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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

Table of Contents

Dairy village: the role of veterinary services in unlocking dairy industry potential through assisted reproductive technologies
Sayid Ali, Mosisa Dire, Tamrat Degefa, Asnaku Funga, Hamid Jemal, Geda Regassa, and Curtis R. Youngs.....1
https://dx.doi.org/10.4314/evj.v28i1.1

Assessment of community knowledge, attitude, and practice towards rabies and its determinants in Kersa District, East Hararghe Zone, Oromia Region, Ethiopia
Fami Adem, Abebe Belete Bitew, Takele Adugna, and Solomon Lulie Abey.....15
https://dx.doi.org/10.4314/evj.v28i1.2

Biosecurity level assessment in commercial poultry farms of Central Ethiopia
Dejene Tadesse, Zerihun Aseffa Abebe Wirtu and Teshale Sori 36
https://dx.doi.org/10.4314/evj.v28i1.3

Detection of aflatoxin in raw and pasteurized milk by high-performance liquid chromatography (HPLC) in central Ethiopia
Sitena Kebede, Belachew Bacha, Belachew Tefera, and Gezahegne Mamo.....55
https://dx.doi.org/10.4314/evj.v28i1.4

Seroprevalence of bovine viral diarrhea virus and detection of persistently infected (PI) animals in dairy farms of Holeta, Central Ethiopia
Worku Birhanu, Asamenew Tesfaye, Yitbarek Getachew, and Haileleul Negussie.....73
https://dx.doi.org/10.4314/evj.v28i1.5

Effect of supplementing concentrate mixture with field pea hull on yearling ram lambs of local sheep: Nutrient intake, linear body measurements, and changes in body weight
Getachew Adugna, Berhane Mekete Bogale ,and Helen Nigussie.....88
https://dx.doi.org/10.4314/evj.v28i1.6

Gut microbiota and relevant abundances of *Prevotella copri*, Lachnospiraceae, *Collinsella*, *Helicobacter cinaedi, Desulfovibrio*, and *Escherichia coli* among cats with *Pemphigus foliaceus*
Kerem Ural, Hasan Erdoğan, Songül Erdoğan, and Cansu Balikçi.....105
https://dx.doi.org/10.4314/evj.v28i1.7

Isolation and identification of *B. abortus* and *B. melitensis* in ruminants with a history of abortion: the first report from Eritrea
Ghebremeskel Habteyohannes Efrem, Bereket Mihreteab, Michael K. Ghebremariam, Yitbarek Getachew, and Gezahegne Mamo.....122
https://dx.doi.org/10.4314/evj.v28i1.8

Short communication
Identification of major protozoal enteropathogens causing calf diarrhea in dairy farms in and around Holeta Town, Oromia Special Zone, Ethiopia
Diriba Girmaye, Akinaw Wagari, and Teshita Edaso Beriso.....139
https://dx.doi.org/10.4314/evj.v28i1.9

Guidelines to Authors.....149

Ali et al., Ethiop. Vet. J., 2024, 28 (1), 1-14
DOI https://dx.doi.org/10.4314/evj.v28i1.1 *Ethiopian Veterinary Journal*

Dairy village: the role of veterinary services in unlocking dairy industry potential through assisted reproductive technologies

Sayid Ali, Mosisa Dire[1], Tamrat Degefa[1], Asnaku Funga[1], Hamid Jemal[2], Geda Regassa[3], and Curtis R. Youngs[4]*

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Abstract

The "Dairy Village" concept was designed to overcome the constraints of estrus synchronization and AI particularly associated with seletion of the breeding animals and subsequent management of estrous cows in Ethiopia. All necessary inputs, including sexed semen, were acquired before the fieldwork. The knowledge and skill gaps were identified and addressed with the active involvement of veterinarians and AI technicians who underwent specialized training to ensure proper physical and gynecological examinations during the selection of cows and heifers. Accordingly, a refresher training was delivered on topics of reproductive anatomy and physiology, assessment of ovarian status, semen handling, and insemination at the optimal time to establish pregnancy. Besides, awareness creation seminars were held every year on topics of benefits of reproductive technologies and management of breeding cows to 99 smallholder dairy farmers. Only 578 cows/heifers were found fit for breeding out of the 1262 cows/heifers that were brought for evaluation; including 81 animals that spontaneously showed estrus. A total of 497 cows/heifers were synchronized using a single IM injection of Lutalyse. Animals responding to the treatment (404 cow/heifers) and those spontaneously in estrus (81cows/heifers) were inseminated using sex-sorted HF semen 18-24hrs into the standing estrus. Pregnancy rate was 80% (388/485) and 287 calves were born (96% female). Results of this project indicate that rigorous gynecological examination during the selection of cows prior to estrus synchronization and judicious application of hormonal treatments can yield improve the success of ES/AI projects under field conditions. Furthermore, the dairy village approach to en-

hanced milk production through efficient use of sex-sorted semen has generated replacement heifers from superior breed that will substantially improve the livelihoods of village farmers.

Keywords: Artificial Insemination; Dairy village; Estrus synchronization; Genetic improvement; Sex-sorted semen.

Introduction

As the growth of the human population increases, so does the demand for food, particularly for nutritious animal-source foods such as milk and meat (WFP and CSA, 2019; Dror and Allen, 2011). Although Ethiopia has a massive livestock population, there is a mismatch between livestock numbers and quantities of livestock products due to low individual animal productivity' (FAO, 2018; CSA, 2020/21). Hence, meeting the steadily increasing demand for nutritious food like milk, is possible only by increasing individual/herd-level productivity through proper management of improved dairy breeds. Nevertheless, despite the great effort made, the use of artificial insemination (AI) and estrus synchronization (ES) has had a trivial impact dairy cattle genetic improvement through crossbreeding with exotic breeds (Hunde, 2018). Hence milk production in Ethiopia remains low and inefficient (Desalegn, 2009). Several possible interventions have been identified to remedy this situation, including culling unproductive animals and decreasing herd size to retain only highly productive animals. This has significantly enhanced reproductive management (because pregnancy and birth are required to initiate milk production); and increased herd/individual productivity through improved genetics and subsequently reduced headcount with the help of biotechnology tools (LGA, 2016; Tegegne *et al.,* 2016; FAO, 2017; Adegbola *et al.,* 2020; Daba *et al*., 2022). As intensification is becoming the best option to cope with scarcity of grazing land, there is currently a high demand for improved dairy cows on the market. The exorbitant prices became unaffordable for smallholder farmers, and the importation of live cows/heifers from other countries is also unlikely due to unaffordable prices. Therefore, a more rational application of technology, knowledge management, and service delivery system are needed to increase productivity and operational effectiveness.

Field application of reproductive technologies in dairy cattle demands skillful assessment of the suitability of anatomy and physiology of cows presented for breeding, judicious implementation of ES protocols, proper detection of es-

trus, appropriate semen handling, and correct timing of insemination. One of the suggested solutions for this in Ethiopia has been the adoption of a "Model Dairy Village". Two reasons make this essential: increasing the operational efficiency of reproductive technologies in community services, and facilitating the adoption of livestock biotechnologies. AI practices augmented with the use of sex-sorted semen would enhance genetic improvement and further improve subsequent animal/herd productivity. The main objective of this article is to share the best practices that evolved during the implementation of the "Model Dairy Village" approach aimed at increasing the production of female calves with superior genetic capacity for milk production. This approach was implemented for three years using ES and AI with sex-sorted semen.

Materials and methods

Description of the study areas

The study was conducted in selected kebeles found in the East Shewa Zone of Ada'aa woreda of Oromia Regional state, namely Denkaka, Ude, Akaki, and Bishoftu towns of Bishoftu City Administration. The study area, Bishoftu, is located at a 45 km distance from Addis Ababa in the South. The area is located at 9°N latitude and 40°E longitude at an altitude of 1850 m with annual rainfall of 866 mm, of which 84% is in the long rainy season, June to September. The annual average temperature ranges from 12.3°C to 27.7°C with an overall average of 18.7°C. It is an important town where most governmental institutions, including national and international research centers, are located. Cattle, small ruminants, poultry, swine, and equines are the major livestock species kept with fast-growing small-scale dairy production (NMSA, 2010). As Ada'aa is known for its mixed crop-livestock farming systems, Teff, westrus, and legumes are major crops produced in the study area.

Study population

A total of 99 smallholder farms comprising 1262 zebu and zebu*HF cross-bred cows/heifers were included in the pilot study. The cows/heifers were selected for breeding after passing a rigorous clinical and gynecological evaluations. Only those that were apparently healthy, cycling and with a CL on their ovaries were enrolled into the estrus synchronization (ES) and AI project.

Review of previous practice and identification of intervention loop

During the early stages of the "Model Dairy Village" concept, a questionnaire was administered to 99 dairy producers. Additionally, researchers with multi-disciplinary expertise were involved in the field implementation of the technology. A total of five animal health and reproductive biotechnology researchers from Debre Zeit Agricultural Research Center and 35 experts (6 AI technicians and 29 animal production experts) from Ada'aa livestock and fishery sectors developmental agents who had previously participated in ES and AI projects were involved in the study. The experts initially made assessment of the main limiting factors that adversely affected previous efforts in the identified pilot sites. A predesigned data collection format consisting of quires on breed, parity, cyclicity, sire ID, calf sex, and BCS was used for recording.

Previous field implementation reports of ES and AI efforts undertaken in different regions of Ethiopia were also reviewed, and four major areas of interventions were identified to circumvent the factors that rendered the previous ES and AI service delivery system inefficient.

- Creating awareness of the dairy farmers regarding needed technological inputs and the critical role of the animal owners in the success of ES and AI procedures
- Reducing the knowledge gap among AI technicians with regard to a) bovine reproductive anatomy and physiology, and b) selection of physically and physiologically "fit" animals presented for ES and subsequent AI
- Provision of inputs and supplies to ensure that supply limitations would not hinder success
- Practical engagement veterinarian, reproductive technology researchers, and trained AIT in field application

Awareness creation and refreshment training

Training was given to the selected farm owners (n=99) to create awareness on bovine estrus and management of breeding cows (Figure 1). The dairy experts (n=29), and AI technicians (n=6) were also given theoretical (25% of allotted time) and skill (75% of allotted time) refresher training on bovine reproductive anatomy and physiology, skills of determining ovarian status, proper semen handling and insemination techniques for use with sex-sorted semen, gynecological evaluation, estrus synchronization, AI and pregnancy diagnosis (Figure

2). The trainees were expected to implement the knowledge and skills captured through this training during the field application of this pilot work.



Figure 1. Awareness creation seminar for the farmers in Bishoftu *C*ity and Dankaka dairy village



Figure 2. Theoretical and practical training on AI and gynecological evaluation at DZARC

Selection of animals for the ES and AI project

A total of 1262 cows/heifers those were presented as potential candidates underwent a thorough clinical and gynecological examination by the trained experts to determine their suitability for enrollment into the ES and AI demonstration project. Only 578 cows/heifers (45.8%) including 81 cows/heifers that showed natural estrus, were considered fit for breeding based on BCS (≥ 2.75 on a 5 scale) and the presence of an active CL. Cows/heifers that did not show natural estrus (n=497) received a single IM injection of Lutalyse® (Dinoprost tromethamine, equivalent to 5mg per ml dinoprost, Zoetis Inc, Kalamazoo, Spain). Estrus was observed visually by the trained owners and 404

cows/heifers (81.3%) responded to the Lutalyse treatment. A total of 485 cows/heifers (both the induced and natural) were inseminated with Holstein breed sex-sorted semen (ABS Global, Inc. USA; BUTZ-HILL MOOGENE-ET, Reg.# 71088738 and DEEP-RUN-CRK DFR CANYON-ET, Reg#73855681) 18 to 24 hours after the start of standing estrus. Pregnancy was diagnosed 70 days post AI via transrectal palpation.

Results

Review of previous reports

Some of the numerous challenges reported to impede the previous ES and AI success were a lack of understanding of the concept of ES by extension personnel, experts, and farmers, lack of proper selection of animals suitable for ES and AI based on body condition score and/or reproductive tract status; poor estrus detection by farmers; inadequate preparation for the provision of the AI service; poor technical capacity and lack of experience by AI technicians (AIT); and weak support from administrative bodies. Furthermore, 77% of the farmers were not aware of the ES and AI technologies. Only 17% were aware and satisfied with the ES and AI achievement. None of the farmers participated in awareness-creation seminars on ES and AI technologies. All AIT and experts did participate in similar training/seminars but never had practical exercise. There were also limitations in the availability of inputs, quality of semen used, and LN2 for regularly topping up the semen tank.

- **Body condition of heifers/cows:** 1.5% of presented animals had BCS < 2.5, 41.2% had 2.5 to 3.0, 30.3% had >3 to 3.5, 24.8% had >3.5 to 4, and 2.2% possessed a BCS >4 (Figure 3).



Figure 3. A cow (left) and a heifer (right) grouped as unfit for synchronization due to poor body condition (BCS <2.5)

- **Estrus response:** From the 497 cows/heifers that received Lutalyse (PGF2α) for synchronization of estrus, 404 (81.3%) showed overt estrus and were presented for AI. The response of the remaining 93 cows/heifers was undetermined because animals didn't return for AI and/or farmers provided no feedback either. Farmers who assigned children to detect estrus or who entirely neglected estrus detection (n=70) failed to get their cows impregnated in a timely manner. Unlike previous reports on mass ES, no cow/heifer aborted after receiving PFG2α in this project confirming the importance of a thorough gynecological exam prior to ES.
- **Pregnancy detection (PD):** the pregnancy rate was 80% (Table 1) with the average services per conception of 1.14 (excluding those animals with no feedback and with a history of repeat breeding). A total of 285 calves were born (including two twin births, Table 2).

Table 1. Pregnancy test results in areas included in mass estrus synchronization by location

Location	PD test result			Total
	Negative	Pregnant	Unknown	
Ada'aa (Denkaka, Ude, Akaki)	47	247		322
Bishoftu	43	113	7	163
Total	90 (18.6%)	388 (80.0%)	7 (1.4%)	485 (100.0%)

Additionally, the rate of abortion, stillbirth, and neonatal death were 3.1%, 0.25%, and 2.8%, respectively. All of the reproductive losses occurred in Holstein or Holstein crosses cows/heifers.

Table 2. Number of female calves born from the ES and sexed semen AI in pilot dairy villages

Location	Gender of calves born		Total
	Female	Male	
Ada'a	182	7	189
Akaki	19	1	20
Bishoftu	722	4	76
Total	273 (95.79%)	12 (4.21%)	285 (100.00%)

Community outreach

Farmers, professionals, development partners, and policymakers were introduced to reproductive technologies and techniques by arranging farmers' field days (Figure 4). During the event, participants were able to see first-hand when ES and AI with sex-sorted semen were successfully applied under field conditions in the target communities. The field days were organized by DZARC in collaboration with the Ada'a district agricultural development office. The level of satisfaction among participant smallholders with the ES and AI project were 45%, 44%, and 7% as excellent, good and fair, respectively.



Figure 4. First batch of newborn calves visited by Minister dE'tat and other zonal higher officials in Dankaka Dairy Village

Discussion

Different research reports from Ethiopia indicate that both ES and AI services are constrained by multiple factors functioning in cohorts, such as poor infrastructure development (Desalegn, 2009), poor feeding management of breeding animals, and rampant disease problems affecting general health and fertility (Tegegne *et al.,* 2016). However, problems of estrus detection, semen shortage, transportation and semen handling issues, unreliable supply of LN_2, and meager skill of AI technicians have long been identified as major limiting factors (Tegegne *et al.*, 2012). The survey that was administered before the field implementation of the "Model Dairy Village" concept confirmed the extensiveness of some of the previously reported problems. In addition, the high proportion of (>50%) cows/heifers failing the evaluation for breeding fitness in the present were mostly because of the poor body condition (41% of cows/heifers were between 2.5 and 3.0) and anestrous. Past performance of scaled-up ES technology in different parts of Ethiopia produced inconsistent results and was deemed as low efficiency (Azage *et al.,* 1989; Gizaw *et al.,* 2016; Mossie and Wondmnew, 2016). The rapid appraisal survey results in the current study showed that the major limitation of ES programs was the lack of or negligence to identify non-cyclic cows/heifers before initiation of hormonal treatment.

The treatment response obtained in this study was in agreement with previous reports (Aydaiso *et al.,* 2020; Shanku, 2022; Haile *et al*., 2023) but relatively higher than rates reported by Fantahun and Admasu (2017) and Gizaw *et al.* (2016). Estrus detection efficiency was one of the major limiting factors for the success of ES programs (Sveberg *et al*., 2011; Roelofs and Van Erp-van der Kooij, 2018), which goes in line with the present report. Prostaglandin F2α and its analogs are effective luteolytic agents capable of inducing regression of the corpus luteum (CL) and are widely used in ES protocols (Lucy *et al*., 2004; Oaxaca *et al*., 2009; Ahlawat *et al*., 2015; Paul *et al*., 2015). In the current work, both natural and synthetic prostaglandin F2α were administered intramuscularly, effectively inducing estrus within 72 hours of treatment.

Limitations in technical skill have been identified to affect AI service in Ethiopia (Desalegn, 2008; Tegegne *et al.,* 2016; Gizaw *et al.,* 2016). In this study, the refresher theoretical and skill training has greatly impacted the ability of the AI technicians to make decisions and correctly recruit animal fit for breeding (cycling and with active CL on the ovary). Owing to this and accurate detection of estrus has improved both the service per conception and conception rate even with sex-sorted semen. The absence of abortion after PGF2α treatment for ES supported the effectiveness of the training in accurately identifying the physiological status of cows/heifers during gynecological examination. Current findings also proved a high degree of effectiveness of a single-shot prostaglandin-based ES protocol if proper CL identification and estrus detection occurred. The proportion of female calves born in this project (96%) was higher than the vendor's claim (company recommendation, which is about 90%).

Conclusions

Successful implementation of an ES protocol requires the selection of cows/heifers based on their BCS and knowledge of their ovarian status before administration of prostaglandin F2α. On-job training of AI technicians focused on bovine reproductive anatomy and physiology requires the technical contribution of veterinarians in enhancing the skill and knowledge base of those AITs. The success achieved in this pilot demonstration project of a "Model Dairy Village" was also attributed to having all required inputs and determining the logistics of implementation of ES and subsequent AI with sexed semen. Farmer education to create awareness of bovine reproductive technologies and their proper implementation, coupled with farmers sharing their personal experiences (with ES and AI) with other farmers, was another key to success. The

current pilot project unveiled the innovative approach followed in the application of ES and AI with sexed semen for the genetic improvement of dairy cattle. This represents a much-needed and long-overdue technological advancement that can transform the lives of smallholder farmers. Therefore, the recommendations emanating from the current "Model Dairy Village" pilot project are:

- Awareness creation and knowledge transfer are needed to enhance farmers' understanding of ES and AI technologies, which increases their willingness to adopt the technologies.
- Proper nutritional management of cows/heifers to obtain a good body condition score (BCS) is crucial to the success of ES and AI programs.
- Refresher training for AI technicians who are focused on bovine reproductive anatomy and physiology (including identification of ovarian structures via rectal palpation) is essential. Additional training topics should include proper semen handling and insemination technique, timing (especially for sexed semen), and site of artificial insemination, which require active involvement and the leading role of veterinarians to unlock the dairy potential of the country.
- Acquisition of inputs and consumables used for AI service before implementation of ES is vital, as is the logistical planning.
- By implementing ES and AI within a village, community members can share resources among themselves and learn from each other.

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Assessment of community knowledge, attitude, and practice towards rabies and its determinants in Kersa District, East Hararghe Zone, Oromia Region, Ethiopia

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Abstract

Despite the prevention and control efforts against rabies, it remains a health problem for humans and animals in Ethiopia. A cross-sectional study design was employed from February to July 2022 to assess community knowledge, attitudes, and practices towards rabies and associated factors in the Keresa district of Oromia regional state, Ethiopia. Face-to-face interviews were used to collect data from 384 households, and logistic regression was employed in the data analysis. Fifty-three percent (95% CI= 47.9-58.1%), 62% (95% CI= 57.3-66.9%), and 50% (95% CI= 45.1-54.9%) had adequate knowledge, a desirable attitude and good preventative practices towards rabies, respectively. 92.7% (95% CI= 90.1-95.3%) of participants had heard of rabies before, and 52.9% (95% CI= 47.9-58.1) of their primary source of information was the community. Nearly all respondents, 95% (95%CI= 93.8-97.7%), admitted to eating rabid animal flesh, and 56.3% (95%CI= 51-61.5%) believed that eating rabid animal meat may transmit the disease. One-fourth, 25.5% (95%CI=21.1-29.9%) of study participants believed that rabies is related to spirits, and 27.9% (95%CI= 23.4-32.6) claimed that holy water might treat rabies. Sex, level of education, occupation, and training status affect the knowledge of study participants. Age, family size, occupation type, and source of information were all linked to preventive practice, but sex and age were the only variables associated with attitude. Respondents having good knowledge and desirable attitudes had better preventive practices against rabies. Therefore, further awareness and disease-

related training are needed for the district population and beyond to improve their knowledge of rabies prevention and control.

Keywords: Attitude**;** Kersa District**;** Knowledge; Practice; Rabies.

Introduction

Rabies is a neurotropic disease caused by Lyssavirus rabies, which belongs to the genus *Lyssavirus* in the family Rhabdoviridae of the order Mononegavirales and is transmissible to all mammals. It is one of the 20 neglected tropical diseases identified by the WHO, nearly 100% lethal in humans and animals and killing 59,000 people annually (WHO *et al.*, 2018). It also causes a severe and long-term societal and financial burden on patients in poverty-stricken countries (WOAH, 2019). The cost of post-exposure prophylaxis for humans, the loss of livestock, and other rabies-related expenses made an estimated 8.6 billion USD annually in global economic burden (WHO, 2023). In Africa, rabies is an endemic disease in which 99% of human cases are caused by dog bites (WOAH, 2022).

WHO and WOAH are pushing for the elimination of human deaths caused by dog-mediated rabies by the year 2030. Dog rabies vaccines can effectively eradicate the disease at its animal source and are 100% preventive (WOAH, 2022; WHO, 2023). However, without consistent efforts to bridge the gaps in access to dog vaccination and post-exposure treatment for afflicted people and animals, the threat of rabies is anticipated to grow in Africa (WOAH, 2022). Community collaboration involving veterinarians and public health officials is necessary to prevent human rabies. Baseline data on the coverage rates of practices (dog vaccination, dog restraint, correct care of bite wounds, seeking Post-Exposure Prophylaxis, and rabid dog management) are crucial for control and prevention (WOAH, 2017). Mass dog vaccination is the preferred method of eradicating rabies, and vaccination coverage in rabies-endemic areas should be at least 70% to prevent outbreaks (WHO, 2013). On the other hand, veterinary services should take the lead in controlling the dog population to prevent the spread of the rabies virus, coordinating their efforts with other relevant public institutions (WOAH, 2019).

Among African countries, Ethiopia was estimated to have the second-largest number of rabies deaths in the year 2018 (WHO *et al.*, 2018). According to the Centers for Disease Control and Prevention (CDC) report, thousands of people

are infected in Ethiopia, and an estimated 2,700 people die each year due to rabies (CDC, 2017). The high incidence of canine rabies in Ethiopia is due to the presence of a large number of dogs and their poor management (Wario *et al.*, 2018). As reported by Jemberu *et al*. (2013), the estimated yearly incidence of rabies was 2.33 cases per 100,000 people, 412.83 cases per 100,000 dogs, 19.89 cases per 100,000 cattle, 67.68 cases per 100,000 horses, and 14.45 cases per 100,000 goats (Jemberu *et al.*, 2013). A total of 2667 dog brain samples were investigated between 1990 and 2000, and 1951 (73.2%) samples tested positive for rabies (Yimer *et al.*, 2002). Between 2001 and 2009, the Ethiopian Health and Nutrition Research Institute recorded 386 fatal human cases, with an annual range of 35 to 58. During that time, 75% of the 3,460 animal brains tested in the lab were found to be rabies-positive (Deressa *et al.*, 2010). On the other hand, 87 cases of human rabies were clinically identified at the Ethiopian Public Health Institute between 2015 and 2019, with a death rate of 100% (Aklilu *et al.*, 2021). According to Akililu (2021), dogs were responsible for 95.4% (83/87) of human deaths, compared to 1.1% (1/87) for cats and 3.4 (3/87) for wild animals (Aklilu *et al.*, 2021).

Even though vaccines are available for humans and dogs in Ethiopia, Kidane *et al*. (2016) found that just 3.9% of dogs in Addis Ababa, the capital of Ethiopia, had gotten their vaccinations (Kidane *et al.*, 2016). The challenges related to the prevention and control of rabies include the extensive use of traditional medications and religious approaches to treat rabies cases. The majority of rabies victims, particularly those from rural regions, visit health institutions when they give up using traditional treatments or when a family member is lost (Deressa *et al.*, 2010). Therefore, assessing the community's knowledge, attitude, and practice towards rabies at all levels would be nice. To the best of the authors' knowledge, we were unable to track down any previously published studies on community KAP towards rabies in the Kersa district. Therefore, this study aimed to assess the community's KAP towards rabies and associated factors in Kersa district, East Hararghae zone of Oromia region, Ethiopia.

Materials and methods

Study area

Kersa is one of the districts of the Eastern Hararghe zone of the Oromia regional state and is bordered by Bedeno to the south, Meta to the west, Dire Dawa to the north, Haro Maya to the northeast, and Kurfa Chele to the south-

east (Figure 1). It is 465 kilometers east of Addis Abeba, at a latitude of 9°15'0" N and a longitude of 41°40'0" E. Its height above sea level varies from 1400 to 3200 meters. The district receives between 550 and 1300 mm of rainfall each year, and the average yearly temperature is between 15 and 20 °C (Wikipedia, 2023). According to statistics released by the Central Statistical Agency in 2007, an estimated 225,510 people are living in this area, with 113,024 males and 112,486 women (ESPS, 2022).

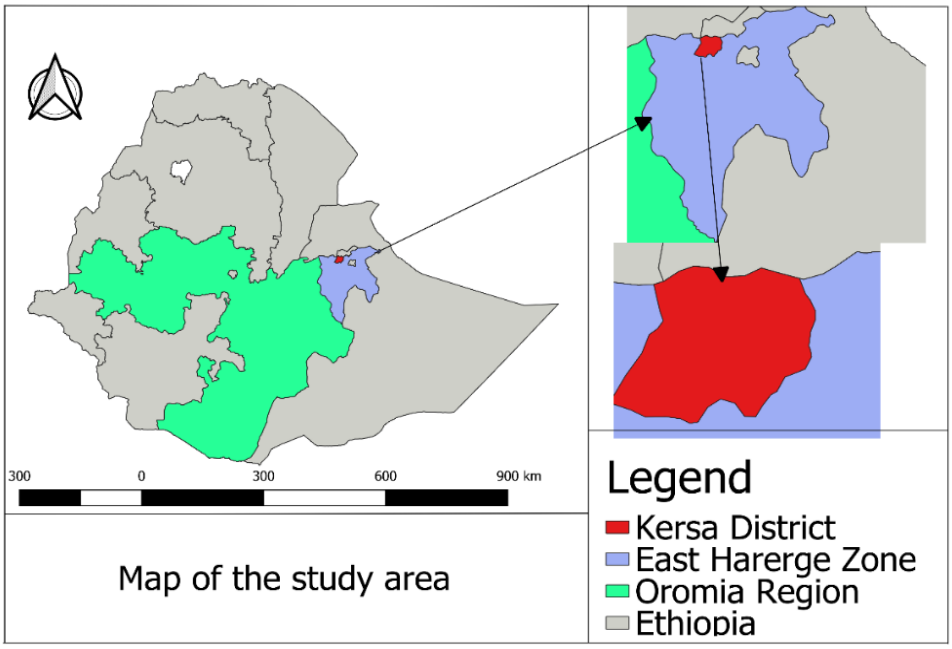


Figure 1. Map of Ethiopia showing the study area

Study population

The study participants include individuals aged ≥ 15 years who have been permanent residents for at least six months in the Kersa district, including urban, peri-urban, and rural residents, who were recruited for the research.

Study design, sample size determination, and sampling technique

The study was carried out using a cross-sectional design from February to June 2022. According to assessments provided by Abdela and Teshome (2017), 57.3% of Munesa district's residents in the Arsi Zone had good KAP for rabies, which was used to determine the required sample size for this study. The Thrusfield (2018) formula was employed to calculate the sample size, assuming a large population proportion (Thrusfield, 2018). Hence, Where n= required sample size; Pexp = population have good expected KAP to rabies (57.3%); d^2= Desired absolute precision (5%); Z- value for 95% confidence interval (1.96). Therefore, = 376, by adding 10% non-response rate, the sample size was 414.

Multi-stage sampling techniques were used for the selection of sampling units. First, areas were selected based on the population's lifestyle to represent urban, peri-urban, and rural areas. Urban and peri-urban areas were represented by one for each, while the rural area was divided into highland, midland, and lowland areas based on agroecology. The five areas, Mada Oda, Weter, Langey, Kersa, and Balina, were purposefully selected based on the above criteria. Each area had an equal number of samples. The households in the selected areas were then further selected via a technique known as systematic random sampling. Finally, one interviewee was chosen at random from each of the selected households.

Data collection tools

For this investigation, a standardized closed-ended questionnaire was used. The questionnaire included questions about knowledge, attitude, practice, and information about the resident's age, sex, education, and religion. It was initially written in English and then translated into Afaan Oromo for the study participants.

Data entry and statistical analysis

Data were entered and coded into Microsoft Excel and transferred to Statistical Packages for Social Science (SPSS) version 23.0 statistical packages. Frequency and percentage were used to present descriptive statistics data. KAP questions were given scores and dichotomized into good and poor KAP levels using a cut-off of 50% of the highest score possible (Bahiru *et al.*, 2022). Binary knowledge, practice, and attitude questions received a score of 1 for a correct response and 0 for an incorrect response. Respondents who had a score of 50%

or more were thought to have adequate knowledge, good practice, and a desirable attitude.

In comparison, those who received a score of less than 50% were thought to have inadequate information, poor practice, and an undesirable attitude (Hagos *et al.*, 2020; Bahiru *et al.*, 2022). Univariable and multivariable logistic regression analyses were performed to figure out the association between the KAP scores and any socio-demographic variables. In the univariable logistic regression, variables with p-values less than 0.2 were fitted into the multiple logistic regression models. Association among KAP of respondents was tested using a Chi-square test. The dependent variables were considered to be significantly associated with variables in multiple logistic regressions when the p-value was less than 0.05.

