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Fax: +387(33) 61 78 50
Address: Sarajevo, 71000
Zmaja od Bosne 90, Bosnia and Herzegovina

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Content


BOSANSKI BRDSKI KONJ

REVIEW ARTICLE

LEGISLATION ON ANIMAL USE IN *IN VIVO* RESEARCH IN BOSNIA AND HERZEGOVINA AND ITS IMPLEMENTATION IN PRACTICE

Lejla Šatrović[1*], Naida Kapo[2], Sanita Maleškić Kapo[3], Martina Perše[4], Aida Kulo Ćesić[3]

[1]Department of Veterinary Public Health and Legislation, University of Sarajevo-Faculty of Veterinary Medicine, Sarajevo, Bosnia and Herzegovina
[2]Department of Veterinary Clinical Sciences, University of Sarajevo-Faculty of Veterinary Medicine, Sarajevo, Bosnia and Herzegovina
[3]Institute of Pharmacology, Clinical Pharmacology and Toxicology, University of Sarajevo-Medical faculty, Sarajevo, Bosnia and Herzegovina
[4]Medical Experimental Centre, Institute of Pathology, University of Ljubljana-Faculty of Medicine, Ljubljana, Slovenia

[*]**Corresponding author:**
Doc. dr. Lejla Šatrović
Adress: University of Sarajevo-Veterinary Faculty, Zmaja od Bosne 90, 71000 Sarajevo/Bosnia and Herzegovina
Phone: +38733729130
ORCID: 0000-0001-5962-6794
E-mail: lejla.satrovic@vfs.unsa.ba

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ABSTRACT

In Bosnia and Herzegovina, the fundamental legal framework for animal protection is the Law on Animal Protection and Welfare (Official Gazette of Bosnia and Herzegovina, No. 25/09 and 9/18). This law represents a significant step forward, establishing basic ethical standards for the treatment of experimental animals. The aim of this paper was to present the current legislative framework governing animal experiments in Bosnia and Herzegovina and its implementation in practice.

Keywords: Bosnia and Herzegovina, experimental animals, legislation, protection, welfare.

INTRODUCTION

Despite progress in non-animal methods, animal experimentation continues to play an indispensable role in research, and it results in legal requirements to implement the 3Rs principles. The codification of the 3R principle - Replacement, Reduction, and Refinement (Russell and Burch, 1959) - into Directive 2010/63/EU underscores the importance of animal welfare in animal research, which is now a central tenet of regulatory frameworks. The legislation regarding the protection of experimental animals is based on the fundamental principle that, under certain conditions, it is morally acceptable to use animals for research or other scientific purposes.

The United Kingdom introduced the first law safeguarding animals used in experimentation in 1876 (The Cruelty to Animals Act 1876). It was produced because of extensive discussions among scientists, politicians, and animal rights activists. For a long time, the United Kingdom was the first and only country that legally protected animals used for scientific purposes. This law prohibited the use of animals for teaching and painful experimentation, emphasizing the responsibility of researchers conducting experiments.

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The regulation of animal experimentation in Europe began in the 1980s with the adoption of two pivotal international agreements: the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Convention ETS 123) in 1985, and the Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Directive 86/609/EEC) in 1986. These two documents initiated significant legislative advancements concerning experimental animals, established a legal framework for the formation of the European Centre for the Validation of Alternative Methods (ECVAM), and stimulated the development of Laboratory Animal Science (LAS). LAS is an interdisciplinary science encompassing a wide range of scientific disciplines concerned with the ethical use, care, and welfare of animals in research on one side and the genetics, molecular biology, immunology, confounding factors, and the quality of scientific studies on the other side. The significant progress of LAS has led to a deeper understanding of the physiological and ethological requirements of experimental animals, resulting in revised housing standards in 2006 (ETS 123-Appendix A, 2006; Euroguide, 2007) and the Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU) in 2010.

Legal regulations establish essential benchmarks based on scientific evidence, with the primary objective of safeguarding animals from unnecessary suffering while simultaneously reinforcing the foundational principles that underpin the validity and reproducibility of scientific research outcomes.

This paper aimed to provide an overview of the current legislative landscape regarding animal experimentation in Bosnia and Herzegovina (BiH), along with an analysis of its practical implementation.

LEGISLATION ON ANIMAL EXPERIMENTATION IN BOSNIA AND HERZEGOVINA

Bosnia and Herzegovina took significant steps to regulate animal experimentation with the adoption of the Law on the Protection and Welfare of Animals, further called LPWA (Official Gazette (OG) of Bosnia and Herzegovina, No. 25/09 and 9/18). Originally enacted in 2009 and revised in 2018, this law highlights the ethical duty of humans to protect the lives, health, and well-being of animals.

The LPWA establishes a comprehensive legal framework for regulating animal experimentation, safeguarding animal welfare, and ensuring the oversight and supervision of experiments involving animals. This framework includes the formation of two national expert bodies tasked with overseeing compliance with the law and ethical standards, i.e. the Ethics Committee responsible for providing advice on ethical issues, and the Advisory Council for the Protection and Welfare of Animals, as outlined in Article 40. Additionally, the LPWA prescribes penalties for those who violate its provisions.

In 2010, two important subsidiary legal acts were introduced to further refine the regulatory landscape. The first, the Regulation on the Protection of Experimental Animals and the Conditions to be Met by Legal Persons Engaged in Carrying Out Animal Experiments (OG of BiH, No. 46/10), further called Regulation 46/10, sets forth the technical and housing requirements that organizations must adhere to when conducting animal experiments. It also outlines the animal species on which experiments are allowed and the specific requirements necessary for the approval of experiments.

The second act, the Regulation on the Method of Record-Keeping for Experimental Animals and Types of Experiments (OG of BiH, No. 19/10), further called Regulation 19/10, mandates documentation practices to ensure transparency and accountability in animal experimentation.

While the national legislation provides a robust framework for the protection of experimental animals, the entity of Republika Srpska (RS) also has its own regulations, specifically the Law on Animal Protection and Welfare of the RS (OG of RS, No. 111/08).

However, this paper will focus exclusively on the regulations established at the state level, since local laws and their accompanying bylaws are expected to be in line with national standards.

ANIMAL EXPERIMENTS

An animal experiment is defined as any intervention or procedure performed on animals for scientific research or educational purposes that may induce pain, suffering, distress, lasting harm, or death. This definition excludes the least painful methods of euthanasia and identification methods (as outlined in the law and regulations) (Article 2, LPWA; Article 2, Regulation 46/10). The legislation regulating the use of animals in *in vivo* research is multifaceted, addressing ethical, scientific, and societal considerations. A key challenge lies in balancing the pursuit of scientific progress with the obligation to ensure the humane treatment of animals.

The regulatory framework in BiH permits animal experimentation conditionally, imposing numerous requirements. As stipulated in Article 31, Paragraphs (2) and (3) LPWA, animal experimentation in BiH is permitted only when the benefit for human or animal health or an advancement in scientific knowledge is expected, and when the research objectives cannot be achieved through acceptable non-animal scientific methods.

For experiments that are likely to cause severe pain, suffering, or distress, a special notification, justification, and approval from the competent authority are required. For example, experiments involving the deprivation of water and/or food beyond the physiological limits necessary for a specific species will not be approved unless they are adequately justified and demonstrate a clear benefit to humans or animals (Article 11, Paragraph (1), (2), Regulation 46/10).

The legislation permits the use of animals for educational purposes only in exceptional circumstances, specifically when satisfactory results cannot be achieved through alternative teaching aids, such as images, models, specimens, or computer programs (Article 33, LPWA). Experiments can only be conducted in organizations or institutions that are registered by the relevant authorities and possess a permit issued by the Veterinary Office of BiH. This permit may be granted for a specific experiment or for a series of experiments over a defined period (Article 31, Paragraph (6), LPWA). Also, approval from the Local Ethics Committee is mandatory prior to conducting any animal experiments (Article 34, Paragraph (4), LPWA). Additionally, designated persons must be responsible for animal welfare and the management of the facilities where the experiments occur.

On the other side, animal experimentation in BiH is explicitly prohibited for research and testing of military weapons, cosmetic products, tobacco, alcoholic beverages, and chemical agents for cleaning and disinfecting (Article 31, Paragraph (5), LPWA).

ANIMALS USED IN EXPERIMENTS

The law in BiH protects animals with developed sensory and nervous systems capable of registering stimuli that may cause pain (Article 1, Paragraph (2), LPWA). Additionally, the legislator mandates compliance with the law's provisions even in cases where there is uncertainty as to whether certain organisms possess the properties of animals (Article 1, Paragraph (3), LPWA).

Usually, the legislation protects animal species for which the scientific community has a consensus that they are sentient animals, i.e. capable of experiencing positive and negative feelings such as pleasure, joy, distress, and pain. European Union (EU) legislation protects all living vertebrates, including cephalopods and larval forms and the last third of foetal and embryonic stages (Directive 2010/63/EU).

The term "experimental animals" encompasses animals used in experiments that are protected by

the legislation, while the term "laboratory animals" refers only to animals that are bred explicitly for experimental purposes in the registered breeding facilities, i.e. mouse (*Mus musculus*), rat (*Rattus norvegicus*), guinea pig (*Cavia porcellus*), golden hamster (*Mesocricetus auratus*), rabbit (*Oryctolagus cuniculus*), quail (*Coturnix coturnix*), dog (*Canis familiaris*), cat (*Felis catus*), and non-human apes (Article 6, Regulation 46/10). Thus, to obtain a permit, the animal species must originate from the registered breeding facilities/organizations.

However, BiH legislation allows that experiments can be carried out also on domestic animals, such as cattle, pigs, poultry, sheep, goats, horses, donkeys, and their hybrids, as well as birds, fish, anthropoid apes, other primates, other mammals, and other carnivores (Article 7, Regulation 46/10). Unlike laboratory animals, domestic animals are not typically housed under strictly controlled conditions, which can make their use in experiments less scientifically preferable.

For experiments involving wild animals, a competent authority can issue a permit only if there are scientifically sound justifications proving that the purpose of the experiment cannot be achieved using other species. Experiments on endangered species are not permitted, except in cases where research is conducted for the conservation of these species, or if the research is biomedical, and the species in question is the only suitable species for that purpose. Additionally, experiments on abandoned and stray animals are strictly forbidden (Article 8, Paragraphs (1), (2), (3) of the Regulation 46/10).

HOUSING CONDITIONS OF EXPERIMENTAL ANIMALS

The environmental conditions in which experimental animals are housed are critical from both welfare and scientific perspectives. The laboratory animal facilities operate under controlled housing conditions and must be constructed to ensure conditions appropriate for the specific species of animals that allows for minimum freedom of movement, adequate food and water, prevent animal escapes, restrict access to unauthorized persons and other animals, incorporate barrier measures to ensure the animals are free of pathogens that may jeopardize human (zoonoses) or animal health (species-specific pathogens) or may confound scientific results and result in misleading research outcomes. (Howard et al., 2004; Cait et al., 2022).

Controlled housing conditions address not only microbiological factors but also other important factors such as temperature, light (Lucas et al., 2024), noise, cage cleaning (Castelhano-Carlos and Baumans, 2009), circadian rhythm (Reardon, 2016), social interactions, nesting material and enrichment (Schipper L et al., 2018; Heimer-McGinn et al., 2020), diet (Pellizzon and Ricci, 2020; Tuck et al., 2020), etc., all of which can influence animal physiology, molecular and immunological mechanisms leading to increased biological and experimental variability as well as increased use of animals. Thus, the conditions under which animals are housed are integral to the integrity of scientific research.

In BiH, Regulations 46/10 outline minimum requirements for space, equipment, and environmental conditions in both breeding and experimental settings. The environment in which the animals are kept, as well as the animals' health and welfare, should be under constant supervision to prevent pain, suffering, distress, exhaustion, or permanent injury. Any restriction on the fulfilment of physiological and ethological needs must be minimized (Article 31, Paragraphs (7), (8), LPWA; Article 19, Regulation 46/10). Animal housing, depending on the species, can be organized in various ways, including cages, pens, and similar options. It is crucial to prevent contact between animals and ensure that the housing does not restrict their ability to satisfy ethological needs, such as climbing, hiding, and providing shelter (Article 35, Regulation 46/10). The legal guidelines regarding the temperature, isolation duration and housing conditions for rodents are presented in Table 1 and 2. Detailed conditions for other animal species are outlined in the appendices of this manuscript (Annex; Tables I–VIII)

Table 1 Temperature and isolation duration in rodent facilities

Animal species	Room temperature (°C)	Quarantine (days)
Mouse, rat, Syrian hamster, albino mouse, guinea pig	20–24	5–15
Rabbit	15–21	20–30

Note: In special cases, such as when housing sick animals, very young animals, or hairless animals, a higher temperature than indicated may be required.

Table 2 Minimum cage area and height for housing small rodents and rabbits

Animal species	Minimum cage area (m²)	Minimum cage height (cm)
Mouse	180	12
Rat	350	14
Syrian hamster	180	12
Guinea pig	600	18
Rabbit		
1 kg	1400	30
2 kg	2000	30
3 kg	2500	35
4 kg	3000	40
5 kg	3600	40

Note: "Cage height" refers to the vertical distance between the bottom of the cage and the upper horizontal part of the lid or cage.

It is important to note that EU legislation sets the highest legal minimal standards on housing conditions worldwide. In EU countries, Annex III of the Directive 2010/63/EU sets minimal housing and accommodation conditions for animal facilities and various species, including recently adopted amendments for Zebrafish and Passerine birds (European Commission, 2024).

Moreover, over 1,100 institutions in 50 countries/regions worldwide earned voluntary accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC, www.aaalac.org) showing their commitment to state-of-the-art animal welfare beyond the laws.

To ensure the welfare of laboratory animals and the integrity of scientific research, the Federation of European Laboratory Animal Science Associations (FELASA), the leading European scientific association for laboratory animals, provided recommendations on health monitoring in breeding and experimental units for mice, rats, hamsters, guinea pig, rabbits (Mahler et al., 2014), rodents for animal transfer (Pritchett-Corning et al., 2014), ruminants and pigs (Caristo et al., 2021), and Zebrafish (Mocho et al., 2022). FELASA emphasizes the importance of health monitoring and the use of specific pathogen-free (SPF) animals to minimize the risk of contamination and to ensure reproducibility of results. For instance,

the composition of microorganisms and microbiota plays a pivotal role in the health and behaviour of animals, affecting their metabolic processes and immune responses (Franklin and Ericsson, 2017).

PERSONNEL QUALIFICATIONS

Current legislation in BiH recognizes the following persons/functions/roles within the organizations/institutions registered for conducting animal experiments, i.e. a person responsible for facility management, a person responsible for experiment management, and an animal welfare specialist. These persons are responsible for the facility, animals, and maintaining the prescribed records. No other position/role is defined.

According to the current legislation in BiH, permission to conduct experiments can be granted to professionals who understand animal physiology and behaviour, such as veterinarians and biologists with specializations in zoology, and, upon request, for a specific experiment, to doctors, agronomists specializing in animal husbandry, pharmacologists, and dentists (Article 32, Paragraph (3), LPWA). Persons conducting or participating or overseeing the experiment, as well as those caring for the animals during the experiment, must have adequate knowledge and a satisfactory level of training for the tasks (Article 32, Paragraph (4), LPWA).

However, even 15 years after the enactment of this Law, the existing practice for assessing the necessary level of knowledge and training for persons involved in experiments has been based solely on qualifications obtained through formal university education, as no official training programs for specialists are established. In this regard, the Ethics Committee's approval for conducting animal experiments is granted by the Law-stipulated professionals, and a veterinarian is considered as an animal welfare specialist (the person responsible for the protection and welfare of animals).

Recognizing that adequate qualifications are crucial for professional and humane animal care, FELASA has developed guidelines with the recommendations for the education and training of persons involved in work with experimental animals (FELASA, 1995; Nevalainen et al., 1999; Nevalainen et al., 2000; Convenor et al., 2010; Abelson et al., 2023) (Categories A-D). These guidelines enabled the establishment of high-quality courses on LAS in various countries in Europe and worldwide and set the standards and foundation for the education and training framework incorporated in the EU legislation. According to FELASA recommendations, BiH can also develop official education/training programs to ensure the licensing and qualification of its experts. These actions are crucial to ensuring good scientific practice and animal welfare in *in vivo* research.

PROCESS OF OBTAINING PERMIT FOR EXPERIMENTS

According to the legislation in BiH, the protection of experimental animals is primarily achieved through a mandatory system of experiment approvals. Experiments can be conducted only after obtaining Veterinary Office permit/approval (Article 9, Paragraphs (1), (2), Regulation 46/19). The permit to conduct animal experiments can be issued only to an organization registered for this purpose (Article 31, Paragraph (1), LPWA) and for a specific experiment or a series of experiments, with a clearly defined validity period (Article 31, Paragraph (6), LPWA). Organizations that conduct experiments are required to employ an animal welfare specialist responsible for managing and preparing the rationale for conducting experiments. Organizations registered for conducting animal experiments are higher education and research institutions, as well as professional institutions (Article 32, Paragraphs (1), (2), LPWA). To obtain a permit for an experiment, registered organizations are required to apply for approval for each planned experiment to the Veterinary Office of BiH (Article 9, Paragraph (2), Regulation 46/10). The application for a permit must include at least the following information:

- location of an experiment, which includes the

name and address of the institution conducting the experiment as well as the name and address of the location where the experiment will be conducted,

- title and the purpose of the planned experiment,
- species, breed, and number of experimental animals,
- name, surname, and professional qualifications of the experiment leader and their deputy,
- name, surname, and professional qualifications of the manager of the facility for experimental animals and his deputy,
- information on the duration of the experiment or the period for which approval is sought (Article 10, Regulation 46/10).

In cases where animals are planned to be subjected to intense pain, suffering, or distress, as previously mentioned, a special notice and justification are required (Article 11, Regulation 46/10).

The issuance of permits for conducting animal experiments is preceded by a process of reviewing the application and providing an expert opinion, i.e. a positive opinion of the Ethics Committee on the justification for animal testing (Article 34, Paragraph (4), LPWA).

To protect animal welfare and ensure control and supervision of experiments involving animals, the LPWA has provided a legal framework for the formation of two national expert bodies, i.e. the Ethics Committee responsible for providing advice on ethical issues, and the Advisory Council for the Protection and Welfare of Animals, an additional advisory expert body that provides opinions and proposals on issues related to the protection and welfare of animals. The law stipulates that the establishment of the Ethics Committee is under the authority of the relevant ministry of BiH, and should consist of seven members, including representative naturalists, medical professionals, veterinarians, scientists, as well as representatives of non-governmental organizations whose legal obligation is animal protection (Article 34, Paragraph (1), LPWA).

However, neither the Ethics Committee nor the Council for the Protection and Welfare of Animals has been established at the national level yet. The conduct of animal experiments in BiH currently occurs based solely on the ethical assessments and positive opinions issued by the Ethics Committees formed as independent expert bodies within the organisations/institutions registered for conducting animal experiments. Those Ethics Committees at the institutional level evaluate requests for approval of animal experiments, considering the ethical aspects of the experiment, taking positions, making decisions, and providing opinions on ethical issues significant for ensuring good scientific practice and preserving the fundamental principles, duties, and obligations established by the LPWA and beyond it.

Although ethical assessment of requests for conducting animal experiments has been ensured at the institutional level, the absence of a unified national Ethics Committee significantly complicates the maintenance of records regarding experiments conducted at the national level. Furthermore, the lack of a request form standardised at the national level prevents a unified approach to the ethical evaluation, which may negatively affect the welfare of experimental animals and the quality of the scientific research. These shortcomings significantly impact the assurance of good scientific practice and the transparency we all strive for.

RECORD KEEPING

The obligation to maintain records, including the format, content, and storage, is also prescribed by the legislation in BiH (Regulation on the Method of Record-Keeping for Experimental Animals and Types of Experiments (OG of BiH, 19/10, further called Regulation 19/10). Within the registered organization, the facility manager is required to maintain following records:

- records of the total number and species of experimental animals used,
- records of the number and species of animals used and the purpose of the experiment,

- records of the number and species of animals used in experiments for the protection of humans, animals, and the environment, and
- records of the number and species of animals used in experiments related to diseases and health disorders in humans (Article 2, Regulation 19/10).

The records must be submitted to the Veterinary Office BiH annually and kept for at least three years from the date of the last entry (Articles 3 and 4, Regulation 19/10), and presented to the competent inspection authority upon request (Article 20, Regulation 19/10).

SUPERVISION OF IMPLEMENTATION OF REGULATIONS

As stipulated by the LPWA, supervision of the implementation of the LPWA and the subordinate legislation adopted under it is carried out by the Veterinary Office BiH and the relevant authorities of the two entities and the Brčko District, through official veterinarians (Article 36, LPWA). As part of the inspection oversight during regular inspections and in cases of suspicion of improper handling of animals, an official veterinarian has the right to access all premises where animals are kept or used (Article 37, LPWA) and to prohibit the conduct of experiments if appropriate approval has not been issued (Article 38, LPWA).

For conducting animal experiments in violation of legal provisions, penalties ranging from 200 to 20,000 BAM are stipulated for both individuals and the directors of organizations involved. In addition to monetary fines that the LPWA provides for offenders, it is important to note that the Criminal Code of the Federation of Bosnia and Herzegovina (Article 318, Criminal Code of FBiH, OG of FBiH, No. 36/2003, 21/2004 - corrected, 69/2004, 18/2005, 42/2010, 42/2011, 59/2014, 76/2014, 46/2016, 75/2017, and 31/2023) recognizes animal cruelty and killing of animals as a separate criminal offense within the category of crimes against the environment, agriculture, and natural resources. Since this law does not impose explicit restrictions regarding an animal type as an object of the criminal act, conducting experiments in a manner that subjects animals to unnecessary pain, prolonged suffering, or that kills animals contrary to regulations can also be categorized as a criminal act and, accordingly, be punished with imprisonment.

CONCLUSION

While non-animal methods are progressing, they cannot yet fully replicate the complexity of *in vivo* systems, necessitating the ongoing use of animal models for specific research areas, such as pharmacokinetics and toxicology. The legal norms incorporated into the legislation of BiH aim to prevent unjustified and excessive exploitation of animals for the purposes of conducting *in vivo* research. These norms aim to ensure compliance with the basic framework for the ethical treatment of animals used in legally permitted, ethically justified, and unavoidable procedures.

However, despite the significant progress achieved with the adoption of the LPWA and its subordinate legislation, further improvements and more efficient practical implementation are needed. While the national regulatory framework provides a broad foundation for the ethical treatment of animals in experiments, it lacks more specific guidelines for ensuring animal welfare, research quality, and result reliability. Key areas, such as clearer guidelines for the application of the 3Rs, stricter monitoring standards, enhanced oversight of experiment implementation, and clearly specific competency requirements for involved personnel remain inadequately addressed compared to EU legislation and the guidelines of leading scientific associations for laboratory animals (FELASA).

Thus, considering that these deficiencies may hinder the consistent application of animal protection measures, and keeping in mind BiH's aspiration for EU integration, there is a pressing need to improve and align legal documents with the standards precisely regulated in the EU.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

CONTRIBUTIONS

Conception: LŠ, NK, SMK, MP, AKĆ; Design: LŠ, NK, SMK, MP, AKĆ; Supervision: LŠ, NK, MP, AKĆ; Fundings: AKĆ; Materials: LŠ, MP; Data Collection and/or Processing: LŠ, NK; Analysis and/or Interpretation of the Data: LŠ, NK, MP, AKĆ; Literature Review: LŠ; Writing: LŠ, NK, MP, AKĆ; Critical Review: MP, AKĆ

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ANNEX

Table I Temperature in facilities for various experimental animals (cages, sections/boxes, or barns)

Animal species	Room temperature (°C)
Non-human primates	20–28
Cat, dog, African ferret, poultry, pigeon	15–21
Pig, goat, sheep, cattle, horse	10–24

Note: In special cases, such as when housing sick, very young animals or hairless animals, a higher temperature in facilities than indicated is required.

Table II Duration of isolation for various experimental animals

Animal species	Isolation Duration (days)
Cat	20–30
Dog	20–30
Non-human primate	20–30

Table III Housing of cats in experimental animal facilities

Cat weight (kg)	Minimum cage area (m^2)	Minimum cage height (cm)	Minimum cage area and bedding (m^2)	Minimum box area and bedding (m^2)
0.5–1	0.2	50	-	-
1–3	0.3	50	0.58	2
3–4	0.4	50	0.58	2
4–5	0.6	50	0.58	2

Note: Keeping cats in cages should be strictly limited. Cats confined in this way should be allowed out at least once a day to stretch, unless this is contrary to the purpose of the experiment. Cages for cats should be equipped with litter boxes, resting areas, and climbing and scratching items. "Cage height" refers to the vertical distance between the highest point at the bottom and the lowest point at the top of the cage. When calculating the minimum area, the space with bedding is also considered. The minimum area of the cage for a cat and bedding includes 0.18 m^2 for kitten cages.

Table IV Housing of dogs in experimental animal facilities

Dog height to shoulder level (cm)	Minimum cage area per dog (m^2)	Minimum cage height (cm)
30	0.75	60
40	1.00	80
70	1.75	140

Note: Dogs may be kept in cages only for as long as required by the experiment. If this does not conflict with the purpose of the experiment, closed dogs should be allowed out at least once a day to stretch. The supervisor of the experimental animal facility determines the maximum time dogs can be confined without daily stretching. The exercise area should be spacious enough to allow freedom of movement for the dog. Cages in which dogs are housed must not use wire mesh at the bottom, unless required by the type of experiment. The minimum height of the cage should be twice the height of the animal, measured to the shoulder level.

