. When the distribution of time to ovulation after GnRH injection on day 9 was considered, more heifers (52.6%) from the GPG group ovulated within the first 24h than the remaining groups (Table 4). In about 73.7% of heifers from the GPG group, 78.6% from the CIDR-GPG group, and 83.3% from the CIDR-P-G group that ovulated to day 9 GnRH injection, the ovulations were from a follicle other than the large follicle that was present at the start date.

Diameter of follicles by treatment group

The mean size of the large follicle on day 0 did not differ ($P>0.05$) among the treatment groups (Table 4). Similarly, the mean size of the larger follicle at day 7 (48h of day 0 GnRH injection), the mean size of the large follicle of on day 9 GnRH injection, and the mean size of pre-ovulatory follicles that eventually ovulated did not statistically differ ($P>0.05$) among the treatment groups (Table 4). However, when the data was combined with heifers ovulating from natural estrus (from experiment one), the mean diameter of the pre-ovulatory

follicle that eventually ovulated was significantly greater ($P< 0.05$) in natural ovulation (18.43±1.53) than hormone-treated groups.

Corpus luteum status by treatments

A large majority of heifers (60.4%) had visible CL on day 0 indicating that they were cyclic. The presence of a new CL on day 7 was greater ($P \leq 0.05$) for the CIDR-GPG group than the CIDR-P-G group. Ovulation to treatment with GnRH on day 0 resulted in a greater proportion of heifers with visible CL in the CIDR-GPG group than the CIDR-P-G group on day 7 (Table 4). The number of total CLs after PGF2α injection on day 7 was significantly greater ($P<0.05$) in heifers receiving GnRH on day 0 (CIDR-GPG) than those without GnRH treatment on day 0. Similarly, the proportion of heifers that have new CL on day 7 was significantly higher ($P<0.05$) in heifers that received GnRH on day 0.

Table 4. Ovarian characteristic and response to treatments

Ovarian status	Treatment group		
	GPG	CIDR-GPG	CIDR-P-G
PGF2***α*** **–** ***ovulation*** interval (h)†	80.9± 2.7	99.5 ± 2.8	82.7 ±1.3
Ovulation due to D0 GnRH††	28.6%[a]	12.5%[b]	0%[c]
Ovulation due to D9 GnRH	67.9%[a]	87.5%[b]	77.4%[c]
D9 GnRH to ovulation interval(h) †	24.3±1.6	28.5±1.01	27.4±1.3
Ovulation in ≤ 24h of GnRH treatment (%)	52.6%	42.9%	45.8%
Ovulation in >24 of GnRH treatment (%)	47.4%	57.1%	54.2%
Follicle diameter at PGF2α	8.4±0.2	9.2±0.3	9.4±0.3
Follicle diameter 24h after PGF2α	10.0±1.2	10.2±0.6	11±1.4
Follicle diameter at D9 GnRH	11.8±1.7	12.7±2.5	12.5±0.7
Follicle diameter immediate to ovulation†	14.3±2.2	15.2±1.9	13.5±1.7
CL presence on d0 (%)	60.7%	65.6%	61.3%
New CL on D7(%) †††	35.7%[a]	47.5%[b]	16.1%[c]
Total CL on D7 (%)	82.1%[a]	62.5%[b]	51.6%[c]
D12 CL diameter†	16.3±2.4	17.6±1.9	16.5±2.7

a,b,c = within the row, cells with superscripts a,b,c differ (P<0.05) from each other, †= measurements were in mean ±SE, †† = heifers with follicle ≥ 10mm on D0 and CL on the same site 48h later,†††= heifers that had no CL on D0 but with new CL on D7

Conception rate

Among heifers inseminated, the pregnancy rate on day 32 was significantly less (P<0.05) in the GPG group than in CIDR-GPG and CIDR-P-G groups (Table 5). Similarly, pregnancy was significantly higher (P<0.05) in heifers that received GnRH at CIDR insertion than heifers without CIDR insert.

In all the 3 treatment protocols, heifers that ovulated within the first 24h after TAI had a higher pregnancy rate than heifers ovulated after 24h of insemination (Table 5). Cycling heifers at day 0 had a greater pregnancy rate compared to non-cycling heifers.

Table5. Pregnancy rates in the three groups as affected by different factors

Factors considered	Pregnancy (%, N0)		
	GPG	**CIDR-GPG**	**CIDR-P-G**
Ovulation within 24h before TAI			
No of heifers (%)	4(21.1%)	7(25%)	5(20.8%)
No pregnant ***(%)***	3(75%)	4(57.1%)	2(40%)
Ovulation within 24h after TAI			
No of heifers (%)	15(78.9%)	21(75%)	19(71.2%)
No pregnant ***(%)***	8(53.3)	14(66.7%)	13(68.4%)
P. value	0.002	0.01	0.003
D0 luteal activity			
No cycling (% pregnant)	8 (44.4%)	12(57.1%)	10(52.6%)
No non-cycling (% pregnant)	3 (30.0%)	6 (54.5%)	5(41.7%)
P. value	0.014	0.07	0.001
Overall P/TAI	39.3% (11/28)	56.3(18/32)	48.4% (15/31)

Discussion

The slightly shorter estrus onset to ovulation in induced estrus compared to natural estrus was probably due to exogenous PGF2α which causes luteolysis and reduced progesterone concentration, which allows heifers to express estrus earlier than heifers under natural luteolysis. The estrus duration in both induced (18.4±4.2h) and natural estrus (20.2 ± 3.9h) groups were longer than of the previous report of Roelof *et al*. (2004) and Stevenson *et al*. (1998) who studied pure Holstein dairy heifers. Animal breed, management, and the environment may be among the factors that majorly contribute to the difference. The

disparity of results may also be due to differences in scoring estrus as Roelof *et al*. (2004) considered estrus when the sum of points during consecutive observation periods exceeded 100 as compared to 50 points considered in this study.

According to Yoshida and Nakao (2005), the average duration of standing estrus was 6.2 ± 3.9 h in Japan's dairy heifers which was slightly shorter than the average duration of standing estrus (7.1h in induced and 8.6h in natural estrus) of the present study. The same authors reported all the 10 heifers observed exhibited standing estrus. Heifers are considered to experience lesser stresses and therefore, should express strong estrus signs. However, in our study, only 63.5% from induced and 54.5% from the natural estrus group exhibited standing estrus behavior. Other authors (van Eerdenburg *et al*., 1996; Le Blanc *et al*., 1998; Lyimo *et al*., 2000; van Eerdenburge *et al*., 2002) reported that standing estrus behavior is not seen in many heifers and cows at different dairy farms. Some of the possible reasons for the lack of standing estrus behavior may be due to inherent breed effects and/or excessive animal handling for daily transrectal ultrasonic evaluation.

The present time interval from estrus onset to ovulation in induced (30.7 ± 1.6) and natural estrus (32 ±0.8hrs) was longer than the 27.7±2.4h and 26.1±1.2 h previously reported for induced and natural estrus groups, respectively (Pinheiro *et al*., 1998). Similarly, Son *et al*. (2007) reported that ovulation occurred at 25.55±5.72h after the onset of estrus in dairy heifers. The differences may be possibly due to breed, and nutrition effects. Energy balance is known to affect reproduction in dairy cattle as reported by some authors (Wathes *et al*., 2007; Van Hoeck *et al*., 2014).

In the current study, the time interval between onset and end of most sexual estrus behaviors to ovulation showed large gap (range). The exceptions were standing to be mounted and mounting other heifers which showed lower variation and were easily detectable visually. Since all heifers that manifested mounting activities finally ovulated, signs related to mounting activities better-predicted ovulation time in this study. However, only 63.6% of heifers from induced and 54.5% from natural estrus groups were in standing estrus, which means when only standing estrus was considered heifers not in standing estrus could be missed. Lyimo *et al*. (2000) concluded that standing estrus, the standard symptom of estrus, was not the primary symptom for detecting estrus in cows as it was observed in only 53% of the cows.

Ovulatory follicle size is known to be affected by animal breed. In the present study, ovulation occurred at ovulatory follicle diameters range of 10mm to 19mm. The study of Gimenes *et al.* (2008) indicated that *B. indicus* cattle can ovulate from the follicle as small as 7mm in contrast to Holstein cattle which only ovulate from follicles with 10mm or more. Studies of Sartorelli *et al.* (2005) and Carvalho *et al.* (2008) indicate that ovulatory follicle diameter frequently ranges from 10 to 13mm in Bos *indicus*. In Holstein cattle, ovulation frequently occurs at a diameter of between 12 and 22mm (Savio *et al.*, 1988; Ginther *et al.*, 1989; Carvalho *et al.*, 2008). In one study (Alvarez *et al.*, 2000); ovulatory follicle was larger in Bos *indicus* than in *Bos taurus* cattle.

In the present study, the day 10 corpus luteum size of crossbred heifers ranged from 10mm to 22.6mm. The study of Carvalho *et al.* (2008) indicated that on day 10 after ovulation corpus luteum was smaller (15.3mm) in *B. indicus* cattle than in *B. taurus* heifers (18.4mm). Corpora lutea size can be as large as 24.1mm diameter for Holstein heifers (Sartori *et al.* (2004). In Nellore cattle (*B. indicus*) corpus luteum size range from 15.6mm diameter to 21.5mm diameter in the reports of Figueiredo *et al.* (1997) and Mollo *et al.* (2007).

Lima *et al.* (2010) compared the cost of timed AI and natural service using as inputs of reproductive results and economical information and, reported that the cost of pregnancy was less for timed AI than natural service. In the present study, Ovsynch protocol in absence of progestin (CIDR) resulted in a low conception rate to TAI (39.3%) in crossbred heifers. Similarly, previous studies (Pursley *et al.*, 1995; Schmitt *et al.*, 1996; Pursley *et al.*, 1997; Tenhagen *et al.*, 2005) indicated low pregnancy to TAI in Holstein heifers synchronized by Ovsynch protocol in absence of progestin. Tenhagen *et al.* (2005) proposed the limited success of Ovsynch protocol in heifers is suspected to be caused by the follicular dynamics of heifers that differs from that of lactating dairy cows.