Ethical considerations

An ethical approval letter was obtained from the University of Gondar, College of Veterinary Medicine and Animal Sciences Institutional Ethical Review Committee with reference number CVMAS.sc-05/2022. Before the interview began, they were briefed about the purpose of the study and asked for their consent, and only volunteer participants were recruited for the study. The responses of study participants were kept completely confidential, and their identities did not appear in the research report.

Results

Socio-demographic characteristics

Of the total 414 study participants approached for an interview, only 384 completed the interview; hence, the response rate was 92.8%. More than half of the respondents, 53.6% (95% CI= 48.7-58.6%), were males. The majority of study participants, 46.4% (95% CI= 41.2-51.3%), were in the age group between 30-45 years old. Nearly half of the study participants, 47.1% (95% CI= 42.4-52.3%), had no formal education, and only 12.2% (95% CI= 8.9-15.9%) of the participants had completed college and above. Half of the respondents, 50% (95% CI= 45.1-55.2%), were farmers, and 11.2% (95% CI= 8.3-14.6%) were government employees. About 62.2% (95% CI= 57.6-67.2%) respondents have gotten- married, and 43.5 % (95% CI= 38.8-48.7%) respondents have a family size of three to six (Table 1).

One-third of respondents, 31.3% (95% CI= 26.3-36.2%), kept dogs in their house, but only 17.5% (21/120) dogs were vaccinated. The 8.6% (95% CI= 6-11.5%) of respondents had health-related training, and 44% (95% CI= 39.1-49.5%) had experience with rabid animals. The majority of participants 92.7% (95%CI= 90.1-95.3%) had heard of rabies previously, and more than half of them 52.9% (95% CI= 47.9-58.1%), and one-fifth of them 21.6% (95% CI= 17.4-25.8) had the source of information from the community and mass media, respectively (Figure 2).

Table 1. Socio-demographic and KAP information of the study participants in the Kersa district (N= 384)

Variables		N (%) Good (%)	Knowledge	Attitude	Practices
			Good (%)	Good (%)	
Sex	Male	206(53.6)	133(64.6)	143(69.4)	110(53.4)
	Female	178(46.4)	71(39.9)	96(53.9)	82(46.1)
Age (in years)	15-29	151(39.3)	90(59.6)	113(74.8)	93(61.6)
	30-45	178(46.4)	87(48.9)	103(57.9)	83(46.6)
	≥46	55(14.3)	27(49.1)	23(41.8)	16(29.1)
Educational status	Illiterate	181(47.1)	70(38.7)	86(47.5)	71(39.2)
	Primary school	78(20.3)	24(30.8)	51(65.4)	47(60.3)
	Secondary school	78(20.3)	66(84.6)	55(70.5)	46(60)
	Collage and above	47(12.2)	44(93.6)	47(100)	28(59.6)
Religion	Orthodox	146(38)	73(50)	86(58.9)	77(52.7)
	Muslim	238(62)	131(55)	153(64.3)	115(48.3)
Occupation	Unemployed	29(7.6)	27(91.1)	24(82.8)	23(79.3)
	Farmer	192(50)	75(39.1)	95(49.5)	78(40.6)
	Trader	76(19.8)	44(57.9)	52(68.4)	48(63.2)
	Employed	43(11.2)	37(86)	40(93)	22(51.2)
	Student	44(11.5)	21(47.7)	28(63.6)	21(47.7)
Family member	1-3	77(20.1)	51(66.2)	52(67.5)	38(49.4)
	4-6	167(43.5)	116(69.5)	106(63.5)	101(60.5)
	>6	140(36.5)	75(53.6)	81(57.9)	53(37.9)
Marital status	Single	127(33.1)	75(59.1)	94(74)	74(58.3)
	Married	239(62.2)	115(48.1)	131(54.8)	107(44.8)
	Divorced	13(3.4)	11(84.6)	11(84.6)	11(84.6)
	Widowed	5(1.3)	3(60)	3(60)	0

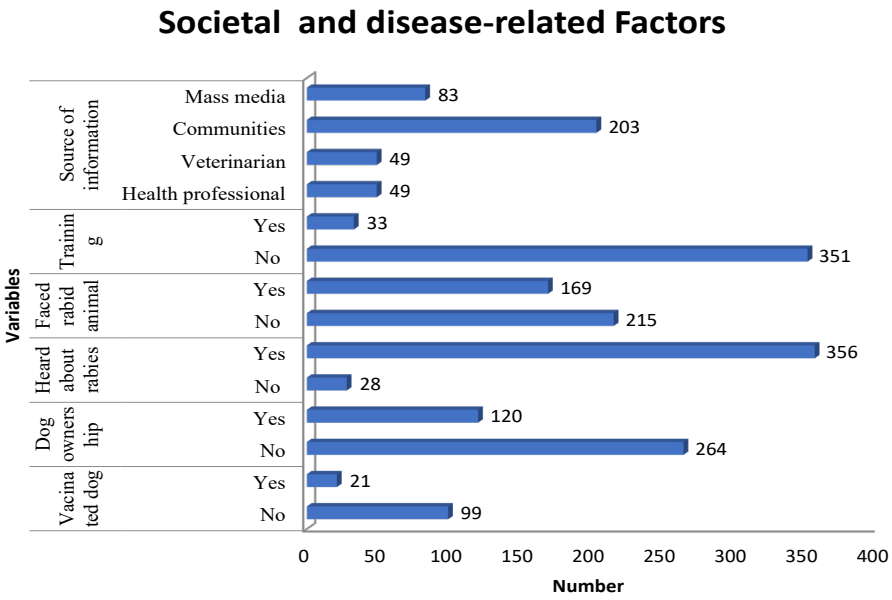


Figure 2. Societal and disease-related factors towards rabies in Kersa district

Knowledge of study participants towards rabies

More than half of the respondents, 53% (95% CI= 47.9-58.1%), had adequate knowledge about rabies in the Kersa district (Figure 3). Almost two-thirds, 65.1% (95% CI=60.4-69.8%) of respondents knew about zoonotic disease, and 69.8% (95%CI= 65.4-74%) think rabies is zoonotic. Slightly higher than one-fourth, 26.8% (95% CI= 22.4-31.3%) of respondents knew the cause of rabies is a virus, and 82% (95% CI= 77.9-85.7%) and 74.7% (95% CI= 70.3-79.1%), knew the clinical sign of rabid animals and the way of transmission from an infected animal to healthy, respectively. About half of the respondents, 51% (95% CI= 46.4-56.3%) and 51.6% (95% CI= 46.6-56.8%), knew dogs as the reservoir of rabies and the time of vaccination for humans after being bitten by a rabid dog, respectively (Table 2).

Table 2. Knowledge of respondents about rabies in the Kersa district

Questions	No (%)	Yes (%)
Do you know about zoonotic diseases?	133(34.6)	250(65.1)
Do you think rabies is zoonotic?	116(30.2)	268(69.8)
Do you know the indications/signs of a rabid animal?	69(18)	315(82)
Do you know how rabies transmits from an infected animal to another animal or human?	97(25.3)	287(74.7)
Do you know the main reservoir of rabies?	188(49)	196(51)
Do you think stray dogs contribute to rabies outbreaks?	202(52.6)	182(47.4)
Do you know the cause of rabies?	281(73.2)	103(26.8)
Do you know when a human be vaccinated against rabies after being exposed?	186(48.4)	198(51.6)

The attitude of respondents towards rabies

The overall attitude of the community of Kersa district revealed that 62.2% (95% CI= 57.3-66.9%) had a desirable attitude of rabies disease (Figure 3). Out of the total participants, 61.7% (95% CI= 57-67.4%) considered rabies as fatal, and 45.1% (95% CI= 40.1-50%) assumed that rabies is treatable after the onset of the clinical sign. Most study participants, 58.9% (95%CI= 54.4-63.8%) and 63% (95% CI= 57.8-68), respond rabies can be prevented by vaccination and educating the community, respectively. One-fourth of respondents, 25.5% (95% CI=21.1-29.9), thought that rabies is related to spirit, and 27.9% (95% CI= 23.4-32.6) believed it is treatable by holly water. More than half of respondents, 56.3 % (95% CI= 51-61.5%), assumed that eating rabid animal meat could transmit the disease, and 21.1% (95% CI= 16.9-25.5%) think that eating rabid animal meat might be used as a vaccine (Table 3).

Table 3. Community attitudes regarding rabies.

Variables	No (%)	Yes (%)
Do you think rabies is a fatal disease	146(38)	238(62)
Rabies is easily treatable after the onset of clinical signs	211(54.9)	173(45.1)
Do you think rabies can be prevented by vaccination?	158(41.1)	226(58.9)
Do you think rabies can be prevented by educating people	142(37)	242(63)
Do you think holly water can treat rabies disease	277(72.1)	107(27.9)
Rabies is related to the spirit	286(74.5)	98(25.5)
Do you think eating rabid animal meat can transmit rabies?	168(43.8)	216(56.3)
Do you think eating rabid animal meat can be used as a vaccine?	303(78.9)	81(21.1)

Community preventive practice toward rabies

Results of this study revealed that half of the respondents, 50% (95% CI= 45.1-54.9%), had good preventive practices towards rabies disease (Figure 3). Three-fourth of respondents, 75% (95% CI= 70.3-79.2%), responded that they visited the health center after being bitten by a rabid animal, and only 31.3% (95% CI= 26.8-36.2%) participants practiced washing the wound with water and soap as an immediate action (first aid) for the victim. The majority of respondents, 82.3% (95% CI= 78.4-86.2%), took immediate action (kill/tie) on the rabid animal, and 56% (95% CI= 50.8-61.2%) reported it to responsible authorities. About 95% (95% CI= 93.8-97.7) of respondents answered that they would eat meat from a rabid animal (Table 4).

Table 4. Community practice toward rabies

Variables	No (%)	Yes (%)
Do you take action (kill/tie) on the rabid dog/animals?	68 (17.7)	316 (82.3)
Have you ever eaten rabid animal meat?	16 (4.2)	368 (95.8)
Would you notify authorities if you came across a rabid dog?	169 (44.0)	215 (56.0)
If a dog bites you, would you visit health centers?	96 (25.0)	288 (75.0)
If a dog bites you, would you wash the wound with water and soap first?	264 (68.8)	120 (31.2)
Have you gone through any disease-related training?	351 (91.4)	33 (8.6)

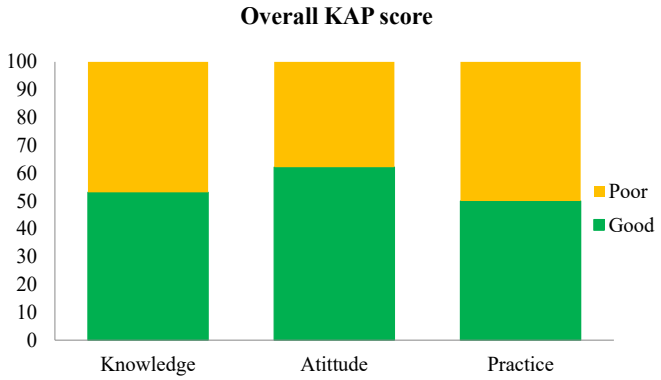


Figure 3. Overall KAP score of the community of Kersa district

Factors associated with community knowledge, attitude, and preventive practice toward rabies

Sex, educational level, occupation, and training had statistically significant associations with knowledge scores. Fifty percent of females were less likely (adjusted odds ratio [AOR]=0.5, CI= 0.3-0.8) to have adequate knowledge about rabies than males. Respondents having secondary school education, college, and above education level had six and more times (AOR=6.7, CI= 2.4-18.8) and thirteen and more times (AOR=13.4, CI= 2.5-71.0) more likely to have adequate knowledge than illiterates, respectively. Farmers and students had 80% (AOR=0.2, CI= 0.03-0.99) and 90% (AOR=0.1, CI= 0.01-0.6) less likely to have adequate knowledge than unemployed respondents. Respondents who took training had seventeen times (AOR=17.0, CI= 2.0-141.1) more likely adequate knowledge than those who didn't take training (Table 5).

Sex and age had a statistically significant association with the attitude score. Females had 40% (AOR=0.6, CI= (0.4-0.96) less likely to have a desirable attitude than males. Respondents having the age group 30-45 years and >=46 years had 50% (AOR=0.5, CI= 0.2-0.9) and 60 % (AOR=0.4, CI= 0.2-0.9) less likely to have a desirable attitude than respondents having age group 15-29 years old (Table 6).

Age, family size, occupation, and source of information had a statistically significant association with outcome variable practice. Respondents having age group 30-45 years and >=46 years had 60% (AOR=0.4, CI= (0.2-0.8) and 70 % (AOR=0.3, CI= 0.1-0.8) less likely to have good preventive practices than respondents having age group 15-29 years old. Respondents with family sizes of 4-6 members had two or more times (AOR=2.1, CI=1.1-4.2) more likely to have good preventive practice than respondents who have 1-3 family members. Farmers, workers, and students had 70% (AOR=0.3, CI=0.1-0.98) and 90% (AOR=0.1, CI=0.04-0.5) less likely to have good preventive practices for rabies than unemployed, respectively. Respondents having sources of information about rabies from veterinarians, communities, and mass media have five and more times (AOR=5.2, CI=2.0-11.8), two and more times (AOR=2.4, CI= 1.1-5.5) and one and more times (AOR= 1.5, CI=1.4-8.1), respectively more likely preventive practices than who have the source of information from health professionals (Table 7).

Table 5. Factors associated with community knowledge of rabies

Variables	Good	Poor	COR (95%CI)	AOR (95%CI)
Sex				
Male	133	73	Reference	Reference
Female	71	107	0.4(0.24-0.6)	0.5(0.3-0.8) **
Age (in years)				
15-29	90	61	Reference	Reference
30-45	87	91	0.7(0.4-1.0)	1.4(0.7-2.8)
>=46	27	28	0.7(0.4-1.2)	2.4(0.9-6.0)
Educational status				
Illiterate	70	111	Reference	Reference
Primary school	24	54	0.7(0.4-1.2)	0.6(0.3-1.3)
Secondary school	66	12	8.7(4.4-17.3)	6.7(2.4-18.8) **
College and above	44	3	23.3(7.0-77.8)	13.4(2.5-71.0) **
Occupation				
Unemployed	27	2	Reference	Reference
Farmer	75	117	0.1(0.01-0.2)	0.2(0.03-0.99) *
Trader	44	32	0.1(0.02-0.5)	0.2(0.03-1.3)
Employed	37	6	0.5(0.1-2.4)	0.1(0.01-1.1)
Student	21	23	0.1(0.01-0.3)	0.1(0.01-0.6) *
Family member				
1-3	51	26	Reference	Reference
4-6	116	51	0.7(0.4-1.1)	0.4(0.2-0.9) *
>6	75	65	0.5(0.3-0.9)	0.5(0.2-1.2)
Source of rabies information				
Health professionals	37	12	Reference	Reference
Veterinarians	41	8	1.7(0.6-4.5)	1.3(0.4-4.5)
Community	73	130	0.2(0.1-0.4)	0.4(0.2-1.98)
Mass media	53	30	0.6(0.3-1.3)	1.1(0.4-3.1)
Faced rabid animal				
No	107	108	Reference	Reference
Yes	97	72	1.4(0.9-2.0)	1.7(0.98-2.9)
Training				
No	172	179	Reference	Reference
Yes	32	1	33.3(4.5-246.4)	17.0(2.0-141.1) **

*p-value <0.05, ** p-value <0.01

Table 6. Factors associated with community attitude towards rabies

Variables	Good	Poor	COR (95%CI)	AOR (95%CI)
Sex				
Male	143	63	Reference	Reference
Female	96	82	0.5(0.3-0.9)	0.6(0.4-0.96) *
Age (in years)				
15-29	113	38	Reference	Reference
30-45	103	75	0.5(0.3-0.9)	0.5(0.2-0.9) *
>=46	23	32	0.2(0.1-0.6)	0.4(0.2-0.9) *
Educational status				
Illiterate	86	95	Reference	Reference
Primary school	51	27	2.1(1.204-3.617))	1.8(0.9-3.6)
Secondary school	55	23	2.6(1.5-4.7)	1.5(0.6-3.4)
College and above	47	0	-	-
Occupation				
Employed	24	5	Reference	Reference
Farmer	95	97	0.2(0.1-0.6)	0.6(0.2-2.3)
Trader	52	24	0.5(0.2-1.3)	0.7(0.2-2.6)
Unemployed	40	3	2.8(0.6-12.7)	2.1(0.4-12.4)
Students	28	16	0.4(0.1-1.1)	0.3(0.1-1.2)
Family member				
1-3	52	25	Reference	2.007
4-6	106	61	0.8(0.5-1.5)	1.3(0.7-2.6)
>=7	81	59	0.7(0.4-1.2)	1.5(0.7-3.3)
Source of rabies information	36	13	Reference	Reference
Health professionals	38	11	1.3(0.5-3.1)	0.7(0.2-2.3)
Veterinarian	105	98	0.4(0.2-0.8)	0.9(0.4-2.1)
Community	60	23	0.9(0.4-2.1)	1.2(0.5-3.2)
Mass media				
Faced rabid animal				
No	144	71	Reference	Reference
Yes	95	74	0.633(0.418-0.960)	0.8(0.5-1.3)

*p-value <0.05

Table 7. Factors associated with community preventive practices towards rabies

Variables	Good	Poor	COR	AOR (95%CI)
Sex				
Male	110	96	Reference	Reference
Female	82	96	0.8(0.5-1.1)	0.8(0.5-1.3)
Age				
15-30	93	58	Reference	Reference
31-45	83	95	0.6(0.4-0.9)	0.4(0.2-0.8) *
>=46	16	39	0.3(0.1-0.5)	0.3(0.1-0.8) *
Educational status				
Illiterate	71	110	Reference	Reference
Primary school	47	31	2.4(1.4-4.04)	1.6(0.8-3.2)
Secondary school	46	32	2.2(1.3-3.8)	1.3(0.6-3.0)
College and above	28	19	2.3(1.2-4.4)	1.0(0.3-3.0)
Family size				
1-3	38	39	Reference	Reference
4-6	101	66	1.6(0.9-2.7)	2.1(1.1-4.2) *
>=7	53	87	0.6(0.4-1.1)	1.2(0.6-2.4)
Occupation				
Unemployed	23	6	Reference	Reference
Farmer	78	114	0.2(0.1-0.5)	0.3(0.1-0.98) *
Trader	48	28	0.5(0.2-1.2)	0.4(0.1-1.4))
Employed	22	21	0.3(0.1-0.8)	0.4(0.1-1.3)
Student	21	23	0.2(0.1-0.7)	0.1(0.04-0.5) **
Source of rabies information				
Health professionals	16	33	Reference	Reference
Veterinarians	35	14	5.2(2.2-12.2)	5.2(2.0-11.8) **
Community	87	116	1.6(0.8-2.99)	2.4(1.1-5.5) *
Mass media	54	29	3.8(1.8-8.1)	1.5(1.4-8.1) **

*p-value <0.05, ** p-value <0.01

Association of preventive practices of rabies with knowledge and attitude

Pearson's chi-square test revealed that both knowledge and attitude had significant effects on preventive practices of rabies (Table 8). There was a strong association between preventive practice and knowledge of respondents ($\chi 2=6.0$, $p=0.014$). Respondents with good knowledge (55.9%) had better preventive practices than respondents with poor knowledge (43.3%). Attitude also had a strong association with good preventive practice ($\chi 2=28.8$, $p\leq 0.001$). Respondents with desirable attitudes (60.7%) had better preventive practices than respondents with undesirable attitudes (43%).

Table 8. Chi-square test showing association of KAP

Variables		Preventive practice		Chi-square	P-value
		Good (%)	Poor (%)		
Knowledge	Good	114 (55.9)	90 (44.1)	6.0	0.014
	Poor	78 (43.3)	102 (56.7)		
Attitude	Desirable	145 (60.7)	94 (39.3)	28.8	≤ 0.001
	Undesirable	74 (43)	98 (57)		

Discussion

The finding of the present study revealed that 53% of the study subjects from the Kersa district had adequate knowledge, and the result was in line with the reports for the Dedo district of the Jima zone (Nejash *et al.*, 2017). The current study and the Dedo district are similar since the majority of the study participants were from rural areas and because both have a similar culture and language. On the other hand, higher results of 56.1, 61.3, 64.1, and 85.7% were reported from households of Mekelle City, residents of Amhara region, and communities of Bahir Dar City and Kombolcha town, respectively (Guadu *et al.*, 2014; Addis *et al.*, 2019; Hagos *et al.*, 2020; Bahiru *et al.*, 2022). The fact that the majority of the study's participants in the current study area resided in rural areas, where information sources and levels of education were lower, might be the cause of the study's lower scores.

The majority of study participants (92.7%) heard about rabies, which indicates respondents are aware of the presence of rabies in their area, and this helps with the control strategy of the disease. This result was in line with the findings of several scholars from different areas of Ethiopia (Digafe *et al.*, 2015; Gebeyaw and Teshome, 2016; Bihon *et al.*, 2020). Over 90% of the respondents of Awash- Basin, Eastern Ethiopia, claimed to know about rabies, but 79.2% did not know how dogs acquire the disease (Tschopp *et al.*, 2016). This may be the consequence of the disease being a highly fatal disease with prominent symptoms to which everyone has been exposed.

More than half of the respondents (52.9%) in this study obtained information from the community; this is because discussion about the disease is good among communities, and influential persons in the community should get awareness of the disease, and they can create awareness in their community. The result was in line with respondents from Addis Ababa City (Ali *et al.*, 2014). Slightly

higher than one-fourth (26.8%) of the study participants knew that the cause of rabies was a virus; this is due to the fact that most respondents are uneducated and have no access to differentiate the causative agents as viruses, bacteria, and other agent. Different studies in and nearby Gondar City, Dedo district of Jima zone, and around Dessie City, Munisa district of Arisi zone and Debre Tabor City reported a higher rate of 60.7, 67.6, 67.6, 78 and 78.4 % of the respondents knew about the causative agents of rabies, respectively (Serebe *et al.*, 2014; Alie *et al.*, 2015; Gebeyaw and Teshome, 2016; Nejash *et al.*, 2017; Nejash and Endale, 2017). The fact that respondents from urban areas tend to have better information sources and educational levels may be attributable to study area variation.

The majority of respondents (74.7%) knew the way of transmission was through the bite of a rabid animal and saliva contact. This suggests that respondents are aware of the transmission of the disease and controlling the disease might be easy as the means of transmission were known; it was higher than the report from Debre Tabor (57.8%) (Alie *et al.*, 2015) and Bahir Dar City (21.4%) (Guadu *et al.*, 2014). Nearly one-fifth of the respondents (18%) did not know the indications and signs of rabid animals, which is lower than 37.3% and 23% of urban and pastoralists of the Awash basin did not know the symptoms of rabies in dogs, respectively (Tschopp *et al.*, 2016).

The result of the current study has also revealed that the majority (62%) of the respondents had a desirable attitude toward rabies disease. A lower result was reported by Wolelaw *et al*. (2022) in the Bure Zuria district, North West Ethiopia, with 51.2% of participants having a good attitude towards rabies (Wolelaw *et al.*, 2022). The majority of study participants, 58.9 and 63%, had positive perceptions about vaccination and educating the community could control rabies disease, respectively; slightly higher results, 65.9%, were reported by Bihon *et al.* (2020) in and Around South Gondar. More than half of respondents (56.3%) assumed rabid animal meat could be used as medicine. This is not a good perception since it might not be used as a medicine, and this may affect the use of modern treatment and increase the mortality rate. The current finding is slightly higher than 48.2% of the community of south Gondar (Bihon *et al.*, 2020).

In the current study, preventive practices towards rabies in Kersa district revealed that 50% of the respondents had good preventive practices, and a lower finding of 45.46% in the residents of Amhara region was reported by Bahiru

et al. (2022). Among the interviewed participants, nearly one-third (31.2%) reported a practice of washing the wound using soap and water. This result agreed with results reported from the Gondar Zuria district (30.7%) of Ethiopia (Digafe *et al.*, 2015). It was higher in a study conducted in Kombolcha town, which reported 92.4% (Addis *et al.*, 2019). The difference can be attributed to the difference in the awareness status of the community and the lack of training in the current study area. The WHO also recommends wound washing and vaccination immediately after contact with a suspected rabid animal, which can prevent almost 100% of rabies deaths (WHO, 2013).

Regarding the preferred action taken for bitten humans, 75% of participants responded to visiting hospitals for post-exposure vaccine, and the rest preferred traditional treatment and religious places. Even though most respondents prefer hospitals, awareness should be mandatory for the rest as traditional treatment and religious places are not guaranteed for the treatment of the disease. This result is higher when compared to a study conducted in Debre Tabor, which was 37.5% preference (Alie *et al.*, 2015).

This study revealed that around 95.8% of respondents consumed meat from rabid animals. It was a poor practice even though the disease is not transmitted through eating meat. There might be accidents during the slaughtering process, and this leads to contact of the virus with the damaged part of the human body, as well as affecting the control and prevention of the disease. This result is high when compared with that of Debark (64.9%) of respondents and reported in the Gondar Zuria district (Digafe *et al.*, 2015), which revealed consumption of cooked or boiled meat from rabid animals was considered safe by 67.0% of the respondents. Cooked meat does not transmit rabies; however, it is not advisable to consume meat from an infected animal (WHO, 2013).

In this study, sex, educational level, occupation, and training were determinant factors for knowledge. Males had more knowledge than females, and this might be because males are more educated than females. On the other hand, as the education level increased, the knowledge about rabies disease also increased, and this might be due to educated people having more chances to know about the disease through different means; this was in agreement with the study conducted in the Arsi zone Munesa district (Nejash and Endale, 2017). The possible explanation might be that an educated person has better access to information and can easily understand the disease.

This finding revealed that a good practice score was significantly associated with age, family size, occupation, and source of information. The age group from 15-29 years old has better practice than the rest of the age groups, and this is because most respondents from 15-29 years old were students, and they have better knowledge and understanding than the other age group. As the family size increased, the good preventive practices also increased, and this might be due to different demographic characteristics of the family members, which increased the chance of good practice. Regarding occupation, farmers and students have poor preventive practices as compared to unemployed respondents; this might be due to farmers having very close contact with dogs as dogs are used as guards for them; on the other hand, students have poor practices as they have youngsters and interact with dogs. Wolelaw *et al.* (2022) reported similar results, namely that the source of information and age had a statistically significant association with practices. An individual whose age group was 18–29 years was 2.7 times more likely to have good prevention practices towards rabies than those in an age group more significant than 45 years (Wolelaw *et al.*, 2022).

Preventive practice is significantly associated with the knowledge and attitude of respondents. As the knowledge increased, the preventive practice also increased, and as the attitude increased, the preventive practice also increased. This suggests that working on the knowledge and attitude of respondents will increase the preventive practice of the disease.

Conclusions

As this study indicates, more than half of the participants in the Kersa district had good KAP. However, there was an information gap regarding the cause of the disease, the first action taken after being bitten by a suspected dog, and the existence of undesirable perception with regard to relating rabies to the spirit as a means of transmission and belief in holy water as a means of treatment. The presence of low vaccination coverage and high practice of traditional medicine was another bottleneck. Educational status, training, and occupational background were the most critical factors that have a significant role in the community's KAP score. Respondents with good knowledge and desirable attitudes had good preventive practices against rabies disease; hence, awareness and disease-related training should be given to increase the knowledge and attitude of society, which will help the preventive practice of the disease.

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Biosecurity level assessment in commercial poultry farms of central Ethiopia

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Abstract

Proper biosecurity practice is crucial in poultry farming to reduce the risk of diseases. A study was conducted in Adama, Ada'a, and Lume districts, central Ethiopia, to assess the biosecurity measures of commercial poultry farms. A total of 51 farms were randomly selected, and their geographical locations were recorded using GPS devices. The biosecurity level of each farm was assessed using an observation checklist and a biosecurity score. The collected data were analyzed using Stata version 16 for binary data, and QGIS version 3.24.1 was used to map the farms. The results of the study showed that the farms were located close together within 5 km square, 3 km square, and 6 km square areas in Ada'a, Lume, and Adama, respectively. The average distance among the farms was 933.8 meters. The majority of the farms were located within 100 meters of residential areas. Only 58.8% of the farms had adequate space between sheds; 60.8% did not have an adequate drainage system. Ninety-eight percent of the farms obtain chicken from certified breeding enterprises; 92.2% provide underground water for their chickens; 80.4% report frequent cleaning and disinfection of farms; and 82.4% disclose vehicle movement control. Nearly one-third (31.4%) of the farms practice isolation of sick birds but keep them within the same shed where healthy birds are kept. The assessment of biosecurity levels of the study farms revealed that 23.5% (n=12) of the farms had a good biosecurity score, while 76.5% (n=39) had a poor score. The biosecurity scores of the farms showed a statistically significant difference among the study districts (p=0.040), with the Lume district having higher biosecurity scores than Adama and Ada'a districts. The age of the farm owners was significantly associated with the biosecurity score (p=0.003); all farms (100%) owned by individuals aged 20 - 39 had good biosecurity scores, and 80% of those farms owned by individuals aged 40 - 59 had good biosecurity. Farms owned by individuals who have previous chicken-rearing experience had better biosecurity

scores than those farms owned by non-experienced owners (p<0.001). Among the farms having professional consultants, 57.7% of them had good biosecurity scores, whereas only 12.0% of farms lacking professional consultants had good biosecurity scores (p=0.015). In conclusion, the study revealed that biosecurity measures were not adequately implemented in poultry farms in central Ethiopia. Farm owners, veterinarians, and livestock authorities must collaborate to implement biosecurity measures to minimize the risk of losses and public health.

Keywords: Biosecurity; commercial poultry farm; spatial distribution; central Ethiopia.