Table V Housing of dogs in sections/boxes

Dog weight (kg)	Minimum box area per dog (m²)	Minimum adjacent exercise space per dog
Up to 3 dogs per m²	More than 3 dogs per m²	<6
6–10	0.7	1.4 (2.1)
10–20	1.2	1.6 (2.8)
20–30	1.7	1.9 (3.6)
>30	2.0	2.0 (4.0)

Note: The numbers in parentheses indicate the total area per dog, i.e., the area of the box plus the area of the adjacent exercise space. Dogs kept outdoors must have access to a sheltered area to protect themselves from adverse weather conditions. If the bottom of the box is mesh, dogs must be provided with a solid surface for sleeping. Wire mesh at the bottom must not be used unless required by the type of experiment. Dividers between boxes must prevent mutual injury among dogs. All boxes must have appropriate drainage.

Table VI Housing of non-human primates in cages

Monkey weight (kg)	Minimum cage area for one or two animals (m²)	Minimum cage height (cm)
<1	0.25	60
1–3	0.35	75
3–5	0.50	80
5–7	0.70	85
7–9	0.90	90
9–15	1.10	125
15–25	1.50	125

Note: Due to large differences in sizes and characteristics of monkeys, it is crucial that the shape, internal equipment, and dimensions of the cage meet their needs. For monkeys, both the total volume and the surface area of the cage are equally important. In principle, the greatest dimension should be the height of the cage. The height of the cage must allow animals to stand upright. The minimum height of the cage for swinging monkeys must be such that they can swing from the ceiling at full swing without touching the bottom of the cage with their legs. Perches can be installed as needed to allow monkeys to use the upper part of the cage. Sociable monkeys can be housed in pairs. If they cannot be housed in pairs, their cages should be arranged so they can see each other. Likewise, if required by the type of experiment, measures should be taken to prevent monkeys from seeing each other.

Table VII Housing of pigs in cages

Pig weight (kg)	Minimum cage area per pig (m²)	Minimum cage height (cm)
5–15	0.35	50
15–25	0.55	60
25–40	0.80	80

Note: The indicated values also apply to piglets. In principle, pigs should not be confined in cages unless required by the type of experiment, and the duration of confinement should be as short as possible.

Table VIII Housing of domestic animals in sections/boxes

Animal type and dog weight (kg)	Minimum box area (m²)	Minimum box length (m)	Minimum box divider height (m)	Minimum box area for groups (m²/animal)	Minimum feeder length per head (m)
Pigs	10–30	2	1.6	0.2	0.20
	30–50	2	1.6	0.3	0.25
	50–100	3	2.1	0.8	0.30
	100–150	5	2.5	1.2	0.35
	>150	5	2.5	2.5	0.40
Sheep	<70	1.4	1.8	1.2	0.35
Goats	<70	1.6	1.8	2.0	0.35
Cattle	<60	2.0	1.1	1.0	0.30
	60–100	2.2	1.8	1.0	0.30
	100–150	2.4	2.8	1.2	0.35

PRAVNI PROPISI O UPOTREBI ŽIVOTINJA U *IN VIVO* ISTRAŽIVANJIMA U BOSNI I HERCEGOVINI I NJIHOVA PRIMJENA U PRAKSI

SAŽETAK

U Bosni i Hercegovini, osnovni pravni okvir za zaštitu životinja čini Zakon o zaštiti i dobrobiti životinja ("Službeni glasnik Bosne i Hercegovine", br. 25/09 i 9/18). Ovaj zakon predstavlja značajan iskorak, uspostavljajući osnovne etičke standarde tretiranja eksperimentalnih životinja. Cilj ovog rada jeste predstaviti postojeći zakonodavni okvir koji regulira upotrebu životinja u eksperimentalnim istraživanjima u Bosni i Hercegovini i njegovu provedbu u praksi.

Ključne riječi: Bosna i Hercegovina, dobrobit, eksperimentalne životinje, legislativa, zaštita

RESEARCH ARTICLE

PRELIMINARY RESULTS OF RADIONUCLIDE MONITORING AND RISK ASSESSMENT FOR BIOTA IN THE MARINE ECOSYSTEM OF BOSNIA AND HERZEGOVINA

Mirza Čelebičić[1*], Nejra Karaman[2], Nedim Mujić[2], Avdul Adrović[1], Nedžad Gradaščević[2]

[1]Department of Biology, Faculty of Science, University of Tuzla, Tuzla, Bosnia and Herzegovina
[2]Department of Animal Production and Biotechnology - Laboratory for Radioactivity Control, University of Sarajevo - Veterinary faculty, Sarajevo, Bosnia and Herzegovina

Corresponding author: MA Mirza Čelebičić
Address: University of Tuzla, Faculty of Science, Univerzitetska 4, Tuzla 75000, Bosnia and Herzegovina
Phone: +38762072157
ORCID: 0000-0003-3403-931X
E-mail: mirzacelebicic24@gmail.com

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ABSTRACT

The purpose of this paper is to provide an example of radioecological monitoring using the *Mytilus galloprovincialis* L. as an indicator organism, following the example of many Mussel Watch monitoring programs in the world. Using gamma spectrometric measurements and ERICA software for risk assessment, we offer the first results in a radioecological sense for the marine ecosystem of Bosnia and Herzegovina. Organisms that are at risk of manifesting the effects of higher radionuclide activities are birds, mammals, phytoplankton and reptiles. The highest risk coefficient of 5.73E+01 is for phytoplankton. If 5 kg of mussels are consumed by humans on an annual level, observed radionuclides would produce an effective dose of 25.6 μSv/a.

Keywords: ERICA risk assessment, Mussel Watch, Neum Bay, radioactivity

INTRODUCTION

The marine ecosystem of Bosnia and Herzegovina is insufficiently researched and monitored in every sense. This ecosystem, which is under great anthropogenic pressure, is actually a determinant of Bosnia and Herzegovina as a Mediterranean country. It is an essential part of the landscape and species biodiversity, but also an important source of renewable resources from aquaculture. As a prerequisite for coastal tourism, it is one of the essential determinants of the economy and income for the local community. Radiation monitoring in the Adriatic Sea has been taking place for many years, with the exception of our country. In neighboring Croatia, it takes place for ecological, sanitary and state security reasons.

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The Neum and Mali Ston Bays, or rather, the part of them that belongs to BiH, are examples of semi-enclosed, large, shallow bays (Natura 2000 code 116), which are under great anthropogenic pressure. These two bays were protected as "special marine reserve" in 1983. The area that belongs to BiH was under the second class of protection. Unfortunately, after the establishment of borders between the newly formed states in the early 1990s, protection on the Croatian side was continued and improved, while it was completely suspended in Bosnia and Herzegovina. The Mali Ston Bay, as a special marine reserve, was declared a strict reserve (Milanović et al. 2015; Official Gazette of the Dubrovnik County, No. 09/02).

The influence of the Neretva river and other inland water bodies on this area which fully belongs to the category of coastal waters, is manifested by the lower salinity of the water and the presence of a large number of hot springs. Brackish water favors the development of bivalves (Copeland, 1996). In addition to the fact that inland waters largely determine the specificity of the coastal ecosystem, i.e. its physical and biocenological characteristics, they are also a potential source of contamination with heavy metals, plastics, radionuclides, organic substances, and microbiological pathogens (Jurina et al., 2013). Every year, 283 tons of plastic waste reaches the Adriatic Sea via the Neretva River, which is the fifth largest polluter in the Mediterranean (Guerranti et al., 2020). With its flow through terrestrial ecosystems that are contaminated in different ways, the river erodes waste and creates conditions for the accumulation of pollutants in sediment and water, and then for bioaccumulation and biomagnification in biocenoses (Chen et al., 2009).

Waste is so present in the seabed of the bay that it seems to be an indispensable part of the underwater landscape up to 200 meters from the shore. Untreated wastewater and a large number of bathers in the season are a potential source of microbiological contamination of the bay. Algal blooms are always a danger to humans, swimmers or consumers of aquaculture products. Also, heavy metals, radionuclides and microbes are potential hazards if they accumulate in aquaculture products. Just as these pollutants accumulate and increase in the biota, climate change increases all these pressures through changes in the biotope and then in the biocenoses. As an example, we cite the effects of climate change and invasive alien species that can change relationships in ecosystems on a large scale. There are two reasons why biomonitoring is necessary in the management of this marine resource. The first is sanitary, that is, the safety of people during their stay in the water and when consuming food from local aquaculture. Another reason is the preservation of ecosystems and associated biocenoses. Assessment of the quality of the coastal ecosystem and the conservation status of the species in it are mandatory for BiH as a Mediterranean country. First of all, the first step is to choose the most efficient and economically acceptable programs and monitoring methods. This is actually the goal of this paper, to propose one such method (Bechard, 2007; Burgiel and Muir, 2010; Carvalho, 2018).

In 1975, Professor Edward D. Goldberg proposed the "Mussel Watch" program, which would use mussels to assess trends in chemicals of environmental importance in the coastal ocean, and after that, many "Mussel Watch"- type monitoring programs appeared on the local, regional, national and international levels with success, but within the initial limitations pointed out by Professor Goldberg.

Mussel *Mytilus galloprovincialis* L. is sedentary, widely distributed marine organism able to filter up to 80 l of seawater per day in optimal conditions (water temperature, food availability, reproductive cycle). High rates of accumulation of dissolved and particulate matter from seawater makes them a good bio-indicator species for environmental pollution studies as they directly reflect the level of contamination in habitat (Brenner et al., 2014; Krmpotić et al., 2015). Mussels extract and concentrate elements from the environment in which they grow but lack the ability to eliminate radioisotopes from its body (Assunta et al., 2008).

Most programs have evolved with their own

characteristics, building on experience from previous or ongoing programs. In the neighboring Mediterranean countries, the monitoring of radionuclides, heavy metals, plastics, and the state of the ecosystem was carried out based on the principles and recommendations of Professor Goldberg (Babarro et al., 2020; De Donno et al., 2008; Kilic et al., 2014; Krmpotić et al., 2015; Nonova and Tosheva, 2016; Oreščanin et al., 2006; Rozmarić et al., 2013; Thebault et al., 2008; Winterbourn et al., 2016). NOAA's National Status and Trends Program's Mussel Watch is still active in the USA (Farrington et al., 2016).

Considering these facts and the fact that until now there has been no monitoring of this type in Bosnia and Herzegovina, in the year 2022 we conducted experimental seasonal monitoring for spring and autumn at four locations in the Bosnia and Herzegovina's coastal waters. This study presents preliminary results of radionuclide monitoring and biota impact assessment to demonstrate the simplicity and effectiveness of this method in monitoring selected descriptors of marine ecosystem quality in biological and public health terms.

MATERIALS AND METHODS

Sampling and sample preparation

Sampling of sediment and mussels was carried out at the locations shown in Figure 1.

Seawater samples were collected in 25 l containers at a depth of 1 m and evaporated to approximately 250 ml, resulting in a mixture of water and salt. The residue was weighed and sealed in 200 ml

Figure 1 Map of the investigated area with sampling sites

cylindrical plastic containers. The samples were stored for one month before measurement (Petrinec et al., 2012).

Sediment samples were collected in a canister for underwater sampling, then dried in the laboratory at 80-105°C and homogenized. The dried samples were subsequently packed into cylindrical plastic containers, sealed and measured. Mussels were sampled twice, in spring and autumn, at the location shown in Figure 1. Approximately 7 kg of fresh mussels were collected per sample. The shells were opened using a microwave oven, and soft tissue was extracted for analysis. The mussel tissue was homogenized and air-dried before being further homogenized in a blender and fully dried in an oven at 80-105°C. Finally, the dried mussels were homogenized again and packed into appropriate containers, as described in detail in the next section (Petrinec et al., 2012).

Measurements - Gamma ray spectrometry

For gamma spectrometry analysis, samples were packed in 200 ml cylindrical plastic containers 36 mm high and 90 mm in diameter, sealed and measured for 350000 s. Gamma spectrometry analysis was used to determine the activity concentrations of observed radionuclides. The gamma spectrometric analysis was conducted using a BSI (Baltic Scientific Instruments) HPGe gamma spectrometer equipped with a P-type detector. The detector was protected by a 15 cm thick lead shield lined inside with copper and cadmium foil. The detector has a relative efficiency of 50% at the energy of 1332 keV emitted by Co-60, and it provides a resolution (FWHM) of 1.9 keV at 1332keV. The levels of all reported radionuclides were determined from their specific gamma lines or from gamma lines of their daughter products. Quality control procedures were included in the operating activities of the laboratory accredited by BAS EN ISO/IEC 17025:2018. Gamma spectrometric system was calibrated for energy and absolute efficiency using standard "multinuclide mix" certified reference material (CRM) containing energies from 59.54 keV (Am-241) to 1836.07 keV (Y-88). This procedure was validated using IAEA reference materials of different composition collected by participation in ALMERA proficiency tests, which is mandatory for ISO/IEC 17025:2018 accredited laboratory. The activity concentrations of radionuclides were determined using calculations previously described in the article by Gradaščević et al., 2023.

Dose estimation and modeling

The ERICA assessment tool was one of the products of the ERICA project (Environmental Risk from Ionizing Pollutants: Assessment and Management, 2004–2007). The ERICA Integrated Approach and the ERICA Tool are two important outcomes of the project. Three components make up the ERICA integrated approach: assessment, risk characterization and management. ERICA tool consists of three compartments (Tiers), designed for use in all ecosystems with multiple scenarios. ERICA Integrated Approach and the ERICA Tool offer an affordable, accessible and user-friendly method of conducting a radiological risk assessment, while still providing a very significant scientific basis for a complex decision-making process in an interdisciplinary context of environmental issues (Prlić et al., 2017).

Level (Tier) 2 of the ERICA Risk Assessment Tool was used for dose calculations and risk assessment. Average sediment and mussels' activity concentrations for specific radionuclides and concentration of Cs-137 in seawater are used as input data for estimating concentration rates and activity dose to marine biota represented by all available reference organisms in ERICA database (Table 1, Table 2). Since the ERICA Assessment Tool database lacks data for concentration ratios for all reference organisms for K-40, the isotope is excluded from the calculation. All parameters are left as predefined. This includes all concentration ratios for the various reference organisms, occupancy factors, which indicate which habitat a particular organism lives in (water surface, water, sediment surface, and sediment), and radiation weighing factors (10 for alpha, 1 for beta and gamma, and 3 for low beta). Dose rate screening

values are set to 40 μGy/h for terrestrial animals, birds, amphibians and reptiles, and 400 μGy/h for plants and other aquatic organisms. It has previously been suggested that below these values (of chronic exposures) no measurable population effects would occur. Uncertainty factor is set to UF=3, this tests for 5% probability of exceeding the dose value, assuming that the risk quotient distribution is exponential (IAEA 1992; USDOE 2002; UNSCEAR 1996).

RESULTS

The results of radionuclide activity concentrations and dose assessments are summarized in Tables 1 to 3. Table 1 shows ERICA assessment outcomes, including measured activity concentrations in sediment, Cs-137 levels in seawater, and estimated activities for other radionuclides in water medium. Table 2 presents radionuclide activity concentrations in mussels (*Mytilus galloprovincialis* L) collected during spring and autumn, allowing comparison of seasonal variations in this area. Table 3 summarizes the assessment of activity concentrations in marine organisms (in Bq/kg fresh weight), including the calculated risk quotient and total dose rate. This allows evaluation of potential radiological risks to marine biota.

Table 1 ERICA - Results for measurement of activity concentration in sediment and for Cs-137 in water, and assessment of activities for water medium (all others).

Isotope	Activity Concentration in water [Bq L-1]	Activity Concentration in sediment [Bq kg-1 d.w.]
Th-234	3.84E-01	4.08E+01
Cs-137	3.62E+00	3.86E+04
Pb-210	1.75E-01	5.94E+01
Ra-226	1.78E-01	3.11E+01
Ac-228	2.11E+01	9.76E+00
U-238	3.51E-01	9.35E+02
Ba-137m	3.42E+00	3.65E+04
Bi-210	1.75E-01	5.94E+01
Po-218	1.78E-01	3.11E+01
Bi-214	1.78E-01	3.11E+01
Pb-214	1.78E-01	3.11E+01
Po-214	1.78E-01	3.11E+01
Pa-234m	3.84E-01	4.08E+01

Table 2 Results of radionuclide measurements in mussels for spring and autumn

Isotop	Spring Bq/Kg f.w.	uncertainty	Autumn Bq/Kg f.w.	uncertainty	Mean	SEM
BE-7	9.81	0.98	5.80	0.84	**7.80**	0.64
TH-234	18.51	2.74	14.06	3.40	**16.28**	2.18
K-40	117.34	6.88	136.24	6.19	**126.79**	4.63
CS-137	0.03	0.02	0.04	0.02	**0.04**	0.01
PB-210	6.12	1.04	6.25	0.96	**6.18**	0.71
RA-226	0.18	0.06	0.27	0.24	**0.22**	0.12
AC-228	0.13	0.12	0.17	0.10	**0.15**	0.08
U-238	1.11	0.67	1.26	0.68	**1.19**	0.47

Table 3 Assessment of activity concentrations in organisms (Bq/kg fresh weight) with Risk quotient and Total Dose Rate

ncentration in organisms Bq/Kg f.w.	Benthic fish	Bird	Crustacean	Macroalgae	Mammal	Mollusc-bivalve	Pelagic fish	Phyto-plankton	Polychaete	Reptile	Anemones	V. plant	Zooplankton
Th-234	2.14E+01	2.14E+01	1.26E+02	5.33E+02	2.14E+01	1.63E+01	2.14E+01	1.96E+05	1.12E+02	2.14E+01	1.68E+02	5.33E+02	1.20E+03
Cs-137	2.95E+02	1.72E+03	1.88E+02	3.08E+02	7.65E+02	4.00E-02	2.95E+02	3.08E+01	6.48E+02	1.72E+03	8.41E+02	3.76E+01	4.73E+02
Pb-210	5.29E+03	3.30E+03	3.53E+03	1.65E+02	3.30E+03	6.18E+00	5.29E+03	7.72E+04	6.98E+03	3.30E+03	6.98E+03	1.65E+02	2.85E+03
Ra-226	2.45E+01	2.85E+01	1.53E+01	2.96E+01	2.85E+01	2.20E-01	2.45E+01	1.20E+02	8.34E+01	2.85E+01	1.53E+01	2.96E+01	5.35E+00
Ac-228	2.45E+02	2.22E+02	1.32E+02	6.42E+03	2.22E+02	1.50E-01	2.45E+02	3.81E+05	1.77E+02	2.22E+02	1.76E+02	6.42E+03	1.26E+03
U-238	1.61E+00	4.39E+01	2.21E+00	2.39E+01	4.39E+01	1.19E+00	1.61E+00	7.66E+01	3.45E+02	4.39E+01	3.45E+02	8.25E+01	9.03E-01
Ba-137m	3.84E-03	3.08E-02	9.03E-02	3.05E-02	3.08E-02	1.20E-01	3.84E-03	2.95E-02	1.20E-01	3.08E-02	9.03E-02	1.16E-01	9.03E-02
Bi-210	4.50E+00	4.50E+00	1.90E+00	5.48E-01	4.50E+00	1.90E+00	4.50E+00	5.48E-01	1.90E+00	4.50E+00	1.90E+00	5.48E-01	1.90E+00
Po-218	9.65E-01	1.18E+00	2.69E+00	1.07E-01	1.18E+00	7.28E-01	9.65E-01	2.75E+00	6.07E+00	1.18E+00	6.07E+00	1.07E-01	1.28E+00
Bi-214	1.47E-02	1.47E-02	6.21E-03	2.30E-03	1.47E-02	6.21E-03	1.47E-02	2.30E-03	6.21E-03	1.47E-02	6.21E-03	2.30E-03	6.21E-03
Pb-214	3.34E+00	2.09E+00	2.23E+00	3.11E-01	2.09E+00	1.95E-03	3.34E+00	1.46E+02	4.41E+00	2.09E+00	4.41E+00	3.11E-01	1.80E+00
Po-214	8.53E-07	1.04E-06	2.38E-06	9.47E-08	1.04E-06	6.43E-07	8.53E-07	2.43E-06	5.36E-06	1.04E-06	5.36E-06	9.47E-08	1.13E-06
Pa-234m	1.42E-02	1.29E-02	7.69E-03	3.79E-01	1.29E-02	6.80E-03	1.42E-02	2.26E+01	1.03E-02	1.29E-02	1.02E-02	3.79E-01	7.31E-02
Risk quotient (Unitless)	2.80E-01	1.84E+00	1.87E-01	9.47E-01	1.84E+00	1.62E-02	2.65E-01	5.73E+01	4.00E-01	1.84E+00	3.61E-01	9.50E-01	3.00E-01
Total Dose Rate per organism µGy/**h**	1.12E+02	7.34E+01	7.47E+01	3.79E+02	7.34E+01	6.48E+00	1.06E+02	2.29E+04	1.60E+02	7.37E+01	1.44E+02	3.80E+02	1.20E+02

DISCUSSION AND CONCLUSION

Activity concentrations of natural radionuclides Be-7, K-40, Th-234, Ra–226, Ac-228, Pb-210, Tl-208, U-238 and anthropogenic Cs-137 were determined in dry soft tissue of mussels collected in Neum Bay in 2022. Be-7 activity concentrations for all sampling locations were found to range between 31.87 and 14.49 Bq/kg f.w. Activities determined in early spring are higher than those measured in early autumn (40,89% decrease), which can be attributed to its input into the marine environment almost exclusively by atmospheric precipitation (rain/snow) and freshwater from the interior. Seasonal spring-autumn decrease in activity concentration of Be-7 was 40,89 %. The reason for that could be seasonal variation in Be-7 air concentration recorded by the other authors and explained by increased growth of Be-7 aerosols in humid conditions, and consequent higher precipitation on the surface (Young Hyun Cho, 2007). K-40 activity concentrations did not differ significantly, and average activities are 360.99 ± 4.63 Bq/kg f.w. This behavior of K-40 can be attributed to its high and uniform concentration in the oceans, which consequently does not change levels in coastal marine waters (De Donno et al., 2020). As can be seen in the comparative table (Table 4), our results are in agreement with the results of monitoring in Mali Ston Bay, which was carried out from 2009-2013 as part of the project "Radionuclides and trace elements in environmental systems" published in 2015 in the work of Krmpotić et al.

Table 4 Comparison of activity concentrations in *Mytilus galloprovincialis* from Croatian and BiH part of the Adriatic Sea

			Activity concentration Bq/kg dry weight				
	Authors	Location	Be-7	K-40	Ra-226	U-238	Cs-137
Spring	Krmpotić et al. 2009-2013	Mali Ston bay CRO	78.3 ± 45.9	283 ± 58	<3.1	<10.6	<0.3
	Our results 2022	Neum, BiH	31.9 ± 0.9	381.4 ± 6.8	0.6 ± 0.1	3.6 ± 0.7	0.1 ± 0.02
Autumn	Krmpotić et al. 2009-2013	CRO	23.1 ± 12.7	292 ± 42	<2.9	<13.1	<0.4
	Our results 2022	Neum, BiH	14.5 ±0.8	340.6 ± 6.2	0.7 ± 0.2	3.2 ± 0.7	0.1 ± 0.02

Organisms that are at risk of manifesting the effects of higher radionuclide activities are birds, mammals, phytoplankton and reptiles. The highest risk coefficient is for phytoplankton. The risk coefficient for organisms is in the range of 1.62E-02 for molluscs-bivalvia, and up to 5.73E+01 for phytoplankton. Total dose rates are highest for phytoplankton 2.29E+04 and lowest for molluscs 6.48E+00. Exceeded doses were in the interval of 10 - 100 μSv/h for birds, mammals and reptiles. For all of these three groups Pb-210 accounts for 74.84 %, Ac-228 for 17.28 % and Ra-226 for 5.42 % of the total dose. In the case of phytoplankton, the doses are exceeded 50 times than the screening limits are set. Ac-228 accounts for 94,24 % of the total dose measured for phytoplankton (22903,38 μGy / h), and Pb-210 accounts for 5.57 % or 1275.48 μGy/h, which is still 3 times higher than the screening limit of 400 μGy/h.