The conception rate to TAI (56.3%) in the CIDR-GPG group in the present study was consistent with Colli *et al.* (2016) who reported 57.3% and 57.1% in crossbred (Angus *X* Nelore) heifers. However, in the work of Colli *et al.* (2016), the PGF2α was given twice, on day 0 and on the day of CIDR removal in the first group and two days prior to device removal in the second group heifers. Mendanha *et al.* (2012) reported lower conception to TAI (40.3% *and* 42.1%) in Girolando heifer when the PGF was given as in the work of Colli *et al.* (2016). The result indicates that animal breed and treatment protocol among the oth-

ers affect conception rate to TAI. Many other factors like sexual maturity of heifers, weight after weaning, and development of the reproductive tract may be the source of difference.

The lower pregnancy rate (48.4%) in CIDR-P-G heifers with a moderate ovulation rate (77.4%) may be due to the absence of treatment with GnRH on day 0 in this group. GnRH ovulates the dominant follicle (Pursley *et al*., 1995) and prevents the formation of a persistent dominant follicle in heifers without CL at CIDR insert. Progestin treatment in the absence of a functional CL has been shown to result in the development of a persistent follicle resulting in poor pregnancy rates and oocytes ovulated from persistent follicles are known to be less fertile (Revah and Butler, 1996; Mihm *et al*., 1999). Similarly, Bello *et al*. (2006) reported that synchronization response to Ovsynch was higher (87.9%) in cows that ovulated in response to the first GnRH of Ovsynch compared with those that did not (62.9%).

The variation in ovulation to treatment with GnRH on day 0 was probably due to the stage of follicular wave development at the time of GnRH injection and/or differences in concentrations of gonadotropins, estrogen, inhibin, or progesterone at GnRH injection (Ireland *et al*., 2000; Thatcher *et al*., 2002). The work of Moreira *et al*. (2000) showed that initiation of Ovsynch on days 5 to 9 of the estrous cycle was a key to successful synchronization of ovulation.

Conclusions

Ovulation synchronization without CIDR insert leads to premature estrus manifestation and reduced fertility to timed insemination in crossbred heifers. Combining GnRH and CIDR at the start of ovulation synchronization increase follicle turnover, induce ovulation, and enhance new corpus luteum formation and increases fertility. Standing to be mounted and mounting other heifers are the best predictor for the time of ovulation, however, these behaviors were not seen in some heifers.

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Anthelminthic Effects of Extracts of Indigenous Browses from Mid Rift Valley of Ethiopia

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Abstract

This study was conducted to evaluate the potential anthelminthic properties of extracts of leaves of indigenous browses (*Acacia seyal, Acacia senegal, Acacia tortilis, Millettia ferruginea,* and *Vernonia amygadalina*) based on three in vitro assays. Acetone extracts of browses at different concentrations (75 to 1200 µg/ml, for egg and larvae and 100mg/ml for an adult) were tested on three developmental stages of *Haemonchus contortus* (eggs, infective larvae, and adult worms) using egg hatch assay (EHA), larval migration inhibition assay (LMIA) and adult worm motility inhibition assay (AMIA). Significant effects were obtained with all five browses but differences were observed depending on the parasitic stages. The effects of five browse extracts on egg hatching were concentration-dependent, the highest (P<0.05) egg hatch inhibition rate was observed at 1200 µg/ml concentration for all browses. All extracts had a higher effect (P<0.01) than that of the negative control, phosphate buffer saline (PBS). In contrast, no concentration-response relationship was found for infective larvae and adult worms, although more potent effects were observed with the highest concentrations. The LMI rate (70%) induced by *Vernonia amygadalina* extract, at a concentration of 300 µg/ml, was the highest (P<0.05) of all other browses, even at higher concentrations. The highest LMI rate (62%) induced by *Acacia senegal* extract at higher concentration, was lower than that of LMI rate (70%) induced by *Vernonia amygadalina*, at 300 µg/ml concentration. *Vernonia amygadalina* was found to be highly and rapidly effective against adult worms inducing the highest mortality rate (90%) as soon as 4 hrs after incubation. Overall, the in vitro results suggest that these five browses do possess anti-parasitic properties and *Vernonia amygadalina* showed the most effective anti-parasitic property. These effects remain to be confirmed through in vivo study.

Keywords: *Haemonchus contortus*; *Acacia seyal; Acacia senegal; Acacia tortilis; Millettia ferruginea; Vernonia amygadalina;* in vitro method; anti-parasitic.

Introduction

Helminthosis is one of the major problems of livestock production throughout the world, particularly in tropical and subtropical areas. In tropical and subtropical regions, where the parasites are more abundant due to favorable environmental conditions, helminthosis is even more devastating (Pathak 2013). Moreover, extensive grazing on native pastures, together with a lack of supplemental nutrients to animals in these areas leads to a low plane of nutrition and, therefore, increased susceptibility. The gastrointestinal parasite has resulted in up to 40% reduction in live weight gain and a 6-30% reduction in feed intake by lambs (Pathak, 2011).

Haemonchus contortus is a blood-sucking nematode parasite. Both the larvae (L4) and the adults of *Haemonchus* species suck blood (Qamar and Maqbool, 2012), primarily occurring in the abomasum of small ruminants, notably sheep and goats. The parasites cause retarded growth, loss of appetite, anemia, edema, decrease in protein, and even mortality in young animals (Qamar and Maqbool, 2012).

The increasing prevalence of anthelmintic resistance in nematodes in domestic ruminants, combined with rising consumer concerns about chemical use on farms, has encouraged research into alternative strategies for the control of internal parasites (Niezen *et al.,* 1993). Apart from anthelmintic resistance, poor availability and affordability of anthelmintics to small-scale farmers in developing countries have compounded the problem (Hammond *et al*., 1997). It follows that a search for novel and more sustainable anthelmintics is the best approach to the control of helminthosis.

The vast majority of the *in-vivo* and *in-vitro* studies examining the effects of browses against the gastrointestinal nematodes have been performed on temperate legume forages (Hoste *et al.*, 2009). Although indigenous browses are largely distributed in the tropics, especially in East Africa, only a limited number of *in vitro* studies have addressed the question of their potential anthelmintic properties (Papachristou *et al*., 2005). Particularly, indigenous browses from the Rift Valley of Ethiopia are not studied for their potentials to control gastrointestinal parasites (GIP) in the ruminants. The current study was therefore conducted to explore the potential anti-parasitic properties of five indigenous browses from the Rift Valley of Ethiopia against *H. contortus*. *In*

vitro methods were employed targeting three life-cycle stages of *H. contortus*, i.e., the eggs, the infective larvae, and the adult worms.

Materials and Methods

Plant Extract Preparation

Leaf samples of experimental browses were collected from the Mid Rift Valley of Ethiopia at the end of the main rainy season (September 2017). The harvested fresh samples were shed dried and then oven-dried at 60 °C for 48h and ground using 1.0 mm sieve size for extraction. Plant leaf extracts were prepared using the method of Makkar (2003). Accordingly, 5 g of ground plant material were taken in a glass beaker of 125 ml capacity. Then 100 ml of aqueous acetone (70%) was added and the beakers were suspended in an ultrasonic water bath and subjected to ultrasonic treatment for 20 minutes at room temperature (20 ^{0}C). The contents of the beakers were then transferred to centrifuge tubes and subjected to centrifugation for 10 minutes at approximately 3000g at 4 °C. The supernatant was collected and concentrated in a rotary evaporator at 40 ^{0}C under reduced pressure. Thereafter, the concentrated filtrate was freeze-dried to obtain a grounded sample. To prepare the extract solutions applied in the bioassays, the powders were dissolved in phosphate buffer saline (PBS 0.1M, pH 7.2) to make the following different concentrations: 75, 300, and 1200 µg/ml for EH test; 150, 300, and 600 µg/ml for LMI test; and 100 mg/ml for AMI test.

Egg Hatch Assay (EHA):

This test was performed according to the procedure described by Coles *et al.* (2006). Briefly, parasite eggs were freshly obtained from donor goats experimentally infected with *H. contortus.* The eggs were extracted by simple flotation method, washed repeatedly with saline, and distributed on 24 multi-well plates at a concentration of 100 eggs/well in 200µl. For the treatment, 1ml of plant extract prepared with PBS at different concentrations (75, 300, and 1200 µg/ml) were added to well plates containing eggs. An oxfendazole at concentrations of 10µg/ml was used as positive control and an egg with PBS was used as a negative control. Each concentration was tested in triplicates.

The eggs were incubated for 48 hr at 24 ^{0}C. Thereafter, the number of larvae present per well was counted under the microscope and the hatching percent-

age was determined as the ratio between the number of larvae to the number of eggs deposited per well. A mean value was calculated for each concentration of the different plants.

Larval Migration Inhibition Assay (LMIA):

Collection of Larvae: Infective larvae of *H. contortus* (L3) were obtained by fecal culture of eggs collected from an experimentally infected goat. After egg hatching, the infective stage was reached after 10 days. The L3 were then collected by sedimentation using Baermann's apparatus.

The larval migration inhibition (LMI) bioassay was used as described by Rabel *et al.* (1994) to measure inhibiting activity against infective larvae (L3). Briefly, larvae were incubated for 3 h at 20 ^{0}C in phosphate buffer solutions (PBS) of plant extracts, at concentrations of 150, 300, or 600 µg/ml. The larvae were then washed three times in PBS and centrifuged. After the last washing, 500 µl of larvae at a concentration of 1000 L3/ml was pipetted onto a 20 µm mesh. The sieve was inserted into a conical tube so that it just touched the surface of the PBS contained therein. Three replicates were run at room temperature for each plant concentration. In addition, negative control (larvae incubated in PBS) and positive control (larvae incubated in levamisole at a concentration of 500µg/ml) were run in parallel. After 3 h, the L3 above the sieve were discarded and those which had actively migrated through the mesh into the PBS below were counted under the Microscope.