Introduction

Biosecurity is defined in the European Animal Health Law as: "the sum of management and physical measures designed to reduce the risk of the introduction, development and spread of diseases to, from and within: (a) an animal population, or (b) an establishment, zone, compartment, means of transport or any other facilities, premises or location". The Food and Agriculture Organization of the United Nations (FAO) defines biosecurity as the "implementation of measures that reduce the risk of the introduction and spread of disease agents; it requires the adoption of a set of attitudes and behaviors by people to reduce risk in all activities involving domestic, captive/exotic and wild animals and their products" (FAO/OIE/WB, 2010). The World Organization for Animal Health defines biosecurity in the Terrestrial Animal Health code as "a set of management and physical measures designed to reduce the risk of introduction, establishment and spread of animal diseases, infections or infestations to, from and within an animal population". According to these definitions at the farm level, biosecurity measures may focus either on reducing the risk of entry of new pathogens (external biosecurity) or on reducing the internal dissemination of pathogens (internal biosecurity). It became an integral part of several strategic policy documents in animal and public health and policy for livestock production (Vanlangendonck *et al.,* 2021).

Biosecurity at a poultry farm comprises all measures and actions implemented to reduce the risk of introduction and spread of disease agents and keep poultry and people healthy. By applying these biosecurity measures and improving the efficiency of management, the chickens are protected against a range of infectious diseases (Dewulf *et al.,* 2020). Globally, the poultry sector has evolved

from a small-scale to an industrial production over the years. The aim is to have a thorough production through the optimization of components such as nutrition and genetics (Sahlström *et al.,* 2014; Alawneh *et al.,* 2014). However, higher densities of animals per production site and associated contact and operational structures have been incriminated as drivers for the occurrence of infectious diseases (Tenorio, 2022), which are responsible for substantial economic losses. The most effective and economically feasible method to prevent and control poultry diseases is the implementation of biosecurity measures (Butcher and Miles, 2012). Biosecurity aims to prevent infectious diseases and their spread to animals (both domestic and wildlife) and humans (Gilbert *et al.,* 2020). That is, it curtails the impact of infectious diseases on the economy, society, and environment to safeguard health and wellbeing. Biosecurity became one of the essential components of the One Health concept (Renault *et al.,* 2022).

Implementation of biosecurity measures resulted in several consequences in addition to minimizing risks for disease outbreaks. Studies have shown that biosecurity is positively associated with increased production, such as daily growth and reduction in mortality in broilers (Wijesinghe *et al.,* 2017). Additionally, the application of biosecurity measures dramatically reduces the use of antibiotics (Nöremark, 2010; Laanen *et al.,* 2013), which in turn reduces the development of antibiotic resistance and improves both animal and human health (Conan *et al.,* 2012). In contrast, high mortality and morbidity of chickens, high medication costs, loss of production, denied access to the market, and a high risk of public health from zoonotic diseases have been associated with farms that did not implement biosecurity measures (Wubet *et al.,* 2019). Diseases such as Newcastle disease, salmonellosis, fowl cholera, coccidiosis, and fowl pox were reported to cause high morbidity and mortality in village and commercial chicken (Wubet *et al.,* 2019) where biosecurity measures were not implemented properly (Cardona and Kuney, 2002; Mazengia *et al.,* 2012).

In Ethiopia, the evolution of the poultry sector from scavenging to small-scale and industrial systems has led to an increased need for biosecurity measures to prevent disease outbreaks that can have a significant impact on farm profitability and the national poultry sector. However, information on the levels of biosecurity measures exercised in commercial poultry farms is limited. Therefore, the objective of this study was to provide spatial maps of commercial poultry farms and quantify the biosecurity levels practiced in central Ethiopia.

Materials and methods

Study area

The study was conducted on medium and large-scale commercial poultry farms located in three districts in central Ethiopia, namely Ada'a (47 km southeast of Addis Ababa), Adama (100 km southeast of the capital, Addis Ababa), and Lume (73 km southeast of Addis Ababa). Figure 1 depicts the geographical locations of the study districts. These districts are located in the mid-Rift valley along two highways: one connecting Ethiopia to Kenya and the other connecting Ethiopia to Djibouti. The area is endowed with a number of creator lakes serving as recreational centers. As a result, there is high human traffic in the areas. Based on data acquired from the Ministry of Agriculture, these districts have emerged as significant contributors to the poultry industry, representing the majority of poultry production in the country. The districts were selected due to the presence of a high number of poultry farms, proximity to the commercial centers, and increased commercial activities.

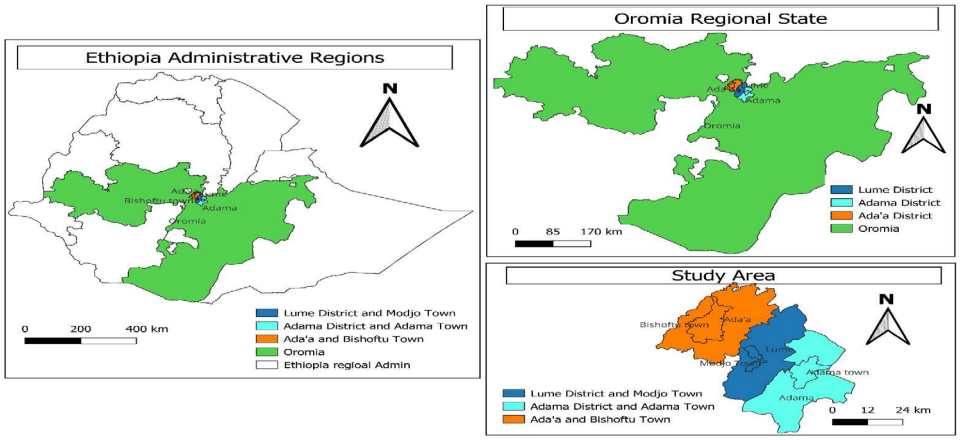


Figure 1. Map of Ethiopia and Oromia Regional State displaying the locations of study districts

Study design

This study utilized a cross-sectional study design. The study population comprised chickens raised under medium and large-scale poultry farms located in the Ada'a, Adama, and Lume districts. Data obtained from the agricultural offices showed that there were 378 (303 layer and 75 broiler) registered com-

mercial poultry farms in the study districts. Out of these, ten broiler farms (5 small, three medium, and two large scale) and 64 layer farms (48 small, 12 medium, and four large scale) were in Adama. In Ada'a district, there were 57 broiler farms (22 small, 30 medium, and five large scale) and 187 layer farms (100 small, 78 medium, and nine large scale). A total of 52 layer farms (40 small, 10 medium, and two large scale) and eight broiler farms (5 small and three medium scale) were registered in the Lume district. For this study, 51 medium and large-scale poultry farms were randomly selected. GPS was used to collect coordinates of the farms, nearby residence areas, highways, open water sources, and live bird markets. Data collection was carried out using semi-structured questionnaires comprising both closed and open-ended questions to gather all the information regarding biosecurity measures and farm characteristics. The questionnaire was pretested before the final survey on a few farms in order to make the necessary refinements for better clarity and completeness. A face-to-face interview was conducted with farm owners, managers, or attendants. Biosecurity measures in each farm were evaluated using Chowdhury *et al*. (2020) poultry farm biosecurity scoring system, which categorized indicators and definitions of biosecurity scores at conceptual, operational, and structural levels.

Data collection

Data on the house design and orientation, types of construction, availability of enough spaces, hygiene status, frequency of cleaning and disinfection, traffic control, health management, and other biosecurity measures of the commercial poultry farms studied were collected using questionnaires and observations. In addition, measurements of variables such as distance among the farms, distance of the farms from main roads, and distance of the farms from residential areas were taken. Information on the demographic features of the farm owners and managers was also collected. All variables collected were categorized.

Operational definition

Large-scale poultry farm: A farm having ≥ 10,000 chicken
Medium-scale poultry farm: A farm rearing 5,000 – 9999 chicken
Small-scale poultry farm: A farm holding less than 5000 chicken
Good biosecurity: when the biosecurity score is above 50%
Poor biosecurity: when the biosecurity score is less than 50%

Data management and analysis

Data were analyzed using STATA version 16, and descriptive and analytical statistics were used. These include frequency summary, proportion, and Pearson's chi-square test, which were used to estimate associations between farm characteristics, demography of farm owners, and biosecurity status. The overall biosecurity status was estimated using the poultry farm biosecurity score system described by Gelaude *et al*. (2014), with farms scoring over 75% of the total score (>33) considered standard and farms scoring less than or equal to 33 considered "below standard." A significance level of $p < 0.05$ was used. Finally, spatial queries of QGIS version 3.24.1 were used to address environmental biosecurity issues, such as proximity to lakes, roads, and live poultry markets.

Ethical clearance

This study was approved by the ethical review board of Addis Ababa University, College of Veterinary Medicine and Agriculture (Certificate Ref. No: VM/ERC/08/02/14/2022). The owners or managers of each farm were informed about the aim of the study, and verbal informed consent was obtained from each respondent. Participation in the study was voluntary, and respondents were free to withdraw from the study at any time. Their consent was recorded (marked) on the questionnaire paper, the interview was anonymous, and data remained confidential throughout the study.

Results

Spatial distribution of commercial poultry farms in the study areas

The farms were found in clusters within areas of 5, 3, and 6 square km in the Ada'a, Lume, and Adama districts, respectively. In Ada'a, the majority of the commercial poultry farms were located in the central, northwest, and eastern parts of the district. Similarly, in Adama, the farms were located in the central and northeastern parts of the district. In contrast, almost all of the farms in Lume district were situated in the northern parts of the district. The relative locations of the commercial poultry farms are depicted in Figure 2.

The average distance between poultry farms in the three districts was 933.8m, with a median of 205.4m. Distances between farms ranged from 16.39 to 4920.67 meters. The average distance between farms in Ada'a was 544.08m, the average distance in Lume district was 1991.59m, and it was 265.88m in

Adama. The study revealed that poultry farms in the selected districts were clustered in some areas of the districts and were located very close to each other. The majority of the farms were within 100 meters of residential areas, with only 9.8% located farther than 100 meters away from residential areas. In terms of proximity to main roads, 19.61% of the poultry farms were located within 300 meters of the main roads, while the rest (80.39%) were situated farther than 300 meters away from the main roads. In Lume district, however, none of the farms were located within 300 meters of the main road (Figure 2).

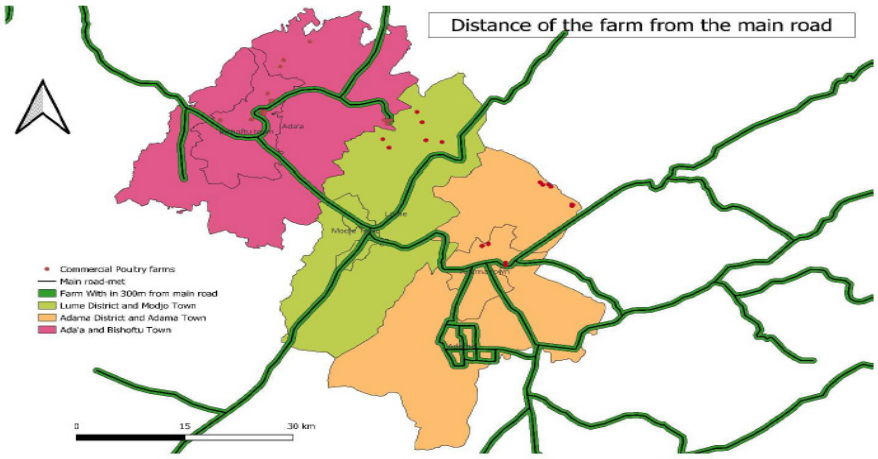


Figure 2. Map of the three study districts depicting the locations of the poultry farms. The top is the Ada'a district, the middle one is the Lume district, and the bottom is the Adama district. The poultry farms are indicated with red dots. The green bold lines represent the main roads.

Biosecurity measures adopted by poultry farms

House design and orientation

The orientation of the poultry houses in the majority of the farms (78.43%) was east-west, while the rest were oriented north-south. The medium-scale farms did not have well-defined physical boundaries. All of the farms were accessible to dogs, cats, and vermin. Although all of the farms had sick bird isolation rooms, the isolation rooms are located within the farms and have the potential to spread disease agents. More than half of the farms did not have washable housing surfaces, although all (100%) of them had concrete floors. More than half (62.75%) of the farms had adequate space between sheds, while

the remaining 37.25% did not have adequate space. The results also showed that 21.57% of the farms had no restrictions on the entrance of humans, vehicles, and animals, while 37.25% of the farms had room for their staff inside the farm compound. Table 2 presents the details of the relative farm location and house design.

Table 2. Results of assessment of the relative farm location and house design of the commercial poultry farms in the three selected districts

Variable	Category	Number of farms	Proportion
Residential areas	None within l00m	5	9.80
	Located within 200m	14	27.45
	Located within 100m	32	62.75
Backyard poultry (chicken, ducks, pigeons)	None within 200m	38	74.51
	Present within 100m	13	25.49
Orientation	East–West direction	40	78.43
	North–South direction	11	21.57
Public Places (Schools, Bazar, Bridge, etc.)	None within 200m	48	94.12
	Present within 200m	3	5.18
Staff rooms	Present inside the Farms	19	37.25
	None inside the Farms	32	62.75
Adequate Space	Present between sheds	32	62.75
	Not present between sheds	19	37.25
Width of sheds	>30 Feet	51	100.00
Floor-type	Concrete	51	100.00
Farm Environment	Vegetation around the shed	36	70.59
	None	15	29.41
Entry restriction	Yes	40	78.43
	No	**11**	21.57

Sources of farm inputs

The majority of the poultry farms (96.08%) in the selected districts obtained their feed from registered companies; 92.16% of the farms provided underground water to their chickens. Nearly all (98.04%) of the farms sourced their chickens from reliable, certified breeding centers and conducted health inspections upon receipt. However, 37.25% of the farms did not have stores for poultry feed. It was found that 39.22% of the farms implemented bait and traps as a rodent control strategy; 23.53% used feed spill-outs, while the remaining

farms used both methods (Table 3). None of the farms studied traced poultry trucks and had very loose control over the transport routes.

Cleaning and disinfection

Most of the farms did not clean and disinfect their farm compounds regularly. Many of them (80.39%) had footbaths at the gates, although none of them regularly cleaned the bath and replenished the disinfectants. The cleaning and disinfection method used by the farms was also not appropriate. It consisted of removing dirt followed by application of disinfectants. The frequency of cleaning and disinfection was low, mainly covering only a portion of the premises. Most (60.78%) of the farms did not have an adequate drainage system (Table 3). The results also showed that none of the farms had a written biosecurity plan or records of vaccination, surveillance, and disinfection for each batch of chickens.

Table 3. Sanitation status, cleaning frequency of sheds, and sources of farm inputs for the commercial poultry farms studied

Parameter	Category	Number of farms	Percentage
Source of feed	Feed Company	49	96.08
	Prepared on farm	2	3.92
Source of water	Underground water	47	92.16
	Rain, nearby spring	3	5.88
	Surface water	1	1.96
Health check on recipient of chicks	Yes by veterinarian	50	98.04
	No	1	1.96
Waste /litter Management	Use for compost	1	1.96
	Stored and sold	28	54.90
	Contracted to farmers	22	43.14
Cleaning and Disinfection	Foot bath	41	80.39
	None	10	19.61
Feed management	Good storage system	32	62.75
	Poor storage system	19	37.25
Water management	Plastic material	51	100.00
Drainage Management	Adequate	20	39.22
	Inadequate	31	60.78
Waste disposal	Vermicomposting	6	11.76
	Composting in a pit	2	3.92
	Sale	43	84.31
Pest control	Bait and traps	20	39.22
	Feed spills out	12	23.53
	Both	19	37.25
Cleaning and disinfection of farm compound	Clean and disinfect the whole compound every week	10	19.61
	Clean and disinfect part of the compound regularly	16	31.37
	Clean and disinfect occasionally	23	45.10
	Not done	2	3.92
Cleaning and disinfection of utensils and shed	Done regularly	12	28.53
	Done when required	17	33.33
	Done sometimes	21	41.18
	Not done	1	1.96

Traffic control practices

The majority of the poultry farms (82.35%) had control measures in place for vehicle movement within the farm, including vehicle disinfection before and after visiting the farm. Almost two-thirds (64.71%) of the farms had a designated area for vehicle parking and cleaning. Most farms had strict control measures for human traffic, with only 3.92% of farms allowing visitors to enter freely. However, 15.69% of the farms did not have proper biosecurity inspections for farm attendants and other staff (Table 4). None of the farms had staff/workers training programs in biosecurity, and as a result, the staff/workers lacked a vigilant attitude towards its implementation. None of the farms studied had standard operating procedures for their workers.

Table 4. The cleaning and disinfection of the farm environment and traffic control status of the poultry farms in the study areas

Variable	Category	Number of farms	Percentage
Vehicle Movement	No movement	18	35.29
	Disinfection upon entry	24	47.06
	No control of the vehicle	9	17.65
Separate area for vehicles	Yes, for cleaning and disinfection	5	9.80
	Yes, only for cleaning	33	64.71
	Not effective	1	1.96
	None	12	23.53
Farm compound	Brick wall with a gate for recording visitors	19	37.25
	Porous wall with footwear and disinfection at the entrance	30	58.82
	No wall with free entrance	2	3.92
Human traffic control	Strict cleaning, disinfection, and PPE required	11	21.57
	Only disinfection	39	76.47
	None	1	1.96
Residence on the farm	Yes permanently	20	39.22
	None	31	60.78
Management of egg trays	Use and dispose	20	39.22
	Clean and disinfect after each use	19	37.25
	Clean and disinfect when dirt is visible	12	23.53

Isolation of sick and disposal of dead birds

The majority of the farms (88.24%) in the selected districts practice the proper disposal of dead birds and the isolation of sick ones. All farms disclosed that they practice vaccination of chickens following the recommendations of veterinarians and the instructions of vaccine manufacturers. However, none of the farms had records of vaccination over the years. About two-thirds (66.67%) of the farm owners were aware of the importance of biosecurity protocols. However, none of them had an epidemiological unit for tracing and monitoring diseases, including regulating the removal and disposal of dead birds or the isolation of sick ones. A separate disposal pit, use of an incinerator, and burning of dead birds were practiced by 84.31% of the farms, while the remaining 15.69% did not disclose their disposal system (Table 5). Most of these actions were taken without consulting veterinarians.

Table 5. Isolation of sick and disposal of dead birds by the selected farms in the study area

Variable	Category	Number of farms	Percentage
Disposal of dead and isolation of sick birds	Quick disposal and isolation	45	88.24
	Disposal of dead birds but keep sick ones in separate rooms in the shed	6	11.76
Vaccination	Yes	51	100.00
All in all, all-out practice	Yes	51	100.00
Awareness about the importance of biosecurity	Yes, all farmworkers	34	66.67
	Only some of the workers	17	33.33
Flock monitoring	Regularly by employed Vet. and government authority	6	11.76
	Only by employed Vet.	45	88.24
Facility for personnel hygiene	Shower facility and disinfection	12	23.53
	Hand wash and foot bath at the gate	1	1.96
	Foot bath and spray system at the gate	2	3.92
	Only foot bath	19	37.25
	None	17	33.33
Management of dead birds	Disposal pit /incinerator /burring	43	84.31
	Not disclosed	8	15.69
Management of sick birds	Isolation room away from shed	35	68.63
	Isolation room in the same shed	16	31.37

Biosecurity scores of the farms

The assessment of biosecurity levels of the study farms revealed that 23.53% (n=12) of the farms had a good biosecurity score, while 76.47% (n=39) had a poor score. The biosecurity scores of the farms showed a statistically significant difference among the study districts (p=0.040), with the Lume district having higher biosecurity scores than Adama and Ada'a districts. The age of the farm owners was significantly associated with the biosecurity score (p=0.003); all farms (100%) owned by individuals aged 20 - 39 had good biosecurity scores, and 80% of those farms owned by individuals aged 40 - 59 had good biosecurity. Farms owned by individuals who have previous chicken-rearing experience, as was the previous chicken-rearing experience, had better biosecurity scores than those farms owned by non-experienced owners (p<0.001). Among the farms having professional consultants, 57.69% of them had good biosecurity scores, whereas only 12.00% of farms lacking professional consultants had good biosecurity scores (p=0.015). All (100%) of the large-scale farms had good biosecurity scores, while only 21.28% of medium-scale farms had good biosecurity scores. The result of the biosecurity scores of the study farms is given in Table 6.

Table 6. Association between biosecurity score and various variables studied in commercial poultry farms in the study areas

Variable	Category	Number of farms	Biosecurity level		Chi-square	p-value
			Poor	Good		
District	Adama	16	14 (87.50%)	2 (12.50%)	4.931	0.040
	Ada	29	21 (72.41%)	8 (27.59%)		
	Lume	6	4 (66.67%)	2 (33.33%)		
	Overall	51	39 (76.47%)	12 (23.53%)		
Age of owners (in years)	13-19	3	0 (0.00)	3 (100.00%)	11.3322	0.003
	20-39	40	8 (20.00%)	32 (80.00%)		
	40-59	8	6 (5.00%)	2 (25.00%)		
Sex of owner	F	20	15 (25.75%)	5 (25.00%)	0.0992	0.753
	M	31	9 (29.03%)	22 (70.97%)		
Owner's educational status	Primary	10	8 (80.00%)	2 (20.00%)	2.546	0.116
	Secondary	18	2 (11.11%)	16 (88.89%)		
	College	8	4 (50.00%)	4 (50.00%)		
	University	15	6 (40.00%)	9 (60.00%)		

Variable	Category	Number of farms	Biosecurity level		Chi-square	p-value
			Poor	Good		
Previous chicken-rearing experience	Yes	21	12 (57.14%)	9 (42.86%)	15.8035	≤ 0.001
	No	30	28 (93.33%)	2 (6.67%)		
Having professional consultant	Yes	26	11 (42.31%)	15 (57.69%)	5.8784	0.015
	No	25	22 (88.00%)	3 (12.00%)		
flock size	Large	4	0 (0.00%)	4 (100.00%)	5.978	0.011
	Medium	47	37 (78.72%)	10 (21.28%)		
House type	Deep litter	50	37 (74.00%)	13 (26.00%)	2.6957	0.101
	Cage system	1	1 (0.00%)	1 (100.00%)		

Discussion

Poultry production requires strict implementation of efficient biosecurity measures to prevent the entry of disease agents to manage a disease once it is on the premises. The goal is to minimize losses due to diseases, optimize production, and improve food security and safety. These measures can be split into bio-exclusion and biocontainment, covering a range of actions such as housing with appropriate ventilation, proper floor systems, sufficient and secure isolation areas, and effective traffic control, among others. Biosecurity practices have been proven effective and inexpensive in disease control measures in other parts of the world (Wijesinghe *et al*., 2017; Van Limbergen *et al*., 2018; Tasie *et al*., 2020). In Ethiopia, the Ministry of Agriculture has identified poultry production as a critical priority sector in its endeavors to enhance household food security and promote overall wellbeing (Hailemichael *et al*., 2016). However, infectious diseases are widespread on commercial farms, resulting in significant financial losses (Asfaw *et al*., 2021). This study revealed poor biosecurity scores among commercial poultry farms, highlighting the need for the implementation of biosecurity procedures to prevent the introduction and spread of infectious agents.

The study found that commercial poultry farms were concentrated in certain parts of the districts and located very close to each other, mostly less than one kilometer apart. This proximity puts the farms at high risk for the introduction of pathogens from nearby farms, carried by vehicles, humans, farm implements, feral birds, or scavenging chickens. Previous studies have reported that

the proximity of farms to neighboring same-species units and significant transport routes influence the risk of disease introduction (Gibbens *et al*., 2001). Standard poultry operations require an isolated geographical location in relation to other poultry and livestock establishments' and wild bird concentrations (OIE, 2022), and the recommended relative locations of the farms are not followed (Gelaude *et al*., 2014). The location of farms within 100 meters of residential areas puts them at high risk of the introduction of pathogens from surrounding waste, free-range chickens reared by households, and human movement. Moreover, this location is repulsive to residents and puts them at risk of contracting diseases (Iyiola-Tunji *et al*., 2013). Observations during this study showed that several chicken farms were located within 0-20 meters of residential areas.

The majority of the farms lack adequate drainage systems, which favors the discharge of untreated wastewater or run-off into chicken habitats, creating favorable conditions for pathogen survival and multiplication on the farm and potentially contaminating chicken feed and/or water. Although most farms indicated that they frequently clean and disinfect their farms, the design and construction materials used are impervious, hindering efficient cleaning and disinfection. Areas surrounding the poultry houses were not paved with concrete or other waterproof material, making effective cleaning and disinfection difficult and putting the farms at risk of disease outbreaks. Disinfection requires proper cleaning and removal of all organic matter and the saturation of all surfaces, sticking to the five sequences of cleaning and disinfection (Kustritz, 2022). Similar observations were reported in northern Ethiopia, where the majority of farms practiced cleaning and disinfection of farms despite unsuitable house design and construction (Birhanu *et al*., 2015). Effective removal of pathogens or niches favorable for their survival is critical to prevent disease spread to neighbors and maintain good hygiene.

Although the majority of the poultry farms in the study area reported practicing vehicle movement control, they did not pay due attention to farm workers and visitors, who were allowed to access the shed and flock without proper inspection. Personnel and visitors did not take showers, change their clothes, or wear provided footwear. The farms did not have a registry for the entry of visitors and vehicles. Hence, the traffic control practices reported by the farms were only for formality, and such practices put the farms at risk of the introduction and dissemination of pathogens. Coupled with the farms' proximity to one another and residence areas, loose traffic control is favorable for outbreaks

of infectious diseases. Similar observations were reported in Sudan (Mustafa *et al*., 2018). Poor biosecurity can result in reduced financial gain, increased disease outbreaks, and negative impacts on the health and welfare of poultry (East, 2007; Fasina *et al*., 2013).

Disposal of dead birds and isolation of sick ones are practiced in the poultry farms in the study areas. Although some farms use disposal pits and incinerators, they are practiced by farm personnel and not supervised by veterinarians. Isolation of sick birds was done in a separate room in the same shed. The absence of a separate and secured isolation house for sick birds increases the risk of pathogen transmission to susceptible birds. Given the farms' proximity to one another, the spread of pathogens to neighboring farms is also possible. In contrast, the vaccination coverage reported in this study is higher than the reports of previous authors (Birhanu *et al*., 2015).

Interestingly, the majority of the farm managers and owners reported being aware of biosecurity issues such as cleaning and disinfection, traffic control, and isolation practices. However, none of them had a written biosecurity plan, and personnel did not receive training in biosecurity relevant to chicken health, human health, and food safety. In addition, there appears to be poor communication among personnel involved in the poultry production chain, such as farmers, veterinarians, and extension workers. This lack of communication and coordination can lead to inadequate disease surveillance, poor implementation of biosecurity measures, and ineffective disease control.

Conclusions

This study shows the prevalence of poor biosecurity levels in commercial poultry farms in the study areas. The farms did not have standard operating procedures for the implementation of biosecurity measures. Biosecurity score was statistically associated with the age of owners, chicken rearing experience of the owners, having a professional farm consultant, and flock size. Collaboration of farm owners and veterinary and livestock authorities is recommended to implement biosecurity measures to minimize the risk of losses and public health.

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Detection of aflatoxin in raw and pasteurized milk by high-performance liquid chromatography (HPLC) in central Ethiopia

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Abstract

Aflatoxins are a group of structurally related mycotoxins produced by the *Aspergillus flavus, Aspergillus parasiticus,* and *Aspergillus nomius species* as secondary metabolites. Well-known forms of Aflatoxin are AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2. aflatoxin B1 is the most prevalent one. It can be metabolized in the liver and excreted as aflatoxin M1 in milk. Both forms have mutagenic and carcinogenic effects. In Ethiopia, there is limited information on the occurrence and contamination level of aflatoxin in raw and pasteurized milk. The present study was conducted in the Addis Ababa milk shed area to detect and quantify the level of Aflatoxin M1 in raw and pasteurized milk. A cross-sectional study was conducted from October 2020 to May 2021 on a total of 114 cow milk samples consisting of 60 raw milk and 54 pasteurized milk samples with the aim of detecting and quantifying the amount of aflatoxin M1 in raw and pasteurized milk in central Ethiopia by high-performance liquid chromatography (HPLC) using C18 column with fluorescence detector. AFM1 was detected in 79 (69.3%) of the 114 tested milk samples. The maximum and mean concentrations were 0.893 µg/L and 0.0465 µg/L, respectively. 25.4% of them contain AFM1 above the maximum limit of EU (0.05 µg/L), and 1.8% contained above the maximum limit of CES278 and FDA (0.5 µg/L). 26.7% of the samples from Sebata and 6.7% from Sululta contain above the maximum limit of EU (0.05 µg/L). Higher contamination of AFM1 was detected in pasteurized milk (96.3%) than in raw milk (16.7%). 35.2% of pasteurized and 16% of raw milk contained AFM1 above the maximum limit of EU0.05 µg /L. The study results showed a significant difference in AFM1 occurrence with feed type,

storage time, and milk type. The current AFM1 concentration level in raw and pasteurized milk was not safe for human consumption in the study areas. Due to its heat resistance, AFM1 is found in pasteurized milk and has great health effects. Creating awareness of feed management for feed producers and farmers and developing risk mitigation methods are crucial in order to reduce public health threats.

Keywords: AFM1; Central Ethiopia; Dairy Farm; HPLC; Pasteurized milk.

Introduction

Aflatoxins are a group of structurally related mycotoxins produced by certain species of fungi in the genus Aspergillus, particularly Aspergillus flavus, Aspergillus parasiticus, and Aspergillus nomius. It was first discovered in 1960 when approximately 100,000 turkeys died in the UK, and the cause was identified as Aspergillus flavus. The name was given from its cause (Bennett et al., 2007). Aflatoxins are produced as a secondary metabolite; the most commonly known are B1, B2, G1, G2, M1, and M2. The letters B and M represent their color under fluorescence detection, where B is blue, and G is green. Those M1 and M2 are metabolites of B1 and B2, respectively. M1 and M2 are found in food of animal origin, such as milk and milk products, meat, and eggs (Jaimez et al., 2000). AFB1 is a well-known and the most prevalent toxin. Its target organ is the liver, which has teratogenic, mutagenic, and carcinogenic effects in animals and humans. The level of toxicity is AFTs-B1 > AFTs-G1 > AFTs-B2 > AFTs- G2 (Ismail et al., 2015). Aflatoxin M1 (AFM1) is four times hydroxylated and produced by hepatic biotransformation of AFB1 in the liver of animals consuming feed contaminated with AFB1 and secreted in milk. The extent of contamination depends on the season (higher in winter than summer), environmental conditions, and genetic conditions of the animals (Fallah *et al.,* 2011; Abyaneh *et al.,* 2019).