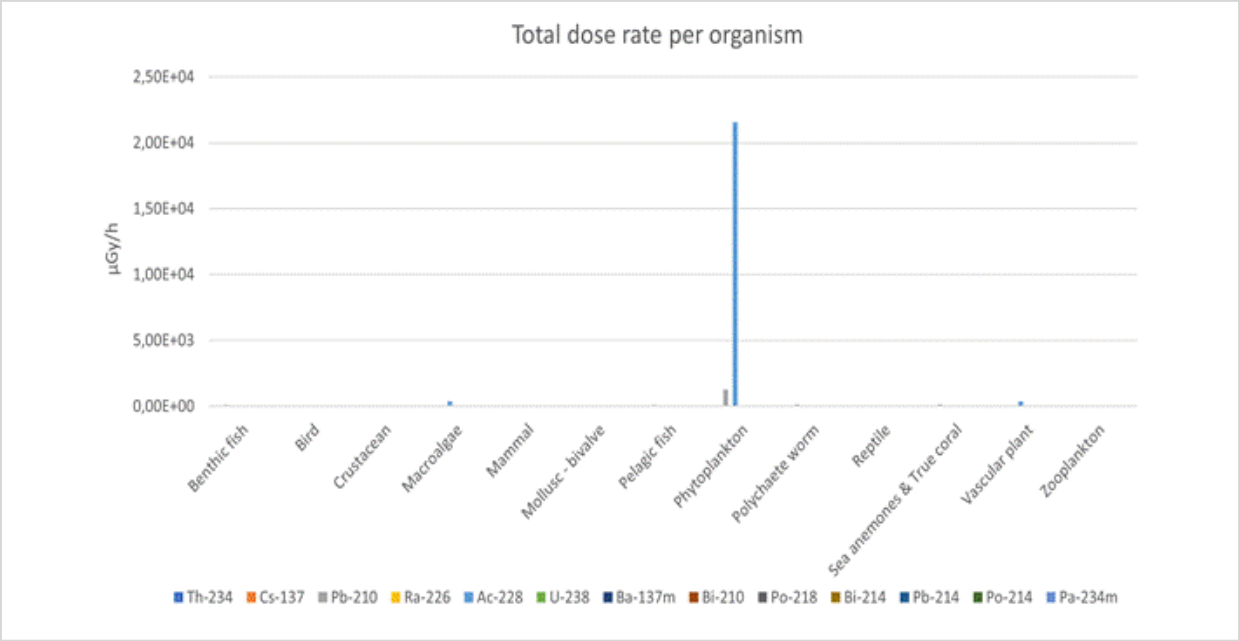

Figure 2 Total dose rate for individual organisms

If 5 kg of mussels are consumed on an annual level, observed radionuclides would produce an effective dose of 25.6 µSv a. The value indicated higher intake concerning the fact the Po-210, as the primary contributor, has not been included in dose-calculation. It can be concluded on the basis of results shown in Table 5. By comparing the annual effective ingestion doses (µSv/a) from various radionuclides received through mussel consumption in different regions for the adult population, it can be concluded that the value obtained in this study generally aligns with the expected results. Effective dose coefficients for ingestion of radionuclides for members of the public from ICRP 119 (2012) show that Po-210 has a much higher dose coefficient due to its alpha emissions, making it more dangerous isotope in terms of ingestion exposure. Since the polonium is not observed in this study, it is expected that the dose from gamma-emitting radionuclides in this study will be lower than doses calculated in other studies that included Po-210 as well. For comparison, Rožmarić (2012) calculated the dose due to Po-210 and Pb-210 as 202 ± 99 µSv/a, with an assumption of 2 kg of mussels being consumed. In this study, the dose was calculated on the basis of ingestion of 5 kg of mussels annually, which is the highest value expected. Estimates and further measurements of the annual doses ingested by our citizens and tourists through the ingestion of aquaculture food are necessary.

Table 5 Annual effective ingestion doses (μSv/a) from various radionuclides received through mussel consumption in different regions for the adult population

Study	Region	Observed radionuclides	Dose μSv/a	m (kg) for dose calculation
Rožmarić (2012)	Croatian Adriatic coast	Po-210, Pb-210	202 ± 99	2
Štrok (2011)	Slovenian part of the Adriatic Sea	Po-210, Pb-210	8.7	0.09
Trotta (2024)	Apulian coast (Italy)	U-238,U-234, U-235, Po-210,Pb-210, Sr-90	5.3	1
Jia (2020)	Six sampling sites in Italy	U-238,U-234, U-235, Po-210,Pb-210, Th-234, Th-230, Th-228, Ra-226, Ra-224, Ra-228, K-40	131-765	6.16
Fonollosa (2016)	Ebro Delta area, Spain	U-238,U-234, Po-210,Pb-210	100.7	1.15
This study	Adriatic Sea, Bosnia and Herzegovina	Be-7, Th-234, K-40, Cs-137, Pb-210, Ra-226, Ac-228, U-238	25.6	5

Table 6 Proven effects of doses on individual organisms

	Effects
Birds	Increase in infestations with parasites of feather and gastroenterine (no value given)
Mammals	Major effect in percentage of voles infected with ectoparasites and low-fatness voles in population (3- fold increase)
	Moderate decrease of life-span (30% decrease)
	Significant increase of life span (1.3 times the control value) – Mice
	Moderate decrease of otter population density (33% reduction)
	Minor decrease of peripheral blood cells (15-50% reversible reduction)
	Minor decrease of body weight (10% reduction). No statistically significant effect on hair density
Reptiles	No data in FREDERICA for effects observed at this dose rate range
Phytoplankton	No data in FREDERICA for effects observed at this dose rate range
> 10000	Minor stimulating effect on growth (1.2-fold)

The assessment of the risk coefficient indicates several groups at risk that should be paid attention to. If we take into account that phytoplankton is the basis of the trophic chain of the marine ecosystem, we can conclude that the danger of potential bioaccumulation of radionuclides in higher levels of the trophic chain is a serious and already obvious problem that needs attention. Insufficient data on the effects of radiation on living organisms at these doses and for individual groups does not mean that there is no risk for the biota (Table 6), but that we should pay more attention in accordance with the consensus symposium organized by the International Union of Radioecology (IUR) in November, 2015 (Prlić et al., 2017), which offered strong statements regarding the ecological effects of radiation on populations and ecosystems while moving towards from an anthropocentric to an ecocentric approach in environmental protection.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Conception: MČ; Design: MČ; Supervision: AA, NG; Materials: MČ, NG; Data Collection and/or Processing: MČ, NM; Analysis and/or Interpretation: MČ, NK, NM, AA; Literature Search: MČ, NK, NM; Writing– Original Draft: MČ, NK, NG; Critical Review: AA, NG

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PRELIMINARNI REZULTATI PRAĆENJA RADIONUKLIDA I PROCJENA RIZIKA ZA BIOTU MORSKOG EKOSISTEMA BOSNE I HERCEGOVINE

SAŽETAK

Cilj istraživanja je, slijedeći primjer mnogih svjetskih programa za praćenje dagnji, prikazati primjer radioekološkog praćenja korištenjem *Mytilus galloprovincialis* L. kao indikatora. Koristeći gama spektrometrijska mjerenja i ERICA softver za procjenu rizika, prikazujemo prve rezultate u radioekološkom smislu koji se odnose na morski ekosistem Bosne i Hercegovine. Organizmi koji su pod rizikom manifestiranja efekata pojačane radionuklidne aktivnosti uključuju ptice, sisare, fitoplankton i reptili. Najveći koeficijent rizika iznosi 5.73E+01 za fitoplankton. U slučaju godišnje konzumacije 5 kg dagnji od strane čovjeka, ispitivani radionuklidi bi proizveli efektivnu dozu zračenja od 25.6 µSv/a.

Ključne riječi: ERICA procjena rizika, Neumski zaljev, praćenje dagnji, radioaktivnost

RESEARCH ARTICLE

STUDY OF THE PREVALENCE OF *CRYPTOSPORIDIUM* SPP. IN CATTLE FARMS IN THE LAGHOUAT REGION, SOUTHERN ALGERIA

Radhwane Saidi[1], Mourad Becheur[2], Mourad Hamiroune[3*], Khadidja Bailiche[4], Lilia Yasmine Laméche[4]

[1] Laboratory of Biological and Agronomic Sciences, Department of Agronomic Sciences, Faculty of Sciences, University of Laghouat, Route de Ghardaia, Laghouat 03000, Algeria
[2] Department of Agronomic Sciences, Faculty of Nature and Life Sciences, University of Laghouat, Route de Ghardaia, Laghouat 03000, Algeria
[3] Laboratory of Exploration and Valorization of Steppe Ecosystems, Faculty of Natural and Life Sciences, University of Djelfa, PO Box 3117, Djelfa 17000, Algeria
[4] Department of Biological Sciences, Faculty of Sciences, University of Laghouat, Route de Ghardaia, Laghouat 03000, Algeria

[*]**Corresponding author:**
Dr. Mourad Hamiroune
Address: Laboratory of Exploration and Valorization of Steppe Ecosystems, Faculty of Natural and Life Sciences, University of Djelfa, PO Box 3117, Djelfa 17000, Algeria
Phone: +213 27900302
ORCID: 0000-0003-0704-595X
E-mail: m.hamiroune@univ-djelfa.dz

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ABSTRACT

The objective of the present work was to detect *Cryptosporidium* spp. infection in cattle farms in Laghouat, Algeria. Our study was carried out for 3 months (February, March and April 2018) and on 300 faecal samples taken from 300 cattle. For this purpose, we used the modified Ziehl–Neelsen staining technique to identify cryptosporidium spp. The parasite prevalence rate was 43.5%. The statistical analysis of the influence of certain variation factors (sex, age, type of breeding, presence of diarrhea and breed) on the prevalence of *Cryptosporidium* spp. revealed no significant effect ($p>0.05$) for the breed, sex, type of breeding and deworming. However, the effect of age was very significant ($p<0.01$) and that of the presence of diarrhea was also significant ($p<0.05$). These results reflect the risk posed by *Cryptosporidium* spp. to cattle and to public health. This requires the implementation of a disease control and awareness program to avoid various accidents and significant economic losses. In addition, early diagnosis of the disease helps preserve animal and human health and the national economy, subsequently.

Keywords: Bovine, Algeria, *Cryptosporidium* spp., prevalence

INTRODUCTION

Cattle breeding represents one of the most important agricultural activities in the world. It plays a fundamental role at the economic, ecological, environmental and cultural levels (Faye, 2001). In Algeria, like all Maghreb countries, cattle breeding is among the oldest activities; it plays a relatively important role both in the national agricultural economy and for breeders, thus providing a considerable financial reserve (Benghida, 2021).

However, this breeding is subject to nutritional, environmental or pathological risks. Among the pathologies which can cause mortality

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and/or poor zootechnical performance, we can cite parasitic diseases, including cryptosporidiosis.

Cryptosporidiosis is of considerable importance in newborn ruminants, in which it is characterized by mild to severe diarrhea, lethargy and poor growth rate (Paraud et al., 2009). The source of cryptosporidian infection is oocysts that are fully spore-forming and infectious when shed in the feces. Transmission can occur directly from calf to calf, indirectly through passive or human transmission, through environmental contamination, or through fecal contamination of food or water supplies. Diagnosis of cryptosporidiosis is based on detection of oocysts by examination of fecal smears with Ziehl-Neelsen stains, fecal flotation techniques, ELISA, fluorescently labeled antibodies, rapid immunochromatographic testing, and PCR (Witola, 2021).

Cryptosporidiosis is an emerging opportunistic parasitic disease (Chermette et Boufassa-Ouzrout, 1988). Most often asymptomatic, it can sometimes manifest clinically as digestive disorders in immunocompromised subjects in the presence of intercurrent conditions. The causative agent is an intracellular protozoan of the coccidia family belonging to the genus *Cryptospridium* (Fayer and Santin, 2009; Squire et al., 2013). Several species have been isolated from a large number of vertebrates, including humans, in whom the interest in this parasite is directly linked to the occurrence of epidemics since 1980.

Cryptosporidiosis has been described in many animal species, both domestic and wild. In ruminants, more precisely the bovine species, it is generally the youngest animals which are the most receptive and most sensitive to infection, while infected adult animals are few in number and asymptomatic (Fayer and Santín, 2009; Paraud et al., 2009; Silverlå at al., 2010). Currently, cryptosporidiosis is one of the leading causes of diarrheal enteritis in newborn calves (Silverlås et al., 2010). This infection causes significant economic losses in newborn ruminants due to the mortality and morbidity it causes. The average prevalence of this infection varies widely between countries and different ruminant host species (Silverlås et al., 2010).

To our knowledge, there have been only two studies on cryptosporidiosis in cattle in the Laghouat region (Chikhaoui and Touhami, 2015). Also, the national bibliography has been enriched in recent years with some research work on bovine cryptosporidiosis, particularly in some regions of the East and central North (Khelef et al., 2007; Ouchene et al., 2014). Therefore, the present study aims to evaluate the prevalence of *Cryptosporidium* spp. and risk factors implicated on cryptosporidian infection in cattle in the wilaya of Laghouat.

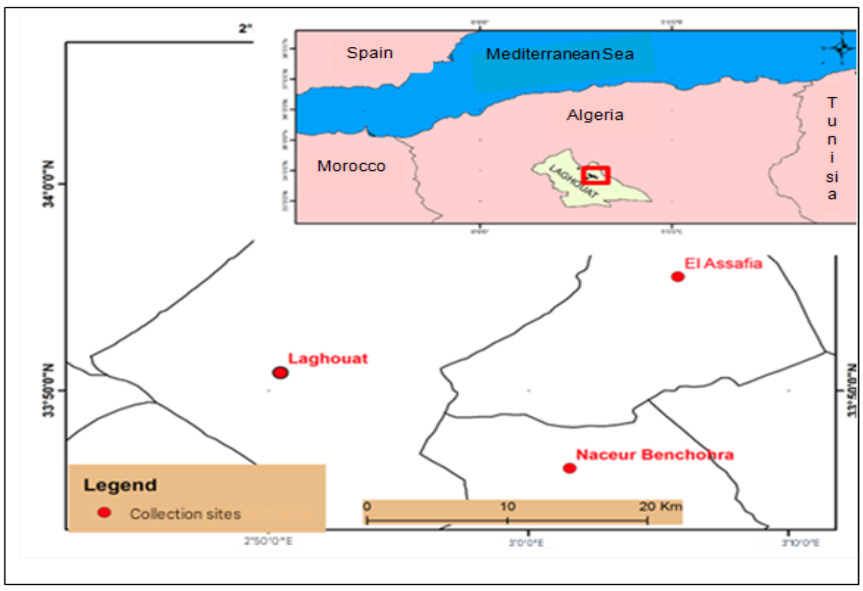

Figure 1 Location map of sampling sites (ARCGIS V10.2 software)

MATERIAL AND METHODS

Location and period of study

This study was carried out at three sites in the Laghouat region in the south of Algeria: Laghouat-City center, El-Assafia, and Bennaceur Benchohra (Figure 1). Our study was carried out for 3 months, from February to April 2018.

The coprological analyzes took place in Parasitology Laboratory of the Biology Department of Amar Telidji University of Laghouat and Laghouat Regional Veterinary Laboratory.

Animal material

The study was carried out on 13 farms, including 4 large livestock farms and 9 small dairy cow farms. A total of 300 cattle were sampled in this study. The characteristics of the animals studied are presented in Table 1.

Table 1 Characteristics of the cattle studied

Characteristics		Number of animals per study site (%)			Total
		Municipality of Laghouat	Municipality of Bennaceur Benchohra	Municipality of El-Assafia	
Gender	Female	141(47%)	35(11.7%)	20(6.7%)	196(65.3%)
	Male	76(25.3%)	13(4.3%)	15(5%)	104(34.7%)
Race	Prim'Holstein	160(53.3%)	26(8.7%)	19(6.3%)	205(68.3%)
	Montbéliarde	57(19%)	22(7.3%)	16(5.3%)	95(31.7%)
Age	From 14 days to 30 days	14(4.7%)	0(0%)	6(2%)	20(6.7%)
	From 1 month to 3 months	53(17.7%)	17(5.7%)	7(2.3%)	77(25.7%)
	From 4 months to 12 months	43(14.3%)	1(0.3%)	1(0.3%)	45(15%)
	From 1 year to 2 years	43(14.3%)	7(2.3%)	12(4%)	62(20.7%)
	More than 2 years	64(21.3%)	23(7.7%)	9(3%)	96(32%)

The characteristics of the farms visited are presented in Table 2.

Table 2 Characteristics of the farms visited during this study

Characteristics		Number of animals per study site (%)			Total
		Municipality of Laghouat	Municipality of Bennaceur Benchohra	Municipality of El-Assafia	
Type of farming	**Intensive**	127 (42.3%)	31 (10.3%)	0 (0%)	158 (52.7%)
	Semi-intensive	90 (3%)	17 (5.7%)	35 (11.7%)	142 (47.3%)
Deworming	**Dewormed animals**	105 (35%)	31 (10.3%)	15 (5%)	151 (50.3%)
	Animals not dewormed	112 (37%)	17 (5.7%)	20 (6.7%)	149 (49.7%)

Parasitology

Sampling and laboratory examination

Sampling was carried out randomly, depending on the availability and cooperation of the breeders contacted. The samples were based on animal feces, taken directly from the rectum. After collecting the feces, they were placed in sterile collection boxes, labeled (the date of collection, age, sex and possibly the breed of the animal are mentioned) then transported in a cooler to the laboratory. Samples that are not analyzed the same day are stored cold at 4°C.

In the laboratory, observation of stools with the naked eye was carried out, in order to assess in particular the color, the appearance of stools, the presence of blood, pus or mucus and observation of different forms of parasites (eggs, larvae, worms etc.). Second, to test for *Cryptosporidium* spp. oocysts, a smear was prepared for each sample. Then, the smear was colored using the Ziehl-Neelsen staining technique modified by Henriksen and Pohlenz (1981), starting by lethally spreading a drop of stool as finely as possible on a slide which will be fixed in ethanol at 95% for 5 minutes. The blade was then flamed with a Bunsen burner and covered while still hot with Ziehl fuchsin and left to act for 5 min. Then, it was rinsed with tap water until excess fuchsin was eliminated, then sprayed with one or two jets of 3% HCL in 95% ethanol, rinsing with water each time. After rinsing with water, the slide was soaked in 0.25% malachite green or methylene blue for 30 seconds. Finally, after drying, an optical microscope observation (×100 objective) was carried out without covering a coverslip (using an immersion oil). When positive, the oocysts are colored red or pink on a green or blue background.

Calculation of total prevalence

It is the percentage ratio P (%) of the number of hosts infested by a given species of HP parasite to the total number of hosts examined HE (Margolis et al., 1982).

P (%) = HP/HE x 100

Statistical processing of data

The recorded results were grouped in an Excel 2007 file for creating graphs and calculating prevalences. The effect of the variation factors was analyzed using SPSS software (Version 20) using the Chi-square test. The difference is considered significant at a threshold of $p < 0.05$.

RESULTS

***Cryptosporidium* spp. observed in the animals studied**

Oocysts colored bright red or pink on a green background. Some may appear as empty discs,

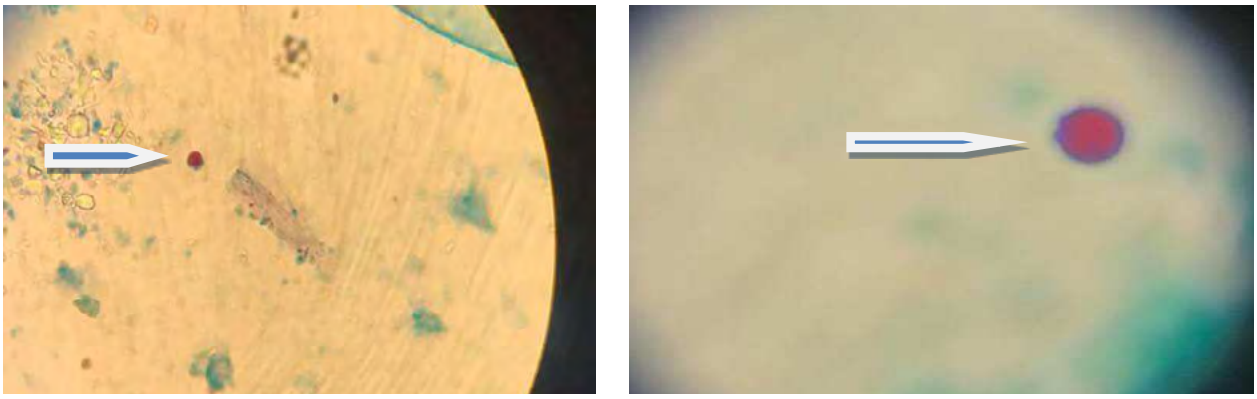

Figure 2 Oocysts of *Cryptosporidium* spp. Observed under the optical microscope: G x 1000, immersion oil (modified Ziehl-Neelsen coloring)

while others may contain crescent-shaped elements that are characteristic of sporozoites (Figure 2).

General prevalence of *Cryptosporidium* spp.

Of the 300 cattle sampled, 164 were infected with *Cryptosporidium* spp., representing an overall prevalence rate of 45.3%

Study of the influence of certain parameters on the rate of infection by *Cryptosporidium* spp.

Influence of age

The rate of parasitism according to age shows that the infection rate of animals from 14 days to 30 days (100%) was higher than that of the other groups. Statistical analysis revealed that the difference was very significant ($p<0.001$), Table 3.

Table 3 Representation of the parasitism rate among age groups

	Number of positive cases/total number	Prevalence of *Cryptosporidia* (%)
G1 : From 14 days to 30 days	20/20	100
G2 : From 1 month to 3 months	63/77	81.8
G3 : From 4 months to 12 months	21/45	46.7
G4 : From 1 year to 2 years	24/62	38.7
G5 : More than 2 years	8/96	8.3

Influence of gender

Generally speaking, the rate of parasitism in females (47.4%) was higher than that in males (41.3%) (Table 4). However, statistical analysis revealed that the difference was not significant.

Table 4 Representation of the parasitism rate according to sex, breed, type of breeding and antiparasitic treatment

Characteristics		Number of positive cases/ total number	Prevalence of *Cryptosporidia* (%)
Gender	**Female**	93/196	47.4
	Male	43/104	41.3
Race	**Prim'Holstein**	102/205	49.8
	Montbéliarde	34/95	35.8
Type of farming	**Intensive**	74/158	46.8
	Semi-intensive	62/142	43.7
Deworming	**Dewormed animals**	69/151	45.7
	Animals not dewormed	67/149	45

Influence of race

The rate of parasitism depending on the breed, as illustrated in Table 4, shows that the infection rate in cattle of the Prim'Holstein breed (49.8%) was higher than that of the Montbéliarde breed (35.8%). However, statistical analysis did not reveal a statistically significant difference ($p>0.05$).

Influence of the type of breeding

The rate of parasitism depending on the type of breeding, as illustrated in Table 4, shows that the infection rate in intensive breeding (46.8%) was higher than that of semi-intensive breeding (43.7%). However, statistical analysis revealed that the difference was not significant ($p>0.05$).

Influence of deworming

The rate of parasitism as a function of deworming, as illustrated in Table 4, shows that there is no big difference between the infection rate in dewormed cattle (45.7%) and non-dewormed cattle (45.0%). In this sense, the statistical analysis revealed that the difference was not significant

Influence of diarrhea

The rate of parasitism depending on the presence of diarrhea or not, as illustrated in Figure 3, shows

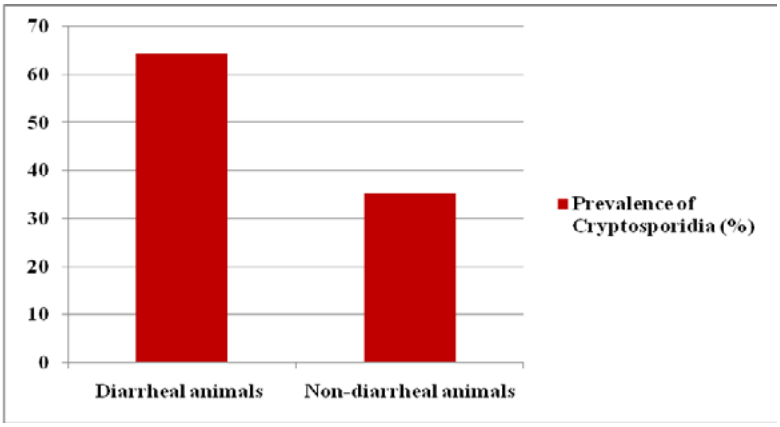

Figure 3 Graphical representation of the parasitism rate according to the presence of diarrhea

that the infection rate of diarrheal animals (64.4%) was higher than that of non-diarrheal animals (35.2%). In addition, the statistical analysis revealed that the difference is very significant ($p < 0.001$).

DISCUSSION AND CONCLUSION

Our results revealed a high presence of *Cryptosporidium*-infection in cattle in the studied region. Indeed, the presence of the parasite is noted in all the sites studied but in varying proportions.

The stool examination revealed a rate of 45.3% for *Cryptosporidium* spp. This value is close to that recorded by Chikhaoui and Touhami (2015) (45%) in the same region, therefore under the same climatic conditions. This last result is explained by its authors by the fact that the animal population studied was essentially composed of adults, knowing that excretion in young animals is significantly higher than in adults (Khelef et al, 2007). Our result is slightly higher than that found by Khelef et al., (2007) in eastern and central Algeria (17%). However, it is lower than that recorded by Ouchane et al. (2014) in Sétif (69.2%).

The difference in the prevalence of *Cryptosporidium* spp. recorded during our survey compared to the results of other studies must be related to the breeding management practiced, the choice of the population studied, and the climatic factors which condition the epidemiology of the parasite, such as temperature, humidity and oxygenation.

The evolution of the incidence of cryptosporidiosis over time showed that parasitic infections were higher in spring and winter during our study period (February-March-April). This could be linked less to the season factor, but to the greater concentration

of calvings over time, which would be responsible for massive contamination of calves via oocysts pre-existing in the boxes (or premises) and those newly released by young and /or adults ; this would also be linked to the great promiscuity that results. Similar observations are reported by Henriksen and Krogh (1985), Ongerth and Stibb (1989) and Dărăbuş et al., (2001). Indeed, according to these authors, the high incidence recorded in winter is mainly attributed to the mode of animal breeding, a period during which animals are grouped in stables, which increases the spread of the parasite between conspecifics.