The percentage of LMI was calculated as = (T – M/ T)*100; where T is the total number of L3 deposited in the sieve and M is the number of L3 having migrated through the mesh into the PBS.

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Adult Worm Motility Inhibition Assay (AMIA):

The adult motility inhibition (AMI) bioassay was used as described by Tariq *et al*. (2009). The adult worms were collected from the abomasa of freshly slaughtered goats, washed, and finally suspended in PBS. The 20–25 adult, actively moving *H. contortus* worms were exposed in triplicate in each Petridish containing 100 mg/ml concentration of the plant extract prepared in 5 ml of PBS and PBS alone for the negative control groups. Levamisole at the rate

of 0.5 mg/ml was used as a reference drug of positive control. The Petri dishes were kept in an incubator at 37 °C for 8-hrs. The inhibition of motility, activeness, and mortality of the worms was observed at an interval of 0.5, 1, 2, 4, 6, 8 h. The number of motile (alive) and nonmotile (dead) worms were counted under the stereomicroscope and recorded for each concentration. The death of worms was ascertained by the absence of motility for an observation period of 30 seconds in the lukewarm fresh PBS.

Statistical Analysis

Data were analyzed using the general linear model (GLM) procedure of SAS, Version 9.2. The effects of browse extract, concentration, and the interaction effects on EHI, LMI, and AMI were analyzed by two-factor ANOVA with the following model; $Y_{ij} = \mu + B_i + C_j + B * C_{ij} + e_{ij.}$ Where: Y_{ij} is an observation, μ is the overall mean, B_i is the effect of browse extracts, C_j is the effect of concentration, $B*C_{ij}$ is the interaction between browse species and concentration, and e_{ij} is the experimental error. All laboratory analyses of each browse extracts were conducted in triplicates. The means were separated by Duncan multiple range test. Differences between means were considered statistically significant if $P < 0.05$.

Results

Egg hatch inhibition assay

The percentage of inhibition of egg hatching at different concentrations of plant extracts and their overall means are indicated in Table-1. More than 90% of eggs in negative control were hatched. Compared to the negative control, all plant extracts showed ($P < 0.05$) higher egg hatching inhibition rates. All extracts inhibited egg hatching in a concentration-dependent manner with a maximum inhibition of 50% at the highest concentration (1200 µg/ml) for *Acacia tortilis* and lowest inhibition of 17% at the lowest concentration (75 µg/ml) for *Acacia seyal*. Oxfendazole used as positive control significantly ($P< 0.001$) inhibited egg hatching with an inhibition rate of 85 %.

Table -1. Percentage egg hatch inhibition of extracts of five browse leaves from Rift Valley of Ethiopia at different concentrations (µg/ml) (2017, G.C)

		Concentration (µg/ml)		
Plant extract	**Overall mean**	**75**	**300**	**1200**
Oxfendazole (10µg/ml)	85.00[a]	-	-	-
Millettia ferruginea	31.67[b]	20.00[B]	35.00[A]	40.00[A]
Acacia senegal	33.00[b]	20.00[B]	38.00[A]	41.00[A]
Vernonia amygadalina	35.00[b]	23.00[B]	40.00[A]	42.00[A]
Acacia tortilis	37.00[b]	22.00[C]	39.00[B]	50.00[A]
Acacia. seyal	31.00[b]	17.00[B]	34.00[A]	42.00[A]
PBS	10.00[c]	-	-	-
SE	2.5	3.0	3.0	3.0

Means with different superscripts (a, b, c) in the same column and (A, B, C) in the same row differ significantly (P<0.05). PBS = phosphate buffer saline

Larval migration inhibition assay

The positive control, levamisole, was effective since the larval migration inhibition (LMI) rate was 85%. The migration rate observed for the larvae of the negative control was 90% (Table 2). A significant effect of the extracts of the five **browse** on larval migration (P<0.01) was observed. Overall, the inhibition rates obtained with the *Acacia senegal, Acacia tortilis,* and *Vernonia amygadalina* extracts (P<0.01) were highest as compared to other **browse** extracts. The inhibition rate of *Vernonia amygadalina* extract was (P<0.01) highest at 300 µg/ml concentration. The inhibition rate obtained with the *Acacia senegal* extract (P<0.01) was higher than others except for *Acacia tortilis* at 150 µg/ml concentration. Although a tendency was observed for the highest effects to be associated with the highest concentrations, whatever the plant, a dose-response relationship was similar among the plant extracts.

Table 2. Percentage Larval migration inhibition of extracts of five browse leaves Rift Valley of Ethiopia at different concentrations (µg/ml) (2017, G.C)

Plant extract/control	Overall mean	Concentration (µg/ml)		
		150	300	600
Levamisole (0.5mg/ml)	85.00^{a}	-	-	-
Millettia ferruginea	19.00^{d}	5.00^{cB}	2.00^{cB}	50.00^{bA}
Acacia senegal	55.67^{b}	45.00^{aB}	60.00^{abA}	62.00^{aA}
Vernonia amygadalina	50.00^{bc}	25.00^{bC}	70.00^{aA}	55.00^{abB}
Acacia tortilis	51.33^{bc}	35.00^{abB}	62.00^{abA}	57.00^{abA}
Acacia. seyal	46.33^{c}	30.00^{bB}	57.00^{bA}	52.00^{abA}
PBS	10.00^{e}	-	-	-
SEM	2.5	2.6	2.6	2.6

Means with different superscripts (a, b, c) in the same column and (A, B, C) in the same row differ significantly (P <0.05). PBS = phosphate buffer saline

Adult worms' motility inhibition assay

The efficacy of the five plant extracts on adult *H. contortus* is indicated in Table 3. The effects of extracts on survival of *H. contortus* were time-dependent and there were differences (P<0.05) among plant extracts in the rate of worm mortality at all times after 2h of incubation. All plant extracts except *Acacia seyal* caused a greater than 70% mortality rate at 4 hr of the incubation time. The extract from *Vernonia amygadalina* showed 90% mortality of the adult *H. contortus* worms at 4 h of the incubation time and this was the highest (P<0.05) rate of all other plant extracts followed by *Millettia ferruginea* at this time. The results revealed that parasites were sluggish and movement was little at 4 hr post-incubation for all plant extracts. Extract from *Acacia tortilis* induced the highest (P<0.05) mortality rate at 2 hr post-incubation. Levamisole at a concentration of 0.5 mg/ml caused 50 % mortality at 2 h post-incubation and 100 % mortality at 4 h post-incubation. The control (PBS) recorded the lowest mortality rate (1%). The differences between plant extracts and control were highly significant (P<0.001). Moreover, all extracts except extract of *Acacia seyal* induced 100% adult mortality at 8 hr post-incubation.

Table 3. Percentage of mortality of adult worms of *H. contortus* at different times after incubation with plant extracts at 100mg/ml concentration. (2017, G.C)

Plant extract	Time (h)					
	0.5h	1h	2h	4h	6h	8h
Acacia tortilis	0.00	0.00	25.00^{b}	75.00^{d}	92.00^{c}	100^{a}
Acacia seyal	0.00	0.00	8.00^{e}	17.00^{e}	50.00^{e}	50.0^{b}
Acacia senegal	0.00	0.00	15.00^{d}	77.00^{d}	85.00^{d}	100^{a}
Millettia ferruginea	0.00	0.00	10.00^{e}	85.00^{c}	95.00^{b}	100^{a}
Vernonia amygadalina	0.00	0.00	20.00^{c}	90.00^{b}	90.00^{c}	100^{a}
PBS	0.00	0.00	0.00^{f}	0.00^{f}	0.00^{f}	1.00^{e}
Levamisole (0.5mg/ml)	0.00	20^{a}	50^{a}	100^{a}	100^{a}	100^{a}
SEM	0.00	0.22	0.54	0.51	0.51	0.42

Means in the same column with different superscripts differ significantly (P<0.05). SEM = standard error of means, PBS = phosphate buffer saline

Discussion

Overall, the extracts of the five tropical plants, which were examined in the current study, had *in vitro* effects on the different *H. contortus* life-cycle stages. Recent *in vivo* studies in goats suggest that the consequences of consumption of tannin-containing plants on the biology of worm populations could differ depending on the parasitic stage present in the host (Paolini *et al*., 2003a, b).

The results obtained from EHA indicated that the five plant extracts have in vitro ovicidal activity. Concentration-dependent effects on egg hatching were found with the extracts of the five plants. The plant extracts induced inhibition of egg hatching from 17 to 50 % at the concentration of 75 to1200 µg/ml. These results are comparable to the findings of Bogning *et al*. (2016) who reported a range of egg hatching inhibition rates from 22 to 51% with leaf extracts of *Crassocaphalum crepidioides* at a concentration of 75 to 2400 µg/ml. Similar results were reported on *H. contortus* eggs by Hounzangbe-Adote *et al.* (2005) with extracts from four plants of South of Benin (*Morinda lucida, Carica papaya, Newbouldia laevis,* and *Zanthoxylum zanthoxyloïdes*) where the inhibition rates ranged between 40 and 60%. In general, the extracts of the 5 browses in the current study had ovicidal activity. However, the reductions observed remained relatively limited, since maximal reductions in egg hatching were around 50%.