About 0.3% to 6.2% of AFB1 is converted to AFM1 and classified as a cause of human liver cancer. Excretion of AFM1 in milk can take 12 - 24 hours after the ingestion of AFB1; however, there is a decrease in concentration after 72 hours (Fallah *et al.*, 2011; Alahlah *et al.*, 2020; Bukari *et al.,* 2020). AFM1 is resistant to autoclaving, pasteurization, and thermal inactivation. It has both acute and chronic effects (Sani and Nikpooyan, 2013). AFM1 is classified as group one causative agent of human liver cancer by the International Agency for Re-

search on Cancer (IARC) (Sharma *et al.,* 2019). It has a potency which is close to that of aflatoxin B1 (AFB1). Milk and milk products are some of the most important diets of humans worldwide. Aflatoxin in milk and milk products is one of the most severe problems of food safety and security (Polak-Śliwińska, 2020). Therefore, it is essential to determine aflatoxin M1 levels in milk to protect children and adults from its potential health hazards (Dehcheshmeh *et al.*, 2020).

Considering its profound health effects, many countries have set limits for its presence in feed and foods. Commission Regulation of the European Union (EU) states that the maximum level of AFM1 in liquid milk should not exceed 0.05 μg/L(ppb). The US Food and Drug Administration (USFDA) standard indicates that Aflatoxin should not be higher than 0.5 μg/L in liquid milk (Markaki and Melissari, 1997; Maqbool *et al.*, 2009).

Ethiopia produces approximately 3.2 billion liters of milk per year (Desalegn, 2018). However, different factors, like the presence of aflatoxin, can affect its safety and pose health risks to the consumer. Gizachew and his colleagues reported a high contamination level of AFM1 in milk in 2016 in the Addis Ababa milk shade area, which was a significant national issue and increased awareness of aflatoxin (Gizachew *et al.*, 2016). After that, there was no report about the status or contamination level of AFM1 in central Ethiopia, including the current study areas (Sebata and Sululta in the North Showa zone of Oromia region, Ethiopia). It is known that aflatoxin is not entirely removed by pasteurization and other thermal treatments. However, there is no report on the contamination level of AFM1 in pasteurized milk in Ethiopia. Therefore, there is a need to study the current status of AFM1 in this area, which is designated as a significant Addis Ababa milk-shed area. The present study attempted to detect and quantify the contamination level of AFM1 in raw milk and pasteurized milk from the Sebata and Sululta areas.

Materials and methods

Study area description

The study was conducted in the significant Addis Ababa milkshed area (Sululta and Sebata). These areas were selected because they are among the major milk supplier sites in Addis Ababa, both for household raw milk consumption and for milk processing plants.

Sebata is located in a special zone of the Oromia regional state in the central highlands of Ethiopia, 24 km west of Addis Ababa, on the main road to Jimma. The average annual rainfall is 1100 mm, more than 85% of which falls in the main rainy season (June to September). The area's altitude ranges from 2200-2600 meters above sea level, and the average annual temperature ranges from 6-21°C (Desalegn, 2018). According to the Sebeta-Hawas district's livestock agency, there are 310 dairy farms managed by both intensive and semi-intensive farming systems. Daily milk production in the area was about 20,000 liters, of which only half is marketed to Addis Ababa through the formal market. The rest is either consumed at the household level or processed as traditional dairy products (Brandsma *et al.,* 2012).

Sululta district is located between 9° 13′–10° 57′N latitude and 37° 57′–39° 33′E longitude. It is 40 km north of Addis Ababa at an average altitude of 2,550 masl. The annual rainfall is a minimum of 834 mm and a maximum of 1,447 mm. The area's mean minimum and maximum temperatures are 4.4 and 22.5 °C, respectively (Beyecha *et al.,* 2012). According to the Sululta district Livestock Production, Marketing, and Health Agency office, the total cattle population of the district for the year 2019 was 210,211 heads. Intensive and semi-intensive farming systems are practiced in the study area, and there are about 500 dairy farms. From 2018 to 2019, the average annual milk production of the area was 6,694,750 liters, and the average daily milk production was 21,950 liters. From this, about 16,462 liters were marketed formally, and about 5,488 liters were retained at home (Brandsma *et al.,* 2012). More than six milk processing plants receive raw milk from both study areas.

Study design and sample size determination

A cross-sectional study design was used to determine aflatoxin levels in raw and pasteurized milk in central Ethiopia from October 2020 to May 2021. The desired sample size was calculated by using the formula given by Thrusfield (2005) with a 95% confidence interval, 5% precision, and 91.8% expected prevalence based on a previous study by ILRI (Gizachew *et al.*, 2016).

$$n=(Z^2 Pexp(1-Pexp))/d^2$$

Where **n** = required sample size

Z=statistic for the level of confidence at 95% CI, which is 1.96

d= desired absolute precision or margin of error = 0.05

Pexp=expected prevalence, which is 91.8%

$$\mathbf{n} = \frac{(1.96)^2 [0.918(1-0.918)]}{(0.05)^2}$$

n =113

For this study, 114 samples were collected based on previous prevalence by simple random sampling (60 raw milk samples from farms and 54 pasteurized milk samples from different brands). Simple random sampling was used to select the farms in the districts, and each district's sample size was proportionally allocated.

Sample collection method

About half a liter (500 ml) of pooled raw milk was collected from 60 dairy farms. The sample was taken after the milk was mixed well in a container. This helped to homogenize the milk and take appropriate samples. For the pasteurized milk, 54 packs of 500 ml were purchased from supermarkets from three different brands and transported (at a temperature of +4°C in an ice box) to the Ethiopian Agricultural Authority, Animal Products and Inputs quality testing center, former (VDFACA) and stored at -20°C until analysis. Each farm owner had a questionnaire/interview on the type of feed they used, their feed management practice (storage place, time, and method of storage), the feed source, and their knowledge of aflatoxin.

Sample preparation procedures

The extraction and clean-up procedures for sample preparation were based on the association of official analytical chemists (AOAC, 2002). Frozen milk samples of 100 ml were thawed using a water bath at 40 °C for 30 minutes. After heating and bringing it to room temperature, it was centrifuged at 4500rpm for 15 minutes. This helps to separate the fat from the milk and remove it quickly using a spoon. The fat was then filtered using syringe filters through Whatman No.4 filter paper and transferred into a 50 mL tube. Then, 50 mL of defatted (skim) milk was ultimately passed through the Afla M1^{TM} Immunoaffinity Column (Afla CLEAN produced by LCTech GmbH Company of Germany) at a rate of about 1-2 drops/second. All samples were allowed to drain through the column until there was no more sample in the column. At this time, antigen-antibody bonds were formed. Then, the column was

washed with 10 ml of distilled water at a rate of 1-2 drops/second. A gentle gas stream or vacuum removes the residual water. Then, 3 ml of acetonitrile was added, and the analyte-antibody bond was waited for 5 minutes to break. After 5 min, the column was opened, transferred into a 10 ml centrifuge tube, and evaporated/concentrated under the nitrogen stream. Finally, the samples were reconstituted in 1 ml of the mobile phase solution of water-acetonitrile-methanol (60:25:15), transferred to amber glass vials, and ready for HPLC detection (AOAC, 2002).

HPLC conditioning and injection procedures

The HPLC machine was conditioned by pumping a mobile phase solution of water-acetonitrile-methanol (60:25:15) at a steady flow rate until a stable baseline developed. Working standard solutions were prepared at concentrations of 0.05, 0.1, 0.5, 1, 1.25, 1.5, 2, and 4 AFM1 µg/L in the mobile phase to construct the calibration curve. The optimal instrument conditions were checked with an aflatoxin M1 calibrant solution before analyzing the test sample. Then, the linearity of the injection of calibrant solutions and the stability of the chromatographic system were checked. A fixed amount of Aflatoxin M1 calibrant solution was repeatedly injected until stable peak areas were obtained. Peak areas corresponding to consecutive injections were within ± 5%. After the HPLC output, the calibration graph was prepared by plotting the peak area against the mass of injected aflatoxin M1. By following the stipulated injection scheme or an ordered sequence, the test samples were injected using the same conditions as for the calibrant solutions (AOAC, 2002).

Once the aflatoxin M1 peak area was determined, the aflatoxin M1 concentration in the test samples was calculated from the calibration graph in µg/L. The formula/conversion factor used to calculate the actual AFM1 was Wm = Wa x (Vf/Vi) x(1/Vs). Where Wm = the numerical value of aflatoxin M1 in the test sample in ng/ml or ug/L, Wa = the numerical value of the amount of aflatoxin M1 corresponding to the area or height of the aflatoxin M1 peak of the test extract (ng), Vf = the numerical value of the final volume of redissolved elute (uL), Vi = the numerical value of the volume of injected elute(uL) and Vs = the numerical value of the volume of prepared test portion passing through the column(ml). The HPLC system was interfaced via network chromatographic software (Agilent Chem Station) to a personal computer for instrumentation control, data acquisition, and processing (AOAC, 2002). The result was interpreted according to the Ethiopian Standard Agency regulatory limit (CES278),

which is 0.5 µg/L in raw/liquid milk, and other country standards were also used for comparison.

Questionnaire survey method

A questionnaire-based survey was used to assess the potential risk factors associated with the contamination level of AFM1 at the individual farm level. The questionnaire was prepared by targeting farm owners concerning the significant risk factors of aflatoxin, like storage time of the feed, moisture content, ventilation of feed storing room, type of commonly used feed, quality of the feed, and the knowledge of farmers on aflatoxin. All necessary information was gathered through this structured questionnaire and was accompanied by direct observation of the farm.

Ethics approval and consent to participate

Ethical clearance was obtained from the animal research ethical review committee of Addis Ababa University College of Veterinary Medicine (Certificate Ref. No: VM/ERC/28/06/13/2021, Date: 28/03/2021). All methods were carried out accordingly.

Data management and analysis

All collected data were organized, coded, and entered into an Excel spreadsheet (Microsoft® Office Excel 2016) and exported to R-statistical software (version R-3.5.1) for analysis. Descriptive statistics (maximum, minimum, mean, SD) were used to present the result of AFM1 contamination level of milk source and milk type. A table of frequency was used to figure out the findings of the questionnaire. Logistic regression was used to analyze the association between AFM1 contamination levels and the considered risk factors. For variables with small positive or negative results, a Fisher exact test was used.

Results

The HPLC analytical results showed high contamination of milk samples with AFM1. The minimum AFM1 was 0, and the maximum was 0.893 µg/L, with mean and SD of 0.0465 and 0.102, respectively. From a total of 114 analyzed milk samples, 1.8% contained AFM1 above the maximum limit of (CES278(0.5 µg/L), and 29(25.4%) of them had AFM1 above the permissible level of the European Community recommended limit, which is 0.05 µg/L in liquid milk. Of

114 samples, 79(69.3%) were contaminated by AFM1 or contained detectable amounts of aflatoxin M1. Only two samples (1.8%) of 114 analyzed milk samples exceeded the maximum limit set by the USFDA, which is 0.5 µg/L in liquid milk. An example of the HPLC results in ppb is indicated below in Figure 1.

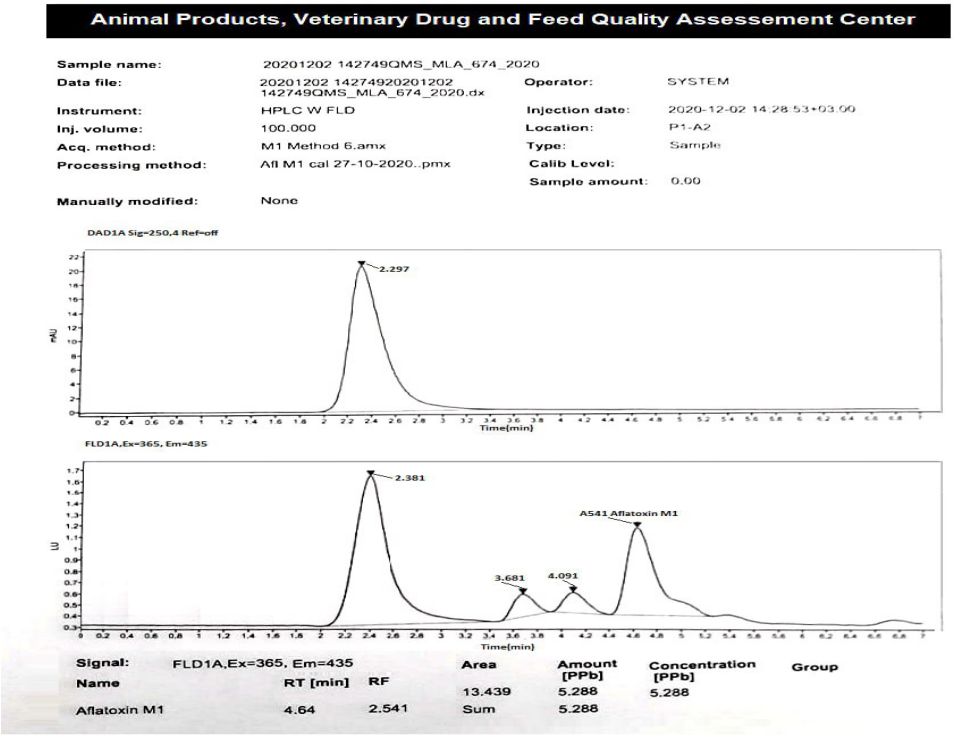


Figure 1. HPLC analytical results in ppb

Raw milk analysis

Of 60 raw milk samples, 10 (16.7%) had AFM1 above the permissible level of 0.05 µg/L. The mean and SD were 0.0469 and 0.1367 respectively. About 50% of the analyzed raw milk samples were positive or had detectable amounts of aflatoxin M1, as indicated in Table 1.

Table 1. Descriptive statistics of AFM1 level in raw milk in the study area

Location	No sample	Positive	> 0.05 µg/L	min	max	Mean ±SD
Sebata	30	20	8	0	0.893	0.082±0.184
Sululta	30	10	2	0	0.2018	0.012±0.038

The samples from Sebata were more contaminated, with 66.7% positive and 26.7% above the permissible level, than those from the Sululta. Still, it is not statistically significant, with 33.3% of the samples positive and 6.7% above the permissible level. As shown in Table 2 below, the considered risk factors (feed source, presence of noug seed cake in feed, type of milk, and storage time of feed) had a statistically significant difference at 95%CI.

Table 2. Contamination level of AFM1 in raw milk with considered risk factors

Risk factors	Variables	No of sample	Positive	>0.05 µg/L	p value
Location	Sebata	30	20	8	
	Sululta	30	10	2	0.079
	Graze	37	11	2	
Feed source	Not use grazing	23	19	8	0.005
Type of milk	Raw	60	30	10	
	Pasteurized	54	52	19	0.023
Industry byproduct	Without	2634			
			19	9	0.001
	Without noug		11	1	
Storage time	1week	37	8	1	
	Above1 week	23	22	9	0.001

Pasteurized milk analysis

A total of 54 pasteurized milk samples from different brands were analyzed. Of these,19(35.2%) specimens had AFM1 above the permissible level of 0.05 µg/L. The minimum AFM1 detected was 0, and the maximum was 0.119, with a mean and SD of 0.046 and 0.037, respectively. The toxin (AFM1) was detected in almost all of them (52/54 or 96% of them).

The test statistics showed that there was no statistically significant difference ($p>0.05$) among the pasteurized milk brands. This means that AFM1 was found in all brands of pasteurized milk included in this study. This can prove the heat-resistant properties of AFM1, as stated in different studies, which

have significant public health effects. Table 3 shows the result of logistic regression of AFM1 for pasteurized milk from different brands.

Table 3. Contamination level of AFM1 in pasteurized milk by their respective brands

Brands	No sample	Positive	>0.05ug/L	Mean	SD	p value
1	13	12	6	0.052	0.032	0.782
2	26	20	6	0.038	0.037	0.148
3	15	14	7	0.055	0.041	0.978

Comparing AFM1 contamination levels in raw and pasteurized milk

There was a statistically significant difference between raw and pasteurized milk contamination by aflatoxin M1 at 95% CI (p-value = 0.0233). As indicated in Table 4, 50% of raw and 96.4% of pasteurized milk were contaminated by AFM1, and 16.7% of raw and 35.2% of pasteurized milk contained AFM1, which is above the maximum limit of 0.05 µg/L.

Table 4. Contamination level of AFM1 by type of milk (logistic regression analysis)

Brands	No sample	Positive	>0.05ug/L	Mean	SD	p value
Raw	60	30 (50%)	10 (16.7%)	0	0.893	0.023
Pasteurized	54	52 (96.4%)	19 (35.2%)	0	0.119	

Farmer's knowledge and awareness of aflatoxin

Survey data were collected during sample collection from the dairy farm owners to obtain complete information; the result of the survey indicates from 60 interviewed farmers, 15(%) were females, and 45(%) were males. Their educational background:6.7% of farmers were illiterate, 41.7% attended primary school, 45% attended secondary school, and 6.6% attended higher education. Indirect observation was also performed on their feed management. The results are summarized in Table 5.

Table 5. Knowledge and practice (KP) of dairy farm owners on aflatoxin contamination.

Knowledge of Aflatoxin	Response	Frequency	Percentage %
Knowledge of mold growth and formation of the toxin No	Yes	41	68.3
	No	19	31.6
Knowledge of favorable conditions for mold growth on animal feed No	Yes	41	68.33
	No	19	31.66
Do you know or heard of Aflatoxin No	Yes	6	10
	No	54	90
Do you know that Aflatoxin causes disease in animals	Yes	6	10
	No	54	90
Do you know Aflatoxin can pass through milk to consumers and have an effect on human	Yes	6	10
	No	54	90
Do you think that Aflatoxin can be destroyed by pasteurization of the milk	Yes	49	81.7
	No	11	18.3
Knowledge and practice on feed management practice			
How do you store the feed	In house	50	83.3
	In shade	8	13.3
	Open field	2	3.4
Is there a ventilator in the feed storing house	Yes	60	100%
	No	0	
Do you control the moisture content of the feed	Yes	45	75
	No	15	25
Do you check the quality of the feed while buying and feeding	Yes	6	10
	No	54	90

Discussion

The present study showed that there is widespread contamination of raw and pasteurized milk by aflatoxin originating from the Sebata and Sululta milk shed areas. From a total of 114 analyzed milk samples, 25.4% exceeded the maximum permissible level of the EU standard (0.05 µg/L). This result is lower than the result of previous studies of Gizachew and his friends in 2016 in the Addis Ababa milk-shed area in which 91.8% of analyzed samples contained above the permissible level of 0.05 µg/L (Gizachew *et al.*, 2016). This difference could be due to changes in the farmers' practices, especially regarding the feed

type used since 2016. Most farmers reduce the amount of noug seed cake in their dairy feed and reduce the time of storage of the feed that is purchased from feed retailers. A study from Injibara reported a low contamination level of AFM1. According to this report, 10% of analyzed samples were above permissible levels, and AFM1 contaminated 15% of the sample (Kassa *et al.,* 2020). This discrepancy could be due to the differences in environmental conditions or climate change, the type of feed used, and the feed management practices of the farmers in Injibara.

The current study showed that 79(69.3%) of 114 analyzed samples were contaminated by AFM1. This result was much lower than the study from Bishoftu (Tadesse *et al.*, 2020), who reported that, from 108 analyzed milk samples, all samples(100%) were found to be contaminated by AFM1 with a mean value of 0.835 µg/l. The result of this study was in line with a study from Kenya by (Anyango *et al.,* 2018), who reported that 26.4% of the analyzed sample exceeded the limit of the EU. A study from Pakistan by (Ahmad *et al.,* 2018) reported that AFM1 contaminated 93% of the analyzed samples, and 69% of the samples exceeded the EU ML (0.05 µg/L), which is much higher than the result of this study.

In the present study, AFM1 was detected in 50% of raw milk samples. Of those, 16.7% were above the permissible level of 0.05 µg/L. This is lower than the study from Kenya by Kagera and his friend (Kagera *et al.,* 2018), who reported that 99% of analyzed milk samples were positive for AFM1 and 64% exceeded the permissible level of EU 0.05 µg/L. The results of this study were also much lower than the study report of Asghar and his colleagues in Pakistan (Asghar *et al*., 2018), who reported 91.7% contamination and 80.1% above the permissible level from 156 tested raw milk samples.

When comparing the contamination level of raw milk by location of sample collection, raw milk collected from Sebata district had a higher contamination level of AFM1(26.7%) than that of Sululta district (2/30 or 6.7%). This can be due to environmental temperature (high temperature in Sebata), feed type used, and farming system. Most farmers in Sebata district do not have free grazing land for their dairy cattle, so they use purchased feed under an intensive farming system. Farmers from the Sululta district use semi-intensive systems, and they use grazing in their backyard around the home and crop by-

products during harvesting of the crops. This can help to reduce the exposure of dairy cows to Aflatoxin originated from feed.

The present study revealed that high AFM1 contamination was found in pasteurized milk compared with raw milk. However, the maximum AFM1 concentration was found in raw milk. This result was in line with a study in Iran in 2017 (Abyaneh *et al*., 2019), which reported high AFM1 contamination in pasteurized and heat-treated milk. A report by (Sharma *et al*., 2019) showed that a high contamination level of AFM1 was found in pasteurized, which indicates thermostability of AFM1, which is in line with this study. An earlier study by (Sani and Nikpooyan,2013) showed a low contamination level of AFM1 in pasteurized milk, but all samples were positive for AFM1. A study report from Iran by(Taherabadi *et al*., 2016) indicated lower contamination in pasteurized milk (5%) than in raw milk (9.2%). The results of the present study showed that considered risk factors such as storage time, feed type, and grazing have significant effects on aflatoxin production in feed and milk. The storage time of feed has a significant effect on mold growth and aflatoxin production. Feed that was stored for more than one week had more AFM1 than feed stored for less than one week, but in the present study, the toxin was detected at both storage times. This can be due to the moisture content of the feed, climate conditions, ventilation, and other factors. This result was also supported by the study performed by (Abyaneh *et al*., 2019), who reported that the storage condition of feed can increase the chance of mold growth.

Feed type had a significant effect on aflatoxin production. Some feed can easily be contaminated by AFB1, which can be transformed into AFM1 in lactating cows. In this study, milk from farmers who fed their lactating cows feed containing noug seed cake had a higher contamination level of AFM1 than milk from cows fed other feed types, such as brewery grain and wheat bran. This is in agreement with the previous study by (Gizachew *et al*., 2016), who reported a high contamination level in feed containing noug seed cake. All (100%) dairy farm owners offered concentrate feed to their lactating cows. The majority (76.7%) of farmers from Sebata district practice zero grazing due to a lack of grazing land in the area, and those farmers were obligated to rely on purchased pasture, fodder, and concentrates, which increases the chance of aflatoxin contamination. Grazing also has a significant effect on the contamination level of AFM1. In this study, a high contamination level was recorded in milk originating from farmers who did not use grazing and low AFM1 con-

tamination in milk originating from farmers who practice an open grazing system in the backyard or grazing field, in addition, to concentrate feeds for their dairy cows. According to the survey results, 68.3% of the farmers were aware of favorable conditions for mold growth and aflatoxin formation. Only 10% of interviewed farmers had heard the word aflatoxin before and knew that it could cause disease in humans through milk from intoxicated cows. 81.7% of the farmers assume that aflatoxin M1 can be destroyed by pasteurization or other heat treatment. This assumption is based on the fact that pasteurized milk is safe from bacteria, but AFM1 is a toxin, not a living organism. The feed management practices of the farmers were observed during the sample collection, and the majority (83.3% of them stored their feed in houses with ventilation, and 75% of them checked the moisture content of the stored feed. Only 10% of them can check the quality of feed by physical observation while buying the feed. They check the physical appearance of the feed and try to buy from good feed retailers in the area.

Conclusions

The present study showed that there is a widespread aflatoxin M1 contamination of milk produced in the study area, with 69.3% of the milk having a detectable amount of AFM1 even though only 1.8% of the milk had a contamination level above the maximum limit (0.5μg/L) as per the compulsory Ethiopian standard (CES)-278 and USFAD regulation level. High AFM1 contamination was found in pasteurized milk compared with raw milk. In this study, risk factors such as storage time, feed type, and grazing have shown a significant association with aflatoxin M1 contamination level in milk. In general, the consumption of milk above the maximum tolerance level might cause a severe public health risk to the community, especially to children who consume milk on a daily basis. Hence, specific regulations and compulsory food safety standards should be put in place by the national regulatory authorities to control or regulate AFB1 in animal feeds and AFM1 in milk and milk products. Awareness and training on feed management, primarily on feed storage and quality feed purchasing, should be provided to farmers. Screening and monitoring of AFB1 in concentrate feed and AFM1 in milk should be performed regularly. Pasteurized milk should be monitored and checked frequently since AFM1 was not removed by pasteurization. A further comprehensive study should be conducted by designing a nationwide survey that encompasses risk mitigation strategies and biodetoxification methods for both AFB1 and AFM1. Moreover,

planned aflatoxin residue surveillance and monitoring activities need to be carried out by the Ethiopian Agricultural Authority.

Declarations

Consent to publication

All authors have agreed to publish the manuscript.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

No conflict of interest.

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Authors contribution

Sitena Kebede was responsible for writing the proposal, collecting data, conducting all laboratory work, performing data analysis, and writing the manuscript. Gezahegne Mamo provided guidance and edited the manuscript. Belachew Bacha and Belachew Tefera contributed by providing comments and editing the manuscript. All authors have thoroughly reviewed and approved the final manuscript.

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Seroprevalence of bovine viral diarrhea virus and detection of persistently infected (PI) animals in dairy farms of Holeta, central Ethiopia

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Abstract

Bovine viral diarrhea virus (BVDV) is one of the most important pathogens of the reproductive systems that have a significant socioeconomic impact on the dairy industry. A cross-sectional study was conducted on 17 randomly selected farms out of 133 registered dairy farms in Holeta, a town in the Walmera district of Ethiopia. The study aimed to detect BVDV antigen-positive animals and investigate the seroprevalence of BVDV infection. A total of 337 serum samples were collected from selected farms with no history of BVDV vaccination. The indirect enzyme-linked immunosorbent assay (I-ELISA) test was the screening test used to detect antibodies against BVDV. In contrast, antigen-capture ELISA was used for the detection of BVDV antigens in seronegative animals. A logistic regression model was used to assess the association between potential predictors and BVDV exposure. The overall animal level seroprevalence was 15.4%, and 64.7% of the herds had at least one seropositive animal. From 285 seronegative animals, one animal (0.4%) was found positive for BVDV antigen. The same animal was found positive in a double check 21 days later. In this study, cows with a history of abortion (OR = 6.3; 95% CI: 1.61 -13.1), history of repeated breeding (OR = 7; 95% CI: 2.5 - 14.3), animals managed intensively (OR = 4.6; 95% CI: 1.6 - 13.0) and multiparous cows (OR = 3.6; 95% CI: 1.5 - 8.9) had higher proportion of seroreactors in their respective comparison category ($p<0.05$). Besides, cows with a history of congenital defective calve birth (OR = 15.2; 95% CI: 3.2 - 73.6), adult age groups (OR = 2.9; 95% CI: 1.0-7.9), and cows bred both artificially and natural mating (OR = 4.6; 95% CI: 1.7 - 12.6) were statistically associated with BVDV seropositivity ($p<0.05$). In conclusion, this report demonstrated the presence of persistently infected (PI) dairy cattle in Ethiopia. Thus, the findings warrant the need for

immediate control intervention that involves both screening and culling of PI animals and vaccination.

Keywords: BVDV; Dairy Cattle; ELISA; Seroepidemiology; Holeta; Ethiopia.

Introduction

Efficient reproduction in cattle is critical for the economic success of the dairy industry. Especially in developing nations like Ethiopia, reproductive inefficiency significantly affects the livelihoods of livestock owners. The problem has been linked to multiple infectious and non-infectious causes. Among these, bovine viral diarrhea virus (BVDV) is one of the most important infectious pathogens that impose a significant economic impact on the dairy industry worldwide (Albrecht *et al.*, 2021).

A single-stranded RNA virus causes bovine viral diarrhea in the genus *Pestivirus* of the *Flaviviridae* family (Brodersen, 2014). The virus has two genetically distinct genotypes (BVDV-1 and BVDV-2) and two biotypes (cytopathic and non-cytopathic). Infection with BVDV leads to significant economic losses, both directly and indirectly. Directly, it affects the reproductive performance of dairy herds, causing a decline in conception rates, increased rates of abortion, stillbirths, the birth of weak calves, teratogenesis, and reduced milk yield. Indirectly, the expenses incurred for implementing control programs further contribute to the economic burden (Lindberg, 2003; Pinior *et al.*, 2017). Besides, infection of cattle during the early gestation period may lead to the birth of persistently infected (PI) calves that have great epidemiological relevance in the dynamics of BVD infections (Albrecht *et al.*, 2021).

Bovine viral diarrhea virus survival in the cattle population mainly depends on the characteristics of PI cattle. Persistent infection arises from the unique ability of the BVDV to survive by inducing immune tolerance in the bovine fetus through evasion of both innate and acquired immunity in utero (Brodersen, 2014). The main transmission route in infected herds is direct contact with PI animals (Lindberg, 2003). Viral transmission can also occur through the placenta to the developing fetus (Brownlie, 1987).

Reproductive diseases in dairy cattle can be prevented by vaccination and the implementation of biosecurity measures. Vaccination against BVDV improved

reproductive efficiency parameters in dairy herds (Pereira *et al*., 2013). However, vaccines against BVDV are not available in Ethiopia. Hence, identification of the PI cattle is crucial for the implementation of management practices that decrease the risk of exposure of susceptible dairy cattle. BVDV control and eradication have primarily focused on identifying and removing the PI cattle from the herd. Although PI cattle are the primary reservoir of the virus, their identification and elimination from susceptible cattle populations can be costly in the form of diagnostic testing since less than 1% of the cattle population is PI with BVDV (Newcomer *et al*., 2017; Peddireddi *et al*., 2018).