All of our results suggest the presence of particular receptivity of the newborn calf (from 14 to 30 days) to *Cryptosporidium* spp. in the first weeks of age (100%), with a highly significant difference ($p<0.001$). This is consistent with the observations of numerous authors (Stein, 1982; Sobeih et al., 1987; Ongerth and Stibb, 1989) who report the great receptivity of calves belonging to this age group to cryptosporidia, with, however, a maximum incidence in the second week after birth. Some authors report this receptivity of young people to their deficient immune state in the first days. Colostrum intake at this level plays an important role, if only in the clinical expression of diarrhea. At the end of the first week, and throughout the second and third, is the period of maximum excretion. It explains the strong positivity during this period, which is consistent with several works in the literature (Naciri et al., 1999).

It is, therefore, established that cryptosporidia mainly affects lambs less than one month old (100%) and, to a lesser degree, those aged between 4 and 12 months (46.7%).

However, 32/300 cases of infection are noted in adults. This sensitivity of adults seems to be linked to the stress of peripartum (gestation, lactation and drying off) and calving which affects certain cows, poor hygiene conditions would also be responsible (Villacorta et al, 1991). The low excretion of the parasite by adults over time is also reported in the work of Henriksen and Krogh (1985).

The relationship between the sex of the cattle studied with the rate of parasitism shows that the infection of females in general was not significantly higher (47.4%) compared to males (41.3%). We did not find an explanation for this observation in the literature available to us. The distribution of results according to the clinical status (presence or absence of diarrhea) of cattle is consistent with what is reported by other researchers: cryptosporidia are more isolated in diarrheal subjects (64.4%) compared to those without diarrhea (35.2%). Indeed, among the former, depending on the studies, the positivity rate varies from 10 to 76% (Heine and Boch, 1981; Ongerth and Stibbs, 1989), while among the latter the carriage frequency varies from 4% to 37%. (Nagy et al., 1980; Pivont et al., 1981; Heine and Boch, 1981).

Some authors have shown a significant correlation between the presence of diarrhea and the excretion of *Cryptosporidium* (Naciri et al., 1999; Castro-Hermida et al.,2002).

On the other hand, according to the study by Atwill's team dating from 1999, there is a weak correlation between the presence of liquid faeces and the excretion of the protozoan. In general, the presence of blood and liquid faeces is rather associated with a coexistence between *C. parvum* and other enteropathogenic agents (mixed infection with rotavirus, coronavirus, *Salmonella*, or others) (Naciri et al., 1999).

Regarding the influence of breed on cryptosporidian prevalence, 49.8% of Prim'Holstein breed (black magpie) cattle were infested with *Cryptosporidium* spp. compared to 35.8% in the Montbéliarde breed (red magpie). However, the difference was not significant. The black piebald breed is much more dairy than meat; on the other hand, the red magpie is mixed with a meaty tendency. The absence of the effect of this factor would be explained by the fact that the breeds studied were both improved.

Other authors have also studied the breed effect (Chikhaoui and Touhami, 2015) by comparing receptivity to *Cryptosporidium* spp. between local breed cattle and those of cross breeds. Statistical analysis revealed that the difference was not significant ($p > 0.05$).

The present study revealed no effect of the breeding method on the infection rate of *Cryptosporidium* spp. Indeed, cattle raised in intensive mode were infested in an almost similar manner to those raised in semi-intensive mode. It also diverges with the results of Ghesquier et al. (2003) and Marechal (2004) who confirm that the high concentration of livestock in livestock premises constitutes a contributing cause of the inter-individual spread of this parasite.

The administration of antiparasitic treatment as a preventive measure did not have a significant effect on cryptosporidian prevalence between treated and untreated animals. This confirms the literature data which note the ineffectiveness of various molecules tested to fight against this parasite. Indeed, *Cryptosporidium* holds a unique position in the host cell since the parasite is intracellular but extra-cytoplasmic (Manent-Manent, 2014). It thus escapes the intracellular action of antiparasitic drugs. In addition, controlling environmental contamination is difficult due to the very high resistance of oocysts in a natural environment but also in the presence of common disinfectants (Manent-Manent, 2014).

The results of this study confirm that the prevalence of bovine cryptosporidiosis varies from one animal to another. Infection with this disease is considered an important indicator of insufficient control of *Cryptospridium* spp. which takes animals as its main reservoir, which constitutes a risk for animal and human health in the event of contact or consumption of the oocyst of this parasite. This requires increasing the number of cattle checked each year in order to preserve animal health and consumer health, which is directly linked to the eradication of bovine cryptosporidiosis through medical and sanitary interventions.

For this reason, important are awareness and popularization among breeders, consumers and other stakeholders in the veterinary and public health sector. In addition, it would be very useful to carry out systematic screening on all farms in the country through general and specific laboratory examinations for confirmation. It is obligatory for cattle farms where the breeder has an agreement with the Directorate of Veterinary Services (DVS) for the carrying out of early detection tests for the disease, which will help to better fight against the disease in cattle and preserve the health of consumers and thus, ultimately, bring benefits to the entire economy of the country.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTIONS

Conception: RS; Design: RS, MH; Supervision: RS, MB, MH; Materials: KB, LYM; Data Collection and/or Processing: KB, LYM; Analysis and/or Interpretation: RS, MB, MH; Literature Search: MH; Writing – Original Draft: RS, MB, MH; Critical Review: RS, MH

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STUDIJA PREVALENCE *CRYPTOSPORIDIUM SPP* NA FARMAMA ZA UZGOJ STOKE U REGIJI LAGHOUAT U ALŽIRU

SAŽETAK

Cilj našeg istraživanja je otkrivanje infekcije sa *Cryptosporidium* spp. na farmama stoke u Laghouatu u Alžiru. Istraživanje smo provodili 3 mjeseca (februar, mart i april 2018. godine) na 300 fekalnih uzoraka prikupljenih od 300 goveda. U tu svrhu smo koristili modificiranu tehniku bojenja po Ziehl–Neelsenu sa ciljem identifikacije Cryptosporidium spp. Prevalenca parazita je iznosila 43.5%. Statistička analiza utjecaja pojedinih varijacijskih faktora (spol, starost, način uzgoja, prisustvo dijareje i pasmina) na prevalencu *Cryptosporidium* spp. nije otkrila statistički signifikantan utjecaj ($p>0.05$) pasmine, spola, načina uzgoja i deparazitizacije. Međutim, utjecaj starosti je bio statistički veoma signifikantan ($p<0.01$), kao i prisustvo dijareje ($p<0.05$). Ovi rezultati odražavaju rizik koji *Cryptosporidium* spp. predstavlja kako za stoku tako i za javno zdravstvo. Ovo zahtjeva implementaciju kontrole bolesti i program podizanja svijesti sa ciljem izbjegavanja nesreća i signifikantnih ekonomskih gubitaka. Osim toga, rano dijagnosticiranje bolesti pomaže očuvanju zdravlja životinja i ljudi, a posljedično i ekonomije.

Ključne riječi: Alžir, bovini, *Cryptosporidium* spp, prevalenca

RESEARCH ARTICLE

IMPACT OF ALKALOID OF *PEGANUM HARMALA* EXTRACT ON OXIDATIVE STRESS BIOMARKERS AND HISTOMORPHOMETRICS OF TESTICULAR TISSUES IN MALE MICE *(MUS MUSCULUS)*

Hanane Derbak[1], Amira Chahrazad Benabdelhak[1], Omar Besseboua[3], El-Hacene Balla[1,2], Abdelhanine Ayad[1*]

[1]Department of Biological Sciences of the Environment, Faculty of Nature and Life Sciences, University of Bejaia, 06000 Bejaia, Algeria.
[2]Laboratory of Applied Zoology and Animal Ecophysiology, Faculty of Nature and Life Sciences, University of Bejaia, 06000 Bejaia, Algeria.
[3]Department of Agricultural Science, Faculty of Natural and Life Sciences, Mascara University, 29000 Mascara, Algeria.

***Corresponding author**:
Prof. dr. Abdelhanine Ayad
Address: Faculty of Nature and Life Sciences, University of Bejaia, 06000 Bejaia, Algeria
ORCID: 0000-0002-9325-7889
E-mail:
abdelhanine.ayad@univ-bejaia.dz

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ABSTRACT

The study was designed to determine the impact of administration of *P. harmala* total alkaloid extract on testicular oxidative stress biomarkers and histomorphometrics of testicular tissues in male mice. After two weeks of acclimatization, twenty eight Swiss albino male mice (Mus musculus) aged 8 weeks were randomly divided into equal four groups (n=7). Male mice received daily by gavage an alkaloid extract of P. harmala at different concentrations (0, 6.25, 12.5 and 25 mg/kg BW/day in carboxymethyl cellulose vehicle 0.5%) for 35 days. After the sacrifice of male mice, the testes were collected and stored at -80°C, while another set was immediately preserved in 10% formalin after removal from the animals. At the end of the trial, oxidative stress parameters and histomorphometric analyses of testicular tissues were evaluated. A significantly lower value of testicular malondialdehyde (MDA) was observed in the treated groups with the total alkaloid extract of *P. harmala* compared to the control group ($P<0.05$). The mean level of superoxide dismutase increased in the groups treated with the 6.25 mg/ kg and 25 mg/ kg of the extract (0.44±0.07, 0.5±0.04 U/mg protein, respectively) compared to the control (0.4±0.08 U/ mg protein). The results show a significant ($P<0.05$) increase of catalase activity in the group treated with a concentration of 25 mg/ kg. Additionally, the histological pattern of the testes in male mice treated with P. harmala extracts appears normal. The diameter of seminiferous tubules and germinal epithelium thickness appeared to increase in the *P. harmala*-treated groups with different extract concentrations. It should also be noted that luminal height values were higher in male mice treated with a concentration of 25mg/kg than in mice in the control group ($P> 0.05$). Based on our results, the total alkaloids of P. harmala appear to protect testicular tissues against oxidative stress, suggesting their potential as an antioxidant source. Furthermore, the positive effects of *P. harmala* seeds extract, especially at a dose of 25 mg/kg body weight, are interesting in relation to the histomorphometric measures of testes.

Keywords: Male mice, oxidative stress, Peganum harmala, testicular histomorphometrics

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INTRODUCTION

Infertility is a dysfunction of the reproductive system characterized by the inability to achieve pregnancy after a year of unprotected sexual intercourse. It is a global clinical issue with a rising incidence, affecting millions of couples worldwide involving both sexes (WHO 2021). Male infertility is defined as quantitative or qualitative deficit in male reproductive cells, such as the absence of spermatozoa (azoospermia), reduced sperm mobility (asthenospermia), and abnormal sperm morphology (teratospermia) (Al-Tawalbeh et al., 2023). It can also be caused by a genetic disorder, hormonal imbalance, blockage of the reproductive tubules, erectile dysfunction, systematic disease, or caused by poor lifestyle practices, *i.e.* smoking, stress, obesity, drug use, advanced marital age, and exposure to environmental pollutants. These factors can disrupt the oxidative balance of the testes, as spermatogenesis and Leydig cell steroidogenesis are vulnerable to oxidative stress, resulting in lipid and protein peroxidation as well as DNA damage to sexual cells (Aitken and Roman, 2008; Bisht et al., 2017). Consequently, these factors can lead to histological alterations, a reduction in spermatogenesis, and a decrease in the physiological capacity for fertilization (Agarwal et al., 2014).

Harmel (*Peganum harmala*) is a plant native to the eastern Mediterranean region to East India and belongs to Nitrariaceae family. It is known as Wild rue, Syrian rue and African rue. It is a perennial herb that grows in arid and semi arid regions, steppe areas, and sandy soils (Aslam et al., 2014). *P. harmala* is consumed due to its various nutritional qualities and considered as a universal medicinal plant. It is used to address various problems, including male infertility (Singh et al., 2013). The alkaloid extract of *P.harmala* has a diverse range of pharmacological activities, such as antibacterial (Iranshahy et al., 2019), analgesic (Farouk et al., 2008), anti-inflammatory (Ramadhan et al., 2013), antioxidant (Abbas et al., 2021), hypoglycemic (Ramazani et al., 2014), and antitumor (Zhang et al., 2022). Furthermore, extracts from *P. harmala* seeds have been shown to have positive and varied impacts on male reproductive health. A recent study reported that the alkaloid extract of *P. harmala* seeds grown in Algeria, had an impact on the sexual performance and sperm quality of male mice (Derbak et al., 2014). Moreover, the extracts demonstrated the ability to stimulate sperm motility, preserve membrane integrity, and protect ram spermatozoa against lipid peroxidation (Derbak et al., 2021). However, to the best of our knowledge, the investigation on effect of *P. harmala* seed alkaloids on testicular antioxidant system status and its histological structure have not been reported. Thus, the aim of this study is to determine the impact of administration of *P. harmala* total alkaloid extract on testicular oxidative stress biomarkers (malonaldehyde, superoxide dismutase and catalase activities) and histomorphometrics of testicular tissues in male mice.

MATERIALS AND METHODS

This research adhered to the guidelines for the ethical treatment of laboratory animals. Approval for the proposed experiments was obtained from the Ethics Committee of the Faculty of Natural and Life Sciences, University of Bejaia (Report of Faculty Scientific Council #05 dated 14 December 2016).

Plant material and extraction

Peganum harmala seeds were collected in April 2016 from Ngaoues region (35°32'N, 6°10'E, Batna province, Algeria). The scientific authentication of plant seeds was carried out by the botanist of the Bejaia University. The voucher specimen (PhB080) is kept in the herbarium of the Pharmacy Department (University of Batna, Algeria). 1 kg of air-dried powdered plant material was first exhaustively extracted with ethanol (96%, v/v) in a Soxhlet apparatus (Behr Labor-Technik GmbH, Dusseldorf, Germany) for 8 h. The solvent from the ethanolic extract was completely removed and concentrated using a rotary evaporator (BüchiLabortechnik AG, Flawil, Switzerland). The ethanolic extract was then concentrated and acidified with HCl (2%, v/v), and extracted

with petroleum ether (5000 mL) to remove the apolar phase. The acidic aqueous solution was basified three times to pH 9 with ammoniac and extracted with dichloromethane (1000 mL). The dichloromethane layer was evaporated under vacuum conditions to obtain approximately 0.05 % w/w of alkaloids fraction (Kartal et al., 2003).

Animals and preparation

Twenty-eight adult Swiss albino male mice (*Mus musculus*) aged 8 weeks were obtained from the animal house of the Central Faculty (University of Constantine, Algeria). The animals were kept under standard conditions of temperature, humidity, light/dark (23 ± 2°C, 55 ± 5% and 12:12h, respectively). During this period, the mice were fed on a standard diet and given tap water *ad libitum*. All animal procedures were conducted following the recommendations of the International Ethics Committee. (Directive 2010/63/EU, which updated and replaced the Directive of the European Council 86/609/EC).

After two weeks of acclimatization, twenty-eight animals were randomly divided into four groups per seven mice. Mice received a daily gavage of an alkaloid extract of *P. harmala* at different concentrations (6.25, 12.5 and 25 mg/kg BW/day in carboxymethyl cellulose vehicle 0.5%) for 35 days. Concerning the control group, animals received only carboxymethylcellulose vehicle (0.5%; 10 mL/kg). After the experimental regimen (24 h) following oral administration of the extract, the mice were sacrificed by cervical dislocation under chloroform anesthesia, and their testes were collected. One testicle was preserved at -80°C and another in 10% formalin immediately after removal from the animal.

Preparation of testicular tissue homogenate

The tunica albuginea was manually removed from the testes. Testicular tissues were homogenized in 3 mL of PBS (pH 7.4) for 5 min. Subsequently, the homogenate was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant obtained was used for the determination of lipid peroxidation, superoxide dismutase, and catalase activity.

Biochemical measurements

Total protein estimation

The protein concentrations of gut samples were determined by the method of Bradford (1976) using Coomassie Brilliant Blue G-250. Bovine serum albumin was used as a standard. The absorbance values were measured at 595 nm using a spectrophotometer.

Measurement of lipid peroxidation

Malondialdehyde (MDA) is the end product of lipid peroxidation, serving as an indicator of oxidative stress. MDA was quantified using thiobarbituric acid (TBA) assay according to Buegeand Aust (1978). Briefly, 1 ml of the stock solution TBA-TCA-HCl containing (trichloracetic acid 15% w/v, thiobarbituric acid 0.375% w/v in hydrochloric acid 0.25 N) was added to one volume of the tissue homogenate. The mixture was kept in boiling water bath for 60 min and then cooled in an ice bath. The suspension was centrifuged at 18000 g for 15 min. The thiobarbituric acid reactive substances (TBARS) were measured using a spectrophotometer (Biotech Engineering Management Co. Ltd. UK VIS-7220G) at a wavelength of 532 nm. The molar extinction coefficient for MDA is 1.56×10^5 M^{-1} cm^{-1}. The value is expressed as nmol of MDA equivalent formed/mg of the testis.

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was assayed by the method of pyrogallol autoxidation, according to Marklund and Marklund (1974). Testicular supernatant was prepared in 1440 µL of Tris–HCl buffer (50 mM/L mM EDTA, pH 8.2), 100 µL of pyrogallol solution (15 mM/10 mM HCl). The SOD activity was measured at 420 nm for 3 min. One unit of SOD was determined as the amount of enzyme that inhibited the oxidation of pyrogallol 50 %. The SOD activity obtained was expressed as U/min/mg of protein.

Determination of catalase (CAT) activity

Catalase activity was estimated by measuring the decomposition of hydrogen peroxide (H_2O_2)

at 240 nm absorption (25 °C for 60 s) (Aebi 1984).The reaction mixture consisted of 1255 µL of phosphate buffer (50 mM, pH 7.0), 20 µL of supernatant, and 725 µLof H_2O_2 (54 mM). The molar extinction coefficient for MDA is 43.6 l/ mol per cm. One unit of the catalase activity was expressed as the amount of H_2O_2 consumed per minute per milligram of protein.

Testis histology

The testes from mice were fixed in buffered 10 % formalin and subsequently processed for routine histopathology. The processed testes were dehydrated in a gradient of alcohol (70-95%), followed by clearing and embedding in paraffin wax. Serial sections, each 2-3 µm thick, were meticulously obtained using a rotary microtome (Leica RM2125 RTS). Subsequently, these sections were stained with hematoxylin and eosin. Examination of the slides was conducted under a light microscope (X10 magnification), and measurements were recorded, including the diameter of seminiferous tubules, thickness of germinal epithelium, diameter of the lumen in testes, and thickness of the tunica albuginea. All morphometric analyses were performed using Image J software (Version 1.52, NIH, USA).

Statistical analysis

Results were expressed as means ± standard error (SEM). Experimental data were analyzed using GraphPad Prism Graph Pad Prism 5.0 (San Diego, CA, USA). Analysis of variance (ANOVA) was used, followed by Tukey's test. Results were considered statistically significant at $P<0.05$.

RESULTS

The effect of the total alkaloid extract of *P. harmala* on testicular MDA levels in mice is illustrated in Figure 1. A significantly lower value of testicular MDA was observed in the treated groups with 6.25 mg/kg, 12.25 mg/kg, 25 mg/ kg of the total alkaloid extract of *P. harmala* (52.93 ±5.47, 55.08 ±3.17 and 58.81±3.16 nmol/ mg, respectively) compared to the control group (76.07±4.14 nmol/mg) ($P< 0.05$).

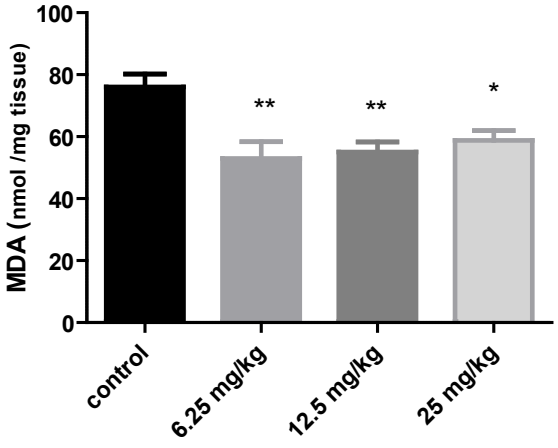

Figure 1 Effect of *Peganum harmala* alkaloid fraction on testicular tissue MDA levels after 35 days of administration. One-way ANOVA followed by Tukey comparison tests was used for statistical significance *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control group

Figure 2 shows the level of Superoxide dismutase (SOD) in different groups after 35 days of administration of total alkaloid extract *P. harmala*. The mean level of superoxide dismutase increased in the groups treated with the 6.25 mg/ kg and 25 mg/ kg of the extract (0.44±0.07, 0.5±0.04 U/mg protein, respectively) compared with the control (0.4±0.08 U/mg protein). However, this rise was statistically non-significant in the treated group with 25 mg/ kg of total alkaloid extract *P. harmala* ($P> 0.05$).

Figure 3 illustrates the effects of different concentrations of total alkaloid extract *P. harmala* on catalase levels in testicular tissues after 35 days of treatment. Results show a significant ($P <0.05$) increase of catalase activity in the group treated with concentration 25 mg/ kg (33.83± 1.7µmol/ H_2O_2/mg prot/min). Mice treated with 6.25 mg/ kg and 12.5 mg/kg concentration of total alkaloid extract *P. harmala* showed a slight, non-significant

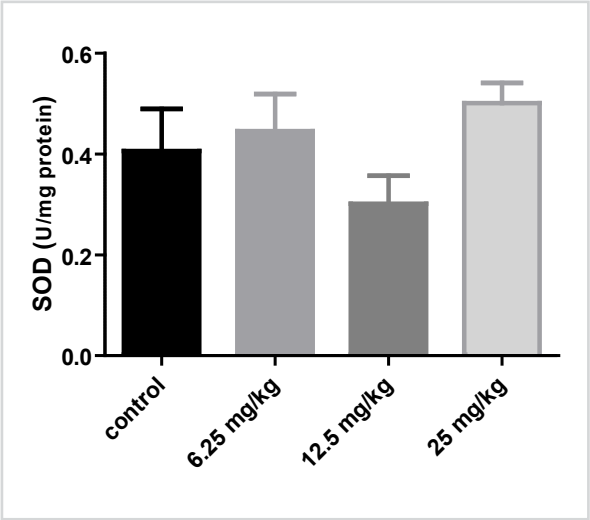

Figure 2 Effect of *Peganum harmala* alkaloid fraction on testicular tissue SOD levels after 35 days of administration. One-way ANOVA followed by Tukey comparison tests was used for statistical significance

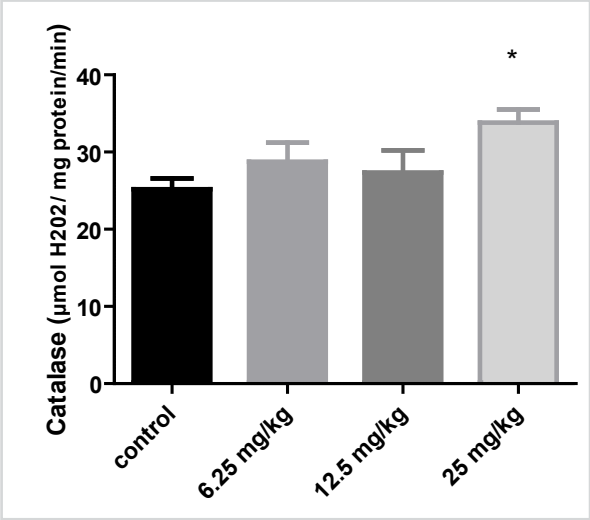

Figure 3 Effect of *Peganum harmala* alkaloid fraction on testicular tissue catalase after 35 days of administration. One-way ANOVA followed by Tukey comparison tests was used for statistical significance **P* < 0.05, ***P* < 0.01, ****P* <0 .001 compared to control group

increase in catalase capacity compared to the control group (25.17± 1.41µmol/ H_2O_2/mg prot/ min).

The histological pattern of the testes of mice treated with total alkaloid extract *P. harmala* appears normal. The spermatogenic cells and Sertoli cells of the seminiferous tubules exhibit regular morphological characteristics. The seminiferous tubules have a regular appearance, lined with well-arranged rows of spermatogenic cells at different stages. Histomorphometric measurements of testicular tissues after 35 days of administration of *P. harmala* extracts in mice were presented in Table 1.

Table 1 Seminiferous tubule diameter, epithelium high, luminal height, and tunica albuginea width of male mice administered with different doses of *Peganum harmala* alkaloid fraction for 35 days. One-way ANOVA followed by Tukey comparison tests was used for statistical significance **P* < 0.05, **^{P} < 0.01, ****P* < .001 compared to control group

Parameters	Experimental groups			
	Control	6.25 mg/kg	12.5 mg/kg	25 mg/kg
Seminiferous tubule diameter (µm)	224.9 ± 3.34	237.5 ± 1.3	235.4 ± 3.2	252.2 ± 4.73***
Epithelial height (µm)	67.9 ± 2.1	68.3 ± 1.86	70.7 ± 2.16	79.8 ± 2.95**
Luminal height (µm)	99.1 ± 2.56	99.4 ±4.41	92.4 ± 2.54	100.6 ± 4.
Width of tunica albuginea (µm)	18.12 ± 0.79	17.9 ± 0.92	18.4 ± 2.2	16.2 ± 1.46

The diameter of seminiferous tubules and germinal epithelium thickness appeared to increase in the total alkaloid extract *P harmala*-treated groups with different extract concentrations. Only the group treated with an extract concentration of 25 mg/kg showed a significant difference compared to the control group. It should also be noted that luminal height values were higher in mice treated with concentration 25 than in mice in the control group, but there was no significant difference ($P > 0.05$). Meanwhile, the thickness of the tunica albuginea does not appear to be affected by the different concentrations of total alkaloid extract *P. harmala*.