The effects of plant extracts on the third-stage larvae were not concentration-dependent. However, the migration inhibition rate of four out of five plant extracts ranged from 57 to 70% at the concentration of 300µg/ml. These results were comparable to the findings of Hounzangbe-Adote *et al.* (2005) who reported a range of larval migration inhibition rates from 50 to 75% with extracts of tropical plants of South of Benin at a concentration of 300µg/ml. Molan *et al.* (2000a) found a 80% inhibition of migration for *H. contortus* with *Hedysarum coronarium* extract at a concentration of 1000µg/ml. Alonso-Diaz *et al.* (2008a, b) tested *Acacia pennatula* and *A. gaumeri* extracts and found an inhibition rate of 51% at 1200mg/ml and 93.5% at 600mg/ml. The 10% inhibition effect observed in the negative control (PBS) could be due to the natural phenomena.

The adult worm mortality rate caused by plant extracts ranged from 17 to 90% and 50 to 95% at 4 and 6 h post-incubation, respectively. Moreover, all extracts except extract of *Acacia seyal* induced 100% adult mortality at 8 h post-incubation. These results are comparable with the findings of Singh *et al.* (2016) who reported a mortality rate of 64 and 100% for adult *H. contortus* at 6 and 8 h post-incubation, respectively, with aqueous extract of *Zanthoxylum armatum* (100mg/ml). Tariq *et al*. (2009) reported an adult worm mortality rate of 55 and 85% at 4 and 8 h post-incubation with ethanolic extracts of *Artemisia absinthium* with the concentration of 25mg/ml. Hounzangbe-Adote *et al.* (2005a) reported the mortality rate of adult *H. contortus* 92% at 6 h post-incubation with *Newbouldia laevis* extract (2.4mg/ml).

In the current study, *Vernonia amygadalina* appeared to be the most active plant against adult worms since the mortality rate was 90% as soon as 4 h after incubation. In contrast, *Acacia seyal* was less efficient under these in vitro conditions.

Conclusion

All extracts of the five browses inhibited egg hatching in a concentration-dependent manner with a maximum inhibition of 50% at the highest concentration. All extracts significantly inhibited larval migration compared to the control and the inhibition rate of *Vernonia amygadalina* was the highest of all other browses at 300µg/ml concentrations. The highest mortality rates (90% and 85%) of adult *H. contortus* were induced after 4h of incubation by extracts

of *Vernonia amygadalina* and *Millettia ferruginea,* respectively. Moreover, all extracts except *Acacia seyal* induced 100% adult mortality at 8 h post-incubation.

From the results of the current study, it can be concluded that significant inhibition effects were obtained with all five browses on all development stages of *H. contortus*. Particularly, *Vernonia amygadalina* appeared to be the most active plant against the infective larvae and adult worms since it induced the highest LMI rate at a lower concentration, and highest adult mortality rate as soon as 4-h after incubation. Hence, the extract from *Vernonia amygadalina* could be used as a locally available treatment for *H. contortus* infestation for small-scale farmers. But this recommendation should be validated by the in-vivo experiment, which the current study lacks.

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Comparative evaluation of the immune responses of seven chicken ecotypes to vaccination against Newcastle disease

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Abstract

Newcastle disease (ND) is a highly contagious viral disease of poultry with high mortality. The local velogenic viral pool of the ND strains influences its severity and occurrence. ND vaccination is the most feasible approach to control the disease. However, some ND-vaccinated groups within chicken populations are susceptible to velogenic ND infection developing outbreaks with marked pathological lesions and shedding of the virus. Vaccine strain-related factors as well as inadequate vaccine application and delivery methods during vaccination might explain the suboptimum ND vaccine efficacy. In this study, however, we propose that host factors may contribute to the suboptimal vaccine efficacy in vaccinated chickens. We, therefore, compared the immune response of five Ethiopian chicken ecotypes to ND immunization in the presence of two reference breeds (Fayoumi and Bovans). All chickens received initial immunization at age of 21 days with HB1 ND vaccine followed by two-times LaSota booster immunization at age 50 and 120 days. Subsequently, serum was collected fortnightly post-vaccination at age 35, 65, and 135 days for immune response analysis using the hemagglutination inhibition (HI) test. HI antibody was significantly higher at days 135 > 65 > 35 in each ecotype following the third, second, and first vaccination, respectively. The different chicken

ecotypes had significant differences in HI antibody response to the ND vaccination. Accordingly, the HI titer was significantly higher in Jarso > Cheffe > Fayoumi > Arsi > Bovans > Tepi > Horro suggesting antibody titer and ND vaccine efficacy of the ND vaccine depends on host factors. Moreover, some chicken groups within each ecotype had low HI titer. Chicken ecotypes with weak immune responses may not completely clear the virus from their body; thus, they can serve as a reservoir host by maintaining the ND virus. We conclude that herd immunity level and blanket vaccination program based on the results of a single host genetic group can be misleading during developing and recommending a new vaccine. Hence, understanding the host determinant factors in the immune response during vaccination can lead to improved efficacy and protection against ND in chicken populations.

Keywords: *Chicken; Ecotypes; Immune Response; Newcastle Disease; Vaccination, HI.*

Introduction

The chicken industry in Ethiopia is dominated by local chicken ecotypes with low input – low output production systems in the villages (Duguma, 2009). Local chicken ecotypes contribute about 97% to the sector whereas the modern commercial production system, however, is very low (2-3%) (Duguma, 2009 ; Gulilat *et al*., 2021). The Ethiopian local chicken ecotypes are a pool of highly heterogeneous genetic resources (Hassen *et al.* 2009; Goraga *et al.,* 2012). They demonstrate heterogeneity in production performance (Duguma 2009), morphology and phenotypes (Duguma, 2006; Dana *et al.,* 2010a; Melesse and Negesse, 2011), and individual and ecotype variation in carrying diverse polypathogens as well as parasite loads (Ashenafi *et al*., 2004; Duguma, 2009; Luu *et al.,* 2013; Bettridge *et al*., 2014; Sarba *et al*., 2019). The heterogeneity of local chickens is a dynamic process attributable to continuous selection pressure imposed on them by two broad forces (Duguma, 2009). These include (1) selection by natural phenomena including diseases, starvation, predators, and ecological stress factors; and (2) selection by production and marketing processes including preferred traits for the market, reproduction (stocking), and slaughter or gift for socio-cultural needs (Duguma, 2009; Dana *et al.,* 2010b).
Pathogens causing diseases can impose selective pressure on their hosts by inducing host genetic diversity subsequently grouping the host population into a continuum, ranging from susceptible to resistant groups (Hamilton, 1982;

Lars *et al.,* 2009; Grubaugh *et al.*, 2015). A marked continuum among Ethiopian chicken ecotypes had been reported in Marek's disease virus infection and in their responses to immunization, ranging from highly susceptible to highly resistant ecotypes (Duguma *et al.,* 2005; Duguma *et al.,* 2006).

One of the major pathogenic diseases of chicken in Ethiopia is ND, which is circulating in all regions causing high morbidity and mortality at particular seasons (Chaka *et al*., 2012, 2013a, b; de Almeida *et al.,* 2013; Fentie *et al.,* 2014a,b; Mulisa *et al.,* 2014; Bettridge *et al.,* 2014). ND outbreaks vary geospatially in village backyard chicken production systems (Miguel *et al*., 2013; Snoeck *et al.,* 2013) because of the composition of the local viral genotype population diversity (Alexander, 2011; Snoeck *et al*., 2013; Fentie *et al*., 2014b; Mulisa *et al.,* 2014). Globally, the ND virus is classified into two classes based on their genome sequence similarity, namely class I and II whereas further phylogenetic analysis of the genome sequence of viral isolates shows class I has a single genotype and class II has 18 genotypes (Dimitrov *et al.,* 2016). Other researchers have previously reported that 16 different ND genotypes are present globally, being distributed distinctly on the different landscapes, resulting in variation in ND outbreaks based on geographic location (Diel *et al.,* 2012; Snoeck *et al.,* 2013). Such spatial variation in local ND strains can contribute to vaccine mismatch (Degefa *et al*., 2004; van Boven *et al.,* 2008; Dortmans *et al.,* 2012), which may impair the effectiveness of ND control program due to low vaccine efficacy in the vaccinated-chickens (partial immunity and viral shedding) (van Boven *et al*., 2008; Dortmans *et al*., 2012; Fentie *et al.,* 2014a). Contrarily, a study suggests that the ND vaccine may be effective (i.e. no mismatch in ND vaccine) but the vaccine failure could be due to inappropriate vaccination practice in the field (Dortmans *et al*., 2012). In addition to viral-specific factors, the heterogeneity of the ND viral population and the evolution of new ND genotypes in a particular area are consistently shaped by bird species-dependent factors through fitness competition among viral genotypes as well as a selective pressure imposed by bird species (Grubaugh *et al.,* 2015). Thus, host genetics is an ecological condition that determines the geospatial variation in the local viral strain pool, ND lineage distributions, and ND outbreaks (Koenen *et al*., 2002; Grubaugh *et al.,* 2015). Conversely, the ND virus creates host genetic diversity in their host population (Hamilton, 1982). Host-viral interaction and/or host-specific factors modify the immune responses and ND outbreaks (Grubaugh *et al*., 2015; Alfonso, 2021), affecting the ND control program of the disease. Disease occurrence and transmission dynamics are affected by variation in genetic diversity within a host species

(Ostfeld and Keesing, 2012). Host genetics have a role in the modulation, mobilization, and operation of the host defense system in multiple ways on varying scales against deadly infectious pathogens to ensure health. Overall, host animals shape the ND viruses by creating genotype diversity (i.e., evolution of new ND virus through mutation) to adapt to the diverse host species in which new genotype can spillover from wild birds to domestic poultry (Alfonso, 2021). However, immunogenetic study on genotypes migrating between different interfaces is scarce. Little is known if such new genotype evolution causes antigenic variation (vaccine mismatch) in ND. Further study is required from both viral factors such as new genotype evolution and host factors on ND vaccine efficacy. The role of chicken genetic polymorphism on ND vaccine immune response is poorly understood in Ethiopia. This study, thus, has evaluated the comparative immune responses of seven chicken ecotypes to ND vaccine vaccination.