In Ethiopia, reproductive abnormalities, including repeat breeding, abortion, stillbirth, fetal mummification, and congenital abnormalities, have been reported in previous studies, and the presence of antibodies against BVDV in dairy cattle has been recorded with variable prevalence (Nigussie *et al*., 2010; Asmare *et al*., 2013; Aragaw *et al*., 2018). However, many epidemiological data gaps still exist, and no information is available on the status of PI cattle. Thus, more investigations need to be done to identify the PI cattle from dairy herds and better understand the epidemiology of BVDV in the different geographical settings of the dairy cattle population, which could help to formulate strategies to mitigate the impact of BVDV on the production and reproduction of dairy cattle in Ethiopia. Hence, the present study aimed to investigate the seroprevalence of BVDV and detect BVDV antigens in dairy cattle in Holeta town.

Materials and methods

Study area and population

The study was conducted in Holeta, a town in the Walmera District, West Shoa Zone, and Oromia regional state (Figure 1). Holeta, a town in central Ethiopia, is known for its abundance of dairy farms and the long tradition of keeping improved dairy cattle. Smallholder dairy farms are major employment areas and sources of income for youths and women in the town. Cattle are the dominant livestock in the area. Holeta town has 133 registered dairy farms, according to the Walemera District Livestock Development and Fishery Office (WDLDFO, 2019).

The target population was Holstein-Friesian crossbreeds with no history of vaccination, breeding females, and reared in smallholder, medium, and large commercial dairy farms.

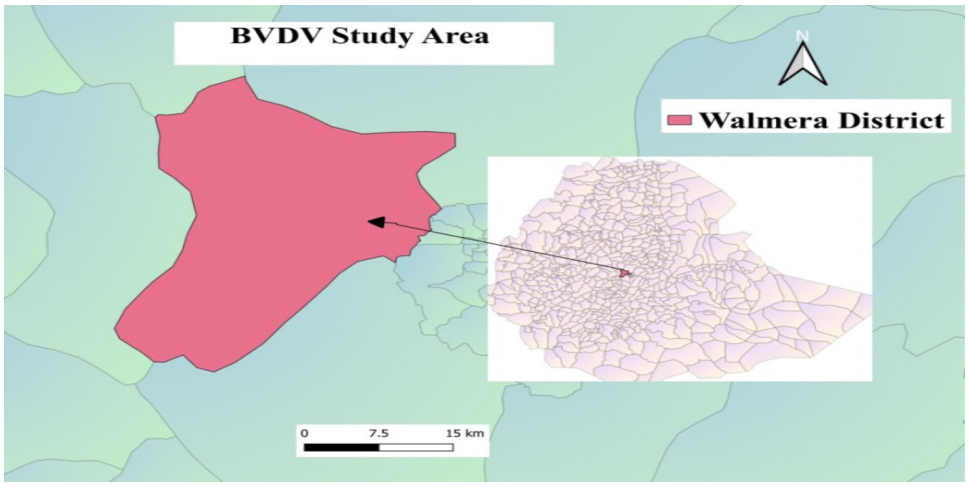


Figure1. Map of Ethiopia that shows the study area.

Study design, sampling, and sample size

A cross-sectional study was conducted on 17 randomly selected farms out of the 133 registered dairy farms in Holeta. Animal biodata and management-related factors were recorded to identify the potential risk factors associated with BVDV, such as age, herd size, farming systems (intensive and semi-intensive), parity [nulliparous (no calving), primiparous (given calving once), and multiparous (given two or more calving)], animal husbandry and housing practices, the structure of the dairy farm, composition of dairy herds, and breeding system (using artificial insemination (AI) and/or bull). Herds were classified into small (1-15 animals), medium (15-25 animals), and large (above 25 animals) herds. The age was grouped into six months to < 1 year, 1-2 years, and above two years.

Simple random sampling techniques were used to select 17 farms from 133 registered dairy farms in the town. The minimum sample size was estimated based on Thrusfield (2005). The predetermined parameters were an expected prevalence of 32.6% (Aragaw *et al.,* 2018), a 95% level of confidence, and 5% absolute precision. Accordingly, a total of 337 blood samples were collected.

All seronegative serum samples were subjected to antigen capture ELISA to detect PI animals. The positive animals were rechecked for their status after 21 days.

Blood sample collection

Ten ml of blood samples were collected using a plain vacutainer tube from the jugular veins of 337 randomly selected dairy cattle. The blood samples were labeled and allowed to stand overnight, and then the serum was decanted into cryovials. The collected serum samples were transported to the Animal Health Institute (AHI) in an icebox and kept at -20 °C until tested.

Laboratory tests

Indirect ELISA assay targeting antibodies

BVDV-specific antibodies were detected using indirect enzyme-linked Immunosorbent Assay (iELISA) (IDEXX, France) that detects antibodies against p80 protein of BVDV with a diagnostic sensitivity of 97.6 % and specificity of 97.27 % according to the information of the manufacturer's validation data report. BioTek EL800 Microplate Reader was used to measure the optical density (OD) of each sample and control at 450nm. The results were interpreted based on the sample-to-positive control OD ratio (S/P). Samples with an S/P ratio greater than or equal to 50 % were considered negative, whereas samples with an S/N ratio less than or equal to 40 % were considered positive; samples with an S/N ratio between 40% and 50 % were considered doubtful.

Antigen capture ELISA assay

Negative serum samples in the iELISA were subjected to antigen capture ELISA assay. The detection of BVDV antigen in serum samples was performed using the manufacturer's instruction (BVDV Ag/Serum Plus, IDEXX, Switzerland). This test kit has a diagnostic specificity of 100% (95% CI: 95.69 - 100) and a sensitivity of 100% (95% CI: 90.5 – 100). The test protocol includes adding a positive and negative control to each plate for every run of the assay. The sample/positive control ratio (S/P) was calculated using the following formula, and the sample was considered BVDV positive if the S/P ratio was ≥ 30.

S/P = (OD of the sample-OD of the negative control)/(mean OD of the positive control-OD of the negative control) x 100

Data analysis

Data were recorded and coded using Microsoft Excel for Windows 2010 and transferred to STATA13.1 (Stata Corp. College Station, Texas) software for analysis. The data were then summarized using descriptive statistics. A univariable logistic regression analysis was used to assess the unconditional association of prevalence with individual risk factors for BVDV exposure. Then, the independent variables were checked for multicollinearity using Variance Inflation Factors (VIF) and tolerance value (Dohoo *et al.*, 2003). Variables with p-value ≤ 0.25 in the univariable logistic regression analysis with no multicollinearity were entered into the multivariable logistic regression model. The forward elimination procedure was used to eliminate the factors that were not significant ($p > 0.05$). The model was finally assessed using the Hosmer-Lemeshow test for goodness-of-fit and the receiver-operating curve (ROC) for reliability. The model with a value of the area under the curve (AUC) of the ROC greater than 0.7 was accepted. In all analyses, the confidence level of 95% and $p < 0.05$ was set as statistically significant.

Results

Out of the total of 337 serum samples tested, 15.4% (95% CI: 11.7 – 19.7%) were positive for antibodies against BVDV. Similarly, from a total of 285 seronegative animals subjected to antigen-capture ELISA, one animal (0.4%) was found positive for BVDV antigen (Table 1). The same animal was found positive in a double check 21 days later. Similarly, the overall seroprevalence of BVDV in herds with at least one positive animal was 64.7% (95% CI: 38.3 - 85.8%).

Table 1. Animal and herd-level prevalence estimate of BVDV in Holeta dairy farms

Variable	No examined	No. of positives	Prevalence (95% CI)
Individual animal level	337	52	15.4 (11.7 – 19.7)
Herd level	17	11	64.7 (38.3 - 85.8)

Cl: Confidence interval

BVDV seroprevalence and associated risk factors

Results from the initial univariable logistic analysis are given in Table 2. The risk factors such as age, herd size, parity, and farming systems were significantly ($p < 0.05$) associated with BVDV seropositivity. In the next multivariable logistic regression model, significant differences in seroprevalence were observed in age, farming system, parity, and breeding system (Table 4). In this model, a significantly higher seroprevalence was observed in animals greater than two years old (OR = 2.9; 95% CI: 1.0 – 7.9; $p = 0.043$), multiparous cows (OR = 3.6; 95% CI: 1.46- 8.59; $p \leq 0.001$), in intensive farms (OR = 4.6; 95% CI: 1.6 - 13.1; $p = 0.004$) and farms using both bulls and AI for breeding (OR = 4.57; 95% CI: 1.7- 12.6; $p = 0.003$) (Table 4).

Table 2. Univariable logistic regression analysis for potential risk factors associated with BVDV exposure status in dairy cattle.

Variables	No. examined	Prevalence (%)	COR (95% CI)	p-value
Age				
Six months - <1 year	95	7.4	Ref	
1 -2 years	56	3.6	0.5 (0.1 - 2.3)	0.351
> 2 years	186	23.1	3.8 (1.6 - 8.8)	0.002
Herd Size				
Small	69	8.7	Ref	
Medium	107	6.5	0.7 (0.2 - 2.3)	0.595
Large	161	24.2	3.4 (1.4 - 8.4)	0.009
Parity				
Nulliparous	154	6.5	Ref	
Primiparous	63	20.6	3.6 (1.4 - 9.3)	0.008
Multiparous	120	24.2	4.4 (1.9 - 10.1)	≤ 0.001
Farming system				
Semi-intensive	197	7.61	Ref	
Intensive	140	26.4	4.4 (2.3 - 8.3)	≤ 0.001
Breeding System				
AI	79	8.9	Ref	
AI and Bull	258	17.4	2.17 (0.9 - 5.0)	0.070

Ref: reference; COR: Crude Odds ratio; CI: Confidence interval

Association of BVDV seroprevalence and reproductive problems

Univariable logistic regression analysis showed that cattle with a history of abortion, repeat breeding, and congenital defects were significantly ($p < 0.05$) associated with BVDV seropositivity, as shown in Table 3. A multivariable logistic regression model was fitted to predict the association of BVDV seropositivity with a history of abortion, repeat breeding, and congenital defects. Within this model, the risk of being BVDV seropositive was six times higher in cows with a history of abortion (OR = 6.3; 95% CI: 2.4 - 16.7; $p \leq 0.001$) compared to cows without a history of abortion. Similarly, the risk of being BVDV seropositive was six times higher in cows with a history of repeat breeding (OR = 5.9; 95% CI: 2.5 – 14.3; $p \leq 0.001$) compared to cows with no history of repeat breeding. The risk of being BVDV seropositive was 15 times higher in cows with a history of congenital defects (OR =15.2; 95%CI: 3.2 – 73.6; $p \leq 0.001$) compared to calves without a history of congenital problems (Table 5).

Table 3. Univariable logistic regression analysis of reproductive problems with BVDV exposure status in dairy cattle.

Variables	No. of tested animals	Prevalence (%)	COR (95% CI)	p-value
Abortion history				
No	312	12.5	Ref	
Yes	25	52.0	7.0 (2.9 - 16.6)	≤ 0.001
History of Repeat Breeding				
No	303	11.9	Ref	
Yes	34	50.0	6.1 (2.9 - 13.1)	≤ 0.001
History of Congenital Defects				
No	325	14.5	Ref	
Yes	12	41.7	4.2 (1.3 - 13.9)	0.017

Ref: reference; COR: Crude Odds ratio; CI: Confidence interval

Table 4. Multivariable logistic regression for the potential risk factors with BVDV exposure status in dairy cattle.

Variables	AOR (95% Confidence interval)	P-value
Age		
≥ 2 years	2.9 (1.0- 7.9)	0.043
1 -2 years	0.3 (0.6 - 1.8)	0.190
Herd size		
Medium	1.1 (0.2- 5.9)	0.874
Large	3.5 (0.5- 22.7)	0.191
Farming system		
Intensive	4.6 (1.6 - 13.1)	0.004
Parity		
Multiparous	3.6 (1.56- 8.6)	0.005
Primiparous	1.6 (0.5- 4.7)	0.408
Breeding system		
AI and Bull	4.57 (1.75- 12.69)	0.003

Table 5. Multivariate logistic regression analysis of reproductive disorders with BVDV exposure status in dairy cattle.

Variables	AOR (95% Confidence interval)	P-value
Abortion history		
Yes	6.3 (2.4 - 16.7)	≤ 0.001
History of Repeat Breeding		
Yes	5.9 (2.59 - 14.3)	≤ 0.001
History of Congenital Defects		
Yes	15.2 (3.2 - 73.6)	0.001

Discussion

In this study, 15.4% animal-level seroprevalence of BVDV was recorded. The animal level seroprevalence of this study was comparable with previous reports of Nigussie et al. (2010), with an overall seroprevalence of 11.5% in Jimma, Shoa, and South Shoa zones of Ethiopia, and Asmare et al. (2013) with a seroprevalence of 11.7% in dairy farms of southern Ethiopia. This study was also in agreement with the previous reports in Sudan (10.7%) (Saeed et al., 2015), Egypt (10.4%) (Soltan et al., 2015), and Kenya (19.8%) (Callaby et al.,

2016). On the other hand, the current findings were lower than the findings reported in Ethiopia (32.6%) (Aragaw et al., 2018), Mexico (78.8%) (Montiel, 2019), Iran (77.9%) (Shirvani et al., 2012), and Nigeria (64.4%) (Bello et al., 2016). In the current study, 64.7% herd-level seroprevalence of BVDV antibodies was reported. This finding is in agreement with a herd prevalence of 69.8% in Ethiopia (Aragaw et al., 2018), 65.5% in Brazil (Fernandes et al., 2016), 66% in Great Britain (Velasova et al., 2017), and 69% in Colombia (Ortega et al., 2020). The difference in animal and herd level seroprevalence might be attributed to differences in animal management systems, the diagnostic test used, sample size, study design, and environmental (agroecology) conditions (Houe, 1999; Saa et al., 2012; Fernandes et al., 2016).

In this study, a significantly higher seroprevalence was observed in the intensive farming system compared to the semi-intensive farming system. This might be due to the high contact rate in intensively managed herds that facilitate virus transmission. Van Campen reported that herd density is a significant predictor of the prevalence of BVDV (Van Campen, 2010).

In the current study, the seroprevalence of BVDV antibodies was significantly associated with dairy cows with a history of abortions. The risk of being BVDV seropositive was six times higher in cows with a history of abortion (OR = 6.3; 95% CI: 2.4- 16.7; $p \leq 0.001$) compared to cows without a history of abortion. This suggests that there is an association between abortion and BVDV infection. This result was in agreement with other studies that reported a higher seroprevalence of BVDV antibodies in cows with a history of abortions (Asmare *et al*., 2013; Derdour *et al*., 2017; Tadesse *et al*., 2019; Thapa *et al*., 2019).

In this study, the risk of being BVDV seropositive was six times higher in cows with a history of repeat breeding (OR = 5.9; 95% CI: 2.49 – 14.30; $p \leq 0.001$) compared to cows with no history of repeat breeding. This suggests that BVDV might be responsible for repeat breeding in dairy cows in Ethiopia. This result is in agreement with previous findings in Ethiopia (Asmare *et al*., 2013; Nigussie *et al*., 2010). Reduced conception rates, early embryonic deaths, abortions, congenital defects, and the birth of weak calves have been associated with BVDV infection in dairy cattle (Kaiser *et al*., 2013).

In this study, BVDV antigen was detected at a prevalence of 0.35%. To the authors' knowledge, this is the first report demonstrating BVDV antigen in dairy cattle in Ethiopia. This study indicates the presence of PI animals in

Ethiopia's dairy herds. A single PI animal can serve as a potential source of infection for the entire herd and contribute to the spread of the virus. PI animals are critical virus transmitters since they continuously shed a large amount of viruses (Lindberg, 2003). A previous study showed that BVDV PI prevalence at the animal level ranged from low (≤ 0.8%) in Europe, North America, and Australia to medium (>0.8 to 1.6%) in East Asia, and high (>1.6%) in West Asia (Scharnböck *et al*., 2018). At present, there is no BVDV vaccine available in Ethiopia, and no control strategies have been implemented. Scharnböck *et al.* (2018) identified the highest PI prevalence at the animal level in countries that had failed to implement any BVDV control and/or eradication programs, including vaccination. Thus, improving the overall biosecurity measures of the farms and early detection of PI animals to eliminate them from the herd is crucial.

Conclusions

In this study, the reported animal level prevalence is moderate, but the herd level prevalence is apparently high. To the author's knowledge, this is also the first report demonstrating PI animals in dairy cattle in Ethiopia. Multiparous cows, cattle reared in an intensive production system, and older age significantly increase the risk of BVDV infection. Moreover, specific reproductive abnormalities such as abortions, congenital defects, and repeat breeding were associated with seropositivity of BVDV. Thus, there is a need for control intervention focusing on screening and culling of PI animals and vaccination to protect the herd, at least in the area.

Data availability

The data collected and used to support this article can be offered by the first or corresponding author upon request.

Competing interest

The authors declare that they have no competing interests.

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tion, design, and interpretation of the study, data collection, analysis, and interpretation of the data reported in this manuscript.

Ethical considerations

Ethical approval for this study was granted by the animal research ethical review committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University (reference number: VM/ERC/19513/2021). Animals were handled following the best veterinary care guidelines. Before conducting the research, animal owners were informed about the objectives and the benefits of the study, and they gave consent for their animal's inclusion in the study. The consent obtained from animal owners was verbal because owners are unable to write and read. Consent was obtained in the presence of a third independent party and approved by the College of Veterinary Medicine and Agriculture of Addis Ababa University Ethics Committee.

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Effect of supplementing concentrate mixture with field pea hull on yearling ram lambs of local sheep: Nutrient intake, linear body measurements, and changes in body weight

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Abstract

The fattening of sheep has been recognized as a potentially profitable activity in the highlands of Ethiopia. However, feed shortage and increasing prices for conventional feed ingredients and commercially formulated concentrate mixtures critically affect the fattening practice of sheep in the country. So, this feeding trial was conducted to evaluate the effect of feeding field pea hull at different inclusion levels with a concentrate mixture on feed intakes, nutrient utilization, linear body measurements, and body weight gain. Eighteen yearling ram lambs were randomly allocated to three dietary treatments using a randomized complete block design. The dietary treatments were T1 (control), fed native hay adlibitum + concentrate mixture formulated using only conventional feed ingredients (0% field pea hull); T2 and T3 were fed native hay adlibitum + concentrate mixture formulated with inclusion of 35% field pea hull and 50% field pea hull, respectively along with other feed ingredients. The feeding trial was conducted for 90 days. Data were analyzed using GLM procedures of SAS, and Turkey's HSD multiple-comparison was used for means separation. The result showed a significant (p<0.001) variation in dry matter and nutrient intake among treatments. The DMI was the highest in T3, followed by T2, while it was the lowest in T1. The average total neutral detergent fiber and acid detergent fiber intakes and metabolizable energy intake were highest in T3 and followed by T2, while they were the lowest in T1. The average total body weight gain (kg) was higher in T3 (9.0kg) and T2 (8.67kg) than in T1 (6.58kg). Similarly, higher average daily body weight gains were recorded in T3 (100g) and T2 (96.3g) than in T1 (73.2g). Sheep that were fed field pea

hull by the inclusion of 50 % in concentrate mixtures (T3) were more efficient in feed utilization (p<0.05) than T1 as they consumed significantly less amount of feed per 1 g of body weight gain. Hence, it is concluded that feeding of 50% field pea hull along with concentrate mixture could be used for better fattening performance of sheep.

Keywords: Concentrate mixtures; feed efficiency; grass hay; highland sheep; field pea hull.

Introduction

Sheep production plays a significant role in the Ethiopian economy (Gizaw *et al.,* 2013); it provides cash income for meat and skin production for the livelihoods of smallholder farmers. Sheep are also a significant means of employment and income in different production systems (Herrero *et al.,* 2013). The total population of sheep is about 42.9 million (CSA, 2021). However, the mutton production of the sheep is low compared to its potential.

Fattening sheep has been recognized as a potentially profitable activity that enhances the income of smallholder farmers in Ethiopia (Pasha, 2006). However, it is a seasonal activity across all the production systems due to the unavailability of feed throughout the year. Feed shortage is one of the limiting factors for increasing the production and productivity of sheep in most agroecological zones in Ethiopia (Bachano and Etefa, 2022). The most commonly used conventional feed ingredients to prepare concentrate mixtures for ruminant animals include Niger seed (*Guizatia abyssinica*) cake, wheat bran, wheat middling, etc. (Berhane *et al.,* 2013). However, the price of these conventional feed ingredients and the formulated concentrate mixtures are expensive and are increasing at an alarming rate from time to time. Gizaw *et al.* (2017) also indicated that a shortage of feed constrains sheep productivity in Ethiopia and higher price increments of feed, among other factors. Hence, the use of non-conventional feed resources could be an alternative feed ingredient to reduce feed costs in fattening sheep, as most of them are relatively cheaper compared to conventional feeds.

According to FAO (2014), Non-Conventional Feed Resources (NCFR) refers to all those feeds that have not been traditionally used for animal feeding either by farmers or by feed manufacturers in commercial feeds, and these are not

used in the ration of commercially produced animals. Non-conventional feeds include agro-industrial by-products, food left over, pulse (such as field peas, grass peas, faba bean, lentils, etc.), hulls, banana peels, and avocado peels, etc. (FAO, 2014). Utilization of non-conventional feeds could be an alternative and relatively cheaper source of supplements (Salem *et al*., 2004).

Pulse hulls are a by-product of pulse seed processing to get pure cracked pulses for human consumption, which are abundantly available in most urban and peri-urban areas of Ethiopia. They are non-conventional feed resources which include field pea (*Pisum sativum* L.) hull, grass pea (*Lathyrus sativus* L.) hull, lentil (*Lens culinaris*) hull, faba bean (*Vicia faba*) hull, etc., where the protein content of these pulse hulls range between 15 to 16% (Berhane *et al*., 2013). Although pulse hulls are available in large quantities in most places of Ethiopia and are good potential non-conventional feed resources, limited research has been conducted on feeding field pea hulls to fatten sheep. Therefore, the objective of the study was to investigate the effect of feeding field pea (*Pisum sativum* L.) hull inclusion level in the concentrate mixtures on feed intake, nutrient intake, body linear measurements, and body weight gain in yearling ram lambs of local sheep.

Materials and methods

Description of the study area

The feeding trial was conducted at Ambo University, Guder Mamo Mezemmir Campus (AUGMC), Guder town, West Shewa Zone, Oromia National Regional State, Ethiopia. Guder town is located 126 km away from Addis Ababa, which is the capital city of Ethiopia, and 12 km away from Ambo Town (capital town of West Shewa Zone). The town of Guder lies with an altitude of about 1850 above sea level, and the temperature varies from 17 to 23.4^{0}C, the average being 20.35^{0}C. The rainfall amounts range between 1200 and 1,700mm (AUGM website, 2021) (https://ambou.edu.et>mmc). The sheep population of Oromia National Regional States is 9,752,385, of which West Shewa Zone, being one of the 21 zones of the region, has a sheep population of 1,074,939 contributes 11.02 % of the population (CSA, 2021).

Experimental animals and their management

For conducting a feeding trial, eighteen-yearling ram lambs were purchased from local markets around Holeta and its surroundings by considering their age of about one year (one pair of permanent teeth), using dentation body weight (17-22 kg), better body condition, and health of the yearling ram lambs. The ram lambs were tagged for identification after they were purchased and were kept in an experimental house. Each sheep was kept in a single pen in an area of 1.1 m^2 with individual feeding troughs. The house was cleaned and washed two times a day, early in the morning and afternoon, to keep the animals healthy during the whole experimental period. All purchased ram lambs were dewormed against internal parasites and injected with ivermectin 2 ml per head against external parasites. The experiment considers the ethical issues of animal handling. All animal handling practices followed the international guiding principles listed by the Council for International Organizations of Medical Sciences and the International Council for Laboratory Animal Science (2012).

Experimental design and treatment allocation

The animals were randomly assigned into six blocks (three animals in one block), representing one animal for each treatment using initial body weight taken after overnight fasting at the end of environmental adaptation. All experimental ram lambs were managed for one month of adaptation by providing the same feed during quarantine, deworming, and confining management system. They also kept ten additional days for experimental feed adaptation. The average initial body weight of experimental animals was 21.06±1.32 kg (mean ± SE). The three treatments were randomly allotted to each block. Then, the animals taking the same treatment were picked up from each block and formed one treatment group, i.e.,e there were six replications per treatment.

The descriptions of the three dietary treatments were as follows:

T1 (**Control) =** Fed native hay *ad libitum* + control concentrate mixture formulated by using only conventional feed ingredients of niger seed cake, maize, and salt at the rate of 49.5, 49.5, and 1%, respectively, according to Solomon *et al.* (1991), without field pea hull;

T2 = Fed native hay *ad libitum* + concentrate mixture formulated with the inclusion of 35% field pea hull along with niger seed cake, wheat bran, and salt at the rate of 34, 30, and 1%, respectively;

T3 = Fed native hay *ad libitum* + concentrate mixture formulated with the inclusion of 50% field pea hull along with niger seed cake, wheat bran, and salt at the rate of 33,16, and 1%, respectively (Table 1).

These three types of concentrated mixtures were formulated based on NRC (1989) requirements so as to have isonitrogenous and isocaloric rations. They had actual average crude protein content of 21.5%, ranging from 21.3 to 21.7% (Table 1).

Table 1.The proportion (%) of feed ingredients used in the formulation of different concentrate mixtures

Feed ingredients	T1	T2	T3
Maize	49.50	-	-
Niger seed cake	49.50	34	33
Wheat bran	-	30	16
Field pea hull	-	35	50
Salt	1	1	1
Total	100	100	100
Calculated CP%	20.30	20.30	20.30
Actual CP %	21.70	21.40	21.30

T1 native hay +standard concentrate mixture formulated from Niger seed cake (49.5%), maize (49.5%) and salt (1%) without pulse hull; T2 native hay + concentrate mixture formulated by inclusion of 35% field pea hull + Niger seed cake (34%), maize (30%) and salt (1%); T3 native hay + concentrate mixture formulated by inclusion of 50% field pea hull + Niger seed cake (33%), maize (16%) and salt (1%).

Feeding of experimental animals

The weighted amount of formulated concentrate mixtures was fed in equal proportion to individual sheep twice a day at 9:00 AM and 3:00 PM. Native hay (at 20% of refusal) and water were provided ad libitum to individual ram lamb. The feeding experiment was conducted for 90 days.

Data recording and measurements

Feed intake

The weighed amount of native hay and different concentrate mixtures were fed based on treatments for each yearling ram lamb on each day, and the data were

recorded. The next day, the feed refusal from each type of concentrate mixture and hay from each sheep were weighed and recorded. The daily feed intake for each sheep was calculated by subtracting the amount of each feed refusal from each type of feed offered. The dry matter intake (DMI) was then calculated by multiplying the feed intake from each type of concentrate mixture and hay by the corresponding dry matter percentages of the consumed types of feed.

Body weight gains and efficiency of feed utilization

The weight of each sheep was taken at the initial period, at fortnightly or 15-day intervals, and at the end of the experimental period after overnight fasting using a spring weighing balance. The body weight change/the body weight gain (BWG) of each yearling ram lamb was calculated as the difference between the final body weight (FBW) and initial body weight (IBW), i.e., BWG = FBW-IBW. Average daily body weight gain (ADWG) was calculated as the difference between the FBW and IBW and divided by the number of experimental days, i.e., ADG = FBW-IBW/the number of experimental days. The feed conversion ratio (FCR) is the parameter used to calculate the efficiency of feed utilization. FCR was calculated as the ratio between the average daily dry matter intake (DMI) per sheep (g) to the average daily gain per sheep (g), i.e., FCR = Average daily DMI of each sheep (g)/ average daily gain per sheep (g) (AOAC, 2005).

Linear body measurements and body condition scores

The body condition score for each sheep was taken at the start of the experiment, at fortnightly intervals, and at the end of the experimental period. The body condition score was done by palpation at the back and loin of the sheep with fingers to estimate the amount of muscle and fat deposited in the animal body using three trained personnel. For each sheep, values were given from one to five by each person, and then the average values were taken. Body surface measurements of body length, body height, chest circumference, and chest width were also taken. Body length was taken from pin bone up to shoulder area. Body height was taken at the wither height (from the ground to the top of the wither), heart girth was measured at the circumference of the sheep behind the front legs, and chest width was measured at the front width of the sheep using centimeters (FAO, 2012). All measured data were taken at the initial of the experimental period, at fortnightly intervals, and at the end of the experimental period using centimeters.

Feed sampling and chemical analysis of experimental feeds

Feed samples for chemical analysis were taken from each feed ingredient of hay, field pea hull, Niger seed cake, wheat bran, and maize. Samples were also taken from three types of formulated concentrate mixtures at fortnightly intervals for two consecutive days till the end of the feeding trial. The collected samples were then pooled per treatment. Composite samples were made after thorough mixing for laboratory analysis and sent to Holeta Agriculture Research Center Animal Nutrition Laboratory for chemical analysis**.** The partially dried samples of each feed were ground using a laboratory mill to pass through a 1-mm sieve screen size. The samples were analyzed for dry matter, organic matter, crude protein, and total ash contents using the proximate method of analysis (AOAC, 2005). Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), Acid Detergent Lignin (ADL), Digestibility of Organic Dry Matter (DOMD), and Metabolizable Energy content of different feeds were determined as per the methods described by Goering and Van Soest (1970).

Data analysis

All the data collected on the different parameters were analyzed using least square analysis of variance for RCBD as per General Linear Models (GLMs) procedures using SAS software packages version 9.2 (SAS, 2008). Means were separated using Tukeys' HSD multiple-comparison techniques whenever ANOVA showed significant variation.

The model used for data analysis was:-

$$Yijk = \mu + \tau i + \beta j + \tau\beta ij + \varepsilon ijk$$

Where yijk = observation k in treatment i and block j (individual measurement on each sheep such as body weight, body length, etc.)
μ = overall mean effect.
τi = fixed effect due to the i^{th} treatment.
βj = fixed block effect due to body weight.
Tβij = the interaction effect of treatment i and block j.
εijk = random error.

Results

Chemical composition of different feed ingredients and concentrate mixtures

The chemical compositions of different feed ingredients used to formulate the concentrate mixture are given in Table 2. The dry matter contents of different feed ingredients ranged from 90.4% in maize to 93.39% in Niger seed cake, while the crude protein (CP) contents ranged from 9.5% in maize to 33% in Niger seed cake. The highest NDF and ADL contents in the feed ingredients were recorded in native hay (74.29 and 5.53%, respectively), while the lowest NDF was recorded in maize (19.11%), and the lowest ADL was observed in wheat bran (2.62%).