DISCUSSION AND CONCLUSION

Plant extracts are widely used to treat or alleviate male reproductive diseases as well as to improve fertility, encompassing all aspects of reproductive health. Male reproductive health may benefit from oxidative stress management and prevention. The main defense against oxidation is the consumption of an antioxidant-rich diet. Rich in phytochemicals, plants can provide an extra layer of protection against oxidative stress and are a strong source of natural antioxidants. This investigation aimed to study testicular histology and antioxidant biomarkers in mice after administration of *P. harmala* alkaloid extracts for 35 days, corresponding to the period of mice spermatogenesis.

It is known that antioxidants are utilized extensively these days to halt the oxidative chain reaction (Bilaspuri and Bansal, 2008). In recent years, herbal therapies have emerged as a potential gold standard for treating male infertility. The primary factor likely responsible for the ability of plants to combat infertility may be related to their antioxidant content (Safarnavadeh and Rastegarpanah, 2011). Common Harmel (*Peganum harmala*), widespread in North Africa, is one of the plants that is frequently used in traditional medicine. The results of the present investigation revealed that *P. harmala* total alkaloid extract possessed antioxidant properties, decreasing MDA levels in testicular tissues. Furthermore, the study demonstrates that *P. harmala* extract has beneficial effects on antioxidant enzymes, as shown by the notable elevation of testicular superoxide dismutase and catalase levels in mice administered with the extract. Male infertility is largely linked to oxidative stress, which can affect the immune-privileged testis. It is important to underline that the testicular microenvironment is characterized by high amounts of unsaturated fatty acids and low oxygen concentration (Aprioku, 2013). Lipid peroxidation, also described as the oxidative destruction of polyunsaturated fatty acids, causes a number of membrane-bound enzymes to become inactive and impairs fluidity of the cell membrane (Taso et al., 2019). MDA is one of the main oxidation products of peroxidized polyunsaturated fatty acids (Partyka et al., 2012). Our results are consistent with those previously published, which showed that total alkaloid extract *P harmala* protects sperm *in vitro* from ROS by reducing MDA levels and preserving sperm membrane integrity (Derbak et al., 2021). Likewise, lipid peroxidation has been linked to poor sperm quality and has an impact on the sperm concentration, motility and morphology (Colagar et al., 2013). In another study, Berrougui et al. (2006) suggested that *P. harmala* alkaloids (harmine and harmaline) compounds could be a major source of compounds that inhibit LDL oxidative modification induced by copper. Furthermore, Rezaei et al. (2016) have reported that in rats with Parkinson's disease induced by 6-hydroxydopamine, the aqueous extract of *P. harmala* decreased oxidative stress and the levels of lipid and protein oxidation in the brain. A significant reduction in MDA is explained by a decrease in lipid peroxidation, resulting in high antioxidant enzyme activity. The total alkaloids extract of *P. harmala* have the power to prevent lipid peroxidation and strengthen the antioxidant defense system of testicular tissues. Our findings are in agreement with those of Shokoohi et al. (2019), who found that the flavonoid-rich *Fumaria parviflora* extract, isoquinoline alkaloids,and phenolic compounds protect the testis tissue and sperm quality in diabetic rats from oxidative stress.

It is important to remember that many complex

antioxidant defense systems, including enzymes superoxide dismutase (SOD) and catalase (CAT), block the initiation of free radical chain reactions. Free radicals can trigger chain reactions by interacting with proteins, lipids, and nucleic acids, leading to cellular malfunction and even death when they are produced in excess or when the antioxidant defense system within the cell is compromised. Antioxidant enzymes have been widely investigated for the prevention and treatment of diseases resulting from oxidative damage. Many studies have investigated on the antioxidant activity of numerous plant extracts on reproductive function (Iamsaard et al., 2014; Rasyidah et al., 2014), Contino et al., 2023, Akbari Bazm et al., 2019). The present study demonstrated the antioxidant properties of *P. harmala* seeds extract at a concentration of 25 mg/kg in mice by an increase in the enzymes catalase and superoxide dismutase in the testicular tissues. This suggested that the usage of *P. harmala* extracts prevented the decrease in SOD and CAT enzyme, which may be due to the elimination of free radicals by alkaloid total extract. As a result, these antioxidant enzymes are preserved and conserved. Alkaloids are known to have analgesic, antioxidant and anti-inflammatory bioactivity, which help to develop endurance against oxidative stress in animals (Singhai and Patil, 2021; Kumar et al., 2015; Elansary et al., 2020). Our findings are consistent with those reported by Hamden et al. (2008) in an *in vivo* study on male rats, who demonstrated the protective effect of *P. harmala* extract (50 mg/kg) against the negative effects of reactive oxygen species by increasing the activity of the antioxidant enzymes SOD and CAT. It is known that SOD and CAT are primary antioxidants that break down superoxide into hydrogen peroxide and hydrogen peroxide into molecular of oxygen and water, respectively (Weydert and Cullen, 2010). Tissue levels of SOD and CAT reflect the degree of oxidative stress experienced (Singh et al., 2017). As illustrated by the results, the increased levels of SOD and CAT in the testes of mice were enhanced after administration of *P. harmala* alkaloid extracts, and this positive effect may be due to the antioxidant effects of its bioactive compounds. Recently, Abbas et al. (2021) revealed that extracts of *P. harmala* exhibited significant antioxidant activity in both *in vitro* and *in vivo* models, justifying the use of this plant in the traditional medicine. In addition, this bioactivity might be attributed to the presence of harmol, harmine, harmaline, and peganine. In another study, the research team members demonstrated the ability of aqueous extract of *Allium sativum*, rich in alkaloids, to restore the antioxidant status of rats testis *in vivo* (Ayoka et al., 2016).

It is also important to mention that spermatocytogenesis in mice starts at age of 30 days. The presence of spermatocytes and spermatids were considered as signs of spermatocytogenesis in the seminiferous epithelium. Histological findings of the present study indicate that alkaloid extract of *P. harmala* has no harmful effect on spermatogenesis and Sertoli cells in mice. The proliferation of spermatogenic cells observed in mice treated with different concentration of *P. haramala* extract was normal. Regarding the histological appearance of seminiferous tubules, epithelial cell layers, the testes of the control group were comparable to those of the treated animals. It has been reported that *Withania somnifera* provokes spermatogenesis in male Wistar rats based on histological observations (Abdel-Magied et al., 2001). However, Benbotta et al. (2018) reported that *P. harmala* alkaloids induced testicular toxicity at higher doses (80, and 120 mg/k) and disturbed the function of hypothalamic pituitary testis axis. It mainly included stop of spermatogenesis, deformation in seminiferous tubules structure, damage to Leydig cells, and absence of Sertoli cells. In another investigation, Adisa et al. (2014) reported that alkaloid extract of *Telfairia occidentallis* leave had the potential for reducing male sexual function considering the observed histological presentation of testis. It may be testiculotoxic, as indicated damage on testicular cells, such as cellular degeneration, hemorrhage, interstitial space exudations and cellular necrosis. These differences may be due to administration dose, culture or growth conditions,

and geographical variations of plants. In addition, the extraction method can alter the total alkaloid content. In this study, the results of the treated mice with high doses of *P. harmala* total alkaloid extract (25 mg/kg) revealed a significant increased seminiferous tubule diameter, epithelium height, and epithelium area. Our results are in accordance with those previously published (Olawuyi et al., 2019), which showed that *Lawsonia inermis* aqueous leaf-extract led to statistically significant changes in the percentage of seminiferous tubular and seminiferous ductal diameter male Wistar rats. Likewise, Akang et al. (2015) reported that *Telfairia occidentalis* protected the seminiferous epithelium, reduced oxidative stress and promoted spermatogenesis mature male Sprague–Dawley rats, according to cross-section testicular histology. It has also been reported that animals treated with *Cassia abbreviata Oliv* showed an increase in seminiferous tubule diameter and germinal epithelial height with all the stages of spermatogenesis (Msiska et al., 2021). In another study, Azu et al. (2010) investigated the short-term effects of *Kigelia africana*, known to be rich in flavonoids and alkaloids, fruit extract on testicular histomorphometric changes in Sprague-Dawley rats, which significantly improved the testicular weight/volume, diameter somniferous tubules and transverse surfaces. This may be due to a direct effect of bioactive components of *P. harmala* on histomorphometric parameters of testis. Our results could be explained by androgen-stimulating activity, which then stimulates spermatogenesis, increasing the number of spermatogenic cells (Smith and Walker, 2014).

Based on our results, the total alkaloids of *P. harmala* protect testicular tissues against oxidative stress, suggesting it as a potential antioxidant source. Furthermore, the positive effect of *P. harmala* seed extract (especially at a dose of 25 mg/kg body weight) is interesting on the histomorphometric measures of testes. Additionally, the present research showed that the histological architecture of the testes was preserved in its physiological form. However, it would be necessary to undertake *in vitro* and *in vivo* toxicological studies on organs such as the liver and spleen as well as hematological parameters.

AUTHOR CONTRIBUTIONS

Conceptualization, writing—original draft preparation, resources, H.D. and A.A.; technical assistance, H.D. and A.C.B.; data validation, review and editing, O.B., E.H.B. and A.A.; supervision, writing—review and editing, A.A. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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UTJECAJ ALKALOIDA EKSTRAKTA *PEGANUM HARMALE* NA BIOMARKERE OKSIDATIVNOG STRESA I HISTOMORFOMETRIJU TESTIKULARNOG TKIVA KOD MUŽJAKA MIŠA *(MUS MUSCULUS)*

SAŽETAK

Istraživanje je provedeno sa ciljem evaluacije utjecaja primjene alkaloida ekstrakta *P. harmale* na biomarkere testikularnog oksidativnog stresa i histomorfometriju testikularnog tkiva kod mužjaka miševa. Nakon dvije sedmice aklimatizacije, dvadeset i osam Švicarskih albino miševa mužjaka (*Mus musculus)* starih 8 sedmica su metodom slučajnog uzorka podijeljeni u četiri jednake grupe (n=7). Mužjaci miševa su svaki dan putem sonde primali ekstrakt alkaloida *P. harmale* u različitim koncentracijama (0, 6.25, 12.5 i 25 mg/kg TT/dnevno u 0.5% karboksimetil celulozi kao nosaču) u trajanju od 35 dana. Nakon žrtvovanja mužjaka miševa, testisi su ispreparirani, pri čemu je jedan iz para uskladišten na -80°C, a drugi odmah prezerviran u 10% formalinu. Na kraju istraživanja su evaluirani parametri oksidativnog stresa i izvršene su histomorfometrijske analize testikularnog tkiva. Od tretiranih grupa, uočena je signifikantno niža vrijednost testikularnog melondialdehida (MDA) kod grupe koja je primala ukupni alkaloid ekstrakta *P. harmale* u usporedbi sa kontrolnom grupom (P<0.05). Srednja vrijednost superoksid dismutaze je povišena u grupi tretiranoj sa 6.25 mg/ kg i 25 mg/ kg ekstrakta (0.44±0.07, 0.5±0.04 U/mg proteina) u usporedbi sa kontrolom (0.4±0.08 U/mg proteina). Rezultati pokazuju signifikantan (P <0.05) porast aktivnosti katalaze u grupi tretiranoj sa koncentracijom od 25 mg/ kg. Osim toga, histološka slika mužjaka miševa tretiranih sa ekstraktima *P. harmale* se *čini* normalnom. *Čini* se da se dijametar sjemenskih kanalića i debljina germinativnog epitela povećavaju u grupama tretiranim sa ekstraktima *P harmale* u različitim koncentracijama. Također je zabilježeno da su vrijednosti visine lumena veće kod mužjaka miševa tretiranih sa koncentracijom od 25mg/kg u odnosu na miševe iz kontrolne grupe (P> 0.05). Naši rezultati pokazuju da ukupni alkaloidi *P. harmale štite* testikularno tkivo od oksidativnog stresa, sugerirajući njihov potencijal kao izvor antioksidansa. Osim toga, pozitivni učinci ekstrakta sjemena *P. harmale*, posebno u dozama od 25 mg/kg tjelesne težine, su zanimljivi u smislu histomorfometrijskih mjera testisa.

Ključne riječi: Mužjaci miševa, oksidativni stress, *Peganum harmala*, testikularna histomorfometrija

RESEARCH ARTICLE

COMPARATIVE 1H-NMR-BASED CEREBROSPINAL AMINO ACID PROFILING IN TICK-PARALYZED AND HEALTHY DOGS

Erdem Gülersoy[1*], Canberk Balıkçı[1], Esma Kısmet[1], Ismail Günal[1], Adem Şahan[1]

[1]Harran University, Veterinary Faculty, Department of Internal Medicine, Şanlıurfa, Türkiye

[*]**Corresponding author:**
Assoc. Prof. Erdem Gülersoy
Address: Department of Internal Medicine, Faculty of Veterinary Medicine, Harran University Şanlıurfa, Türkiye Harran University, Eyyubiye Campus, 63250, Şanlıurfa
Phone: +905333344042
ORCID: 0000-0001-8511-0150
E-mail: egulersoy@harran.edu.tr

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ABSTRACT

Tick paralysis, caused by neurotoxins released by certain tick species during blood feeding, leads to ascending acute flaccid paralysis (AFP) and can result in severe complications, such as respiratory failure and death. Metabolomic profiling of amino acids, particularly using 1H-NMR, is a valuable tool for understanding the mechanisms underlying these conditions. In this study, 92 dogs presenting with clinical signs, including sudden onset of weakness, difficulty moving, and hind limb incoordination indicative of AFP, were evaluated at the Harran University Veterinary Faculty Animal Hospital. 15 dogs were assigned to the Paralysis group and 10 to the Healthy group based on their respective inclusion/exclusion criteria. CSF samples were collected from all dogs, and 1H-NMR-based amino acid profiling was performed on all samples using an Agilent 400 MHz spectrometer. The Paralysis group exhibited higher body temperature, heart rate, and respiratory rate compared to the Healthy group ($p < 0.028$). Paralyzed dogs had a shorter capillary refill time ($p < 0.008$), while healthy dogs had a higher Modified Glasgow Coma Scale (MGCS) score. Regarding amino acid concentrations, the Paralysis group had higher levels of L-phenylalanine, L-isoleucine, L-histidine, lysine, and L-tryptophan ($p < 0.038$), and lower levels of L-threonine, L-leucine, L-methionine, and L-valine ($p < 0.036$). These findings suggest that the increased levels of certain amino acids reflect a neuroprotective response to neuroinflammation, while the decreased levels point to neuronal damage and disrupted transfer mechanisms. Overall, this study enhances the understanding of tick paralysis and may provide insights into other non-infectious AFP conditions.

Keywords: Acute flaccid paralysis, biomarker, dog, tick paralysis

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INTRODUCTION

Certain tick species, such as *Ixodes holocyclus* and *Rhipicephalus sanguineus* (*R. sanguineus*), the most widespread tick of dogs and also known as the brown dog tick, can release neurotoxins through their salivary glands during blood feeding, causing tick paralysis (Otranto et al., 2012). This condition, with tick paralysis as a part of its etiology, is characterized by rapid, progressive acute flaccid paralysis (AFP) and can be life-threatening (Hogan et al., 2019; Gülersoy et al., 2024a). Clinical signs typically begin with weakness and incoordination, progressing to paralysis. Affected dogs may experience difficulty breathing, gagging, coughing, and, in severe cases, respiratory failure and death can occur. Severity of symptoms depends on factors like tick size, attachment duration, and the dog's sensitivity to the toxin (Hogan et al., 2019).

Diagnosis is based on clinical signs and recent tick exposure, supported by identifying ticks on the dog or in its environment. Laboratory tests, including complete blood count (CBC), serum biochemistry, and cerebrospinal fluid (CSF) analysis, help assess neurological function and exclude other conditions (Mackenzie, 2011; Otranto et al., 2012). However, in cases of tick paralysis, CSF analysis findings—typically evaluating parameters such as glucose or total leukocytes—may remain within reference values due to non-infectious nature of tick paralysis (Hogan et al., 2019). Metabolomic profiling of amino acids is a promising approach for uncovering underlying mechanisms, identifying biomarkers, and informing treatment strategies for autoimmune and neurodegenerative diseases (Gülersoy et al., 2024b). Under normal conditions, amino acid concentrations in CSF are significantly lower than in plasma. Minor disturbances in the CSF amino acid profile have been reported in neurological diseases such as Parkinsonism, epilepsy, and Huntington's chorea (Link and Tibbling, 1977). Guillain-Barré syndrome, an acute flaccid paralysis similar to tick paralysis, is also associated with elevated CSF protein concentrations at certain stages, which are believed to result largely from blood-CSF barrier disruption (Hegen et al., 2021).

Promising results have been reported in 1H-NMR-based metabolomics studies of tick salivary neurotoxin in serum samples from dogs with tick paralysis (Simon et al., 2023; Gülersoy et al., 2024c). 1H-NMR-based CSF amino acid profiling may also be a valuable tool for evaluating different stages of the disease. Therefore, this study aims to analyze 1H-NMR-based amino acid profiles in CSF samples from dogs with tick paralysis, investigate potential diagnostic and prognostic markers, and provide insights for further acute flaccid paralysis studies.

MATERIAL AND METHODS

This study was approved by the Local Ethics Committee for Animal Experiments at Harran University (Date: 09.05.2022.; Decision Number: 2022/003–01/06). In addition, informed consent was obtained from all dog owners prior to the commencement of the study. No experimental procedures that could harm the animals or compromise their welfare were conducted.

Animals

Both the paralyzed and healthy animals in this study were selected from 92 dogs admitted to Harran University Veterinary Faculty Animal Hospital between January and October, 2024. Among these dogs, those without any comorbid diseases, as determined by physical and laboratory examinations, and with findings suggestive of AFP, were included in the Paralysis group. Dogs that were determined to be healthy based on physical and laboratory examinations and were admitted for vaccination or routine check-ups were included in the Healthy group.

Physical Examinations and Inclusion/Exclusion Criteria

During the physical examination, body weight, body temperature, heart rate, respiratory rate, and gingival capillary refill time (CRT) were assessed, and Modified Glasgow Coma Scale (MGCS) scores were calculated for all dogs. Tick-paralyzed

dogs were examined for ticks by thumb-counting across anatomical regions, including the head, neck, ears, thorax, abdomen, interdigital areas, forelimbs, hind limbs, tail, axillary, and inguinal regions. Morphological examination of ticks collected from paralyzed dogs was performed by an expert for species identification, and they were identified as *R. sanguineus*.

Inclusion criteria for the study required that the dog had no history of any disease, had not received antiparasitic medication recently (<1 month), had an engorged tick, and displayed signs of AFP. Clinical findings suggestive of AFP included an inability to contract due to motor pathway impairment from the cortex to muscle fibers, absence of spasticity or other signs of disordered central nervous system motor tracts (e.g., hyperreflexia, clonus, or extensor plantar responses), and the sudden onset and progression of weakness, particularly affecting respiratory muscles and swallowing (Growdon and Fink, 1994; Marx et al., 2000). To avoid interfering with the NMR-based CSF amino acid profiling, CBC and microscopic blood smear examinations were performed on all dogs included in the study. Blood and buffy coat smears were examined for Anaplasma platys, Ehrlichia canis, Babesia spp., and Hepatozoon canis inclusions. Each smear was analyzed under a 100× oil immersion objective to ensure optimal morphology using a light microscope. Dogs with blood parasites or abnormalities such as thrombocytopenia or pancytopenia, commonly observed in dogs previously infected with *R. sanguineus* (Otranto et al., 2012), were excluded from the study. Additionally, CBC results, including leukogram and hemogram indices, were used solely as inclusion/exclusion criteria and were not evaluated further within the scope of this study.

Forming Subgroups

15 dogs infested with *R. sanguineus*, with clinical findings compatible with AFP due to tick infestation and with a confirmed tick paralysis diagnosis ex juvantibus, constituted the Paralysis Group of the study. 10 dogs with similar body weights and similar ages ($p < 0.580$), which were admitted either for vaccination and/or check-up purposes, constituted the healthy Control Group.

CSF Sampling

Before cerebrospinal fluid tap, all tick paralyzed dogs were sedated by intramuscular injection at a dose of 1 mg / kg with xylazine (Xylazin Bio® 2%, Bioveta) after blood sampling. CSF samples were taken (1–2 mL) between the occipital and atlas bones with the appropriate procedure (using a 22 gauge, 1.5 inch stylet spinal needle) (Gülersoy et al., 2024b). Excessive flexion of the head was avoided to prevent airway obstruction. There were no complications observed during and after the procedure.

NMR Analysis

CSF samples were prepared as previously reported (Gülersoy et al., 2024b). The 1H-NMR spectra were acquired at 26.5 °C using an Agilent 400 MHz spectrometer operating at 400.13 MHz, equipped with a 5 mm inverse detection probe with z-axis gradients. The samples were placed in 5 mm Wilmad 507 NMR tubes. Spectral data were recorded using the NOESY presaturation pulse sequence, with the following parameters: 32 scans, a 30 s relaxation delay, 4 s acquisition time, 8223 Hz spectral window, and 64 K data points, providing a digital resolution of 0.12 Hz. The Free Induction Decay (FID) was processed with an exponential line broadening factor of 0.3 Hz. Chemical shifts are reported in δ values (ppm), with TSP (0.0 ppm) as the internal reference. Acquisition and data processing were carried out using Agilent SpectrAA software. The NMR protocol ensured complete signal relaxation with an overall relaxation delay of 37 s. The combination of 0.3 Hz line broadening and 37 s relaxation delay allows for the use of signal intensities in quantification, reducing errors from partial signal overlap in crowded spectra. For each NMR measurement, 600 µL of sample (the standard 5 mm NMR tube volume) was used, after reducing the CSF sample to the minimum required volume. Using the full filling volume for one measurement yields better results compared

to two half-volume measurements, as it improves shimming, resulting in a higher signal-to-noise ratio and increased sensitivity (enhanced magnetic field homogeneity, leading to sharper spectral lines). All sample preparations and measurements were performed by a single operator to minimize operator-induced variability. Analytical error was found to be significantly lower than biological variation when the same operator conducted the tests. For four samples prepared from the same batch, the analytical error for various amino acids ranged from 1-5%, while the biological variation (expressed as % RSD) varied between 10-45%.

Identification and Quantification of CSF Amino Acids

The identification and quantification of CSF amino acids from raw 1D NMR spectra (FID files) acquired on the Agilent spectrometer were carried out using BAYESIL software. BAYESIL provides fully automated spectral processing and profiling for 1D and 1H-NMR spectra, regardless of the acquisition frequency, and is compatible with standard NMR instruments. During spectral deconvolution, BAYESIL divides the spectrum into smaller segments and employs a probabilistic graphical model to represent the sparse dependencies between these segments. Approximate inference is then applied to this model, effectively serving as a stand-in for spectral profiling, leading to the most probable amino acid profile. BAYESIL facilitates a range of spectral processing functions, including zero filling, phasing, baseline correction, smoothing, chemical shift referencing, and reference deconvolution, starting with the raw spectrum. The relative concentrations of the quantified amino acids were determined based on the total area of the spectrum. Statistical analysis of the relative concentrations was also performed using BAYESIL. Visualization, simulation, and presentation of the NMR spectra were carried out using MestReNova software (MestreLab Research, Spain).

Statistical Analysis

Data analysis was performed using SPSS 25.00 (SPSS for Windows®). To assess whether the data followed a parametric or non-parametric distribution, a one-sample Kolmogorov-Smirnov test was conducted. Since the data were determined to be non-parametric, they were analyzed as median (min-max) values using the Mann-Whitney U and Kruskal-Wallis tests. Statistical significance was defined as $p < 0.05$.

RESULTS

Animals

All dogs in the present study were domestic, unvaccinated, fed commercial dry dog food, and taken outside for walks 2-3 times a day. Most dogs in the Paralysis group were housed in rural areas (11 out of 15 dogs). In contrast, all dogs in the Healthy group were housed in more urbanized areas (10 out of 10 dogs). Paralyzed dogs exhibited neurological findings sufficient to raise suspicion of AFP, such as sudden onset of weakness, difficulty in movement, hind limb incoordination, or quadriplegia. Anamnestic data revealed that the dogs had no history of disease previously. The symptom duration of tick paralyzed dogs was 3 (2-6) days. These dogs had ticks detected during clinical examination and were admitted for diagnosis and treatment. The physical examination and CBC findings of dogs classified as healthy were within normal limits. Of the 92 dogs evaluated based on all examinations and the specific inclusion/exclusion criteria of each group in this study, 15 were assigned to the Paralysis group and 10 to the Healthy group.

Physical Examination Findings

A thorough inspection revealed a median of 33 ticks (range: 10–65) in the tick-paralyzed dogs. Some owners had attempted to remove a few ticks on their own prior to the dogs' arrival at the hospital. Additional ticks were removed during the clinical examination. To confirm the ex juvantibus diagnostic approach, all dogs were treated with a spot-on formulation containing Fipronil 10% / (S)-Methoprene 9% (Frontline Combo, Merial S.A.S., France). Compared to the Healthy group, the Paralysis group exhibited higher body temperature, heart rate, and respiratory rate values ($p < 0.028$).

The CRT was shorter in paralyzed dogs ($p < 0.008$), while the MGCS score was higher in the healthy dogs. Demographic data and physical examination findings are presented in Table 1.