Materials and methods

Study area

The study was conducted on-station in the College of Veterinary Medicine and Agriculture of the Addis Ababa University (CVMA-AAU) at Bishoftu, Ethiopia. CVMA-AAU is located 47 km southeast of Addis Ababa, the capital city, having an altitude of 1850 meters above sea level and receiving an average rainfall of 866 mm per year. The maximum and minimum average temperatures are 26^0C and 14^0C, respectively. The relative humidity of the study site ranges from 58.6 to 61.3% (Gari *et al*., 2018).

Study animals

Five indigenous chicken ecotypes from distinct locations of Ethiopia were selected for the study. The ecotypes are Arsi, Chefe, Jarso, Horro, and Tepi; located in the west, southwest, southeast, central, and east of the country, respectively (Fig. 1). Bovans and Fayoumi breeds were included in the study as reference groups. Bovans is a commercial breed developed by Institut de Sélection Animale (ISA). Bovans breed is improved (known) for its high egg production. Fayoum chickens are known for their ability to tolerate hot weather, adapt easily to different climatic conditions, resistant to bacterial and viral diseases (Breed Profile: Egyptian Fayoumi Chicken - Backyard Poultry (iamcountryside.com)). Eggs of each ecotype were purchased from their respective local markets and transported to CVMA-AAU premises, where the study was

conducted. At least two persons including DVM and MSc students were assigned to an ecotype to travel and buy eggs from different Ethiopian locations. The travel schedule of all of them was in such a way that they return at the same time to incubate eggs of all ecotypes at a time. We purchased, transported, incubated, and hatched the eggs at the same time. As to the study chicken population, population genetic parameters of the chicken ecotypes (i.e. phenotypic characteristics and genetic distance and heterozygosity) have been previously measured revealing Ethiopian chicken ecotypes are genetically distinct from exotic commercial chickens and each other (Dessie, 2003; Hassen, 2007; Hassen *et al*., 2009; Dana, 2011; Goraga *et al.,* 2012). Fayoumi eggs were obtained from Debre Zeit Agricultural Research Center while Bovans were obtained from Genesis Poultry Farm, franchised private farm with worldwide locations. The eggs were fumigated (2:1 ratio of formalin to potassium permanganate), incubated, and chickens hatched. Then, day-old chicks were transferred to experimental poultry houses of CVMA-AAU from hatcheries.

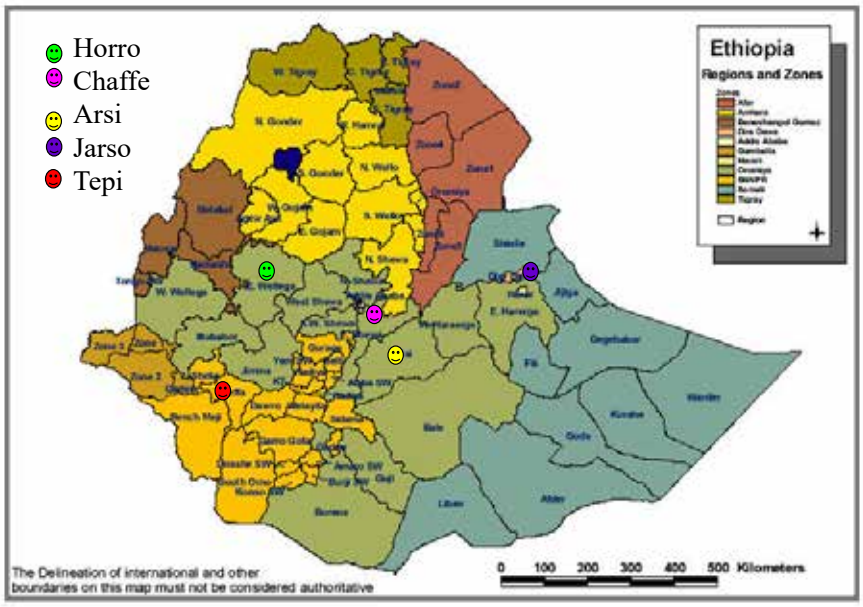


Fig. 1. Map of Ethiopia showing the geographical origin of the selected chicken ecotypes in this study.

Preparation of animal house

A 25m by 8m sized house was constructed on station and equipped with infra-red-light bulbs. The ceiling, walls, and floor were disinfected using 1 % formalin. Cleaned and disinfected local straw (*Eragrostis teff*) was spread over the floor. Drinkers, feeders, and buckets were cleaned and disinfected before the equipment was introduced into the houses.

Study design, study animals' keeping, and vaccination

All day-old chicks were vaccinated against Marek's disease. It was observed that ND HI titer from maternal immunity declines after 14 days in chicks (Abdi *et al*., 2016). HB1 vaccine was administered at 21 days of age whereas two doses of LaSota vaccine were given at days 50 and 120. Both vaccines are produced at the National Veterinary Institute, Bishoftu, Ethiopia. A total of 260 chickens grouped into seven ecotypes were used in this study: 70 Cheffe, 40 Jarso, 21 Horro, 9 Arsi, 30 Tepi, 20 Bevans, and 70 Fayoumi chickens. The experimental design used in this study was a completely randomized design.

Chickens were kept in deep dry formalin disinfected beddings with teff-straw in their pens. Chicks were raised on an electrically heated brooder with infra-red bulbs for light source according to Bovans breed management guide. Artificial lighting was provided for 24 hours starting from the first day until 20 weeks of age decreasing light in equally spaced intensity. The chickens were fed on starter commercial layer ration up to 8 weeks of age, grower commercial ration (9-20 weeks), and layer ration (21 weeks onwards) with standard formulations purchased from Genesis feed processing plant. Feeds and clean potable water were supplied to all stages of birds *ad libitum*.

Serum sampling and antibody titer measurement

Blood was collected from the wing vein at age 35, 65, and 135 days following vaccination on 21, 50, and 120 days of age to measure ND total serum haemagglutination inhibition (HI) as described previously (Abdi *et al*., 2016). The HI antibody titer of $\log_2 \geq 3$ has been used to determine vaccination efficacy. It was reported previously that HI antibody titer of $\log_2 \geq 3$ following vaccination correlated with effective protection to velogenic viral challenge (van Boven *et al*., 2008; Abdi *et al.,* 2016).

Data analysis

We used descriptive statistics (proportions) to summarize the HI antibody data using chicken breed as potential explanatory factors for the induced *HI titer*. The geometric mean value and standard deviation (SD) of HI antibody titers were determined and classified according to chicken ecotypes and a vaccine booster dose (single, second, and third dose). Post-vaccination mean HI antibody titers were analyzed using the General Linear Model of SPSS version 20 (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Where HI results were significant, the least-square difference (LSD) was used to compare HI response by pair-wise breed comparisons. The proportion of chickens with HI antibody titer$\geq \log_2^3$ between breeds was used as a cut-off value to decide the protective level (van Boven *et al.,* 2008; Abdi *et al.,* 2016). Significant differences were tested setting alpha at a 5% significance level.

Results

Immune responses (HI titer) to ND vaccination within and among chicken ecotypes

The immune response (HI titer) of chick ecotypes during ND immunization was heterogeneous; the response varied within and between chicken ecotypes as well as between each of the three-round ND vaccine booster doses (Table 1). The response of individual chickens during 1st, 2nd, and 3rd vaccinations varied among breeds as measured by HI titer as follows. In Arsi chicken, the log HI titer range was: 1st 2-4, 2nd 5-8, 3rd 5-7; Horro 1st 1-4, 2nd 2-8, 3rd 4-9; Cheffe 1st 4-8, 2nd 4-11, 3rd 3-12; Fayoumi 1st 3-9, 2nd 4-11, 3rd 7-12; Tepi 1st 1, 4, 6--8, 2nd 1-8, 3rd 4-9; and Bovans 1st 1-3, 2nd 1,2, 4-8, 3rd 5-12. Some chickens in the Tepi ecotype had low HI titer from the rest of the group after a single vaccination suggesting within ecotype variation. Second and 3rd round LaSota vaccination increased HI titer in all the chicken ecotypes except some in Tepi after 2nd vaccination (Table 1).

These proportions were used to compare the response of ecotype to ND vaccination. The HI log titer value of 2 was measured in 50% of Arsi, 6 in 44% of Cheffe, 2 and 4 in 33% (each) of Horro, 6 in 40% of Tepi, 7 in 35% of Jarso, 6 in 33% of Fayoumi and 2 in 60% of Bovans following the 1st vaccination (Table 1). In general, variation between and within each chicken ecotype in the HI response was consistently observed using proportions (%) of chickens with specific HI

titer following 1st, 2nd, and 3rd vaccination (Table 1). In this study, Bovans had a lower HI titer than some Ethiopian local chicken ecotypes and Fayoumi.

Table 1. Proportion (%) of individual chickens within seven chicken ecotypes with specific HI titer (log_2) and ranges of the HI titers during 1st, 2nd, and 3rd round ND vaccination.