The CP contents of the formulated concentrate mixtures were ranged from 21.31% (T3) to 21.69 (T1). The highest NDF and ADF contents were recorded in T3 (59.39 and 27.88, respectively), while the lowest contents were recorded in T1 (42.94 and 10.19, respectively). The ADL content was slightly higher in T3 (7.38%) and lower in T1 (4.80 %) (Table 2).

Table 2. The chemical composition of different feed ingredients, concentrate mixtures, and hay fed to a yearling ram lamb

Type of Feeds	DM (%)	OM	CP	Ash	NDF	ADF	ADL	DOMD	IVD MD	ME (MJ/kg DM)
T1	91.02	94.45	21.69	5.55	42.94	10.19	4.80	65.66	72.39	10.5
T2	90.86	95.18	21.44	4.82	54.81	22.70	6.05	60.19	67.24	9.63
T3	91.07	96	21.31	4.00	59.39	27.88	7.38	57.31	64.53	9.17
NSC	93.39	89.3	33	10.7	46.38	19.32	9.64	51.30	58.87	8.21
FPH	90.58	95.34	16.52	4.66	68.11	40.05	5.02	58.69	65.83	9.39
WB	90.63	95.45	14.0	4.55	36.33	5.54	2.62	72.71	79.02	11.6
Maize	90.40	93.13	9.5	6.87	19.11	3.03	3.58	79.12	85.05	12.7
NH	90.79	90.61	7.0	9.39	74.29	38.34	5.53	53.11	60.57	8.50

T1: native hay + concentrate mixture formulated from Niger seed cake (49.5%), maize (49.5%) and salt (1%) without pulse hull; T2: native hay + inclusion of 35% field pea hull + concentrate mixture formulated from Niger seed cake (34%), maize (30%) and salt (1%); T3: native hay + inclusion of 50% field pea hull + concentrate mixture formulated from Niger seed cake (33%), maize (16%) and salt (1%); DM: Dry Matter; OM: Organic Matter; CP: Crude protein; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; ADL: Acid detergent lignin; DOMD, ME, MJ, IVDMD: Invitro dry matter digestibility; NSC: Niger seed cake; FPH: Field pea hull; WB: Wheat bran Maize; NH: Native hay

Dry matter intake (DMI) of yearling ram lambs

The average dry matter intake (g/h/d) of yearling ram lambs with different levels of field pea hull in concentrate mixtures is shown in Table 3. A significant difference (p<0.001) was observed in the dry matter intake of yearling ram lambs among the treatments. The average total dry matter intake during the experimental period was the highest (p<0.001) in T3 (714.15g), followed by T2 (705.51g), while the lowest intake (643.22g) was recorded in T1. The dry matter intake of hay followed the same pattern as the total DMI, where the highest dry matter intake of hay (p<0.001) was in T3 (368.2g), followed by T2 (363.56g), while the lowest intake was in T1 (346.39g) treatment. On the other hand, the dry matter intake of the concentrate mixture was higher in T3 and T2 than in T1 (Table 3). The DMI of yearling ram lambs at fortnightly intervals showed that treatment T3 and T2 had higher DMI than the control. The differences in DMI between T3 and T2 were minimal, although T3 showed slightly higher values than T2 in DMI. The DMI of sheep in all the treatments had reduced in the second fortnight, but the latter showed a continuous increase in DMI (Figure 1).

Table 3.The average dry matter intake (g/h/d) of yearling ram lambs during the experimental period

	Treatments (6 animals per treatment)				
Variables	**T1**	**T2**	**T3**	**SEM**	**P-Values**
Hay DMI (g)	346.39^{c}	363.56^{b}	368.2^{a}	0.78	0.001
Con. Mix DMI(g)	296.83^{b}	341.95^{a}	345.95^{a}	1.23	0.001
Total DMI (g)	643.22^{c}	705.51^{b}	714.15^{a}	1. 68	0.001

Con. Mix: Concentrate mixture; DMI: Dry matter intake; g: gram; T1: native hay + concentrate mixture formulated from Niger seed cake (49.5%), maize (49.5%) and salt (1%) without pulse hull; T2: native hay + inclusion of 35% field pea hull + concentrate mixture formulated from Niger seed cake (34%), maize (30%) and salt (1%); T3: native hay + inclusion of 50% field pea hull + concentrate mixture formulated from Niger seed cake (33%), maize (16%) and salt (1%); 0.001: Significant at (p<0.001)

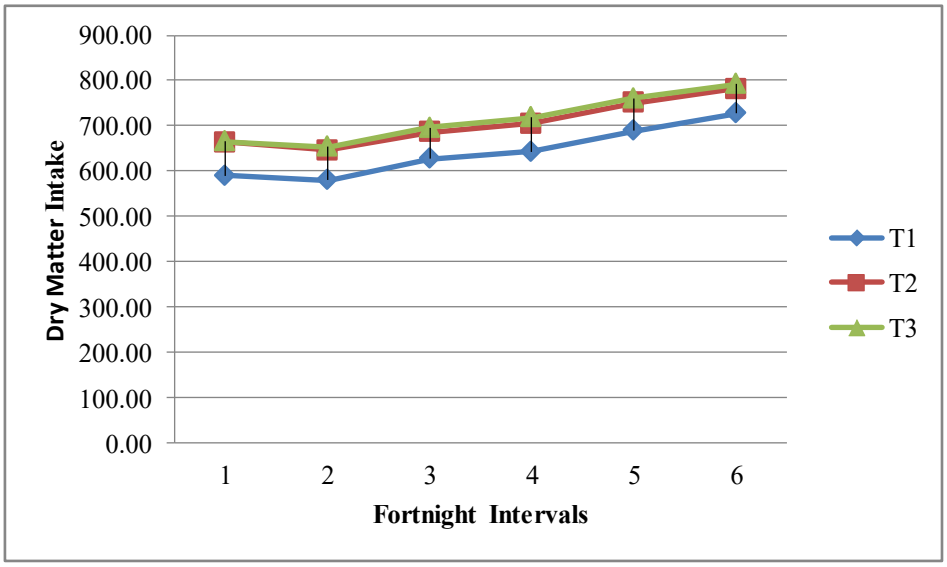


Figure 1. Trends of fortnightly dry matter intake of yearling ram lambs (g/d/h) during the experimental period

Nutrient intake of yearling ram lambs

The average nutrient intake (g/h/d) of yearling ram lambs is shown in Table 4. The average total CP intake of yearling ram lambs was higher (p<0.001) in T3 (99.56) and T2 (98.76) than in T1(88.66). The average total neutral detergent fiber (NDF) intake of yearling ram lambs was the highest (p < 0.001) in T3 (478.99), followed by T2 (457.51). The average hay and concentrate mixtures of neutral detergent fiber intakes followed the same pattern as the average total neutral detergent fiber intake, where the intakes were the highest (p<0.001) in T3, followed by T2. The ADF intakes of hay, concentrate mixtures, and total ADF intakes were the highest (p<0.001) in T3, followed by T2 than the T1 control. Similarly, ME intakes (MJ per sheep per day) were the highest (p<0.001) in T3, followed by T2, while intakes were the lowest (p<0.001) in T1 (Table 4).

Table 4. The average nutrient intake (g/h/d) of yearling ram lambs during the experimental period

Variables	Treatments (6 animals per treatment)			SEM	p-values
	T1	T2	T3		
CPI					
Hay CPI	24.25c	25.45b	25.77a	0.05	0.001
Con. Mix CPI	64.41b	73.31a	73.79a	0.26	0.001
Total CPI	88.66b	98.76a	99.56a	0.28	0.001
NDFI					
Hay NDFI	257.33c	270.09b	273.54a	0.58	0.001
Con. Mix. NDFI	127.46c	187.42b	205.46a	1.01	0.001
Total NDFI	384.79c	457.51b	478.99a	1.35	0.001
ADFI					
Hay ADFI	132.81c	139.39 b	141.17a	0.3	0.001
Con. Mix ADFI	30.25c	77.62b	96.45a	0.73	0.001
Total ADFI	163.05c	217.01b	237.62a	0.89	0.001
MEI(MJ/day)					
Hay MEI	0.041c	0.0428b	0.0433a	0.00	0.001
Con.Mix MEI	0.028c	0.035b	0.038a	0.00	0.001
Total MEI	0.069c	0.078b	0.081a	0.00	0.001

Con.Mix: Concentrate mixtures; MEI: Metabolizable energy intake; CPI: Crude protein intake; ADFI: Acid detergent fiber intake; NDFI: Neutral detergent fiber intake; T1: native hay + concentrate mixture formulated from Niger seed cake (49.5%), maize (49.5%) and salt (1%) without pulse hull; T2: native hay + inclusion of 35% field pea hull + concentrate mixture formulated from Niger seed cake (34%), maize (30%) and salt (1%); T3: native hay + inclusion of 50% field pea hull + concentrate mixture formulated from Niger seed cake (33%), maize (16%) and salt (1%).

Linear body measurements of yearling ram lambs

The average linear body measurements (cm) of yearling ram lambs are shown in Table 5. Body length and height did not show significant variation (p>0.05) among the treatment groups. However, there was a highly significant variation (p<0.001) in heart girth and chest width, which increased steadily throughout the experimental period.

Table 5. The average body linear body measurements (cm) of yearling ram lambs that were taken every 15 days interval during the experimental period

	Treatments (6 animals per treatment)				
Variables	**T1**	**T2**	**T3**	**SEM**	**p-values**
Body length	59.75	59.33	60.61	0.45	0.50
Heart girth	66.17[b]	68.25[a]	69.53[a]	0.30	0.001
Body height	60.28	60.22	61.33	0.29	0.23
Chest width	13.06[b]	13.94[a]	14.03[a]	0.12	0.001

T1: native hay + concentrate mixture formulated from Niger seed cake (49.5%), maize (49.5%) and salt (1%) without pulse hull; T2: native hay + inclusion of 35% field pea hull + concentrate mixture formulated from iger seed cake (34%), maize (30%) and salt (1%); T3: native hay + inclusion of 50% field pea hull + concentrate mixture formulated from Niger seed cake (33%), maize (16%) and salt (1%); SEM: Standard Error Mean.

Body weight gain and efficiency of feed utilization

The effect of feeding field pea hull at different levels of inclusion in concentrate mixtures on body weight gain and the efficiency of feed utilization of yearling ram lambs is presented in Table 6. There was no significant difference (p>0.05) among the dietary treatment groups. However, the total body weight gain (kg) was higher (p<0.01) in T3 and T2, which was 9.0kg and 8.67kg, respectively, than T1 (6.58kg). Similarly, the higher average daily body weight gain was higher (p<0.01) in T3 (100g) and T2 (96.3g) than in T1 (73.2g) during the experimental period. Feed conversion ratio (FCR) showed significant (p<0.05) variation among the treatment groups, where the yearling ram lambs in T2 and T3 were more efficient in feed utilization than the control group (T1).

Table 6. Average body weight gain of yearling ram lambs during the experimental period

Variables	**Treatments (6 animals per treatment)**				
	T1	**T2**	**T3**	**SEM**	**p-Values**
IBW (kg)	19.33	19.08	19.33	0.36	0.95
FW (kg)	25.92	27.75	28.33	0.53	0.16
TBWG (kg)	6.58[b]	8.67[a]	9.00[a]	0.39	0.01
ADBWG (g)	73.15[b]	96.30[a]	100.00[a]	4.29	0.01
FCR	8.93[a]	7.39[b]	7.38[b]	0.46	0.05

T1: native hay + concentrate mixture formulated from Niger seed cake (49.5%), maize (49.5%) and salt (1%) without pulse hull; T2: native hay + inclusion of 35% field pea hull + concentrate mixture formulated from Niger seed cake (34%), maize (30%) and salt (1%); T3: native hay + inclusion of 50% field pea hull + concentrate mixture formulated from Niger seed cake (33%), maize (16%) and salt (1%); SEM: Standard Error Mean; IBW: Intial body weight; FW: Final body weight; TBWG: Total Body weight gain; ADBWG: Average daily body weight gain; FCR: Feed conversion ratio.

Discussion

Chemical composition of experimental feeds

Dietary nutrients, especially energy and protein, are the significant factors affecting the productivity of sheep (Yimenu and Abebe, 2023). The CP content of native hay in the experimental feed is 7%, which is lower than the minimum maintenance requirement of sheep (7.5% on DM) (Van Soest, 1994). However, the CP content of the filed pea hull is 16.52, which was higher than the reported value for the filed pea hull (Yimenu and Abebe, 2023). The CP content of T2 (35% of field pea hull) and T3 (50% of field pea hull is comparable with T1 (concentrate mix), 21.31, 21.44, and 21.69, respectively. The level of CP contents for all concentrate mixtures in the present study was above the CP content of 12.5 % required to satisfy rumen microbial demands for nitrogen that would provide sufficient CP for the maintenance requirement of the animal (Van Soest, 1994). The better protein content of the filed pea hull and its inclusion with concentrate will support the fattening of ram lamb and could be included in the fattening ration. However, the value of NDF of the field pea hull was high. Still, its level in the concentrate mix T2 (54.81), and T3 (59.39) is considered as a medium, which indicates that the inclusion of filed pea with other concentrates will help to lower the NDF content instead of using it alone. Singh and Oosting (1992) pointed out that roughage feeds containing NDF values of less than 45% are classified as high, those with values ranging from 45 to 65% as medium, and those with values higher than 65% as low quality. The values of ADF contents of experiment feed ingredients are also considered low. Kellems and Church (1998) indicated that roughage with less than 40% ADF is categorized as better quality and those with greater than 40% as poor quality. The ADF of filed pea hull values were lower than the earlier reports of 63.9% ADF reported by Seyoum *et al.* (2007) and higher than 30.4-42.4% and 43.3% of ADF reported by Yetimwork (2005) and Ermias (2008) in that order for faba bean haulms.

Dry matter and nutrient intake

The present findings of higher total DMI and CP intake in T3 and T2 agree with the findings of Yoseph *et al.* (2002), who reported that feeding of pulse hulls (lentil, grass pea, and field pea hull) to the sheep improved total DMI and CP intake than the control group (feeding of native hay). Habte (2010) also revealed that supplementation of different levels of lentil hull to lambs resulted in higher DMI and CPI than the control group (feeding of native hay).

The higher crude protein intake in T3 and T2 in the current study compared to the control might be due to the optimal tannin content present in the treatment concentrate mixtures (T3 and T2), a result of the inclusion various levels of field pea hull, in the concentrate mixtures. Berhane *et al*. (2013) reported that field pea hull has a tannin content of 6.16%. But, when this field pea hull was included at the rate of 35 and 50% in concentrate mixtures formulated for dairy cows, the tannin content in the formulated concentrate mixtures was 0.88 and 1.57%, respectively. This indicated that the rate of tannin content in the formulated concentrate mixtures was lower than 4% and resulted in the beneficial effect of the bypass protein in the present experiment. Different authors also reported that the optimum tannin content (≤ 4%) in formulated concentrate mixtures facilitates the bypass protein by binding with macromolecules such as protein in the rumen; thereby, amino acids are more effectively utilized in the lower digestive tract improve the performance of the sheep (Barman and Rai, 2006; Dubey, 2007).

Body weight gain, efficiency of feed utilization, and linear body measurement changes

The current findings of the average daily body weight gains recorded in T3 (100g) and T2 (96.30g) were higher than the reported values of Yimenu and Abebe (2023), who reported an average daily body weight gain of 74.82 g for the Washera sheep supplemented with 400g concentrate feeds over the basal diet of natural pasture grass hay and 50% field pea hull with traditional brewery dried residue and concentrate feeds. On the other hand, the average daily body weight gain of T1 (73.2g) in the current study was similar to the reported values of 74.82g Hirute *et al*., 2011) in which Arsi bale sheep fed on urea-treated barley straw and 400g concentrate. Feed utilization efficiency variation among the treatments showed that T3 and T2 were more efficient in feed utilization than T1. This indicated that the amount of feed consumed per 1 g of body weight gain was higher in T1 (8.93g) than T2 (7.39g) and T3 (7.38g), indicating feeding of field pea hull at an inclusion level of both 35 and 50% is more efficient than the control.

Significant variation in Heart girth and Chest Width in the current study agreed with the report of Ayele and Urge (2019), who reported that there was a significant difference in linear body measurements between experimental groups of three sheep breeds. Higher values of Heart girth and Chest Width in T2 and T3 might be due to the compensatory growth of yearling ram lambs

in higher inclusion levels of 35 and 50% field pea hull. The same authors also indicated that higher CP and ME intake of sheep might have contributed to enhanced skeletal growth. Luzardo *et al.* (2019) also indicated that higher feed intake and growth efficacy were associated with compensatory growth.

Conclusions

There was the highest total dry matter intake in those yearling ram lambs that were fed 50% field pea hull in concentrate mixture (T3), followed by those fed 35% field pea hull (T2), while it was significantly the least in control (T1). On the other hand, total crude protein intake was higher in both T2 and T3 than in the control. The total body weight gain and the average daily body weight gain (g) during the experimental period were significantly higher both in those yearling ram lambs that were fed concentrates mixtures formulated by the inclusion of 50% and 35% field pea hull (T3 and T2, respectively) than in the control group (T1). Feed utilization efficiency was higher in T2 and T3 than the control group (T1), which indicates that the amount of feed consumed (g) per g of body weight gain was the lowest in T3 and T2 than the control. Heart girth and chest width were also higher in T3 and T2 than in the control group (T1). Generally, it has been concluded that feeding of field pea hull at the inclusion level of 50% in concentrate mixture along with 33% Niger seed cake, 16% wheat bran, and 1% salt could be used for better fattening performance of sheep. The current result is limited to two levels of inclusion of field pea hull. However, further investigation could be conducted to evaluate the effects of feeding peal hull in concentrate mixture at different levels of inclusion for efficient utilization of field pea hull.

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Gut microbiota and relevant abundances of *Prevotella copri*, Lachnospiraceae, *Collinsella*, *Helicobacter cinaedi*, *Desulfovibrio,* and *Escherichia coli* among cats with *Pemphigus foliaceus*

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Abstract

The cat gut-inhabitant conjoint microbiota is a peculiar ecosystem in relationship with several bodily functions and immunity. Gut microbiota dysbiosis could participate within autoimmune disease pathogenesis, whereas its niche, whether causative or influencing role regarding systemic immunity in autoimmune diseases, remains fugitive. The purpose of the present study was to identify gut microbiota alterations and probable mechanisms that participated in *the development of Pemphigus foliaceus for a better understanding of future effective therapeutical armamentarium based on* gut microbiota. In the present article, the authors investigated gut microbiota alterations to those of eight cats diagnosed with Pemphigus foliaceus. Furthermore, the study thoroughly analyzed pathogenic bacteria species as triggers of autoimmunity. The diagnostic algorithm involved two distinct sides: i) on referral with the first criteria was proof of evidence for possible autoimmunity, and ii) Pemphigus foliaceus diagnosis with relevant analytes. A total of eight cats were enrolled, and gut microbiomes were detected by the use of the MIDOG All-in-One Microbial Test targeting Next-Generation DNA Sequencing. By this methodology, we analyzed the dysbiosis network for bacterial kingdoms and determined the relationship between disease activity related to *Pemphigus foliaceus* and gut microbiota. The data showed increasing abundances of *Collinsella*, Lachnospiroceae, and *Escherichia coli* and decreasing *Desulfovibrio piger*, *Prevotella copri,* and *Helicobacter cianedi* among cats with pemphigus foliaceus. For the first time in Turkey, the gut microbiota of cats with pemphigus foliaceus were detected, the results of which could be cautiously taken into consideration for novel and effective therapeutical approaches.

Keywords: Cat; Microbiota; Pemphigus.

Introduction

Autoimmune diseases are composed of an assorted cloud of disorders presented by abnormal B/T cell reactivity to normal host tissue (Arbuckle *et al*., 2003; Mu *et al*., 2020; Volkov *et al*., 2020). For the vast majority of this group of disorders, frequently recognized immunological reflection is autoantibody production, allowing clinicians to provide important biomarkers for diagnosis, classification, and activity of disease (Arbuckle *et al*., 2003; Mu *et al*., 2020; Volkov *et al*., 2020; Psetsky, 2023). Infection has been postulated as a frequent common trigger of autoimmunity, whereas gut microbiota might influence pathogenesis (Ercolini *et al*., 2009; Christovich and Luo, 2022; Qui *et al*., 2019; Xu *et al*., 2019).

The core microbiome pool of the intestine has been detected to alter disease conditions (Christovich and Luo, 2022) in association with the stage of systemic lupus erythematosus participation of regulatory B (Breg) cells altered. Given the onset of the disease, the latter cells exhibited protection, whereas later on, they seemed to aggravate the condition (Mu *et al*., 2020). This data is linked to the B cells' tendency to exhibit autoantigens to T cells and existing autoantibodies (Jacob and Stohl, 2010; Dörner *et al*., 2011). Moreover, bacteria appeared to participate in this protection, through which oral usage of bacterial DNA was capable of inducing Breg cells and diminished autoimmunity (Mu *et al*., 2020). It was suggested that the selected gut microbiota pool of lupus-prone mice contributed to pathogenesis (Johnson *et al*., 2020). Fecal microbiota transplantation showed that the female microbiota fostered, whereas male ones deliberated the disease (Johnson *et al*., 2020). There still exists a question of whether alterations in gut microbiota composition have a causality or effective interaction with systemic lupus erythematosus and even if it contributed to the onset of the disorder or aggravated disease activity (Mu *et al*., 2020). Moreover, there is lacking data regarding the relationship between gut microbiota and pemphigus foliaceus among cats, which prompted us to perform this study aiming at this route.

Material and methods

The diagnostic algorithm involved two distinct sides. On referral, the first (I.) criterion was proof of evidence for possible autoimmunity (Huve *et al*., 2020) (Table 1). Any cat that met this criterion was enrolled in this study.

Cats enrolled in the present study were retrospectively archived between February 2017 and May 2022. Other relevant diagnostic criteria (II.) were previously described (Bizikova and Burrow, 2019).

Data regarding lesion location/distribution, the existence of systemic clinical findings, therapy outcome, and duration of the follow-up period were all recorded by a well-experienced assistant and simultaneously extracted to an Excel sheet.

Sampling procedure

Microbiome analysis was conducted using collected fecal material. Experienced veterinary surgeons participated in manual withdrawal of gaita through rectal swabbing at a veterinary healthcare setting (Aydin Adnan Menderes University, Faculty of Veterinary) (Ural *et al.*, 2023). Briefly, test material obtained from Midog Center was composed of sterile swabs, which were used to take rectal sampling. The MiDOG® All-in-One Microbial Test, executed at the MiDOG® Test Center located in Irvine, CA, is recognized for its precise NextGeneration DNA Sequencing capabilities. This analytical service is adept at pinpointing and profiling unique molecular patterns that are indicative of specific microbial entities. Ensured by rigorous methods of sample preservation and transport by our investigative team, the process includes comprehensive DNA extraction from the entirety of the microbial population in the specimen. This is succeeded by the selective amplification of microbial DNA and subsequent sequencing using cutting-edge technologies provided by Illumina (Illumina, Inc., San Diego, CA). The data processing is conducted through well-maintained microbial databases, which facilitate the accurate alignment of DNA sequences, thereby enabling the precise identification of fungi down to the species level within the sample. The absence of fungal detection in the sample could be attributed to an extremely low microbial load or a diminutive concentration of microbial DNA.

Mycobiome analysis-methodology

The MiDOG® All-in-One Microbial Test is denoted as a targeted, Next-generation DNA sequencing testing. This testing service is capable of identifying molecular signatures dedicated to the identity and character of a specific microorganism. The latter test is based on safeguarded preservation and transportation of obtained samples (by our research group), thoroughly extracted DNA (from entire microbes that existed within the specimen), selected am-

plification of microbial DNA followed by Next-generation DNA sequencing [by use of the latest/novelty technologies from Illumina (Illumina, Inc., San Diego, CA). Data maneuver is performed via curated microbial databases to align DNA sequences accurately to ensure precise and accurate (species-level) identification of all fungi present in the collected sample. Even if no fungal species were detected, relevant reasons should, therefore, involve a very low microbial load and/or low concentration of microbial DNA within the sample sent to the testing center. Briefly, Each specimen was immediately secured in DNA/RNA Shield™ (supplied by Zymo Research Corp.; Catalog No. R1108, Irvine, CA, USA) and remained there until it was time for processing at MiDOG LLC's laboratory located in Tustin, CA, USA. The ZymoBIOMICS™-96 DNA kit (Catalog No. D4304, Zymo Research Corp., Irvine, CA, USA) was utilized to purify the genomic DNA. Zymo Research Corp. handled the library construction and subsequent data interpretation for microbial assessments, employing the Quick-16S NGS Library Prep Kit (Catalog No. D6400, Zymo Research Corp., Irvine, CA, USA) and making necessary adaptations. The primer sequences, exclusive to MiDOG LLC, are designed to amplify the 16S rDNA V1–V3 regions for bacteria and the ITS-2 regions for fungi. Sequencing was undertaken with the Illumina HiSeq 1500 system, followed by data curation using the Dada2 (R package version 3.4). Microbial composition was quantified as a percentage relative to the aggregate sequence count per sample. The comparative abundance of bacteria and fungi was estimated by equating a single 16S rDNA instance to one fungal ITS copy. The precision at the species level afforded by the sequencing technique herein has been corroborated by prior shotgun sequencing studies (Callahan *et al.,* 2016; Tang *et al.,* 2020).

Antinuclear antibody (ANA) and other clinical assessments were done using a routine clinical and laboratory approach. Our research was conducted as part of routine clinical evaluations and involved obtaining informed consent forms from the animal owners after providing them with the necessary information. All cases were represented individually, and the relative abundance of microbial diversity was represented in graphs using Microsoft Excel (Office 365).

Results

In a total of eight cats from various breeds at the age of 2 to 7 years old of both sexes (5 female and two male) diagnostic tree with clinical findings and their distribution based on individual (Figure 1) along with *Pemphigus foliaceus* lesion distribution diagram (Figure 2), clinical photos (Figure 3-5) and

gut microbiota analytes (Figure 6-11) at phylum, genus, family and species level with special reference to abundant bacterial agents. For the diagnosis of *Pemphigus foliaceous,* the method recommended by Bizikova *et al.* (2019) was adopted (Table 1).

Table 1. The second diagnostic tree (Huve *et al.*, 2020) on behalf of referral to our clinic with a presumptive diagnosis of autoimmune dermatitis among cats herein involved. These criteria were adopted in an attempt to exclude cases with *Pemphigus foliaceus.*

Pemphigus foliaceous diagnosis (Bizikova* *et al.*, 2019) was adopted in this study.	clinical proof of superficial pustule/erosion or crust
	b) unresponsive antibacterial trial (21 days and above)
	microscopical evidence of acantholytic cells [histopathology or cytology]
	monitorization for 90 days or more

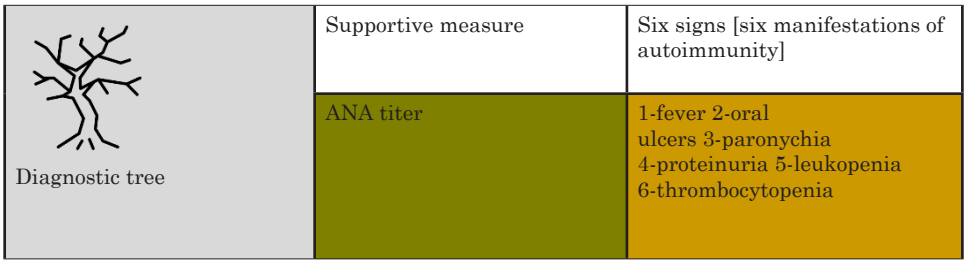


Figure 1. First diagnostic tree (Huve *et al.*, 2020) on behalf of referral to our clinic with a presumptive diagnosis of autoimmune dermatitis among cats herein involved.

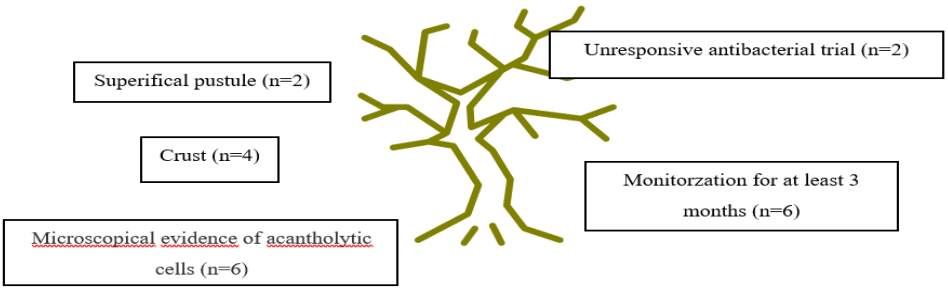


Figure 2. Diagnostic tree with lesional invasion mapping of individual distribution.

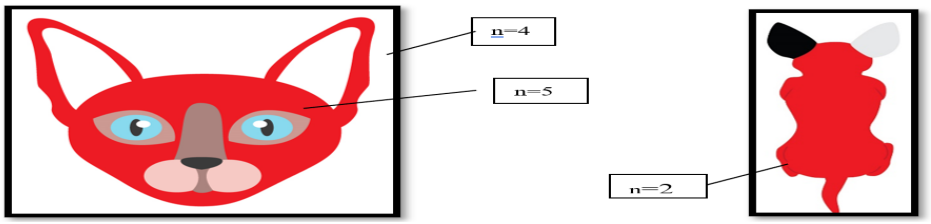


Figure 3. Feline *Pemphigus foliaceus* lesion distribution diagram and individual data regarding lesional distribution in a total of 8 cats involved herein.



Figure 4. One of the eight cats involved presenting clinical photos depicting characteristic dermatological invasion with multifocal coalescing erosions and crusts on the face.



Figure 5. Thick crusting and eroded pustules on a pawpad of a cat involved herein.