Table 1 Physical examination findings

Parameters	Paralysis Group n:15 median (min-max)	Healthy Group n:10 median (min-max)	p value
Body temperature (°C)	39.6 (38.7-40.5)	38.1 (37.7-38.5)	0.0001
Heart rate (beats/min)	104 (90-152)	78 (65-96)	0.0001
Respiratory rate (breaths/min)	54 (40-68)	42 (33-55)	0.009
CRT (sec)	1 (1-2)	2 (1-3)	0.001
MGCS	6 (3-13)	16 (15-18)	0.0001
Body weight (kg)	6.22 (4.05-8.15)	6.18 (4.35-8.11)	0.769
Age (months)	5 (4-6.5)	5.75 (5.5-6.5)	0.457

CRT: Capillary Refill Time, MGCS: Modified Glasgow Coma Scale.

1H-NMR-based CSF Amino Acid Profiling Results

Compared to the Healthy group, the Paralysis group had higher concentrations of L-phenylalanine, L-isoleucine, L-histidine, lysine, and L-tryptophan ($p < 0.038$), and lower concentrations of L-threonine, L-leucine, L-methionine, and L-valine ($p < 0.036$). The CSF amino acid profiling results are presented in Table 2. The identified and quantified metabolites are visualized in Figure 1 and 2.

Table 2 1H-NMR-based CSF Amino Acid Profiling

Parameters*	Paralysis Group n:15 median (min-max)	Healthy Group n:10 median (min-max)	p value
L-phenylalanine	4.72 (0.11-8.44)	0.22 (0.13-0.37)	0.017
L-threonine	14.35 (10.2-69.9)	33.69 (18.1-50.98)	0.036
L-isoleucine	2.32 (1.33-3.92)	1.73 (0.27-3.88)	0.055
L-histidine	4.65 (0.1-7.1)	0.5 (0.2-0.8)	0.010
Lysine	35.56 (14.6-57.61)	29.98 (24.73-36.74)	0.038

Parameters*	Paralysis Group n:15 median (min-max)	Healthy Group n:10 median (min-max)	p value
L-leucine	5.5 (3.8-8.7)	19.03 (7.71-39.95)	0.016
L-methionine	39.56 (15.45-65.14)	215.73 (56.41-272.03)	0.001
L-valine	7.7 (6.2-11.4)	26.97 (15.2-43.11)	0.004
L-tryptophan	2.8 (1.4-4.1)	0.29 (0.14-0.64)	0.0001

*Concentrations are in arbitrary units

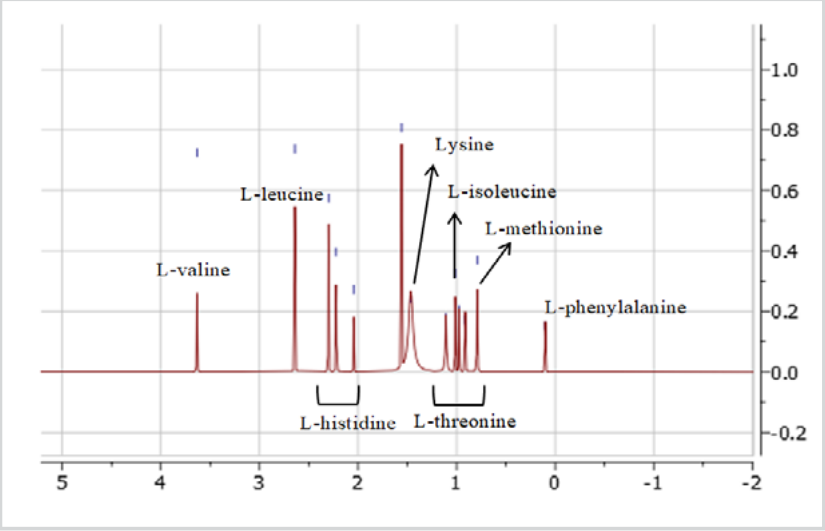

Figure 11H-NMR spectrum of Paralysis group's cerebrospinal fluid. Detected chemical shifts (ppm) for L-phenylalanine (0.06-0.25), L-isoleucine (0.73-2.46, methine group), L-histidine (0.78-2.34, imidazole ring), Lysine (0.73-2.19), L-valine (1.46-3.65), L-tryptophan (0.13-0.65, amine group), L-threonine (0.59-2.38, methine group), L-leucine (0.66-2.64), L-methionine (0.29-1.46).

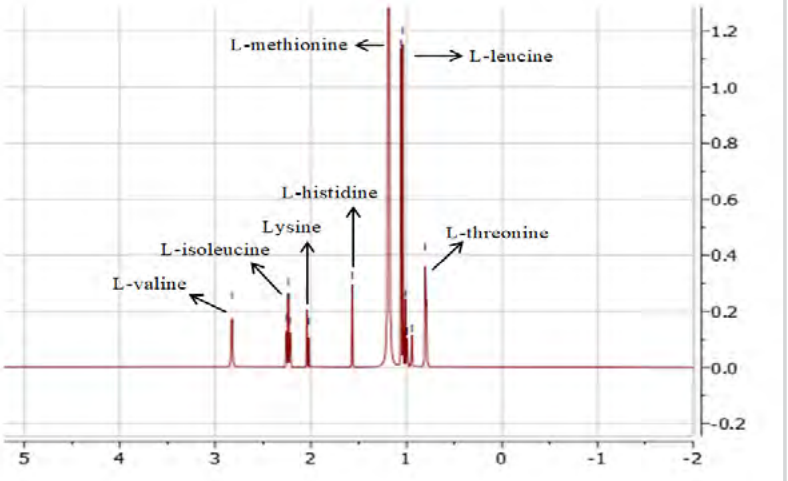

Figure 2 1H-NMR spectrum of Healthy group's cerebrospinal fluid. Detected chemical shifts (ppm) for L-phenylalanine (0.06-0.25), L-isoleucine (0.73-2.46, methine group), L-histidine (0.78-2.34, imidazole ring), Lysine (0.73-2.19), L-valine (1.46-3.65), L-tryptophan (0.13-0.65, amine group), L-threonine (0.59-2.38, methine group), L-leucine (0.66-2.64), L-methionine (0.29-1.46). L-phenylalanine cannot be visualized due to its low concentrations.

DISCUSSION AND CONCLUSION

Tick paralysis is caused by neurotoxins secreted by adult female ticks, primarily *Ixodes holocyclus*. However, there are some reports of *R. sanguineus* being a cause of paralysis in dogs (Otranto et al., 2012). These toxins inhibit the presynaptic release of acetylcholine into the synaptic space of the neuromuscular junction, leading to various clinical manifestations, ranging from rare isolated cranial nerve involvement to severe quadriplegia and paralysis of the respiratory muscles. In critical cases, paralysis of the respiratory muscles without access to mechanical ventilation can result in death. Therefore, early diagnosis of tick paralysis is vital due to its highly variable prognosis (Gülersoy et al., 2024c). While certain omics approaches have been explored in the context of tick vaccination and potential diagnostic or prognostic metabolomics in affected dogs, research on 1H-NMR-based CSF evaluation in cases of tick paralysis has been notably absent (de la Fuente and Merino, 2013). This study, in which potential diagnostic and/or prognostic markers were investigated in CSF samples of dogs affected by tick paralysis using a 1H-NMR based approach, has yielded important results. Tick-paralyzed dogs were observed to have higher concentrations of L-phenylalanine, L-isoleucine, L-histidine, lysine, and L-tryptophan, likely as a neuroprotective response to neuroinflammation, and lower concentrations of L-threonine, L-leucine, L-methionine, and L-valine, attributed to an impaired transfer mechanism and ongoing neuronal damage. The amino acids with altered expression detected in this study, along with this approach, may provide insights into the pathological mechanisms underlying tick paralysis. Additionally, these metabolites with altered expression could serve as potential diagnostic and prognostic markers, as

the prognosis of tick paralysis is highly variable, especially when diagnosis is delayed, particularly in atypical cases where ticks cannot be found on the host. Furthermore, the findings may also contribute to research on other non-infectious acute flaccid paralysis conditions.

If left untreated, tick paralysis can progress to respiratory failure and potentially result in death. Therefore, healthcare workers must be well-acquainted with this relatively rare but treatable cause of acute motor weakness, maintaining a high level of suspicion to avoid delays in diagnosis and treatment (Padula et al., 2020). Unlike assessments that focus solely on gait and respiratory scores, the MGCS evaluates the overall neurological status, making it a more comprehensive tool for monitoring patient condition (O'Keeffe and Donaldson, 2023). Thus, the MGCS was chosen for its ability to provide vital prognostic assessment for both veterinarians and owners, allowing for the grading of initial neurological status, including gait, respiratory pattern, and serial patient follow-up (Platt et al., 2001). Clinical examinations of dogs with tick paralysis often reveal fever, altered mental status, and increased respiratory rate, all of which were evaluated using the MGCS in the present study. The cardiovascular system is also adversely affected, leading to an increased heart rate, elevated blood pressure, irregular heart rhythms, and coagulopathies (Shaffran, 2008). The elevated body temperature, pulse, respiratory rate, and shortened CRT observed in the tick-paralyzed dogs in the present study align with previous findings and may be attributed to a hypermetabolic reaction of skeletal muscles to the tick neurotoxin (Padula, 2016).

Little is known about the pharmacological mechanism of tick toxin action (Simpson, 1996; Padula, 2016). The toxin binds with high affinity to receptors on nerve endings, penetrates the cell membrane via receptor-mediated endocytosis, and crosses the endosomal membrane through pH-dependent translocation. Once in the cytosol, the toxin acts as a zinc-dependent endoprotease, cleaving polypeptides essential for exocytosis. The absence of these polypeptides prevents nerve action potentials from triggering acetylcholine release, a process thought to be enzymatically mediated (Simpson, 1986; Padula 2016; Simon et al., 2023). Acute flaccid paralysis (AFP) typically reaches maximum severity within days to weeks, with the progression timeline depending on the specific etiology (Bowley and Chad, 2019). In the brain, supraphysiological glutamate release induces neurotoxicity. Therefore, the search for effective neuroprotective agents has centered on compounds that block glutamate receptors or inhibit glutamate release. Drugs capable of targeting multiple excitotoxic pathways may offer superior neuroprotective efficacy. Previous studies suggest that the aromatic amino acid L-phenylalanine, an endogenous substance, may exhibit such properties (Kagiyama et al., 2004). Although tick paralysis primarily affects peripheral neurons, brainstem dysfunction and autonomic dysregulation can also occur (Hegen et al., 2021; Gülersoy et al., 2024c). The elevated CSF L-phenylalanine concentrations observed in this study may represent a neuroprotective response by the body. Therefore, L-phenylalanine supplementation, along with monitoring its concentrations in both serum and CSF, may serve therapeutic and prognostic purposes. However, it should be kept in mind that a key challenge for neuroprotective compounds is achieving concentrations sufficient for neuroprotection without causing adverse effects on the cardiovascular or nervous systems.

Histidine is an essential amino acid in dogs, playing critical roles in various metabolic processes, including its involvement in the histaminergic system of the central nervous system. It also serves as a precursor to histamine, a molecule that contributes to inflammatory processes and the pathogenesis of multiple sclerosis (Shmalberg, 2015; Židó et al., 2023). Elevated L-histidine concentrations in cerebrospinal fluid (CSF) have also been reported in individuals with Alzheimer's disease, although the specific nature of this role remains unclear (Kaiser et al., 2007). Animal models suggest that increased histamine levels may drive the synthesis of pro-inflammatory

cytokines, such as TNF and interferon gamma (Židó et al., 2023). In the present study, higher CSF L-histidine concentrations were observed in tick-paralyzed dogs compared to healthy controls. This increase may reflect the dual roles of microglia, which contribute to both neuroprotection and neurodegeneration (Smith et al., 2012). Additionally, this finding aligns with the elevated CSF L-phenylalanine concentrations observed in this study, suggesting a broader metabolic response to tick paralysis.

Lysine is an essential amino acid known to promote protein synthesis and improve neurological function. Previous studies have shown that lysine enhances cerebral blood flow and supports recovery in patients with ischemic stroke through its neuroprotective and neurotrophic effects (Kondoh et al., 2010). Elevated CSF lysine concentrations have also been linked to mental retardation and motor neuron diseases (Cheng et al., 2020). Additionally, increased nitrogen excretion has been observed in certain neuronal diseases. In the present study, the elevated CSF lysine concentrations in tick-paralyzed dogs may be associated with enhanced nitrogen excretion, microglial polarization, neuroprotection, and the prevention of brain cell death (Kondoh et al., 2010; Cheng et al., 2020).

Isoleucine, leucine, valine, phenylalanine, tyrosine, and lysine compete for transport across the blood-CSF barrier via a common carrier system. In humans, this amino acid transport system differs from the neutral amino acid carrier observed at the blood-brain barrier in rats (Oldendorf and Szabo, 1976). Methionine and tryptophan, however, do not compete with other neutral amino acids for this system. Interestingly, lysine, a basic amino acid, has been associated with the same transport mechanism as the five neutral amino acids. Patients with blood-CSF barrier dysfunction for proteins may exhibit partly normal, increased, or decreased CSF concentration quotients for these amino acids (Kruse et al., 1985). Amino acid transport across the blood-CSF barrier is crucial for neuronal metabolism and neurotransmission. The synthesis of neuronal glutamate from α-ketoglutarate requires an amino group nitrogen donor. Among the branched-chain amino acids (BCAAs)—leucine, isoleucine, and valine-these amino acids can serve as nitrogen donors for vesicular neurotransmitter glutamate synthesis. However, a previous study found that only valine sufficiently supports the increased demand for vesicular glutamate synthesis (Bak et al., 2012). In conditions involving blood-CSF or blood-nerve barrier dysfunction, such as Guillain-Barré syndrome (GBS), which is an acute flaccid paralysis similar to tick paralysis, CSF total protein levels are elevated due to the release of myelin proteins from inflamed spinal nerve roots (Hegen et al., 2021). In the present study, increased L-phenylalanine levels, which may serve a neuroprotective function, alongside elevated L-isoleucine and L-valine concentrations and reduced L-leucine levels, suggest an underlying dysfunction of the blood-nerve barrier or blood-CSF barrier.

Threonine serves as a phosphorylation site for numerous enzymes, playing several vital roles in the body. Additionally, it acts as a precursor to glycine, an inhibitory neurotransmitter, thereby influencing neurotransmitter balance in the brain (Shmalberg, 2015). Studies have shown a significant correlation between threonine consumption and its plasma, brainstem, and cortical levels, which are closely associated with cortical concentrations of threonine and glycine (Kaiser et al., 2007). Furthermore, certain genes are implicated in the transport of amino acids, such as serine and threonine, from astrocytes to neurons. While elevated CSF L-threonine levels are linked to neurodegeneration of meningeal cells, neurons, granular cells, and Purkinje cells, the reduced CSF L-threonine concentrations observed in the tick-paralyzed dogs in this study may result from impaired transport mechanisms (Swanson et al., 2022; Gülersoy et al., 2024c). However, these mechanisms need to be investigated in cases of acute flaccid paralysis.

The principal role of tryptophan is as a constituent of protein synthesis. Because it is found in

the lowest concentrations among amino acids, tryptophan is relatively less available and is considered to play a rate-limiting role during protein synthesis. Tryptophan is also the precursor to two important metabolic pathways: kynurenine synthesis and serotonin synthesis (Dougherty et al., 2008; Richard et al., 2009). The kynurenine pathway is a major route for tryptophan metabolism, producing a range of biologically active molecules with properties including oxidants, antioxidants, immunomodulators, neurotoxins, and neuroprotectants. Disruption of the tryptophan-kynurenine metabolism is strongly associated with neuroinflammation and immune activation. Increased production of pro-inflammatory cytokines activates the kynurenine pathway's regulatory enzyme, indoleamine-2,3-dioxygenase (IDO1), along with related enzymes. This causes dysregulation of the pathway, leading to depletion of tryptophan and an imbalance in the formation of neuroprotective (kynurenic acid) and neurotoxic (quinolinic acid, 3-hydroxykynurenine) metabolites (Richard et al., 2009; Mithaiwala et al., 2021). Given that L-tryptophan can cross the blood-brain barrier (Richard et al., 2009), the elevated concentrations of CSF L-tryptophan observed in the tick-paralyzed dogs of the present study may be due to an increased transfer of tryptophan from the blood to the CSF to provide neuroprotection in response to neuroinflammation.

L-methionine is an essential amino acid and a key component of one-carbon metabolism. It is necessary for the production of S-adenosyl methionine, the primary methyl donor in the body, which is involved in nearly all methylation reactions. These reactions target substrates such as ribonucleic acids, proteins, carbohydrates, phospholipids, and neurotransmitters (Trivedi and Deth, 2012). Methylation is a universal biological process critical for cell proliferation, differentiation, survival, and other cellular functions (Roidland Hacker, 2014). Disruption of methionine metabolism has been linked to various neurological and psychiatric disorders. For instance, altered levels of L-methionine and other one-carbon cycle metabolites have been reported in neurodevelopmental disorders like autism and schizophrenia, as well as age-related neurodegenerative diseases such as Alzheimer's disease and vascular dementia (Zuin et al., 2021). High methionine levels have also been associated with neuronal degeneration and vascular dysfunction (Kalani et al., 2019). The immuno-inflammatory role of methionine has been demonstrated in studies showing that methionine restriction in a mouse model of multiple sclerosis reduces T-cell-mediated inflammation in the brain and spinal cord, delaying disease onset and progression (Agbas and Moskovitz, 2009). In the present study, decreased CSF L-methionine concentrations may reflect a neuroprotective response to tick paralysis. However, most studies have focused on methionine metabolism's impact on T cells, with limited understanding of its effects on innate immune cells, such as microglia in the brain. Further research is needed to elucidate these mechanisms.

Certain tick species can release neurotoxins through their salivary glands during blood feeding, leading to tick paralysis, which is characterized by ascending AFP. In severe cases, respiratory failure and death may occur. Metabolomic profiling of amino acids offers a promising approach for uncovering underlying mechanisms, identifying biomarkers, and informing treatment strategies for autoimmune and neurodegenerative diseases. In this study, 1H-NMR-based amino acid profiling of CSF samples from dogs with tick paralysis revealed notable findings. Tick-paralyzed dogs exhibited higher concentrations of L-phenylalanine, L-isoleucine, L-histidine, lysine, and L-tryptophan, alongside lower concentrations of L-threonine, L-leucine, L-methionine, and L-valine. It was interpreted that the increased levels of amino acids, such as L-phenylalanine, L-isoleucine, L-histidine, lysine, and L-tryptophan reflected a neuroprotective response to neuroinflammation. Conversely, the decreased levels of amino acids, such as L-threonine, L-leucine, L-methionine, and L-valine suggest ongoing neuronal damage and disrupted transfer mechanisms. This approach is concluded to offer valuable insights into the

pathological mechanisms underlying tick paralysis and may also contribute to research on other non-infectious AFP conditions.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHORS CONTRIBUTION

Conception: EG, CB; Design: EG, AŞ; Supervision: EG, İG; Materials: EK, İG; Data Collection and/or Processing EG, CB, AŞ; Analysis and/or Interpretation of the Data; EG, CB, AŞ; Literature Review:EK, İG; Writing:EG; Critical Review: EG, CB, AŞ

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KOMPARATIVNO PROFILIRANJE CEREBROSPINALNIH AMINOKISELINA KORIŠTENJEM 1H-NMR KOD KRPELJSKI PARALIZIRANIH I ZDRAVIH PASA

SAŽETAK

Krpeljska paraliza uzrokovana neurotoksinima, koje pojedine vrste krpelja otpuštaju sišući krv, dovodi do ascendentne akutne flaksidne paralize (AFP) koja može imati ozbiljne komplikacije, kao što su respiratorna insuficijencija i smrt. Metabolomičko profiliranje aminokiselina, posebno korištenjem 1H-NMR, predstavlja dragocjenu alatku za razumijevanje mehanizama koji uzrokuju ovo stanje. Naše istraživanje je obuhvatilo 92 psa sa kliničkim znacima iznenadne slabosti, otežanog kretanja i gubitka koordinacije stražnjih nogu koji su indikativni na AFP, a koji su evaluirani u bolnici za životinje Veterinarskog fakulteta Univerziteta Harran. Prema uključno/isključnim kriterijima, 15 pasa je svrstano u grupu paraliziranih, a 10 u grupu zdravih. Od svih pasa su prikupljeni uzorci cerebrospinalne tečnosti, pri čemu je na svim uzorcima provedeno 1H-NMR bazirano profiliranje aminokiselina korištenjem Agilent 400 MHz spektrometra. Grupa paraliziranih je pokazala višu tjelesnu temperaturu, srčanu frekvencu i frekvencu disanja u odnosu na grupu zdravih ($p<0,028$). Paralizirani psi su imali kraće vrijeme kapilarnog punjenja ($p<0.008$), dok su zdravi psi imali veći skor na Modificiranoj Glasgowskoj skali kome (MGCS). Što se tiče koncentracije aminokiselina, grupa paraliziranih je pokazala više koncentracije L-fenilalanina, L-izoleucina, L-histidina, lizina i L-triptofana ($p<0.038$), a niže koncentracije L-treonina, L-leucina, L-metionina i L-valina ($p<0.036$). Ovi rezultati pokazuju kako povišene koncentracije određenih aminokiselina odražavaju neuroprotektivni odgovor na neuroinflamaciju, dok snižene koncentracije ukazuju na neuronalno oštećenje i prekid transfernih mehanizama. U cjelini, ovo istraživanje produbljuje razumijevanje krpeljske paralize i može pružiti uvid u druga neinfektivna stanja s AFP.

Ključne riječi: Akutna flakcidna paraliza, biomarker, krpeljska paraliza, pas

RESEARCH ARTICLE

PREVALENCE OF GASTROINTESTINAL PARASITES AND ASSOCIATED RISK FACTORS IN SLAUGHTERED TRADE DROMEDARY CAMELS IN KANO, NORTHERN NIGERIA

Mary Oluwatomisin Elijah[1*], Sani Adamu[1], Bisallah Mohammed[1], John Oluwamayokun Elijah[2], James Samson Enam[1], Sherrif Yusuf Idris[1,3], Maryam Nyeta Patrobas[4], John Addra Abari[1], Olumide Odunayo Akinniyi[5]

[1]Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.
[2]Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.
[3]Department of Pathobiology, College of Veterinary Medicine, Tuskegee University, Alabama, USA
[4]Department of Veterinary Parasitology, Faculty of Veterinary Medicine, University of Jos, Jos, Nigeria.
[5]Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria.

***Corresponding author:**
Dr. sc. Mary Oluwatomisin Elijah
Address: Ahmadu Bello University, Zaria, Kaduna State, Nigeria
Phone: +234(0)8142100134
ORCID: 0000-0002-2205-2480
E-mail: maryoluwatomisinelijah@gmail.com

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ABSTRACT

Parasites play a crucial role in animal health due to the numerous substantial challenges they present to the health and well-being of animals. The prevalence and distribution of gastrointestinal parasitic infections among trade dromedary camels in Kano, a major hub for camel trade in Northern Nigeria, are not well documented. This study, which identified gastrointestinal parasites up to the genus level and assessed associated risk factors in camels slaughtered at the Kano Main Abattoir in Nigeria, contributes to developing effective parasite control strategies to improve camel health and productivity. Using a cross-sectional study design, a total of 115 camels were examined for gastrointestinal parasites using faecal flotation and sedimentation techniques. The overall prevalence of gastrointestinal parasites was 84.4%, with 76.3% of infected camels having mixed parasite infections. Seven different parasite types were identified, belonging to nematodes (*Strongyle*-type egg, *Trichuris* spp., *Strongyloides* spp.), cestodes (*Anoplocephala* spp.), trematodes (*Fasciola* spp.), and coccidia (*Eimeria cameli*, *Eimeria rajasthani*). The most prevalent parasites were *Strongyle*-type egg (12.4) and *Trichuris* spp. (7.2%). Risk factors significantly associated with parasite prevalence were sex (males 93.3%, females 74.5%) and faecal consistency (pasty 100%, pelleted 80.9%). Body condition score and presence of mucus in faeces did not show a significant association. The mean faecal egg count was 162.7 eggs per gram, with 92.6% of infected camels having mild infections. This study highlights the high burden of gastrointestinal parasites in camels in this region and the need for improved parasite control strategies.

Keywords: Camels, gastrointestinal parasites, Northern Nigeria, prevalence

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INTRODUCTION

Camel rearing is a crucial economic activity in arid and semi-arid regions worldwide, particularly in Africa and the Middle East (Faye, 2018). Dromedary camels (*Camelus dromedarius*) play a vital role in the livelihoods of pastoralist communities, providing milk, meat, transportation and other products essential for survival in harsh environments (Faye, 2018). However, the health and productivity of camel herds can be significantly impacted by various factors, with gastrointestinal parasitic infections being a major concern (Bekele, 2022).

Gastrointestinal parasites, including nematodes, cestodes and protozoa, pose a substantial threat to camel health and production (El-Khabaz et al., 2019). These parasites can cause a range of clinical signs, from subclinical infections to severe manifestations, including reduced growth rate, decreased milk yield, poor body condition, and even mortality in severe cases (El-Khabaz et al., 2019). The prevalence and impact of camel gastrointestinal parasites have been reported in various regions, with studies conducted in Nigeria (Bamaiyi and Kalu, 2011; Mahmuda et al., 2014), Ethiopia (Birhanu et al., 2014), Tanzania (Swai et al., 2011), and Egypt (El-Dakhly et al., 2020; El-Khabaz et al., 2019).