Breed	Vaccination	HI titer (log_2) ranges from 1 to 12											
		1	2	3	4	5	6	7	8	9	10	11	12
Arsi	1st	-	50	25	25	-	-	-	-	-	-	-	-
	2nd	-	-	-	-	50	-	25	25	-	-	-	-
	3rd	-	-	-	-	13	13	38	-	13	-	25	-
Bovans	1st	30	60	10	-	-	-	-	-	-	-	-	-
	2nd	10	20	-	20	20	10	10	10	-	-	-	-
	3rd	-	-	-	-	16	5	16	-	21	11	5	26
Cheffe	1st	-	-	-	8	8	44	31	8	-	-	-	-
	2nd	-	-	-	3	14	26	23	11	6	11	6	-
	3rd	-	-	1	3	15	12	15	10	10	4	7	22
Fayoumi	1st	-	-	6	8	19	33	8	19	6	-	-	-
	2nd	-	-	-	3	19	17	25	28	3	-	6	-
	3rd	-	-	-	-	-	-	12	20	17	13	10	28
Horro	1st	22	33	11	33	-	-	-	-	-	-	-	-
	2nd	-	11	22	-	56	11	-	-	-	-	-	-
	3rd	-	-	-	5	25	20	35	10	5	-	-	-
Jarso	1st	-	-	5	5	20	10	35	25	-	-	-	-
	2nd	-	-	-	10	5	10	10	30	15	20	-	-
	3rd	-	-	-	-	-	-	14	5	30	8	16	27
Tepi	1st	7	-	-	13	-	40	33	7	-	-	-	-
	2nd	7	21	7	14	21	14	7	7	-	-	-	-
	3rd	-	-	-	14	7	18	25	32	4	-	-	-

Ecotype Differences to ND vaccination (unimodal or bimodal HI responses)

The immune response of each chicken ecotype/breed towards HI titer varied. The frequency of HI titer was different among breeds despite receiving the same vaccination. After the first HB1 vaccination on day 21, the serological response of Bovans had a mono-modal HI titer and peaked at HI titer 2 in 60% of

the chickens. Chaffe also tends to have a uni-modal HI titer of 6. The remaining chicken ecotypes had a bi-modal HI titer; however, peaks fall at different HI titer levels. Tepi ecotype had three peaks. This indicated the presence of subpopulations (diversities) within each ecotype, in addition to ecotype differences. Bovans and Cheffe seem to have less diversity (homogeneity) (Fig. 2).

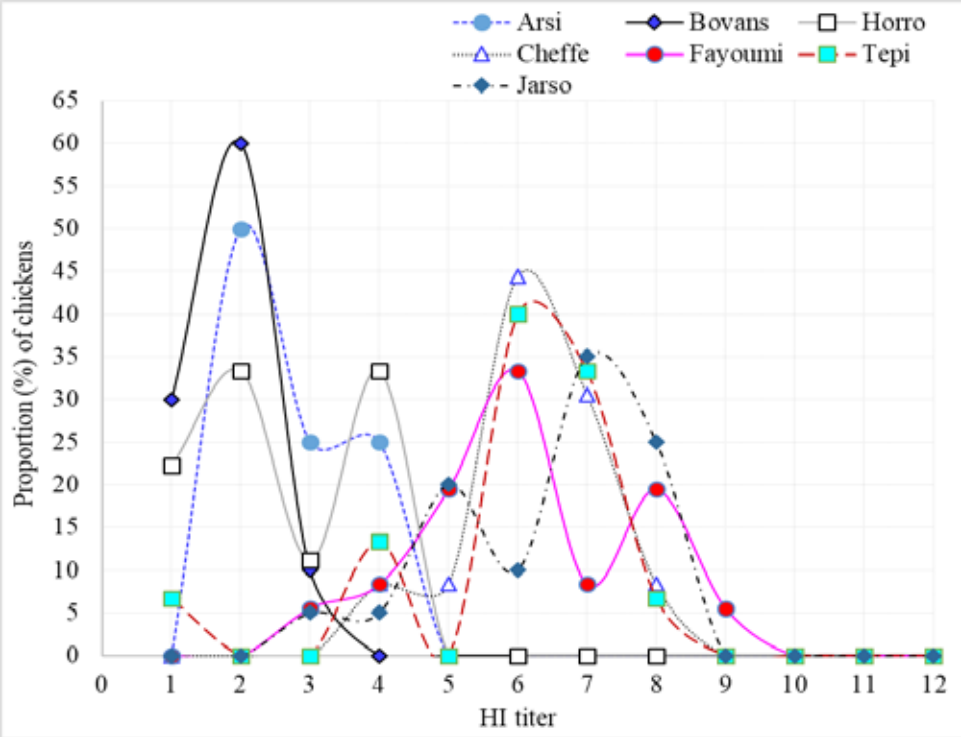


Fig. 2. Differences in proportion (%) of chickens displaying specific HI titer after a single ND vaccination among seven chicken ecotypes. The uni- or bi-modal HI responses within each ecotype revealed the presence of diversities (subpopulations) in each chicken ecotype.

Similarly, following booster vaccination (i.e., 2nd round vaccination), the curve of HI titer of the different ecotypes showed unique patterns for each ecotype whereby two or more models were showing the presence of subpopulations within each ecotype with a varying HI response to the ND vaccination (Fig 3).

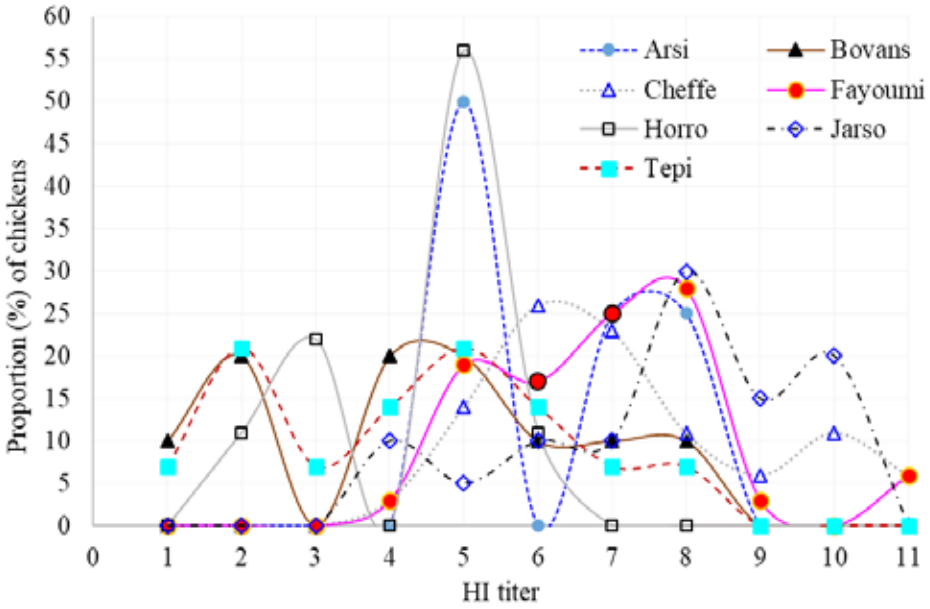


Fig. 3. Differences in proportion (%) of chickens displaying specific HI titer after two-round ND vaccinations among seven chicken ecotypes.

HI antibody titer response of the chicken types to NDV vaccine strain

There was a significant difference in HI titer across the sequential booster dosing of ND vaccination where the HI rises with increasing intensity of booster dosing. In this regard, post-vaccination geometric mean HI titers were significantly (P<0.001) higher within each ecotype at 3-times vaccination > 2-times vaccination > 1-time vaccination (Fig. 4). A significant (P<0.001) difference was noticed in mean log HI titer at 35, 65, and 135 days among chicken ecotypes. At day 35 the mean log HI titer ranked as Jarso > Tepi > Cheffe > Fayoumi > Arsi > Horro > Bovans. Similarly, at day 65 the mean HI titer rank as Jarso > Cheffe > Fayoumi > Arsi > Bovans > Tepi > Horro (Fig. 4). The mean log HI titer for Arsi, Horro, and Bovans was less ($\log_2 < 3$) at day 35 following the first vaccination. The lowest titer was observed in Bovans (1.80), while the highest was observed for Jarso (6.51). Fayoumi, Chefe, Tepi, and Jarso had mean log HI titers above 5. Following booster dose on day 50 of age, serum sampling on day 65 of age showed that Horro ecotypes (3.90) had still lower HI titer followed by Tepi (3.92) and Bovans (4.40). On day 65, Jarso chickens maintained high titer (7.80) followed by Chefe (7.18) and Fayoumi (6.99). The

HI titer in the Arsi ecotype increased (6.25), but the antibody level declined in Tepi at day 65. The third-round vaccination increased the mean HI titer of all chicken breeds above $\log_2^2 > 5$.

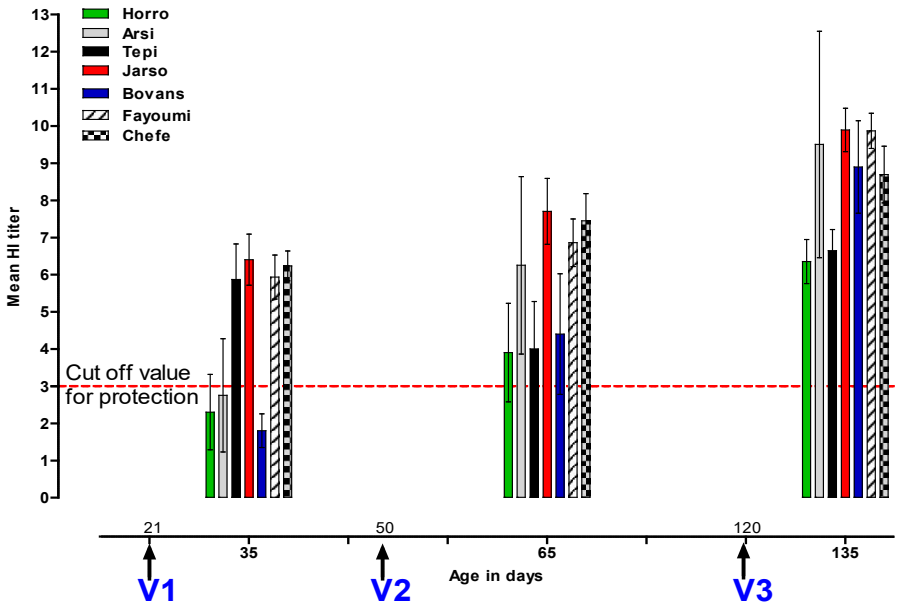


Fig. 4. The HI titer of chicken ecotypes during three times ND immunization (V1 first vaccination, V2 second vaccination, V3 third vaccination).