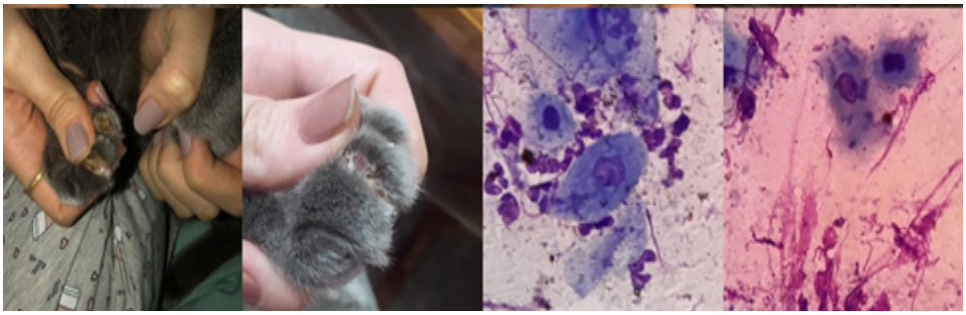


Figure 6. As a part of the diagnostic tree and algorithm adopted in this study, a) and b) paronychia (on the first occasion detective sign) and c) and d) acantholytic cells on microscopy were evident in a cat involved in this study.

k_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__copri							
0.2%	0.0%	0.2%	1.3%	0.0%	0.3%	0.0%	0.0%

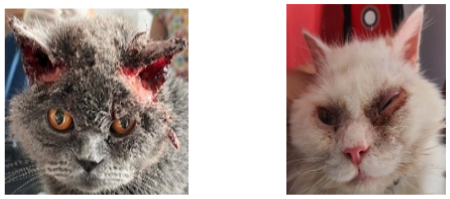


Figure 7. Relative abundance of *Prevotella copri* among cats involved herein.

k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Desulfovibrio;s__piger							
0.3%	0.0%	0.0%	0.0%	2.3%	0.0%	0.0%	0.0%

Figure 8. Relative abundance of *Desulfovibrio piger* among cats involved herein.

k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae;g__Helicobacter;s__cinaedi								
0.2%	0.0%	0.5%	0.2%	0.0%	0.0%	0.0%	0.7%	0.0%

Figure 9. Relative abundance of *Helicobacter cinaedi* among cats involved herein.

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae							
47.4%	2.4%	22.3%	4.7%	25.1%	28.7%	2.3%	5.3%

Figure 10. Relative abundance of *Lachnospiraceae* among cats involved herein.

k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacterales;f__Enterobacteriaceae;g__Escherichia;s__coli							
7.3%	1.3%	0.2%	3.5%	2.1%	6.2%	0.8%	5.3%

Figure 11. Relative abundance of *E. coli* among cats involved herein.

k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Collinsella							
0.0%	0.1%	18.9%	4.4%	16.9%	0.0%	3.1%	1.2%

Figure 12. Relative abundance of *Colinsella* among cats involved herein.

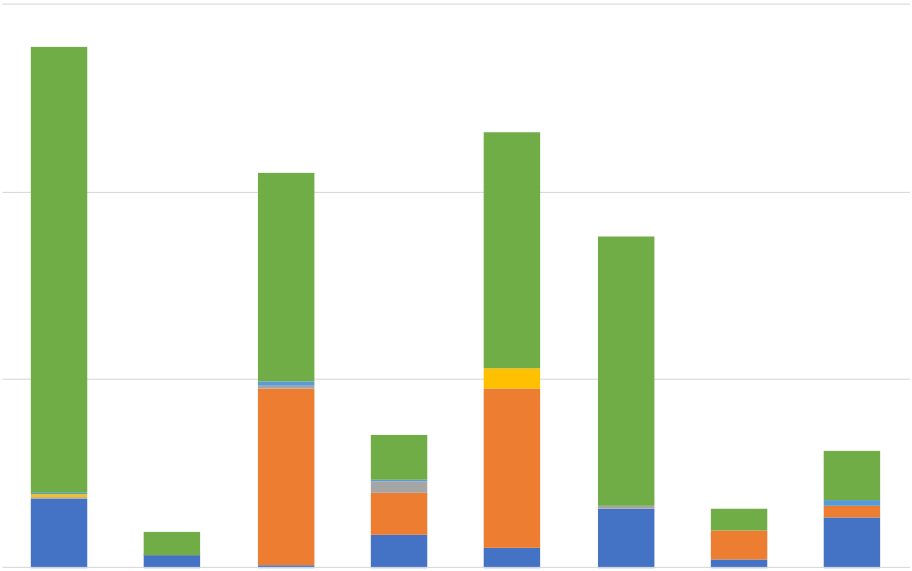


Figure 13. Relative abundances at selected family, genus, and species levels.

Documented clinical findings across the feline subjects were as follows: two exhibited superficial pustules, two others were non-responsive to antibacterial treatment protocols, crust formations were evident in four subjects, and acantholytic cells were detected microscopically in six cases. Figure 4 illustrates a characteristic dermatological manifestation in one feline, presenting with multifocal coalescing erosions and crusts on the facial integument. In Figure 5, we observe substantial crusting and eroded pustules on a paw pad, whereas Figure 6 displays the microscopic visualization of acantholytic cells. Figures 7 through 13 delineate the relative bacterial abundances identified within each case, providing a comprehensive overview of the bacterial profiles encountered in this cohort.

Discussion

As two distinct sides of the diagnostic tree were deemed available in this study (Figure 1 and Table 1), those cats with a presumptive diagnosis of autoimmunity were subjected to ANA titer, which gave positive results. On the other side, classical and mostly adorable clinical signs (Faircloth and Montgomery, 1981; Gabbert, 1983; Scott, 1984; Bennett and Nash, 1988; Pedersen and Barlough, 1991; Day, 1996; Vitale et al., 1997) of systemic lupus erythematosus [i.e., proteinuria and anemia were not evident in any of the cats involved] were not entirely evident. In contrast, cutaneous lesions were evident, mimicking Pemphigus foliaceous on the first occasion, which was then supported by diagnostic tree modality (Table 1 and Figure 12). This diagnostic tree (Figure 1 and Table 1) approach and its consequences (Figures 2 and 3) would have helped veterinary surgeons in field conditions with limited access to diagnostic approaches and laboratory facilities.

Given autoimmune disorders, i) digressive building of autoantibodies, ii) genetic and/or environmental factors [resulted within anomalous existence of autoantibody-producing B cells, autoreactive T cells, and proinflammatory cytokines], iii) hefty shifts among gut microbiome (Xu *et al*., 2019) are all involved within the pathogenesis as usual suspects. Dietary alterations and frequent usage of antibiotics all contribute to the disease state by altering the gut microbiome (Xu *et al*., 2019), of which two factors were evident in all diseased cats herein involved in the present study. Intriguingly, none of the cats were presented to our clinic without previous antibiotic usage. Briefly, all diseased cats were undernourished with high carbohydrate commercial diets (at least % 42; with different brands composed of 42 to 58% carbohydrate) in the pres-

ent study. Taking into account that a rapid and efficacious immune response is demanding, as the body could demolish viral pathogens and combat infection, clinicians must at least understand the relationship between insulin and the immune system. It was suggested that an association had been evidenced among insulin resistance, chronic inflammation, and poor immune response. A group of researchers from Toronto postulated that immune cells, denoted as T cells presented in abdominal fat, exhibited proinflammatory responses, negatively influencing the body's responsiveness to insulin (Tsai *et al*., 2018). As all cats herein involved were fed with high carbohydrates, insulin response might be abolished (although not analyzed), which could have participated in chronic inflammation.

Another interesting study was focused on the hypothesis that the gut microbiota bestow the ordinance of glucose metabolism (in pregnancy). Given the abundance of the genus *Collinsella*, which has been correlated with circulating insulin levels, the factors determining Collinsella's abundance have to be clarified. The latter study aimed to validate the correlation between *Collinsella* and insulin; therefore, the interpretation of whether macronutrient intake alters *Collinsella* abundance. In that study, *Collinsella* abundance positively exhibited a correlation with circulating insulin, whereas it negatively correlated with dietary fiber intake. According to the results obtained, low dietary fiber may hasten Collinsella's abundance (Gomez-Arango *et al*., 2018). As-fed, caloric basis (g/100 kcal) high fiber (3.1) or low-fiber (0.1) were defined in a prior study (Bennett *et al*., 2006). Comparatively, all eight cats involved in this study were fed high carbohydrate-low fiber diets (data not shown due to several different brands), which could all contribute to the overgrowth of *Collinsella* with autoimmune dermatitis in the present study. Relative abundances of *Collinsella (0.0 to 18.9%)* were strikingly high in 5 out of 8 cats involved herein.

Novel research exhibited a probable association between selected Gram-negative anaerobic bacilli and rheumatoid arthritis occurrence in people. The primary periodontal disorder caused by *Porphyromonas gingivalis* (Liao *et al*., 2009; Mikuls *et al*., 2009), followed by and the second is the prevalence of *P. copri* among gut microbiome (Scher *et al*., 2013). In the present study, out of 8 cats involved, 4 of those exhibited increased relative abundances of *P. copri* (Figures 7 and 13).

In the present study, solely *P. copri* (Figures 7 and 13) was evident among diseased cats herein involved. In humans with rheumatoid arthritis, low di-

versity, and gut microbiota exhibited significant participation within disease status (Chen *et al*., 2016; Scher *et al*., 2013), to those of which *P. copri* existed within the pathogenesis. The microbiota analytes presented elevated *P. copri* in new-onset untreated rheumatoid arthritis in comparison to healthy people. *P. copri* is capable of invigorating T-helper 1 cells in the new-onset untreated rheumatoid arthritis group through synthesizing 27 kD protein, consequently influencing the prognosis of the disease. The latter compound is a bracing abundance of specific immunoglobulin A (IgA) and immunoglobulin G (IgG) antibodies (Pianta *et al*., 2017). On the other hand, the latter negative outcome occurred strain-dependently. Moreover, mice with experimentally induced arthritis exhibited *Prevotella histicola*, which impedes the insurrection of arthritis, positively influencing rheumatoid arthritis (Marietta *et al*., 2016).

In the present study, among cats with pemphigus foliaceus *P. copri,* abundance was between 0.2 to 3.2% (Figures 7 and 13). A novel survey published in November 2022 detected the fecal microbial communities, denoting the core microbiome of North American domestic cats. According to the latter study, 30 different bacteria were found in the fecal microbiomes of most cats. The composition of the fecal microbiome depended on the diet of the cat, their age, and whether the cat lived in a private home or a shelter. In that study, 30 core bacterial genera were detected, including *Prevotella*, *Bacteroides*, *Collinsella*, *Blautia*, and *Megasphaera*, with vast majority abundances (Ganz *et al*., 2022). In the present study, 5 out of 8 diseased cats were female; a similar study in mice showed that, among mice, male androgens exhibited impaction on the gut microbiome, leading to diminished immunoreactivity and declined susceptibility against autoimmune diabetes (Gomez-Arango *et al*., 2018). Rheumatoid arthritis is more frequent among females, probably in association with the microbiota (Yurkovetskiy *et al*., 2013).

Given detailed literature corresponding to gut microbiota linked to autoimmunity, MRL/*lpr* mice subjected to mixed antibacterial intervention post-disease onset presented extended lupus-like clinical signs along with elevated *Lactobacillus spp.* and diminished Lachnospiraceae (Mu *et al*., 2017). Moreover, it was suggested that *Lactobacillus* spp. is good while Lachnospiraceae is bad (Mu *et al*., 2017; Christovich and Luo, 2022). In the present study, Lachnospiraceae abundance was significantly high (ranging from 2.3 to 47.4%), indicating the role of this bacteria. The genera of the Lachnospiraceae family, as members of the Firmicutes phylum, are owned by core gut microbiome participate as the vast majority of SCFAs producers (Vacca *et al*., 2020). The human gut has been

detected to exhibit various dominant genera [i.e., the Lachnospiraceae family composed of *Blautia, Roseburia, Lachnospira*, and *L.Ruminococcus]* (Vacca *et al*., 2020) with anti-inflammatory and immunomodulatory efficacy (Abdugheni *et al*., 2022). Arousing interest in the investigation of Lachnospiraceae's participation in the maintenance of intestinal homeostasis is evident. In a prior study composed of 20 humans, systemic lupus erythematosus, diminished Lachnospiraceae, and Ruminococcaceae were detected at the remission stage (Hevia *et al*., 2014). In contrast, in our study, all diseased cats were exhibiting active clinical signs, suggesting not the remission stage but probably the active stage of disease, which could briefly explain the increased relative abundance of Lachnospiraceae for combatting anti-inflammatory efficacy or immune defense in an attempt to overwhelm disease.

In the present study, those cats enrolled with *Pemphigus foliaceus, P. copri*, Lachnospiraceae, *Collinsella*, *Helicobacter cinaedi,* and *Escherichia coli* were exhibited with varying degree of abundances (Figure 7-13). A similar study on systemic Lupus Erythematosus corresponds to elevated intestinal permeability, consequently causing upgraded systemic microbial exposure. Thus, diminishing microbial exposure or modulating barrier function could guide treatment interventions for Systemic Lupus Erythematosus patients (Petri, 2007). Moreover, gut microbiota-focused medical interventions might be beneficial (Li *et al*., 2019). Pronounced dysbiosis of the gut microbiota has been linked to active and remissive Systemic Lupus Erythematosus cases, which may have helped diagnose and predict the activity of the disease. It has been well recognized that systemic lupus erythematosus is associated with dynamic alterations of the gut microbiota in parallel line with disease activity. Dysbiosis in relationship with the latter disease involved the genera *Streptococcus, Campylobacter*, and *Veillonella* in positive correlation with lupus activity (Li *et al*., 2019).

In the present study, the relative abundance of several bacteria was altered, with significant abundances of *E. coli* and Lachnospiraceae (Figure 7-13). In a well-designed research study that attempted to highlight the concept of gut dysbiosis could participate in disease onset in rheumatoid arthritis, DQ8 mice were orally administered with rheumatoid arthritis-associated (*Eggerthella lenta*/*Collinsella aerofaciens*) and non-associated (*Prevotella histicola*/*Bifidobacterium* spp.) bacteria on alternate days for a week in naïve mice. The rheumatoid arthritis-associated bacteria elevated intestinal permeability, whereas rheumatoid arthritis non-associated bacteria surmount other relevant rheu-

matoid arthritis-associated bacteria even if gavaged/cultured together. To those mice administered with rheumatoid arthritis, non-associated bacteria exhibited lower levels of proinflammatory cytokines, suggesting that intestinal commensal bacteria impact immune responses by altering gut permeability and immunity. Dysbiosis might have helped the growth of rheumatoid arthritis-associated bacteria along with diminished beneficial bacteria (Balakrishnan *et al*., 2019).

In the present study, the relative abundance of *E. coli* was significantly increased (ranging from 0.2 to 7.3%), probably causing increased permeability among autoimmune diseased cats herein enrolled. Given obesity modality among rodents, *E. coli* elevated intestinal permeability and circulating levels of bacterial lipopolysaccharide, which could all contribute to systemic low-grade inflammation (Cani *et al*., 2008). Increased abundance of *E. coli* was also determined among humans with type 2 diabetes mellitus (Qin *et al*., 2012).

Conclusions

This study presents the first reported findings on gut microbiota alterations in cats with pemphigus foliaceus within Türkiye. These preliminary observations offer a foundational understanding that may guide future research endeavors. The implications of these alterations in the gut microbiota could potentially inform the development of novel treatment modalities. Further studies are warranted to explore the therapeutic potential and to elucidate the underlying mechanisms that could contribute to the advancement of clinical management strategies for this condition.

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Isolation and identification of *Brucella abortus* and *B. melitensis* in ruminants with a history of abortion: the first report from Eritrea

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Abstract

Brucella species have never been isolated or documented in Eritrea. The present study was initiated to isolate and identify *Brucella* species that infect livestock in Eritrea. A total of 316 blood sera, 137 milk, 71 vaginal swabs, a placental cotyledon, and a fetal stomach content were sampled from 208 goat, 102 sheep, and 6 cattle. Series testing protocol starting with Rose Bengal plate test, followed by c-ELISA, culturing, and then multiplex AMOS-PCR was followed. The purpose-sampling strategy was applied, and animals with a history of abortion in the last three weeks were sampled to increase the probability of capturing *Brucella* species. Isolation was conducted on *Burcella* media agar, and *Brucella* broth was added with *Brucella* selective supplement containing 2,500IU polymyxin B, 12,500IU bacitracin, 50.0 mg cycloheximide, 2.5mg nalidixic acid, 50,000 IU nystatin, and 10.0 mg vancomycin. Accordingly, 41 animals were positive for a series of serological tests. The overall *Brucella* seropositive detection proportion was 13.0%, and goats constituted the majority 78% (n=32). The highest number of positive samples were from the *Maekel* region. Six *Brucella* organisms were successfully isolated from two milk samples, two vaginal swabs, one placental cotyledon, and one fetal stomach content obtained from seropositive animals. Among the *Brucella* isolates, five were *B. melitensis,* while the remaining one was *B. abortus* as per the multiplex AMOS- PCR assay. The present study documented the first *B. abortus* and *B. melitensis* isolates in Eritrea. Milk, vaginal discharges, placental cotyledon, and aborted fetus were identified as sources of *Brucella* for livestock and livestock keepers. Besides, the findings highlight a reasonable proportion of the reproductive disorders in cattle and small ruminants could be due to

brucellosis. This calls for public sector intervention to control the diseases in animals and to enhance community awareness of good practices such as drinking boiled milk and avoiding contact with infected tissues and body discharges to prevent potential public health risks from zoonosis.

Keywords: *Brucella;* Cattle; Eritrea; Goat; Sheep.

Introduction

Brucellosis is a reemerging but neglected zoonotic disease of the tropics (Lopez-Goni *et al.*, 2008; Ghanbari *et al.*, 2020). A global threat, brucellosis, affects both domestic and wild animals, inflicting significant economic damage on livestock production (Godfroid *et al.*, 2013). This bacterial disease inflicts disorder on the reproductive health of animals, leading to infertility, delayed breeding cycles, lost calves, decreased meat and milk yields, and forced culling (Pappas *et al.*, 2005; Gonzalez-Espinoza *et al.*, 2021). Unfortunately, humans are not spared either. Brucellosis in humans, almost invariably linked to infected animals or their products, poses a particular risk to those in close contact, including farmers, animal handlers, slaughterhouse workers, and veterinarians (Zinsstag *et al.*, 2005; Godfroid *et al.*, 2013; Ghanbari *et al.*, 2020).

The genus *Brucella* is responsible for causing brucellosis, with *B. melitensis*, *B. abortus*, and *B. suis* being the most significant species in public health as they pose a high risk of human infection (Alton and Forsyth, 1996). *Brucella* spp. are gram-negative, nonmotile, non-spore-forming, slow-growing, facultative intracellular bacteria. Laboratory diagnosis is based on direct diagnosis by culture, indirect diagnosis by serological tests, and direct rapid diagnosis by molecular methods (Di Bonaventura *et al.*, 2021). Unequivocal diagnosis requires the isolation of the organism; therefore, culture is considered the 'gold standard' in laboratory diagnosis of brucellosis due to its clinical and epidemiological relevance (Ghanbari *et al.*, 2020; Di Bonaventura *et al.*, 2021). Serological tests, although they lack specificity and provide results that may be difficult to interpret in individuals repeatedly exposed to *Brucella* organisms, nevertheless remain a diagnostic cornerstone in resource-poor countries (Yagupsky *et al.*, 2019). *Brucella* DNA detection by polymerase chain reaction (PCR) is a more valid and definitive diagnostic method, and PCR is performed by amplification of specific genomic sequences *in vitro* (Lopez-Goni *et al.*, 2008; Wang *et al.*, 2014).

Brucella species are very difficult to grow, and the process is tedious, time-consuming, requires expensive biosafety facilities, and poses significant risks to the operator and environment (Godfroid *et al.*, 2010; Mathew *et al.*, 2015). Identification of members of the *Brucella* genus at the species level is essential for epidemiological reasons due to the strong association between the individual species and their naturally occurring hosts (Al Dahouk *et al.*, 2010). Moreover, *Brucella* species isolation and identification in a specific area or country is of great importance, as the findings could help the concerned authorities, decision-makers, and experts to design and implement appropriate prevention and control strategies, particularly for potential vaccine design based on the circulating *Brucella* species and strains (Refai, 2002; Zinsstag *et al.*, 2005; Kurmanov *et al.*, 2022).

Brucellosis is endemic to Eritrea (Efrem *et al.*, 2023). The presence of *Brucella* in Eritrea was first reported in 1943 (Cilli and Andolfato, 1943), and the pathogen persists both in livestock and human populations (Efrem *et al.*, 2023; Efrem *et al.*, 2022; Scacchia *et al.*, 2013; Omer *et al.*, 2002; Omer *et al.*, 2000;). All *Brucella* reports in Eritrea were based on indirect evidence generated through serology, except the study (Bereket *et al.*, 2021), which provided genetic evidence based on the PCR method. The present study was initiated to isolate and identify *Brucella* species that infect ruminants in the administrative regions of Maekel, Debub, Anseba, and the Northern Red Sea (NRS) of Eritrea.

Material and methods

Study area

The present study was conducted in four administrative regions of Eritrea, namely, Maekel, Debub, Anseba, and the Northern Red Sea (Figure 1). Eritrea is found in the Horn of Africa, bordering Ethiopia, Sudan, Djibouti, and the Red Sea. Eritrea is located between latitudes 12 ° 42' N to 18° 2' N and longitudes 36° 30' E to 43° 20' E. The country is divided into six administrative regions, namely Anseba, Debub, Gash-Barka, Maekel, Northern Red Sea (NRS), and Southern Red Sea (SRS). The four administrative regions were selected purposively due to the high population of livestock and history of brucellosis (Omer *et al.*, 2000; Omer *et al.*, 2002; Efrem *et al.*, 2022; Efrem *et al.*, 2023). The total estimated number of livestock species is approximately 3.15 million goats, 2.14 million sheep, 1.70 million local cattle, 0.22 million dairy cattle, 0.29 million donkeys, 0.19 million camels, and 0.002 million swine (MOA, 2019).

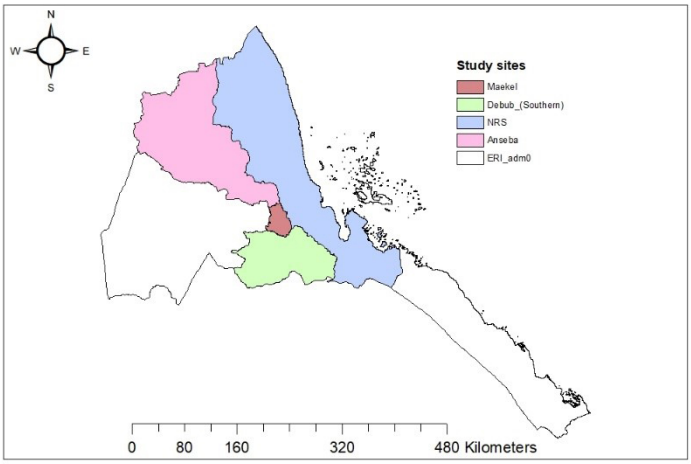


Figure 1. Map of Eritrea showing the study area

Study design and study population

The study was a case series study looking for livestock species with a recent history of reproductive disorder, *i.e.,* abortion/retained fetal membrane. Serological screening tests and bacteriological and molecular methods were deployed for the screening, isolation, and identification of *Brucella* species from the study population. The study population includes cattle, sheep, and goats with a history of reproductive disorder in intensive/semi-intensive and extensive farms of Maekel, Debub, Anseba, and NRS administrative region of Eritrea.

Sample size determination and sampling techniques

To specifically focus on animals potentially infected with *Bucella*, a purposesampling strategy was employed. During farm visits, livestock owners identified animals that had experienced late-term abortion or retained fetal membranes within the past three weeks. The animals were then recruited for specimen collection. Taking into account an expected prevalence of 30% based on Rose Bengal Test (RBT) data (Alsaad, 2022), the required sample size to detect *Brucella*-positive animals with a history of abortion was calculated using Epi InfoTM version 7.2.6 (CDC, USA). When a design effect of 1% was taken into account, a 5% error margin, and a 95% confidence level, the minimum sample size was determined to be 323; however, only 316 animals were

sampled for logistic reasons. In addition to serum samples, biological samples, including milk, vaginal swabs, placenta cotyledon, and abomasa content of aborted fetuses, were collected from 208 goats, 102 sheep, and 6 cattle. Ultimately, the collection consisted of 316 blood serum, 137 milk samples, 71 vaginal swabs, one placental cotyledon, and one fetal stomach content.

Sample collection and processing

For laboratory analysis, a 5 ml blood sample was collected in plain vacutainers. Serum separation was then performed using centrifugation. The resulting clear serum was harvested into cryo-tubes. The remaining whole blood and clear sera were stored at -20 °C until analysis. A pooled 10 ml of milk from the four quarters, a vaginal swab in 2 ml of PBS, placental cotyledon in 50 ml of sterile PBS container, and the content of fetal stomach (2 ml) were collected. All samples were adequately labeled with animal ID, sample type, and collection date. They were then transported in an ice box stored at + 4 °C until processed at the National Animal and Plant Health Laboratory (NAPHL) at the Ministry of Agriculture in Asmara.

Serological test

All sera samples were tested for *Brucella* using the RBPT kit (Animal Health and Veterinary Laboratory Agency; Surry, UK). The RBPT test was carried out following a standard protocol (Diaz *et al.*, 2011; Legesse *et al.*, 2023). In summary, 30µl of serum was mixed with an equal volume of antigen on a white tile that produced a zone of 2 cm in diameter. The mixture was gently agitated for 4 minutes at room temperature and then observed for agglutination. Consequently, serum with any visible agglutination was considered positive for *Brucella* antibodies and further confirmed by ELISA.

Blood sera screened as positive by RBPT were further tested using c-ELISA for confirmation following the protocol supplied with the reagent kit (INGNASA: Adeva Dela Institucion Libre de Ensenanza; Madrid, Spain), using a plate reader machine (Thermo, Multiscan Ascent). Each test result was validated as follows: (i) If the OD in the negative control wells (NC) was greater than 1 and the OD in the positive control wells (PC) was less than 0.35, then the result was valid for calculation. (ii) The percentage of inhibition (PI) of each sample was calculated as PI=100 × [1-(OD sample/OD negative control)]. (iii) Sera with PI≥ 40% were referred to as positive for *Brucella* antibody, while

sera samples with PI <40% were recorded as negative results. The sensitivity and specificity of the c-ELISA test were 95.2% and 99.7%, respectively.

Bacteriological procedures

Serologically positive blood samples and connected specimens were subjected to bacteriological procedures. Samples were processed in the BSC Class II Safety Cabinet (Telstar Bio-II-A) with strict application of personal protection equipment (PPE) for laboratory personnel biosafety. During this operation, access to the entire facility was restricted except for investigators—*Brucella* spp. Isolation and preliminary identification using biochemical tests were carried out as previously described in the WOAH manual (WOAH, 2018) and NAPHL standard operating protocol. The brief description of the bacteriological protocol is as follows:

Media preparation: *Brucella* isolation was performed using *Brucella* selective growth media. This includes *Brucella* media agar (CM 0169, Oxoid, England) and *Brucella* broth added with *Brucella* selective supplement **(**SR0083A, Oxoid, England**)** containing 2,500IU polymyxin B, 12,500IU bacitracin, 50.0 mg cycloheximide, 2.5 mg nalidixic acid, 50,000 IU nystatin, and 10.0 mg vancomycin, to inhibit other contaminates and enhance the growth of *Brucella* species. The medium was further enriched with filter-sterilized heat-inactivated (56 °C /30 min) 5% horse serum plus 0.5% dextrose and 0.5% methanol.

Sample preparation and inoculation**:** Milk samples were centrifuged at 2000 g rpm for 15 minutes at 20 °C to obtain the sediment pellet at the bottom and the supernatant cream at the top. After sucking and removing the middle layer, the fatty layer and sediment were mixed using a sterile swab. Using the same swab, two *Brucella* agar plates were inoculated, and the remaining material was inoculated in 10 ml of *Brucella* broth. The vaginal swab was directly streaked on the prepared solid media (CM 0169, Oxoid, England) and inoculated into *Brucella* broth with supplement **(***SR0083A,* Oxoid, England**)**, similar to that of the milk sample. The surface of the cotyledon samples was flamed with a spatula, and the internal parts were cut into small pieces and homogenized using sterile mortar and pestle with little PBS. Then, from the homogenized sample, two drops were placed on each *Brucella* agar plate and spread using the streaking method, along with 1 ml to 10 ml of *Brucella* broth. Similarly, two drops and 1 ml of the fetal stomach content were inoculated into a *Brucella* agar plate and *Brucella* broth, respectively.

Incubation: *Brucella* media inoculated agar plates and broth bottles were incubated at 37 ^{0}C in the presence and absence of 5-10% CO_2 and checked after 48 hours, then daily for up to 7 days for both primary and consecutive subcultures. After seven days, plates and broth with no signs of growth were discarded. However, broths with signs of growth were cultured in agar plates and followed the same procedure as above to the end.

Brucella isolation: *Colonies:* Colonies with translucent, smooth margins and a light yellow color were suspected to be *Brucella* species. A typical colony was picked and examined using Gram's stain and modified Ziehl-Neelsen stain (MZN) methods. If gram-negative coccobacillus and red coccobacillus on a blue background in MZN staining were observed, then the suspected colony was further sub-cultured and examined.

Biochemical test: The grown subcultures were subjected to different phenotypic tests, including catalase, oxidase, urea hydrolysis, motility, growth in dyes (on 20 and 40 µg /ml of basic fuchsine and thionin), CO_2 requirement, H_2S production, and hemolysis tests were performed following standard protocols for each test. Plates showing positive growth for *Brucella* organism were sub-cultured and, after 48 hours, sent to the molecular biology laboratory for species-level identification.

Molecular protocols

DNA extraction: The DNA genome was extracted from heat-inactivated pure *Brucella* culture using the High Pure template preparation kit (DNA HP kit, Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions.

Brucella AMOS-PCR Assay: *Brucella* species identification was performed using the *Brucella* AMOS-PCR kit as per a published protocol (Bricker and Halling, 1994). The specific primers of *B. abortus, B. melitensis, B. ovis,* and *B. suis* are listed in Table 1.