In Nigeria, several studies have investigated the prevalence of gastrointestinal parasites in camels. Bamaiyi and Kalu (2011) reported a high prevalence (92.4%) of gastrointestinal parasites in camels in Maiduguri, while Mahmuda et al. (2014) found a prevalence of 78% in Sokoto. These studies identified various parasites, including those with *Strongyle*-type egg, *Strongyloides* spp., *Trichuris* spp., and coccidian oocysts. However, these investigations were limited in scope and did not thoroughly explore the factors influencing parasite occurrence or the potential impact on camel health and productivity.

The prevalence and diversity of gastrointestinal parasites in camels are influenced by various factors, including agro-climatic conditions, management practices, and the presence of reservoir hosts (El-Khabaz et al., 2019; El-Naga and Barghash, 2016). The tropical agro-climatic conditions in Nigeria, characterized by wide deserts, high temperatures, and infrequent rainfall seasons, coupled with free-ranging management systems, may contribute to the high prevalence of parasites, particularly Strongyle species (Swai et al., 2011; Mahmuda et al., 2014).

Kano State, Nigeria is significant as it serves as a major hub for camel trade in the northern region, potentially influencing parasite transmission patterns. The findings from this research will contribute to a better understanding of the parasitic burden in camels within the study region and provide valuable insights that can inform more effective parasite control strategies for camel herds. This information is crucial for improving camel health management practices and, ultimately, enhancing the productivity and welfare of these important livestock animals in Nigeria and similar ecological zones. Despite the existing research, there remains a gap in our understanding of the comprehensive parasitic profile of camels in Nigeria, particularly in major trade hubs like Kano. The present study aims to address this gap by providing a detailed assessment of the prevalence and diversity of gastrointestinal parasites in camels slaughtered at the Kano Main Abattoir, Nigeria.

MATERIALS AND METHODS

Ethics Approval

Ethical approval for the use of camels in this study was obtained from Ahmadu Bello University Committee for Animal Use and Care (ABUCAUC) with the approval number: ABUCAUC/2022/055.

Study Area

Camels used in this study were sampled from the Kano Main Abattoir. The abattoir is located in Fagge Local Government, Kano, Kano State. The local government is one of the commercial districts of the city (Sani et al., 2020). Kano is located between latitude 11°59'59.57''N to 12°02'39.57''N and longitude 8°31'19.69''E to 8°33'19.69''E, with urban land area of 137 km²

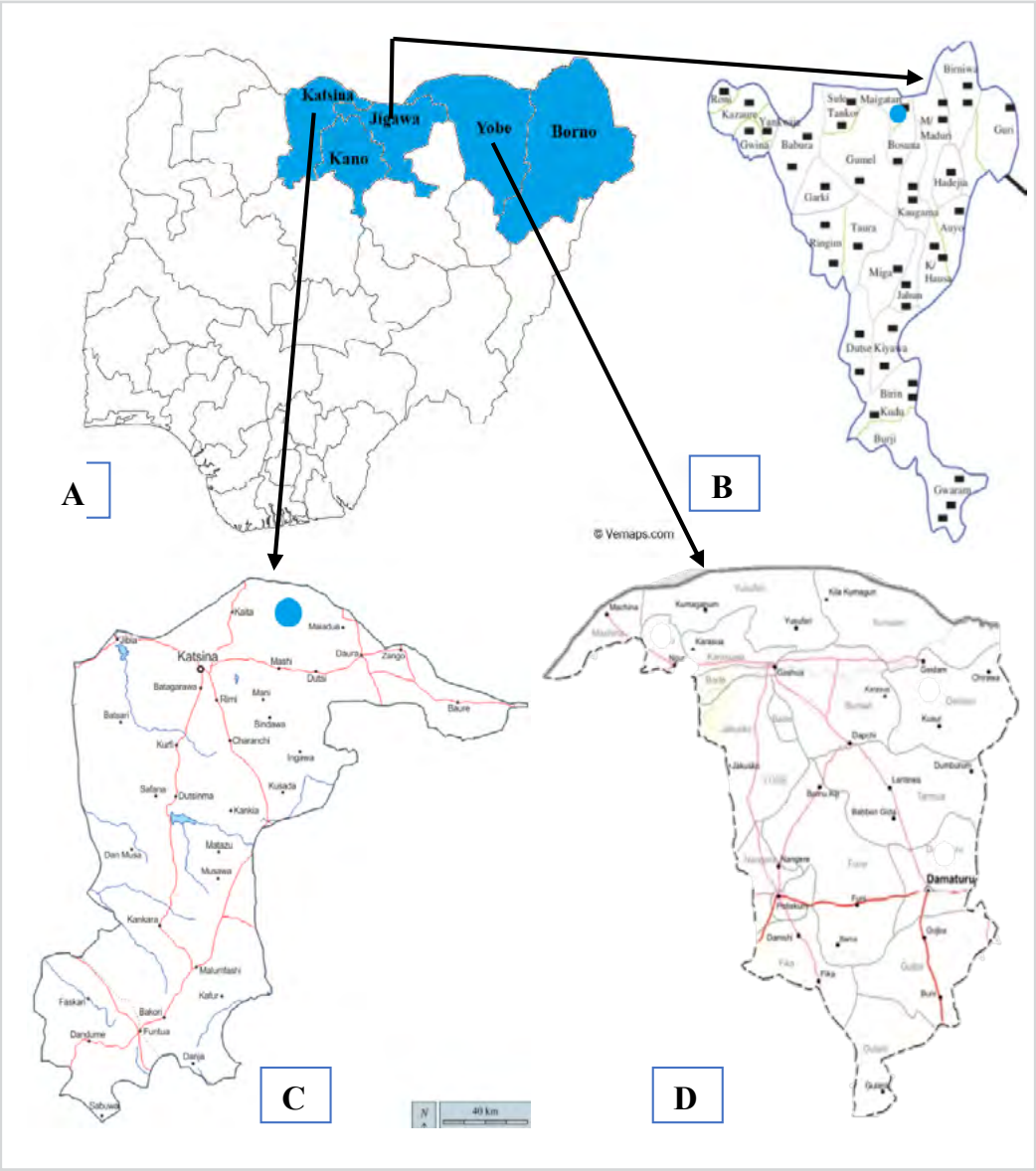

Figure 1 A. Map of Nigeria showing Katsina, Jigawa, Yobe, Borno and Kano States; B. Map of Jigawa State showing Maigatari town; C. Map of Katsina State showing Maiadua town; D. Map of Yobe State showing the Geidam, Nguru and Damaturu towns

and a metropolitan area of 499 km² (Ibrahim, 2014). Most of the camels were said to have been bought and transported from Geidam, Nguru and Damaturu towns of Yobe State; Maigatari in Jigawa State; Maiadua in Katsina State and from Borno State (Figure 1).

Study Design

The study was a cross-sectional study of the prevalence of gastrointestinal parasites and associated factors in camels slaughtered at Kano main abattoir. A purposive sampling method was employed in this study.

Sample Size

The sample size for the prevalence study was calculated according to Thrusfield (Thrusfield, 2018) using the formula below. Using the prevalence of 92.4% (gastrointestinal parasite infection in one-humped camels of Nigeria) reported by Bamaiyi and Kalu (Bamaiyi and Kalu, 2011), the sample size was calculated to be 108. But a total of 115 camels were sampled.

$$n = \frac{1.96^2 \times P_{exp}(1 - P_{exp})}{d^2}$$

where; n = required sample size
Pexp = expected prevalence (0.924)
d = desired absolute precision (0.05).

Sample Collection and Data Recording

Faecal samples were collected directly from the rectum of selected camels immediately after slaughter using disposable gloves. Approximately 10g of faeces were collected from each animal and placed in labelled plastic containers. The samples were then transported in a cool box to the Parasitology Laboratory at the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, for analysis within 24 hours of collection.

During sample collection, data on the sex (based on physical characteristics), estimated age (based on dentition), body condition score (BCS), faecal consistency and faecal mucoid status of each camel were recorded. The body condition score was assessed using a 5-point scale as Faye et al. (2001) described, where 1 = emaciated, 2 = thin, 3 = average, 4 = fat, and 5 = obese. Modifying this scale, emaciated and thin were categorized as a poor condition; average as a moderate condition, and fat as a good condition. A total of 7 (6.09%) were in poor condition, 75 (65.22%) were in moderate condition, and 33 (28.70%) were in good condition. No obese camels were seen. Faecal consistency was categorized as pasty or pelleted. Faecal mucoid status was categorized as mucoid and non-mucoid.

Parasitological Examination

Faecal samples were examined using standard parasitological techniques, including direct smear, sedimentation and flotation techniques, as described by Soulsby (1982).

Direct Smear Technique

A small amount of faeces was mixed with a drop of normal saline on a clean glass slide, covered with a coverslip, and examined under a light microscope at 10x and 40x magnifications for the presence of motile parasites and protozoan trophozoites.

Sedimentation Technique

Two grams of faeces were mixed with 30 ml of water in a beaker. The mixture was filtered through a tea strainer, and the filtrate was allowed to sediment for 30 minutes. The supernatant was discarded, and the sediment was examined under a microscope at 10x magnification for the presence of trematode eggs.

Flotation Technique

Two grams of faeces were mixed with 28 ml of saturated sodium chloride and sugar solution. The mixture was strained through a tea strainer, and the filtrate was poured into a 15 ml test tube. The test tube was filled to the brim with the flotation solution, and a coverslip was placed on top. After 20 minutes, the coverslip was removed and placed on a glass slide for examination under a microscope at 10x and 40x magnifications.

Parasite eggs and oocysts were identified based on their morphological characteristics using a reference guide (Soulsby, 1982).

Quantitative Analysis

Quantitative analysis of parasite eggs was performed using the McMaster technique as described by Hansen and Perry, (1994). Two grams of faeces were mixed with 28 ml of saturated sodium chloride and sugar solution. The mixture was strained, and the filtrate was used to fill both chambers of a McMaster slide. After 5 minutes, the slide was examined under a microscope at 10x magnification. The number of eggs per gram

(EPG) of faeces was calculated by multiplying the total number of eggs in both chambers by 50.

Data Analyses

The collected data were summarised and presented in tables, charts and photomicrographs. Descriptive statistics were used to calculate the prevalence. The Chi-square test for association was used to check for the relationship between the prevalence of the disease and factors such as sex, body condition score, and the nature of faeces. SPSS version 26 from IBM was used for prevalence and risk factors analyses. Values of P less than or equal to 0.05 were considered significant.

RESULTS

In terms of sex, 60 (52.17%) were males, and 55 (47.82%) were females. Regarding body condition score, 7 (6.09%) were in poor condition, 75 (65.22%) were in moderate condition, and 33 (28.70%) were in good condition. No obese camels were seen. For faecal consistency, 21 (18.26%) had pasty faeces, and 94 (81.7%) had pelleted faeces. In terms of faecal mucus status, 20 (17.40%) had mucoid faeces, and 95 (82.61%) had non-mucoid faeces (Table 1).

Ninety-seven (84.35%) of the camels were positive for gastrointestinal parasites, while 18 (15.65%) were negative (Table 2).

In this study, seven (7) different parasite egg/oocyst types were identified based on their egg/oocyst morphology. Parasites belonging to the three classes of helminths namely nematodes, cestodes and trematodes as well as coccidian oocysts were identified in the faecal samples. The eggs of helminths seen were those of *Strongyle*-type egg (Figure 2a), *Strongyloides* spp. (Figure 2b), and *Trichuris* spp. (Figure 2c); *Anoplocephala* spp. (Figure 2d); and *Fasciola* spp. (Figure 2e). Also, *Eimeria camelli* (Figure 3b) and *Eimeria rajasthani* (Figure 3b) oocysts were seen.

Out of the total, 20 individuals (20.62%) were infected with nematodes, 1 (1.03%) with cestodes, none (0%) with trematodes, 2 (2.06%) with coccidia, while 74 (76.29%) had mixed gastrointestinal parasite infections (Table 3). Among nematodes, *Strongyle*-type egg (12 cases, 12.37%), *Trichuris* spp.(7 cases, 7.22%), and *Strongyloides* spp. (1 case, 1.03%) were identified. Among cestodes, *Anoplocephala* spp. (2 cases, 2.06%) was found. Among Protozoan parasites, *Eimeria* spp. (1 case, 1.03%) was identified. Seventy-four cases (76.29%) had mixed infections (Table 4).

There were cases of single infections with parasites like *Eimeria* spp., *Strongyle*-type egg, *Strongyloides* spp., *Trichuris* spp., and *Anoplocephala* spp. Double infections were observed with various combinations of two parasite genera. The most common double infection was *Eimeria* spp. + *Strongyle*-type egg (11 cases). Triple infections were present, with the most prevalent being *Eimeria* spp. + *Strongyle*-type egg + *Trichuris* spp. (15 cases). Quadruple infections involved four different parasite genera co-occurring, with the combination of *Eimeria* spp. + *Strongyle*-type egg + *Strongyloides* spp. + *Trichuris* spp. being the most frequent (7 cases). A few cases (2 cases) of quintuple infections with five different parasite genera were also observed (Table 5).

For body condition score: Poor (7 cases, 100%), Moderate (62 out of 75 cases, 82.67%), Good (28 out of 33 cases, 84.84%). For sex: Male (56 out of 60 cases, 93.33%), Female (41 out of 55 cases, 74.54%). For faecal consistency: Pasty faeces (21 out of 21 cases, 100%), pelleted faeces (76 out of 94 cases, 80.85%). For faecal mucus status: Mucoid faeces (19 out of 20 cases, 95.00%), non-mucoid faeces (78 out of 95 cases, 82.10%). Sex and faecal consistency showed a statistically significant association with gastrointestinal parasite prevalence (p-value < 0.05), while body condition score and faecal mucus status did not show a significant association (p-value > 0.05) (Table 6).

A mean faecal egg count of 162.70 ± 30.55 was recorded in this study. Out of the camels infected with gastrointestinal parasites, 92.63 % (90/97) had a mild infection (mean faecal egg count of 0-500), 3.16 % (3/97) had a moderate infection

(mean faecal egg count of 500-1000), and 4.21% (4/97) of the camels had a severe infection (mean faecal egg count above 1000). The mean faecal egg count was slightly higher (166 ± 41.90) in the female camels than in the male (162.20 ± 46.38). Interestingly, the mean faecal egg count was 250 ± 174.49 in camels with poor body condition and 142.86 ± 45.83 in those with good body condition.

Table 1 Demography of Slaughtered Trade Dromedary Camels in Kano, Northern Nigeria (n= 115)

Demography	Number	Percentage (%)
Sex		
Male	60	52.2
Female	55	47.8
Body condition score		
Poor	7	6.0
Moderate	75	65.2
Good	33	28.7
Faeces consistencies		
Pasty faeces	21	18.3
Pelleted faeces	94	81.7
Faecal mucus status		
Mucoid	20	17.4
Non-mucoid	95	82.6

Table 2 Prevalence of Gastrointestinal Parasite in Slaughtered Trade Dromedary Camels in Kano, Northern Nigeria

Gastrointestinal parasite	Number	Prevalence (%)
Positive	97	84.4
Negative	18	15.6
Total	115	100

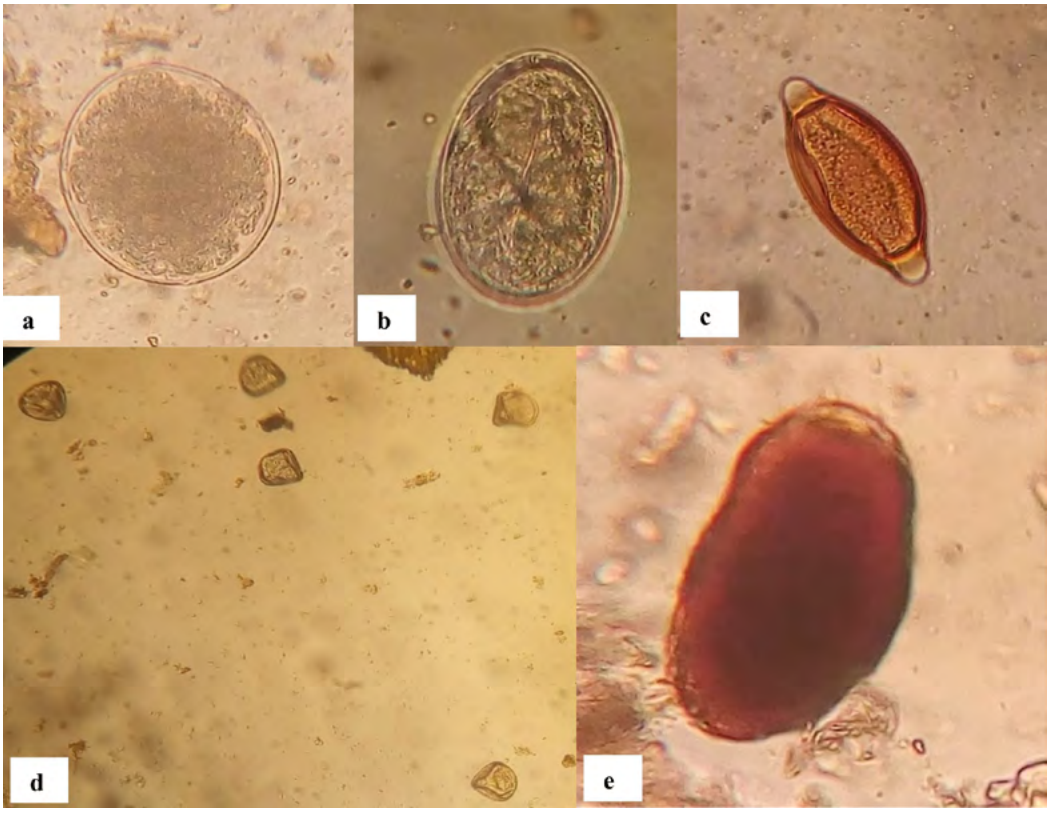

Figure 2 Photomicrographs of a: Strongyle-type egg; b: *Strongyloides* spp.; c: *Trichuris* spp.; d: *Anoplocephala* spp; e: *Fasciola* spp. in the faeces of camels slaughtered in Kano Main Abattoir

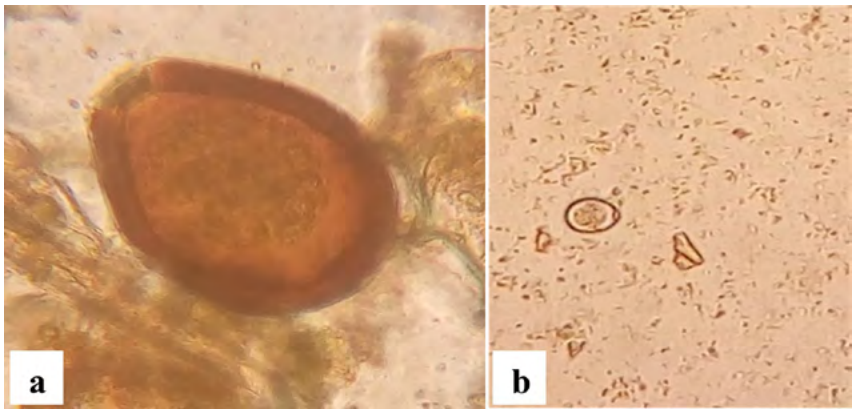

Figure 3 Photomicrographs of a: *E. camelli;* and b: *E. rajasthani* oocysts in the faeces of camels slaughtered at Kano Main Abattoir

Table 3 Summary of Gastrointestinal Parasites Found in Slaughtered Trade Dromedary Camels in Kano, Northern Nigeria

Gastrointestinal parasite	Number	Percentage (%)
Nematodes	20	20.62
Cestodes	1	1.03
Trematodes	0	0
Coccidia	2	2.06
Mixed GIT parasite	74	76.29
Total	97	100

GIT= Gastrointestinal

Table 4 Summary of Genus-Specific Gastrointestinal Parasites Found in Slaughtered Trade Dromedary Camels in Kano, Northern Nigeria

Gastrointestinal parasite	Frequency	Percentage (%)
Single Infections		
Nematodes		
Strongyle-type egg	12	12.37
Trichuris spp.	7	7.22
Strongyloides spp.	1	1.03
Cestodes		
Anoplocephala spp.	2	2.06
Trematodes		
Fasciola spp.	0	0
Coccidia		
Eimeria spp.	1	1.03
Mixed infection	74	76.29
Total	97	100

Table 5 Distribution of Specific Gastrointestinal Parasites Found in Each Slaughtered Trade Dromedary Camel in Kano Main Abattoir

Gastrointestinal parasites	No. positive	Prevalence
Eimeria spp.	2	2.04
Strongyle-type egg	12	12.24
Strongyloides spp.	1	1.02
Trichuris spp.	7	7.14
Anoplocephalide spp.	1	1.02
Eimeria spp. + *Fasciola* spp.	1	1.02
Eimeria spp. + *Strongyloides* spp.	1	1.02
Eimeria spp. +*Strongyle*-type egg	11	11.22
Strongyloides spp. + *Trichuris* spp.	1	1.02
Strongyle-type egg + *Fasciola* spp.	2	2.04

Gastrointestinal parasites	No. positive	Prevalence
Strongyle-type egg + *Strongyloides* spp.	5	5.10
Strongyle-type egg + *Trichuris* spp.	7	7.14
Eimeria spp. + *Strongyle*-type egg + *Trichuris* spp.	15	15.31
Eimeria spp. + *Strongyloides* spp. + *Trichuris* spp.	1	1.02
Eimeria spp. + *Strongyle*-type egg + *Anoplocephalide* spp.	2	2.04
Coccidia spp. + *Strongyle*-type egg + *Fasciola* spp.	1	1.02
Eimeria spp. + *Strongyle*-type egg + *Trichuris* spp.	5	5.10
Strongyle-type egg + *Trichuris* spp. + *Strongyloides* spp.	7	7.14
Eimeria spp. + *Strongyle*-type egg + *Strongyloides* spp. + *Trichuris* spp.	7	7.14
Eimeria spp. + *Strongyle*-type egg + *Strongyloides* spp. +*Anoplocephalide* spp.	1	1.02
Eimeria spp. + *Strongyle*-type egg + *Strongyloides* spp. + *Fasciola* spp.	1	1.02
Eimeria spp. + *Strongyle*-type egg + *Trichuris* spp. + *Anoplocephalide* spp.	2	2.04
Eimeria spp. + *Strongyle*-type egg + *Trichuris* spp. + *Fasciola* spp.	1	1.02
Strongyle-type egg + *Strongyloides* spp. + *Trichuris* spp.+ *Anoplocephalide* spp.	1	1.02
Eimeria spp. + *Strongyle*-type egg + *Strongyloides* spp. + *Trichuris* spp. + *Fasciola* spp.	1	1.02
Strongyle-type egg + *Strongyloides* spp. + *Trichuris* spp. + *Anoplocephala* spp. + *Fasciola* spp.	1	1.02
Total	97	100

Table 6 Evaluation of Body Condition Score, Sex, Faecal Consistency, and Faecal Mucus Status as Potential Risk Factors Associated with the Prevalence of Gastrointestinal Parasites in Slaughtered Trade Dromedary Camels in Kano Main Abattoir

Factor	No. of camel	No. of camel with GIT parasite	Prevalence of GIT parasite (%)	X^2 value	p- value
Body condition score					
Poor	7	7	100	2.5	0.298
Moderate	75	62	82.7		
Good	33	28	84.8		
Sex					
Male	60	56	93.3	7.6	0.006*
Female	55	41	74.5		
Faeces consistencies					
Pasty faeces	21	21	100	4.9	0.027*

Factor	No. of camel	No. of camel with GIT parasite	Prevalence of GIT parasite (%)	X^2 value	p- value
Pelleted faeces	94	76	80.9		
Faecal mucus status					
Mucoid faeces	20	19	95	2.7	0.101
Non-mucoid faeces	95	78	82.1		

* = significant ($p < 0.05$)

The present study revealed a high prevalence (84.35%) of gastrointestinal parasites in camels slaughtered at the Kano Main Abattoir, with Strongyle eggs being the most common (72.17%). This finding is consistent with previous studies conducted in Nigeria and other parts of Africa, highlighting the widespread nature of camel parasitic infections in these regions. The prevalence observed in our study is higher than that reported by Mahmuda et al. (2014) in Sokoto (78%) but lower than the 92.4% reported by Bamaiyi and Kalu (2011) in Maiduguri. These variations may be attributed to differences in geographical location, climate, management practices and sample size.

The high prevalence of parasitic infections, particularly mixed infections (76.29%), is likely attributable to several factors. First, the free-ranging management practices common in camel husbandry in Nigeria provide ample opportunities for parasite transmission (El-Khabaz et al., 2019). Camels often graze on communal pastures, increasing their exposure to infective stages of various parasites. Second, the presence of various reservoir hosts, such as sheep and goats, in the same ecological niche may contribute to cross-species transmission of certain parasites (El-Naga and Barghash, 2016). Third, the climatic conditions in northern Nigeria, characterized by high temperatures and seasonal rainfall, favour the survival and development of parasitic stages in the environment (Swai et al., 2011).