Proportion of chickens with protective titer (log2 ≥ 3) after vaccination

All (100%) of Cheffe, Jarso, and Faoumi chickens acquired a protective HI titer after a single vaccination. However, 10%, 40%, 50%, and 93% of Bovans, Horro, Arsi, and Tepi, respectively, had HI titer above the cut-off value after a single vaccination (Fig 5). Administering the second vaccination significantly increased the proportion of chickens with HI titer above the protective cut-off value in Bovans to 70%, Horro to 80%, and Arsi to 100%. Conversely, in the Tepi ecotype, HI titer decreased below protective level after the second vaccination, even lower than the 1st first immunization. The third vaccination-induced HI titer was above the protective level in all ecotypes.

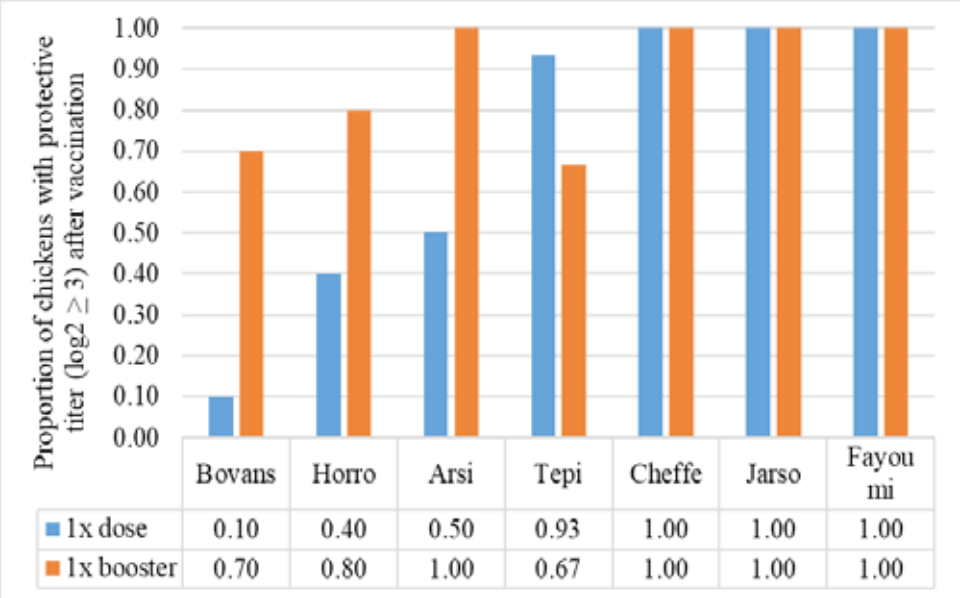


Fig. 5. The proportion of chickens with protective HI titer (log2 ≥ 3) after first (blue color) and second (yellow color) vaccinations. Key: 1x (blue color) indicates one-time first vaccination to naïve chickens. 1x (yellow color) indicates one-time booster vaccination.

Discussion

Seasonal ND outbreaks are common in multiple geographical locations in Ethiopia (Mulisa *et al.,* 2014). This suggests the presence of velogenic strains in the local viral pool (Diel *et al.,* 2012; Snoeck *et al.,* 2013; Fentie *et al.,* 2014b; Mulisa *et al.,* 2014; Bari *et al.*, 2020), or periodic evolution of a novel velogenic strain, or spillover from wild birds (Alexander, 2011; Grubaugh *et al.,* 2015). Ethiopia has never launched vaccination in the village backyard production system (Terfa *et al.,* 2018) although ND vaccine is available in the country against the disease (Nasser *et al.,* 2000; Abdi *et al.,* 2016). Limited field trial on ND vaccine efficacy under field condition has indicated that HB1 and La Sota vaccination induces only 50-60% immunity (Degefa *et al.,* 2004) showing outbreaks and marked pathological lesions (Ezema *et al.,* 2009), and shedding of the virus in the vaccinated chicken population (Fentie *et al.,* 2014a). This indicates that ND vaccines are providing suboptimum efficacy, probably due to vaccine virus-associated factors such as inadequate dose and frequency of vaccine prescription or vaccine mismatch. In the current study, however, we

propose that host genetic differences can be one of the reasons for the low immune response in vaccinated chickens in the field.

Therefore, we compared the HI titer of seven chicken ecotypes to ND immunization at ages 21, 50, and 120 days followed by serum collection for immune response analysis at days 35, 65, and 135. We used HI titer for measuring humoral (antibody) responses. Measuring HI antibody titer directly in sera is widely used for monitoring the induction of antibodies (Yeo *et al.,* 2003). The HI titer of $\log_2 \geq 3$ has been considered the standard cut-off value for deciding effective protective titer against velogenic ND viral challenge (van Boven *et al.,* 2008; Abdi *et al.,* 2016). Further study is required whether the same cut-off value in HI titer works for Ethiopian local chickens. A comparative study among Ethiopian ecotypes on the persistence of maternal immunity in chicks as well as different genotypes infection/challenge study is also required.

In this study, after a single vaccination, 100% of Cheffe, Jarso, and Fayoumi chickens acquired HI titer above the protective level (> $\log_2^3$); however, 10%, 40%, 50%, and 93% of Bovans, Horro, Arsi, and Tepi, respectively, had HI titer > greater than $\log_2^3$. We have reported that the mean HI titer $\log_2^{(3-5)}$ and $\log_2^{(2-3)}$ corresponds to 70–80 and 35–60% survival rates in infected chickens (Abdi *et al.,* 2016). Chickens of different genetic backgrounds have differences in immune response (antibody titer) following vaccination (Newport *et al.,* 2004; Liu *et al.,* 2009). Geographic location, the season of birth, and the timing of vaccination can make the immune response of the host strong or weak during immunization (Hur *et al.,* 2014). In the current study, environmental factors are controlled; the variation in the immune response was thus attributable to host genetics. It has been reported that each pathogen has a continuum of host population groups, ranging from highly susceptible to highly resistant (Hamilton, 1982; Lars *et al.*, 2009; Grubaugh *et al.,* 2015). This study revealed that the seven chicken ecotypes showed differences in the HI response to ND vaccine immunization, suggesting a single vaccination could not provide protective HI titers equally for some chickens of different genetic backgrounds.

After the second vaccination, the proportion of chickens with protective HI titer (> $\log_2^3$) increased: Bovans (70%), Horro (80%), and Arsi (100%); but decreased from 93% (first vaccination) to 67% (second vaccination) in Tepi. The reason for the HI titer declines in the Tepi ecotype was unknown. On the other hand, HI titer in the remaining chicken breeds increased following booster vaccination. Differences in immune induction mechanisms and the persistence of

antibodies post-vaccination have been observed among different breeds (Metcalf *et al.,* 2015). ND antibodies are probably short-lived or incapable of mounting protection under real conditions since outbreaks are reported in vaccinated chickens in the field (Kapczynski and King, 2005; Kapczynski *et al.,* 2013). Another study has similarly indicated that maternal immunity in chicken is depleted within 10 days after hatching (Gharaibeh and Mahmoud, 2013) suggesting the passive immunity from mothers to their neonates (i.e. maternal immunity) is short-lived. However, we poorly know the longitudinal dimension of immune kinetics, dynamics, maintenance, and clearance (decay) of the immune memory and antibodies in different chicken breeds. A study reported that ND antibodies persist for some years in wild birds after a single vaccination (Ramos *et al.,* 2014).

The goal of a successful vaccination program in the field is to control outbreaks through increasing herd immunity. Herd immunity in ND is achieved when greater than 85% of the flocks have HI titer > $\log_2^3$, which is mostly achieved after two vaccinations (van Boven *et al.,* 2008). In this study, two vaccinations were not enough to yield HI titer > $\log_2^3$ among some breeds such as Bovans, Horro, and Tepi compared to the remaining chicken ecotypes. In this regard, after two vaccinations, Bovans, Horro, and Tepi achieved HI titer of only 70%, 80%, and 67%, respectively; thus, required third vaccination to achieve the standard protective HI titer of more than $\log_2^3$. Finally, 100% of chickens vaccinated three times achieved HI titer above the standard cut-off value of more than $\log_2^3$ for protection in this study.

Overall, HI titer above $\log_2^5$ was achieved in Bovans, Horro, and Tepi after the third vaccination; in Arsi ecotype after the second vaccination, and in Cheffe, Jarso, and Fayoumi after a single vaccination; the results of which indicating breed variation in the immune response to ND immunization. We did not investigate the outcome of the challenge group using velogenic ND strain in this study. We determined that the mean HI titer above $\log_2^5$ corresponds to a 100% survival rate (Abdi *et al.,* 2016), caution, though, is needed in grading the survival of chicken breeds using HI titer alone. In this regard, cellular immunity (Ahmed *et al.,* 2007; Dalgaard *et al.,* 2010) or both antibody and cell-mediated immunity contribute to protection against ND in different breeds (Juul-Madsen *et al.,* 2011; Kapczynski *et al.,* 2013). Furthermore, one of the six innate immune genes is highly expressed in response to ND (Deist *et al.,* 2017; Schilling *et al.,* 2018). Protection in animals against disease involves a separate or usually combined interaction of innate and acquired immunity

(humoral and cell-mediated). In this study, we did not address the role of interaction of the innate and acquired immunity in protecting chicken against Newcastle disease; hence further study is required.