Table 1. Primers used for the identification of *Brucella* species

PCR type: AMOS	Primer type	Sequence (5′ to 3′)	Target gene	Product length (bp)
	Forward	GAC GAA CGG AAT TTT TCC AAT CCC	IS711	498
B. abortus	Reverse	TGC CGATCA CTT AAG GGC CTT CAT		
B. melitensis	Forward	AAATCG CGT CCT TGC TGG TCT GA	IS711	731
	Reverse	TGC CGATCA CTT AAG GGC CTT CAT		
	Forward	CGG GTT CTG GCA CCATCG TCG	IS711	976
B. ovis	Reverse	TGC CGATCA CTT AAG GGC CTT CAT		
	Forward	GCG CGG TTT TCT GAA GGT TCA GG	IS711	285
B. suis	Reverse	TGC CGATCA CTT AAG GGC CTT CAT		

DNA amplification was performed using a conventional PCR machine (Eppendorf: Master cycler ingredient). The amplification cycles consisted of initial denaturation at 95 ◦C/5 min, followed by 35 cycles of denaturation at 95 ◦C /60 s, annealing at 58 ◦C for 2 min and then elongation at 72 ◦C for 2 min, and a final elongation step at 72 ◦C for 7min. PCR products were separated by gel electrophoresis using 1.5% agarose gels stained with ethidium bromide. Gel images were captured using UV light in a Gel-Imaging system (BIO-RADTM, USA) at 100 V for 60 min. A 100bp reference DNA marker ladder (BioLabsTM, New England) was used to determine the amplified band size corresponding to each Brucella spp. Furthermore, known strains of B. melitensis and water were used as positive and negative controls, respectively.

Data management and analysis

Laboratory results and related information were recorded in the Microsoft Excel 2016 worksheet. Descriptive statistics were used to report the proportion detected from various specimens. A proportion is a division of the total number of animals or samples determined as positive over the total number of animals or samples examined in a given diagnostic procedure multiplied by 100.

Ethical consideration

The study was approved ethically and authorized by the Ministry of Agriculture of the state of Eritrea. Informed consent was obtained from the herders after the necessary explanation of the study objectives provided by the research team. Participation was voluntary, and participants were informed to withdraw at any time if they decided to do so. Any information acquired from the

participants was kept strictly confidential. Trained animal health technicians and veterinary professionals collected samples.

Results

Serological analysis

Antibodies specific to the *Brucella* genus were detected in 45 (14.2 %) sera samples in RBPT, and 41 (13%) were confirmed positive on consecutive c-ELISA. The positive sera were from 32 of 208 goats (15.4%), 7 of 102 sheep (6.9%), and 2 of 6 cattle (33%). A relatively larger number of positive samples originated from Maekel (17.3%), followed by Debub (9.3 %) administrative regions. The detailed serological results are presented in Table 2.

Table 2. Serological analysis of blood samples from ruminants with a recent history of abortion

Region sampled	No . of seropositive/ total animal tested (%)			
	Goat	Sheep	Cattle	Sub-total
Maekel	25/118 (21.2)	6/62 (9.7)	0/2 (0)	31/182 (17.0)
Debub	7/70 (10.0)	1/35 (2.9)	2/4 (50.0)	10/108 (9.3)
Anseba	0/14 (0)	0/4 (0)	0	0/18 (0)
NRS	0/6(0)	0/2	0	0/8 (0)
Total	32/208 (15.4)	7/102 (6.9)	2/6(33.3)	41/316 (13)

NRS: North Red Sea

Detection and identification of *Brucella* spp.

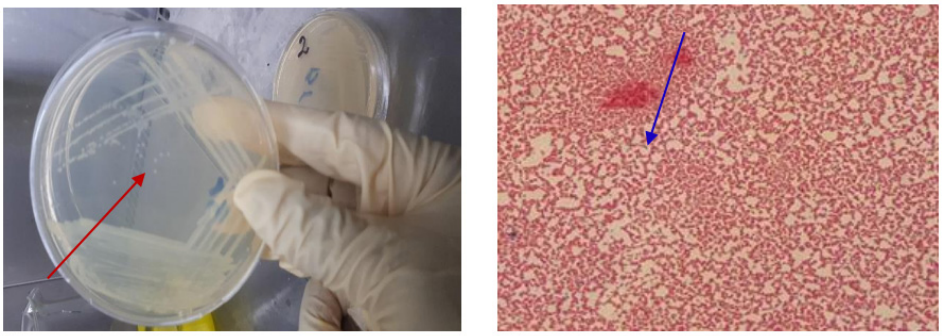
Brucella suspected 253 samples were cultured in a selective medium, and six of the samples produced colonies that resemble *Brucella* spp (Figure 2). Therefore, the overall isolation rate of *Brucella* spp was 2.4%. The sources of positive samples include milk (1.5%), vaginal swabs (2.8%), a placental cotyledon, and one fetal stomach content (Table 3). All culture-positive samples were obtained from seropositive animals. However, none of the blood samples that were seropositive on c-ELISA (n =41) produced *Brucella* colonies. Furthermore, no samples from seronegative animals produced colonies. All samples of sheep origin were negative on the bacteriological protocol.

Table 3. Number and types of samples that produced *Brucella* colonies in culture

Sample source and type Goat	No. Positive samples/No. tested			
	Sheep	Cattle	Sub-total (%)	
Blood	0/32	0/7	0/2	0/41
Milk	2/110	0/25	0/2	2/137 (1.5)
Vaginal swab	2/47	0/24	0/2	2/73 (2.7)
Placental cotyledon	-	-	1/1	1/1
Fetal stomach content	-	-	1/1	1/1
Total (% positive)	**6/253 (2.4)**			

PCR-based *Brucella* species identification

Six *Brucella* spp colonies isolated and identified through biochemical tests were further processed for species-level identification using the published AMOS-PCR protocol (Bricker and Halling, 1994). Consequently, five of the six *Brucella* isolates were identified as *B. melitensis,* and the other isolate was *B. abortus*. Figure 3 depicts the PCR result for the *Brucella* species identification protocol.



(a) (b)

Figure 2. *Brucella* species isolated in Eritrea, 2023*:* (a) *B. melitensis* culture colony grown from goat vaginal swab sampled from a village (Beleza); (b) Gram-negative stained *B. abortus* from cow fetal stomach content sampled from a farm in Dekemhare.

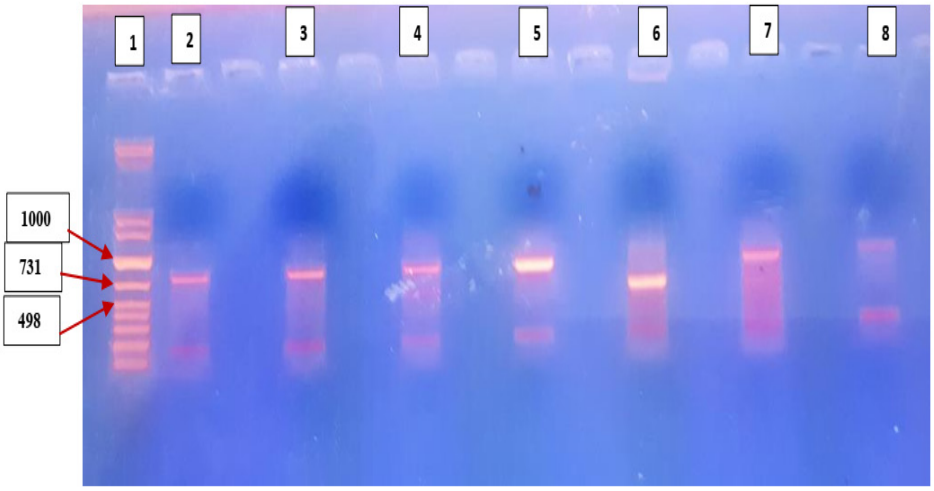


Figure 3. Agarose gel pictures show *Brucella* species-specific bands amplified using AMOS-PCR techniques. Lane 1: DNA marker (100bp); lane 2: *B. melitensis* reference strain 16M; lane 3,4,5,7 and 8 show the DNA band specific (731 bp) for *B. melitensis;* lane 6 showed the DNA band specific (498 bp) for *B. abortus*

Discussion

The present study detected 41 seropositive animals in a series of tests with RBPT and c-ELISA from the administrative regions of Maekel and Debub in Eritrea. The detection rate of *Brucella* in animals with a recent history of abortion was 13%, a relatively higher finding than the 5.6%, 4.3%, 1.4%, and 3.1% rate of detection in local cattle, goats, sheep, and camels, respectively (Omer *et al.*, 2000). A decade ago, a 2.8% seropositive report was made on dairy cattle that had a history of abortion in five regions of Eritrea (Scacchia *et al.,* 2013*).* Our previous study also detected a relatively lower proportion (1.1%) of dairy cattle in the Maekel and Debub regions of Eritrea (Efrem *et al.*, 2023). The disagreement with previous studies could be associated with differences in sampling strategy, or the disease might have advanced. In the present study, only animals with a history of late abortion were sampled to increase the probability of detecting *Brucella*-infected animals. On the other hand, in the previous studies, blood sera were collected from all animals; in most cases, random sampling was employed. Researchers who targeted *Brucella*-suspected goats in the Maekel region reported a comparable result (14.60%) to the present study (Bereket *et al.*, 2021).

A large number of animals (n = 275) with a recent history of late abortion were negative on serology. A possible explanation for this could be the history of abortion recorded in the data set was not necessarily caused by *Brucella* infection, other factors, and animal diseases that can induce abortion at different stages of the gestation period. For example, infectious agents (*Coxiella burnetii*, *Leptospira* spp, *Listeria monocytogenes,* viruses, protozoa like *Neospora caninum* and fungus); noninfectious agents (physical trauma, stress, toxemia, metabolic disorder, hormonal effect, nutritional deficiencies, hereditary factors, housing conditions, and other physical factors) can induce abortion in animals (Beuzón *et al.*, 1997; Vidic *et al.*, 2007; Clothier and Anderson, 2016). Bacteriological cultures (n =253) were carried out for both samples that came from seropositive and seronegative animals. However, *Brucella* colonies were isolated from only six seropositive animals. The remaining 35 seropositive animals did not form colonies in *Brucella* selective media. The detection rate differences observed between the serological test and culture methods could be caused by the cross-reactivity of antibodies against the *Brucella* lipopolysaccharide virulence factor, which is commonly used for the development of *Brucella* ELISA kit (Golchin *et al.*, 2023). Gram-negative bacteria such as *Escherichia coli* O157, *Vibrio cholera*, and *Yersinia enterocolitica* have been reported to result in cross-reactivity (Golchin *et al.*, 2022). Blood cultures from seropositive animals also did not yield *Brucella* colonies. Similar challenges of isolating *Brucella* from blood were reported elsewhere (Pappas and Papadimitriou, 2007; Patel *et al.*, 2017). Failure of isolation may be due to a low number of viable organisms (Warethc *et al.*, 2014), localization of *Brucella* in predilection tissues such as lymph nodes and uterus as an intracellular organism (Pappas *et al.*, 2005), or it could be associated with the intermittent availability of *Brucella* in peripheral blood (Yagupsky, 2015). Technical limitations could be an additional cause of the low isolation rate of *Brucella* from biological specimens in the present study.

Meanwhile, the highest number of isolates was recorded from goat species. Consequently, the highest number of *Brucella* seropositivity (n = 32) and the number of recovered *Brucella* isolates (n = 4) was observed in goats. Most likely, this was associated with the most significant number of goats accounting for the higher proportion of livestock population in the country. Additionally, goats are considered to be more susceptible to brucellosis (Rajala *et al.*, 2016). The detection of *Brucella* in goat milk has been reported elsewhere (Lonkar *et al.*, 2023). The presence of *Brucella* in milk is very concerning as it could

increase the risk of transmission to humans. In a previous community perceptions and practices study conducted on livestock owners in the NRS region, 90.3% (n = 575) of the participants reported that they handled and disposed of birth materials barehanded, which is a high-risk practice for brucellosis infection (Efrem *et al.*, 2022). Therefore, goat herders who assist in parturition without PPE and drink raw milk and dairy products should be aware of this risk and avoid such risky practices.

Five of the six *Brucella* isolates were identified as *B. melitensis* and one *B. abortus* using the AMOS-PCR method. The current result is in agreement with the recent finding in the same study area (Bereket *et al.*, 2021), who detected *B. melitensis* DNA from a vaginal swab of goats with a history of abortion using the duplex PCR method. It is important to note that in the current study, *B. melitensis* was isolated from the placental cotyledon of cattle. In most cases, such cross-infection to a nonspecific host occurred when different animal species were mixed and herded together. In Eritrea, especially in the highlands, rearing different animal species (cattle, sheep, goat, danker, dog) in the same herd/flock was a common practice. In addition, due to limited land area, different livestock species were housed together in a small room or yard, creating favorable conditions for the cross-transmission of *Brucella* species among the various animal species. Herding mixed populations of cattle, sheep, and goats is the main factors that contribute to the emergence and cross-infection of *B. melitensis* and *B. abortus* to nonspecific hosts, as explained elsewhere (Hegazy *et al.*, 2011). It is well documented that *B. melitensis* is the most virulent of all *Brucella* species for humans and animals, followed by *B. abortus* and *B. suis* (Alton and Forsyth, 1996; Mahmoud and Hamdy, 2018). However, the study did not provide molecular evidence for cross-infection to nonspecific hosts. Additionally, the limitation of the present study includes the use of a purposive sampling protocol that limits the extrapolation of the result to the epidemiology of brucellosis in Eritrea.

Conclusions

In this study, both *B. abortus* and *B. melitensis* were isolated and identified successfully for the first time in the country. *B. melitensis* was the most dominant *Brucella* species infecting goats and cattle. Milk, vaginal discharges, placental cotyledons, and fetal stomach content were confirmed as a source of *Brucella* isolates. National initiatives are recommended to commence control interventions in animals, strengthen community awareness, and promote good

practices, such as drinking boiled milk and proper disposal of aborted fetuses and the placenta. Further molecular epidemiological studies on the isolates to understand the cross infections and relatedness of Eritrean isolates with the global database are commendable.

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Data availability

Data are available upon request from Efrem G. (yafet1212@gmail.com)

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Short communication

Identification of major protozoal enteropathogens causing calf diarrhea in dairy farms in and around Holeta Town, Oromia Special Zone, Ethiopia

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Abstract

Calf diarrhea can be caused by a variety of pathogens, including bacteria, viruses, protozoa, and intestinal parasites. *Giardia*, *Eimeria*, and *Cryptosporidium* are the most significant protozoan parasites and are all individually and collectively infectious. A case-series study was carried out in and around Holeta dairy farms, Oromia Special Zone, Ethiopia, from November 2017 to April 2018 to identify the main protozoan enteropathogens from diarrheic calves. Samples were purposely collected from three dairy farms: Serkalem Dairy Farm (SDF), Holeta Agricultural Research Centre (HARC), and Ada Berga Agricultural Research Centre (ABARC). A total of 93 fecal samples were taken from diarrheic calves of up to 4 months of age. Protozoan enteropathogens were identified using flotation and modified Ziehl Neelsen staining methods. *Cryptosporidium*, *Eimeria*, and *Giardia* were detected in 44 (47.3%), 50 (53.8%), and 34 (36.6%) of the diarrheic fecal samples examined, respectively. The findings indicated that there were 17 (18.27%) cases of *Cryptosporidium*, *Eimeria*, and *Giardia* as mixed infections, 16 (17.2%) cases of *Cryptosporidium* and *Eimeria*, 5 (5.4%) cases of *Cryptosporidium* and *Giardia*, and 4 (4.3%) cases of *Eimeria* and *Giardia*, compared to 13 (13.97%), 6 (6.45%), and 5 (5.4%) cases of *Cryptosporidium*, *Eimeria* and *Giardia* as single infections, respectively. The selected farms had significant prevalence levels of three common protozoa: *Eimeria*, *Giardia*, and *Cryptosporidium*. Further molecular research is required to identify the species and genotype levels of protozoal enteropathogens and related risk factors.

Keywords: Calf diarrhea; Protozoal Enteropathogens; Holeta; Dairy farms.

Introduction

Neonatal calf diarrhea is defined as a clinical syndrome with a complex multifactorial etiology that causes enormous economic losses to dairy producers (Atwa *et al*., 2012). *Cryptosporidium*, *Eimeria*, and *Giardia* are common protozoal parasites that cause calf diarrhea. They are among the most important intestinal pathogens of domestic and wild animals worldwide, contributing to significant morbidity and mortality in calves (Savioli *et al*., 2006). In addition, *Cryptosporidium* and *Giardia* are probably the most common protozoal agents of human gastrointestinal diseases worldwide, leading to a significant health burden in both the developing and developed worlds (Cacciò *et al*., 2005). In Ethiopia, *Eimeria* is among the most common diarrhea-causing protozoan enteropathogens in calves and causes severe calf morbidity and mortality (Eyuel, 2016).

Cryptosporidium is another important protozoan parasite that causes morbidity in neonatal calves. Cattle are commonly infected by *Cryptosporidium parvum*, *C. bovis, C. ryanae,* and *C. andersoni*. The most important *Cryptosporidium* species causing economic losses is *C. parvum* (Chalmers *et al*., 2011). It is an obligate intracellular parasite that affects epithelial cells covering the luminal surfaces of the respiratory and digestive systems of a wide range of hosts. It frequently causes diarrhea in newborn calves (Coklin *et al*., 2007).

Giardia is one of the most significant zoonotic protozoan parasites that affect a variety of domestic animals and humans globally (Cacciò *et al*., 2005). It is commonly found alone or in combination with other pathogens as a cause of calf diarrhea, which can have economic significance.

Calf mortality represents an irrefutable and irrevocable financial source of economic loss. Thus, identification of the factors that can alter a calf's risk of death is an essential prerequisite for avoiding excessive calf mortality (Wudu *et al*., 2008). Understanding the significant causes and predisposing factors implicated in calf diarrhea (Lorino *et al*., 2005) is crucial for developing preventative strategies and reducing losses during the first few months of life. This understanding will ultimately enhance productivity per livestock unit. Protozoal enteropathogens have not been studied in dairy farms in and around Holeta, although major enteropathogens pose significant risks to dairy farms.

This study aimed to identify the major protozoal enteropathogens in diarrheic calves in dairy farms in and around Holeta town.

Materials and methods

Study area

The study was conducted in dairy farms in and around Holeta, Oromia Special Zone, Ethiopia. Holeta is located 45 km west of Addis Ababa, at 09°02' N latitude and 38°34 E longitude. The climatic condition of the area is predominantly temperate, and it receives a mean annual rainfall ranging from 84.5 mm to 89.7 mm. The altitude is 2400 m.a.s.l. The annual temperature ranges between 18°C and 24°C (Tiki and Addis, 2011).

Study population

Animals that were included in this study were crossbred dairy calves of both sexes and up to 4 months of age that were clinically affected with diarrhea, exhibited signs of systemic disease, and defecated pasty-watery feces. The ages of diarrheal calves were divided into three age groups: <4 weeks, 5-8 weeks, and greater than nine weeks (Lee *et al*., 2007) by taking into account the age group of the calves housed on farms. The breed of animals found in the farms was a crossbreed of 50% (50% Holstein Friesian and 50% Borana) and 75% (75% Holstein Friesian and 25% Borana).

Study design, sampling technique, and sample size determination

A case-series study was conducted on three dairy farms. The farms (SDF, HARC, and ABARC) and calves were selected purposively based on the availability of clinical cases and the willingness of the farm owners. The total number of calves on the three selected farms was 396, and during this study period, 93 available diarrheic cases were selected.

Sample collection and examination

Sample collection from the study animals was performed according to the methods described by Quinn *et al*. (1999). Fecal samples were collected directly from the rectum of untreated diarrheic calves, preferably soon after the onset of diarrhea, by using sterile wide-mouth screw-capped bottles for parasitological

examination. The sample bottles were labeled with calf identification. Then, samples were transported in an icebox with an ice pack to the Parasitology Laboratory of the National Biotechnology Institute in Holeta and processed within 24 hours. Laboratory analyses of the fecal samples were conducted using a flotation technique. For each sample, 5 ml of feces was processed using sugar solution as the flotation medium to recover *Cryptosporidium* oocysts, *Eimeria* oocysts, and *Giardia* cysts. In addition, the modified Ziehl-Neelsen staining technique was conducted for *Cryptosporidium* oocysts. The sample was considered positive for the respective parasites when *Giardia* cysts, *Cryptosporidium* oocysts, and *Eimeria* oocysts were detected in the specimen.

Data management

The collected data were edited, filtered, coded, and entered into Microsoft Excel® 2007. The data were then exported to SPSS software version 20.0 for appropriate statistical analysis. The proportion of each enteropathogen in the total diarrheic calves was determined by using descriptive statistics.

Results

Cryptosporidium, *Eimeria*, and *Giardia* were found in 44 (47.3%), 50 (53.8%), and 34 (36.6%) of the 93 diarrheic faecal samples that were investigated, respectively. Mixed enteropathogens were found in 18.27% of the cases for *Cryptosporidium*, *Eimeria*, and *Giardia*, 17.2% for *Cryptosporidium* and *Eimeria*, 5.4% for *Cryptosporidium* and *Giardia*, and 4.3% for *Eimeria* and *Giardia*. *Eimeria*, *Cryptosporidium*, and *Giardia* infection rates for single cases were 13.57%, 6.45%, and 5.4%, respectively. Age categories less than four weeks had a higher prevalence than other age groups, and crossbreeds of 75% level were more infected with *Cryptosporidium* infection than crossbreeds of 50% (Table 1).

Table 1. Prevalence of *Cryptosporidium* detection with different variables

Variable	Categories	No of Cases	Positive isolate (%)
Age (weeks)	<4	42	28 (66.7)
	5-8	29	8 (27.6)
	>9	22	8 (36.4)
Breed (cross)	50%	46	13 (28.3)
	75%	47	31 (66.0)
Sex	Male	45	19 (42.2)
	Female	48	25 (52.1)
Birth weight (kg)	16-24	37	20 (54.1)
	25-33	41	16 (39.0)
	34-42	15	8 (53.3)

Calves with birth weight between 16–24 kg and <4 weeks old had a higher proportion of *Eimeria* infection than other birth weight and age groups, respectively (Table 2).

Table 2. Prevalence of *Eimeria* detection with different variables

Variable	Categories	No of Cases	Positive isolate (%)
Age (weeks)	<4	42	30 (71.4)
	5-8	29	10 (34.5)
	> 9	22	10 (45.5)
Breed(cross)	50%	46	20 (43.5)
	75%	47	30 (63.8)
Sex	Male	45	25 (55.6)
	female	48	25 (52.2)
Birth weight	16-24	37	24 (64.9)
	25-33	41	19 (46.3)
	34-42	15	7 (46.7)

When compared to males and other age groups, female calves and calves less than four weeks old had a greater prevalence of *Giardia* infection (Table 3).

Table 3. Prevalence of *Giardia* detection with different variables

Variable	Categories	No of Case	Positive isolate (%)
Age (weeks)	<4	42	24 (57.1)
	5-8	29	5 (17.2)
	>9	22	5 (22.7)
Breed(cross)	50%	46	12 (26.1)
	75%	47	22 (46.8)
Sex	Male	45	15 (33.3)
	female	48	19 (39.6)
Birth weight	16-24	37	12 (32.4)
	25-33	41	15 (36.6)
	34-42	15	7 (46.7)

Discussion

Cryptosporidium, *Eimeria*, and *Giardia* were protozoal enteropathogens isolated at the genus level in the study area. The present findings showed that mixed infections with more than one protozoal genus of enteropathogens were found in diarrheic calves at higher rates (18.27% of the cases for *Cryptosporidium*, *Eimeria*, and *Giardia*) than single infections (*Eimeria*, *Cryptosporidium*, and *Giardia* infection rates for single cases were 13.57%, 6.45%, and 5.4%, respectively). This result was in line with earlier reports in which mixed infections were detected in 64.3% of younger calves, and a single *Cryptosporidium* infection was detected in 52.6% of diarrheic calves (De la Fuente *et al*., 1999). However, this finding disagreed with the report by Hailu *et al*. (2020) in southern Ethiopia, which found that the prevalence of *Cryptosporidium, Giardia,* and mixed infections was 13.03%, 9.7%, and 3.3%, respectively, where mixed infection was lower than single infection. It is suggested that the presence of more than one enteropathogen might be one of the factors determining an infection in a clinical or subclinical disease. Co-infection between pathogens can affect susceptibility to other pathogens and, therefore, can alter host immune responsiveness (Radostits *et al*., 2007).

The present findings revealed that the occurrence of calf diarrhea due to *Cryptosporidium*, *Eimeria*, and *Giardia* infection was more prevalent in calves with an age group of < 4 weeks. This finding is in agreement with the reports in Ethiopia (Wudu, 2004; Yeshwas, 2015; Hailu *et al*., 2020) that the proportion of diarrhea in younger calves is higher than that in older calves. This might be due to several factors, such as early contamination soon after birth by contact with their dams and a contaminated environment (Castro-Hermida *et al*.,

2002). This result indicated that calves from 75% crossbreed level had a higher proportion of *Cryptosporidium, Eimeria, and Giardia* infections. This might be due to calves becoming susceptible as the percentage of exotic breeds' blood levels increases due to environmental and genetic factors (Radostits *et al*., 2007).

Cryptosporidium infection in the present study was 47.3%, which was higher than the previous reports from Ethiopia that ranged from 13.57%–27.8% (Abebe *et al*., 2008; Alemayehu *et al*., 2013; Ayele *et al*., 2018; Manyazewal *et al*., 2018; Hailu *et al*., 2020). On the other hand, there were relatively higher reports of *Cryptosporidium infection*, with a prevalence of 64% in Debre Zeit (Wudu *et al*., 2008), 63.9% in and around Addis Ababa (Demissie, 2007), and 51.79% in Holeta, Debre Zeit, and Muke Turi, Ethiopia (Yenehiwot, 2008) than the current finding. This variation is most likely attributed to the differences in management and husbandry practices of the study animals in different study areas, regions, and countries (Radostits *et al*., 2007).

The present study also showed a 53.8% prevalence of *Eimeria*. This finding was lower than previous reports, with a prevalence of 68.1% in Addis Ababa and Debre Zeit (Abebe *et al*., 2008). In contrast, the current finding was higher than the report in Eastern Ethiopia, with a rate of 22.7% (Dawid *et al*., 2012) and 25.4% in Egypt (Toaleb *et al*., 2011). This variation might be due to husbandry practices, the rate of exposure of calves to oocysts, environmental temperature, humidity, sunlight, and stressors on the calves (Ward *et al*., 1979).

In this study, the detection rate of *Giardia* was 36.6%. This finding was lower than the detection rate of 6.45% in southern Ethiopia (Hailu *et al*., 2020). This variation might be due to differences in research methodology between studies. In addition to the differences inherent to the research methodology employed, several authors highlighted that the differences in management and hygienic-sanitary conditions between the farms could affect the infection rate (Geurden *et al*., 2010). In this study, the use of only one diagnostic technique (microscopy), small sample sizes, and failure to identify the species level were identified as limitations of the current research.

In the present study, major protozoal enteropathogens were isolated from selected dairy farms. *Cryptosporidium*, *Eimeria*, and *Giardia* were identified as common protozoal causative agents of calf diarrhea. Infections were more prevalent in the first few weeks of the age group and 75% of crossbred calves. In most cases, mixed infections of protozoal parasites were isolated. Further

molecular studies should be conducted to identify protozoal enteropathogens at the species and genotype levels. The general health status of the calves should be well maintained, with good hygiene soon after birth to keep calves free of infections due to such pathogens.

Ethical clearance

The Research Animal Ethics and Welfare Committee of Wollega University provided the ethical clearance certificate for this study (Reference RCITT-VP035/01/03/2021). The purpose of the study was explained to farm owners, and the best practices for veterinary care were followed.

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Acknowledgements

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

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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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Table of Contents

Dairy village: the role of veterinary services in unlocking dairy industry potential through assisted reproductive technologies
Sayid Ali, Mosisa Dire, Tamrat Degefa, Asnaku Funga, Hamid Jemal, Geda Regassa, and Curtis R. Youngs.....1
https://dx.doi.org/10.4314/evj.v28i1.1

Assessment of community knowledge, attitude, and practice towards rabies and its determinants in Kersa District, East Hararghe Zone, Oromia Region, Ethiopia
Fami Adem, Abebe Belete Bitew, Takele Adugna, and Solomon Lulie Abey.....15
https://dx.doi.org/10.4314/evj.v28i1.2

Biosecurity level assessment in commercial poultry farms of Central Ethiopia
Dejene Tadesse, Zerihun Aseffa Abebe Wirtu and Teshale Sori 36
https://dx.doi.org/10.4314/evj.v28i1.3

Detection of aflatoxin in raw and pasteurized milk by high-performance liquid chromatography (HPLC) in central Ethiopia
Sitena Kebede, Belachew Bacha, Belachew Tefera, and Gezahegne Mamo.....55
https://dx.doi.org/10.4314/evj.v28i1.4

Seroprevalence of bovine viral diarrhea virus and detection of persistently infected (PI) animals in dairy farms of Holeta, Central Ethiopia
Worku Birhanu, Asamenew Tesfaye, Yitbarek Getachew, and Haileleul Negussie.....73
https://dx.doi.org/10.4314/evj.v28i1.5

Effect of supplementing concentrate mixture with field pea hull on yearling ram lambs of local sheep: Nutrient intake, linear body measurements, and changes in body weight
Getachew Adugna, Berhane Mekete Bogale ,and Helen Nigussie.....88
https://dx.doi.org/10.4314/evj.v28i1.6

Gut microbiota and relevant abundances of *Prevotella copri*, Lachnospiraceae, *Collinsella*, *Helicobacter cinaedi, Desulfovibrio*, and *Escherichia coli* among cats with *Pemphigus foliaceus*
Kerem Ural, Hasan Erdoğan, Songül Erdoğan, and Cansu Balikçi.....105
https://dx.doi.org/10.4314/evj.v28i1.7

Isolation and identification of *B. abortus* and *B. melitensis* in ruminants with a history of abortion: the first report from Eritrea
Ghebremeskel Habteyohannes Efrem, Bereket Mihreteab, Michael K. Ghebremariam, Yitbarek Getachew, and Gezahegne Mamo.....122
https://dx.doi.org/10.4314/evj.v28i1.8

Short communication
Identification of major protozoal enteropathogens causing calf diarrhea in dairy farms in and around Holeta Town, Oromia Special Zone, Ethiopia
Diriba Girmaye, Akinaw Wagari, and Teshita Edaso Beriso.....139
https://dx.doi.org/10.4314/evj.v28i1.9

Guidelines to Authors.....149