In general, commercial Bovans breed had a lower HI titer than some Ethiopian local chicken ecotypes and Fayoumi in this study. The commercial White-leg horn breed had a lower HI titer than the local chicken breed (Liu *et al.,* 2009). The same finding was reported between commercial Fayoumi and local breeds (Deist *et al.,* 2017). This suggests that improved animals for high production are generally weak in producing a protective level of the immune response. Despite selection for specific traits, which increased productivity by 90% in commercial chickens, they lost genetic diversity by over 50% compared to their ancestors indicating an inverse relationship between both traits (Muir *et al.,* 2008). Generally, one of the undesirable side effects of selection for high production efficiency in farm animals is a decline in their disease resistance and immune response to pathogens (Rauw *et al.,* 1998).

Overall, the HI titer against ND was significantly higher in Jarso > Cheffe > Fayoumi > Arsi > Bovans > Tepi > Horro suggesting antibody titer and ND vaccine efficacy of the ND vaccine depends on host factors. Ethiopian chicken ecotypes have demonstrated varying levels of resistance to some diseases such as Marek's disease as well as have had varying levels of production performance (Duguma, 2009). In this regard, the susceptibility of Ethiopian chickens to natural Marek's disease infection under confined management has been ranked previously based on mortality from highest to lowest as follows: Jarso > Tepi > Horro ≥ Konso ≥ Tilili > Fayoumi (Duguma *et al.,* 2005; Duguma, 2009). They have been also ranked based on improved survival rate (i.e. vaccine protection) following vaccination against Marek's disease and showed improved survival rate in the rank order of Jarso > Horro > Fayoumi > Tepi (Duguma, 2006; Duguma, 2009).

Conclusions

Ethiopian chicken ecotypes have demonstrated varying levels of immune response to ND vaccination both within and between each ecotype suggesting the presence of diverse subtypes in a chicken population. The existence of chicken groups with higher, medium, and lower immune responses within each eco-

type can be an indicator of the chickens` strategy for investing their genetic resources in immunity than production. Thus, our result suggests that the chicken ecotypes originated from the different geographical areas may carry a unique set of reservoir genes with special utility in their local ecological conditions, which probably affect vaccine effectiveness and herd immunity; hence invites further studies. We conclude that polymorphism in host genetics influences the mobilization of the host immune defense system for an operation against infection suggesting hosts with a weak immune response may be a good hiding refuge for ND. Thus, a blanket ND vaccination program based on results derived from a single breed might not be realistic to infer for all breeds thriving in different geographical areas. In this regard, a robust comparative evaluation of the innate and acquired immunity responses is essential among chicken ecotypes during comparative experimental infection, immunity, and vaccine trial studies.

Ethical approval

Addis Ababa University College of Veterinary Medicine and Agriculture (Ref. 221/3/4257/2009) provided ethical approval for the experimental trial.

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The Ethiopian Veterinary Journal (*Ethiop. Vet. J.*) is a multidisciplinary peer-reviewed journal intended to promote animal health and production of national and regional/international importance. The journal publishes review articles, original research articles, short communication as well as technical notes in English. Under special circumstances, articles in Amharic may be considered for publication.

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Ethiop. Vet. J., is the Official Scientific Organ of the Ethiopian Veterinary Association

ETHIOPIAN VETERINARY JOURNAL

(*Ethiop. Vet. J.*)

Objectives

- To stand as the official scientific organ of the Ethiopian Veterinary Association and serve as medium of communication with professionals in the fields of animal health and production and other related disciplines.
- To effectively disseminate research outputs in all spheres of veterinary science, veterinary public health, animal production and other related fields of study.
- To help promote agricultural development in Ethiopia and the subregion as a whole through generation via research of technologies, methods etc and dissemination of scientific knowledge to stakeholders and ultimately to end users.

Type of papers

- Original research papers
- Review articles
- Short communications

Original articles

This includes original articles presenting outputs from basic, applied and adaptive research activities related to animal and public health and diverse aspects of animal production in Ethiopia, and from regional and international sources. The material must not have been previously submitted or published elsewhere. The research article should not contain more than 6000 words.

Review articles

These include papers covering baseline data/information as well as advances in the field of veterinary medicine, public health and related fields of research and development. Often review articles will be prepared by specialist or researchers actively engaged in particular area of study for a considerable period of time with proven success. This must be supported by a strong track record

of publications in the area of the proposed review. Before submitting a review, authors must first contact the Editor with an outline of a proposed review. Acquisition of such papers my also be done through invitation. The review article should not contain more than 9000 words

Short communications

This is devoted to entertain results of original research, selected clinical case reports, brief scientific notes including preliminary results, scientific observation, experimental techniques, recent technological advances and news of interest in the field. Short communication must be written in a synthesis form where all necessary information are presented in a condensed manner. The material must not have been previously published elsewhere. Short communication should not contain more than 3000 words

Guidelines for authors

The Ethiopian Veterinary Journal (*Ethiop. Vet. J.*) is a multi-disciplinary and peer-reviewed publication intended to promote animal health and production of national, regional and international importance. All submitted manuscripts should be in English.

Submissions

Electronic copy in Word file should be addressed to the Editorial-Office. All manuscripts should be accompanied with a letter (or a filled Manuscript Submission and Copyright Transfer Form) signed by all authors, who clearly state that the paper, other than oral presentation or abstract, has not been submitted for publication elsewhere in any form. The Editorial Board reserves the right to accept or reject any paper submitted.

Manuscripts

In general, all submitted manuscripts must conform to the requirements set by the Journal. Failure to follow instruction in the preparation of manuscripts may result in total rejection or delay in publication. It should be prepared with Times New Roman 12 pt, double-spaced, minimum 1.5 cm margins. To facili-

tate the review process give line numbers in Arabic numerals. Give also page number in the lower middle of each page, starting from the title page.

Title page

The title page should consist of:

a) Title of the article, which should be concise and descriptive enough; should be in sentence case, and justified left; avoid abbreviations;
b) Name(s) of author (s), institutional affiliation, email addresses of all authors in italics (s); and
c) Name and address of the corresponding author, in case of more than one authors.

Abstract

The second page should provide an abstract of not more than 300 words summarizing the background, objective, materials and methods, major findings and their significance, and conclusions. Avoid the use of undefined abbreviations.

Keywords

Below the abstract, in a separate line, keywords, up to six in number should be given in alphabetical order separated by ";".

Introduction

Give sufficient information on the associated background zooming in to the subject in question; articulate the specific research question, and the contribution of your current research in bridging the gap, followed by statement of objective(s) is in order.

Materials and methods

Under this section, briefly state the materials used, the selection of study animals, subjects, herds or materials, and the design for observational or experimental study and the nature of controls. Briefly describe the methods and procedures followed in sufficient detail and give reference; if new or modified methods are used, give justification for using them. Precisely specify apparatus, instrument or chemicals used in terms of manufacturer's name and ad-

dress in parentheses. Similarly, drugs, biologicals, medicinal plants or others used should be sufficiently described in terms of generic name(s) or scientifically accepted identifications, dose(s), and route(s) of administration. Describe sufficiently the statistical methods used and how the magnitude or precision of analysis results reached.

Results

Quantify qualitative findings and present them with appropriate indicators of measurement of error or uncertainty, such as by using confidence intervals. Likewise, when reporting quantitative data, authors must indicate the extent of variability by either using standard deviation or standard error. Present the results in logical sequence in the text, tables, or illustrations. The results have to be presented in the same order as the questions raised in the objective(s) and methods sections. Results should be concise and no need of interpretation. In clinical or therapeutic trials, report complications or losses or even dropouts of such observations giving numbers. Support all findings by using appropriate statistical analysis.

Tables should be complete enough to be informative. They should be presented in separate pages, numbered consecutively using Arabic numerals and provided with captions. Avoid using vertical lines to separate columns in Tables. Details essential to further explain specific aspects of the Table should be given as a footnote below the table, by using appropriate symbols or lower case letters as superscripts.

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Discussion

Under this heading you should explain the findings and the associated interpretations. Relate the results with the objective of the study and with previ-

ously published work. Here emphasize the new and important aspects of the study and not repeat the results. In the discussion, clearly indicate the significance of the work and implications of the findings for future research.

Conclusion

This section should be separately presented with supporting evidences based on the major findings of the study. Appropriate recommendations can be made if necessary

Acknowledgements

Acknowledgments should be briefly stated after the Conclusions. Under this, technical, financial and material support can be mentioned.

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Authors should declare that they have no conflict of interest.

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Ameni, G., Miorner, H., Roger, F. and Tibbo, M., 2000. Comparison between comparative tuberculin and gamma-interferon tests for the diagnosis of bovine tuberculosis in Ethiopia. *Trop. Anim. Hlth. Prod.*, 32, 267-267.

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Jobre, Y., Malone, J. B., McCarroll, J. C., Erko, B., Mukaratirwa, S. and Xinyu, Z., 2001. Satellite climatology and the environmental risk of Schistosoma mansoni in Ethiopia and east Africa. *Acta Trop.*, (*In press*).

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Tegegne, A., Wirtu, G., Mukasa-Mugerwa, E. and Kassa, T., 1994. Oestrus phenomenon and oestrus detection efficiency using androgenized cows and entire bulls in Boran and Boran x Friesian crossbred cows. In: Proceedings, Advances in Tropical Agriculture in the 20th Century and Prospectus for the 21st: TA 2000, 4-9 September 1994, Port-of- Spain, Trinidad.

D) Books or chapters in books:

Thrusfield, M., 1995. Veterinary Epidemiology, 2nd edition, Blackwell Science Ltd, Oxford, UK.

Malone, J. B and Jobre, Y., 1999. Predicting outbreaks of fasciolosis from Ollernshaw to satellites. In: Dalton, J.P. (Ed.), Fasciolosis. CBA International Publications, Cambridge, Pp. 151-183.

E) Organizations as author:

OIE, 1992. Bovine tuberculosis. OIE manual for diagnostic techniques of livestock diseases. Office International des Epizooties (OIE), Paris, France. Pp. 287-296.

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