The significant associations observed between host factors and the prevalence of specific parasite types provide valuable insights into the epidemiology of these infections. The higher prevalence of Strongyle eggs in male camels and those with pasty faecal consistency is consistent with findings from other studies (Ahmed et al., 2013; Duguma et al., 2014). This may be related to differences in grazing behaviour, stress levels, or hormonal influences between males and females (Benaissa and Iglesias-Patrana, 2024). The association between body condition score and the prevalence of infection with *Strongyloides* and *Fasciola* suggests that these parasites may have a more pronounced impact on the overall health status of infected camels.

The low mean faecal egg counts observed in this study, despite the high prevalence of infections, warrant further investigation. Several factors could influence the number of parasite eggs found in faecal samples, including the stage of infection, host immunity, and the impact of recent anthelmintic treatments (Jesca et al., 2017). It is important to note that faecal egg counts may not always accurately reflect the actual worm burden, particularly in cases of immature or single-sex infections (Hansen and Perry, 1994). Future studies combining faecal egg counts with post-mortem worm counts would provide a more comprehensive understanding of the relationship between egg output and parasite burden in camels.

The identification of six different parasite types in this study (*Strongyle, Strongyloides, Trichuris, Anoplocephala, Fasciola*, and *Eimeria* parasites) highlights the diversity of gastrointestinal parasites affecting camels in the study area. This diversity poses challenges for effective parasite

control, as different parasites may require varying treatment approaches. The high prevalence of multiple infections (76.29%) further complicates the situation, potentially leading to more severe clinical manifestations in affected animals (Swai et al., 2011). Several limitations of this study should be acknowledged. First, the cross-sectional design provides a snapshot of parasite prevalence at a specific time point and does not capture seasonal variations or long-term trends. Second, the study was conducted at a single abattoir, which may not represent the entire camel population in Nigeria. Future studies incorporating multiple sampling sites and longitudinal designs would provide a more comprehensive understanding of camel parasite epidemiology in the region.

In conclusion, this study demonstrates a high prevalence of gastrointestinal parasites, particularly Strongyle species, in camels slaughtered at the Kano Main Abattoir. Further research is needed to elucidate the economic impact of these parasitic infections on camel productivity and to develop evidence-based, sustainable parasite control strategies tailored to the specific challenges faced by camel herders in Nigeria and similar ecological zones.

CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

AUTHORS CONTRIBUTIONS

Concept - MOE; Design – MOE, SA, BM, JOE, MNP; Supervision - SA, BM; Resources – MOE, JOE; Materials – JOE, JSE, SYI; Data Collection and Processing -JAA, SYI, JSE, OOA; Analysis and Interpretation – JOE, MNP, OOA, JAA; Literature Search – MOE, JAA OOA; Writing Manuscript -, MOE, JOE, OOA; Critical Review - MOE, SA, BM, JOE, JSE, SYI, MNP.

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PREVALENCA GASTROINTESTINALNIH PARAZITA I PRIDRUŽENI RIZIKO FAKTORI KOD DROMEDARA IZ KLAONICA U KANOU, SJEVERNA NIGERIJA

SAŽETAK

Paraziti igraju ključnu ulogu u zdravlju životinja zbog mnogobrojnih izazova koje predstavljaju kako za zdravlje tako i za dobrobit životinja. Prevalenca i distribucija gastrointestinalnih parazitskih infekcija kod kamila dromedara s tržišta u Kanou, glavnom središtu trgovine kamilama u Sjevernoj Nigeriji, nisu dovoljno dokumentirane. Ovo istraživanje koje identificira gastrointestinalne parazite do razine roda i procjenjuje udružene riziko faktore kod kamila u glavnoj klaonici Kanoa, u Nigeriji, doprinosi razvoju učinkovitih strategija kontrole parazita kako bi se poboljšali zdravlje i produktivnost kamila. U presječnom istraživanju je pregledano ukupno 115 kamila na gastrointestinalne parazite, pri čemu je korištena metoda fekalne flotacije i sedimentacije. Ukupna prevalenca gastrointestinalnih parazita je iznosila 84.4%, pri čemu je 76.3% inficiranih kamila imalo miješanu parazitsku infekciju. Identificirano je sedam različitih tipova parazita koji su pripadali nematodama (jaja *Strongyle*-tipa, *Trichuris* spp., *Strongyloides* spp.), cestodama (*Anoplocephala* spp.), trematodama (*Fasciola* spp.) i kokcidijama (*Eimeria cameli*, *Eimeria rajasthani*). Najvišu prevalencu su imali paraziti sa jajima *Strongyle*-tipa (12.4) i *Trichuris* spp. (7.2%). Riziko faktori signifikantno povezani sa prevalencom parazita su bili: spol (mužjaci 93.3%, ženke 74.5%) i konzistencija fecesa (pastozni 100%, peletirani 80.9%). Tjelesni skor i prisustvo sluzi u fecesu nisu pokazali signifikantnu povezanost. Srednji broj jajašaca u fecesu je iznosio 162.7 po gramu, sa 92.6% inficiranih kamila koje su imale blagu infekciju. Ovo istraživanje naglašava visoko opterećenje kamila gastrointestinalnim parazitima u regiji, kao i potrebu za poboljšanjem strategija kontrole parazita.

Ključne riječi: Gastrointestinalni paraziti, kamile, prevalenca, Sjeverna Nigerija

RESEARCH ARTICLE

OVARIAN POTENTIAL OF LOCAL GOATS FOR *IN VITRO* EMBRYO PRODUCTION IN THE FAR NORTH OF CAMEROON

Justin Kouamo[1]*, Joseph Baring Mampom[1]

[1]Department of Reproductive Physiology and Biotechnology, School of VeterinaryMedicine and Sciences, The University of Ngaoundere, Ngaoundere, Cameroon.

***Corresponding author:**
Pr. Dr. Justin Kouamo
[1]Department of Reproductive Physiology and Biotechnology, School of Veterinary Medicine and Sciences, The University of Ngaoundere, Ngaoundere, Cameroon.
Adress:
PO BOX 454 Ngaoundere, Cameroon
Phone: +237 675376954
ORCID: 0000-0002-5032-5634
E-mail: justinkouamo@yahoo.fr

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ABSTRACT

The study was carried out on 281 local goats (257 Sahelian and 24 Kirdi) at the municipal small ruminant abattoir in Maroua, in the Far North region of Cameroon. A total of 562 ovaries were collected and transported in 0,9% NaCl solution to the laboratory. After clearing the ovaries of the surrounding tissue, the follicles on each ovary were counted, their diameter (Φ) measured and classified into small (Φ< 3 mm), medium (3 ≤ Φ ≤ 8 mm) and large (8 ≤ Φ< 10 mm). Subsequently, the oocytes were harvested in Dubelcco's Phosphate Buffered-Saline solution using the slicing technique, observed under a light microscope at 40X magnification, and classified into four groups according to the degree of cumulus cell compactness and ooplasm transparency. The mean follicular population was 25.38 ± 6.84 per ovary. Small, medium and large follicles were 5.87 ± 2.90, 15.64 ± 4.71 and 3.86 ± 1.69 in diameter, respectively. Oocyte yield was 33.39 ± 11.71 per ovary. Quality I, II, III, IV oocytes were 11.57 ± 3.33 (34.65%), 9.64 ± 3.05 (28.87%), 8.25 ± 3.26 (24.71%) and 3.93 ± 2.01 (11.77%), respectively. The oocyte quality index was 2.11. Oocytes with quality (grade I and II) acceptable for *in vitro* embryo production (IVEP) constituted 63.52% of the harvest. These results indicate that certain factors such as the age, body condition, pregnancy status and stage, ovarian weight and corpus luteum must be considered to increase the ovary's potential for *in vitro* embryo production.

Keywords: Cameroon, follicular population, goats, ovaries, oocyte quality

INTRODUCTION

Goats in Cameroon represent 64.52% of the 11,080,929 head of small ruminants. The Far North Region alone accounts for more than two-thirds (2 923 312) of the national goat population (INS, 2023). The herd is mainly made up of Sahelian and Kirdi goats. The productivity of these breeds remains under-utilised. Genetic, zootechnical, health and breeding problems are cited as the factors responsible for low productivity (Sousa et al., 2004).

In response to these shortcomings, governments have adopted various techniques through programmes of artificial insemination (AI) decades ago to increase the number of offspring with elite genetic merit by reducing the generation interval. Today, the generation interval has been further narrowed by *in vitro* fertilization. It shows the way in terms of the effectiveness of rapid genetic improvement, especially as farmers aim to speed up genetic selection. These techniques have revolutionized genetic selection by the female route (Leroy, 2022). *In vitro* maturation, positive *in vitro* fertilization and embryo transfer allow producers to make the same genetic progress in one generation that would traditionally take five generations using AI or natural reproduction. In addition, superior individuals from both parents are used to contribute to the genetic improvement of the next generation (Ax et al., 2005).

As oocytes form the basis of biotechnologies applied to the embryo, their collection and evaluation are a prerequisite for the success of these biotechnologies, which are mainly aimed at providing solutions to the lack of protein and the conservation of genetic values. The main objective of this study was to evaluate the ovarian potential of Sahelian and Kirdi goats slaughtered in Diamare for the production of fertilizable oocytes *invitro*. Specifically, the follicular population, oocyte yield and quality will be determined, and the effects of ovarian and non-ovarian factors on the follicular population, oocyte yield and quality will be assessed.

MATERIAL AND METHODS

Study area

This study was carried out at the Diamare municipal small ruminant abattoir in Maroua 2, and the samples were analysed in the laboratory of the 'Centre National de Formation Zootechnique et Vétérinaire' in Maroua in the Far North Region of Cameroon. Located between latitude 10° 35′ 37″ North and longitude 14° 18′ 52″ East (Benjamine and Wadou, 2023), it has a Sahelian climate with a long dry season from October to June and a short rainy season from July to September. Average annual rainfall and annual temperature recorded were 794 mm and 27.5°C, respectively. The 281 goats studied came from the Divisions of Mayo Sava (Tokombere, Mora, Pete, Mayo plata), Diamare and Mayo Kani.

Characteristics of the animals

A total of 281 local goats were studied: 257 of the Sahelian type and 24 of the Kirdi breed.

Before slaughter, thoracic perimeter (TP), height at withers, scapulo-ischial length, horn length, ear length and tail length were measured for each goat using the tape measure to enable us to determine breed (Mani et al., 2014). Then, the weight was measured using a 50 kg Mini Mechanical Scale accurate to 100 g. Body condition score (BCS) was based on determining the amount of muscle and fat over and around the vertebrae. Scoring was performed in goats using a BCS ranging from 1.0 to 5.0, with 0.5 increments, as described by Ghosh et al. (2019).

After slaughter, age was estimated by examining the dentition (the change between the milk incisors and the permanent ones): an animal with two permanent teeth is about 1 year old, four permanent teeth at 2 years, six permanent teeth at about 3 years, and when the goat has eight permanent teeth, the animal is about 4 years old, as described by Hutu (2019). In the case of pregnant goats (characterized by the presence of one or more fetuses), their proportion in relation to the goats studied was determined; the fetal age was determined by the formula $X = 2.1 (Y + 17)$

(Arthur et al., 2001), where Y represented cranio-caudal length in cm and X represented gestation length in days, and gestation length was classified into two groups: ≤ 50 days and 51-100 days.

Ovary collection and handling

After the identification and slaughter of each goat, the right and left ovaries were removed using scissors and placed in separate collection bottles containing an isotonic solution (NaCl, 0.9%). They were transported in an isothermal container (20-30°C) within two hours of slaughter. Ovaries with follicles > 10 mm in the absence of a corpus luteum (Cystic ovaries, Kouamo et al., 2020a) were excluded.

Determination of the weight and size of the ovary

In the laboratory, the ovaries were freed of tissue debris (broad ligament or mesovarium holding the ovary together) and then weighed using an MH-Series Pocket Scale 200g electronic balance with a precision of 0.01. Based on their weight, the ovaries were divided into three groups: small (<1 g), medium (1 to 2 g) and large (> 2 g) (Islam et al., 2007). The size (length, width and thickness) of the ovary was measured using a mechanical calliper. The ovaries were divided into two groups based on mean volume: ovarian volume less than 1.38 x 0.94 x 0.56 cm^3 and greater than 1.38 x 0.94 x 0.56 cm^3 (Ngona et al., 2012).

Determination of the follicular population

After rinsing each ovary in a physiological solution, the visible follicles were counted. Follicular diameters (Φ) were measured with a calliper and classified into 3 categories: small (Φ< 3 mm), medium (3 ≤ Φ ≤ 8 mm) and large (8 <Φ< 10mm), as described by Duygu et al. (2013). They were kept in an isothermal bag at a temperature of 30°C throughout their examination.

Collection and classification of oocytes

Each ovary was placed in a Petri dish containing 5 ml of Dulbecco's phosphate-buffered saline (DPBS). The slicing technique was used to collect the oocytes. The oocytes were examined and counted using a light microscope with a 40X magnification. The oocytes were then classified into 4 qualities (Q) taking into account the homogeneity of the cytoplasm and layers of the cumulus oophorus cells, according to Kouamo et al. (2020b). Quality 1 (Q1): the cumulus (granule cells) were compact and surrounded the oocyte (more than three layers). The oocyte ooplasm had a homogeneous appearance; Quality 2 (Q2): the cumulus was compacted with one or two layers, but the ooplasm had a more irregular appearance, with a darker area visible at its periphery; Quality 3 (Q3): the cumulus had a layer of irregular and less compacted cells, and the ooplasm was less regular with dark areas; Quality 4 (Q4): The cumulus was completely expanded or even absent (naked oocytes), and the ooplasm was irregular with dark areas. To assess overall oocyte quality, an index was calculated: [quality I x 1 + quality II x 2 + quality III x 3 + quality IV x 4] / total number of oocytes], as described by Kouamo et al. (2020b). A value tending towards 1 reflects good overall oocyte quality.

Statistical analysis

Data were analysed using R® software. One-way analysis of variance (ANOVA) was performed to assess the effect of ovarian (ovary localization, corpus luteum, ovary weight, ovary size) and non-ovarian (breed, age, BCS, pregnancy status, pregnancy length) factors on follicular population, yield and oocyte quality. Differences between means were tested by Duncan's test. Differences were significant at $P<0.05$.

RESULTS

Characteristics of the slaughtered goats

The average age (year), BCS and live weight (kg) of the goats were 1.64 ± 0.15, 2.74 ± 0.45 and 24.12 ± 4.62, respectively, with that of Sahelian type goats (24.45 ± 4.3 kg) significantly higher ($P<0.05$) than that of Kirdi type goats (20.64 ± 5.63 kg). A pregnancy rate of 43.45% and an average fetal age of about 61 days were recorded.

The mean weight (g) of the ovary was 1.34 ± 0.34,

and the left ovary (1.43 ± 0.49 g) was significantly (P<0.0001) heavier than the right (1.25 ± 0.40 g). The length, width and thickness (cm) of the ovaries were 1.38 ± 0.19, 0.94 ± 0.14 and 0.56 ± 0.17, respectively. BCS and the presence of the corpus luteum significantly (p<0.05) increased ovarian thickness. The presence of the corpus luteum statistically increased the length, width and thickness. The average ovary weights of non-pregnant were greater (P<0.05) than those of pregnant goats (Table 1).

Follicular population

From 562 harvested ovaries, 14,262 follicles were counted with an average of 25.38 ± 6.84 per ovary. Small (Φ < 3 mm), medium (3 ≤ Φ < 8 mm) and large (8 ≤ Φ <10 mm) follicles accounted for 23.15%, 61.63% and 15.22% of the follicular population, respectively.

Oocyte yield and quality

Eighteen thousand seven hundred and sixty-five (18,765) quality I, II, III and IV oocytes were harvested from 562 ovaries, giving a mean oocyte yield per ovary of 33.39 ± 11.71. The quality of oocytes classified as I, II, III and IV (Figure 1) was 11.62 ± 3.34 (34%); 9.56 ± 3.03 (29%); 8.44 ± 3.45 (25%) and 3.99 ± 2.03 (12%), respectively. Oocytes judged to be of good quality for maturation and fertilization *in vitro* (Q. I and II) represented 63.52% (11,919) of the harvest. The oocyte quality index (OQI) was 2.11.

Effect of ovarian factors on follicular population, oocyte yield and quality

The total number of follicles increased with ovarian weight (g) and size (cm); however, oocyte yield and quality decreased with ovarian weight. Ovaries with a corpus luteum had more follicles and more fertilizable oocytes (Tables 2 and 3).

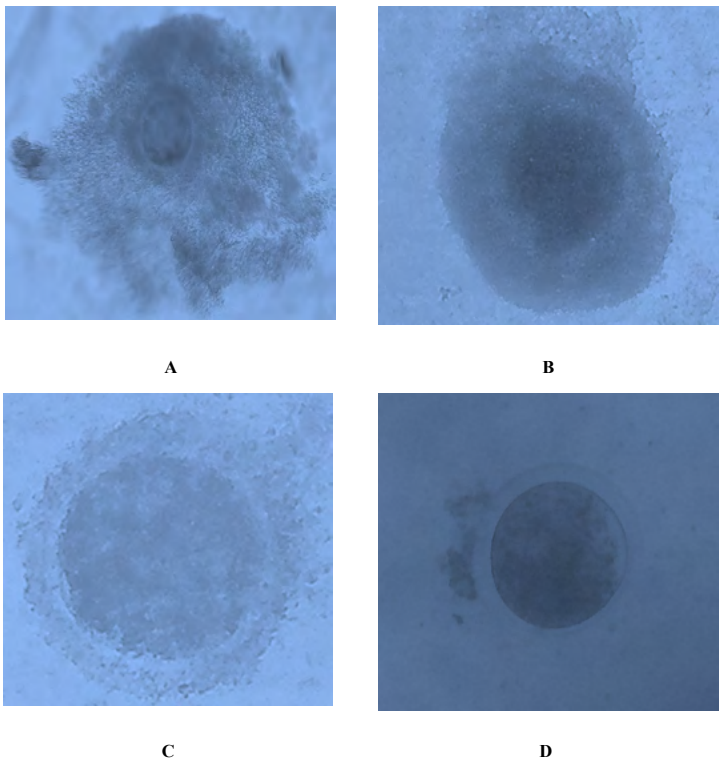

Figure 1 Quality of oocytes.(a) Quality 1: oocyte surrounded by a compact cumulus with more than three layers and presenting a homogeneous ooplasm; (b) Quality 2: oocyte surrounded by a compact cumulus having one or two layers and presenting an ooplasm with an irregular appearance, with a darker zone visible at its periphery; (c) Quality 3: the cumulus has a layer of irregular and less compact cells and the ooplasm is less regular with darker areas; (d) Quality 4 : The cumulus is completely expanded or even absent (naked oocytes) and the ooplasm is irregular with dark areas

Table 1 Variation in ovarian weight and size as a function of breed, BCS, age, pregnancy status and corpora lutea (mean ± SD)

Parameters	Variables	N	Right ovary weight (g)	Left ovary weight (g)	Ovary weight (g) per animal	Ovary length (cm)	Ovary width (cm)	Ovary thickness (cm)
Breed	Kirdi	24	1.42 ± 0.15[a]	1.32 ± 0.26[a]	1.37 ± 0.16[a]	1.41 ± 0.15[a]	0.97 ± 0.12[a]	0.58 ± 0.14[a]
	Sahelian	257	1.29 ± 0.11[a]	1.30 ± 0.19[a]	1.29 ± 0.11[a]	1.35 ± 0.23[a]	0.91 ± 0.16[a]	0.55 ± 0.20[a]
	P-value		***0.262***	***0.911***	***0.530***	***0.3***	***0.051***	***0.2***
BCS	Good (>3)	31	1.38 ± 0.15[a]	1.25 ± 0.26[a]	1.32 ± 0.15[a]	1.36 ± 0.23[a]	0.93 ± 0.22[a]	0.57 ± 0.15[b]
	Medium (2.5-3)	133	1.30 ± 0.12[a]	1.23 ± 0.20[a]	1.26 ± 0.12[a]	1.35 ± 0.21[a]	0.90 ± 0.14[a]	0.51 ± 0.13[a]
	Thin (<2.5)	117	1.37 ± 0.12[a]	1.44 ± 0.22[a]	1.41 ± 0.13[a]	1.36 ± 0.24[a]	0.92 ± 0.16[a]	0.59± 0.25[b]
	P-value		***0.77***	***0.611***	***0.591***	***0.7***	***0.3***	***0.003***
Age (years)	< 1	5	1.48 ± 0.23[a]	1.53 ± 0.41[a]	1.51 ± 0.25[a]	1.45 ± 0.14[a]	0.99 ± 0.07[a]	0.63 ± 0.12[a]
	[1-2[	234	1.30 ± 0.07[a]	1.39 ± 0.13[a]	1.34 ± 0.08[a]	1.36 ± 0.23[a]	0.91 ± 0.16[a]	0.55 ± 0.21[a]
	[2-3[	39	1.34 ± 0.10[a]	1.34 ± 0.17[a]	1.34 ± 0.10[a]	1.31 ± 0.21[a]	0.89 ± 0.16[a]	0.55 ± 0.13[a]
	[3-4]	3	1.29 ± 0.31[a]	0.97 ± 0.55[a]	1.13 ± 0.33[a]	1.37 ± 0.24[a]	0.88 ± 0.12[a]	0.55 ± 0.13[a]
	P-value		***0.96***	***0.924***	***0.905***	***0.5***	***0.5***	***0.5***
Corpus luteum	Absent	106	1.26 ± 0.13[a]	1.17 ± 0.23[a]	1.21 ± 0.14[a]	1.30 ± 0.22[a]	0.87 ± 0.15[a]	0.51 ± 0.13[a]
	Present	175	1.45 ± 0.11[b]	1.44 ± 0.20[b]	1.44 ± 0.12[b]	1.39 ± 0.22[b]	0.94 ± 0.16[b]	0.58 ± 0.22[b]
	P-value		***0.008***	***0.029***	***0.002***	***0.003***	***0.001***	***0.002***
Pregnancystatus	Non-pregnant	159	1.27 ± 0.11[a]	1.51 ± 0.19[a]	1.39 ± 0.11[a]	1.37 ± 0.05[a]	0.91 ± 0.03[a]	0.56 ± 0.04[a]
	Pregnant	122	1.22 ± 0.14[a]	1.34 ± 0.24[a]	1.28 ± 0.14[b]	1.34 ± 0.06[a]	0.92 ± 0.04[b]	0.54 ± 0.05[a]
	P-value		***0.47***	***0.47***	***0.03***	***0.06***	***0.025***	***0.35***

a,b,c : In each column, different letters indicated significant difference between groups ($p<0.05$)

N=number of goats

SD=standard deviation

Table 2 Variation in the number of follicles according to ovarian factors (mean ± SD)

Parameters	Variables	N	Number of follicles			Average number of follicles /ovary
			Small (< 3mm)	Medium (3-8 mm)	Large (> 8 mm)	
Ovary localization	Right	281	5.68 ± 2.91[a]	15.42 ± 4.53[a]	3.84 ± 1.63[a]	24.94 ± 6.40[a]
	Left	281	6.06 ± 2.89[a]	15.86 ± 4.89[a]	3.89 ± 1.74[a]	25.81 ± 7.34[b]
	P-value		***0.894***	***0.254***	***0.299***	***0.035***
Corpus luteum	Absent	106	5.69 ± 2.56[a]	15.57 ± 5.28[a]	3.66 ± 1.51[a]	24.92 ± 7.22[a]
	Present	175	5.99 ± 2.98[a]	15.68 ± 5.08[a]	3.99 ± 1.71[a]	25.66 ± 7.22[a]
	P-value		***0.5***	***0.7***	***0.085***	***0.5***
Ovary weight (g)	<1	56	4.71 ± 3.07[a]	12.68 ± 4.40[a]	2.88 ± 1.48[a]	20.28 ± 6.19[a]
	[1-2]	209	6.05 ± 2.59[b]	16.43 ± 4.99[b]	4.07 ± 1.56[b]	26.56 ± 6.74[b]
	>2	16	7.59 ± 3.67[b]	15.66 ± 6.12[b]	4.59 ± 2.07[b]	27.84 ± 8.93[b]
	P-value		***<0.001***	***<0.001***	***<0.001***	***<0.001***
Ovary size (cm^3)	< 1.38 x 0.94 x 0.56	127	5.10 ± 2.70[a]	14.80 ± 4.90[a]	3.42 ± 1.53[a]	23.31 ± 6.58[a]
	≥ 1.38 x 0.94 x 0.56	154	6.51 ± 2.78[b]	16.34 ± 5.26[b]	4.23 ± 1.65[b]	27.08 ± 7.29[b]
	P-value		***<0.001***	***0.002***	***<0.001***	***<0.001***

a,b,c : In each column, different letters indicated significant difference between groups ($p<0.05$)
N=number of goats
SD=standard deviation

Table 3 Effects of ovarian factors on the number and quality of oocytes (mean ± SD)

Parameters	Variables	N	Average number of oocytes /ovary	Oocyte quality				Selected oocytes for IVEP. (I II)
				I	II	III	IV	
Ovary localization	Right	***281***	33.61 ±11.73[a]	11.61 ±4.38[a]	9.56 ±3.90[a]	8.44 ±4.35[a]	3.99 ±2.57[a]	21.17 ±7.24[a]
	Left	***281***	33.17 ±11.69[a]	11.52 ±4.41[a]	9.72 ±3.99[a]	8.05 ± 3.99[a]	3.86 ± 2.54[a]	21.24 ±7.51[a]
	P-value		***0.581***	***0.963***	***0.848***	***0.087***	***0.807***	***0.807***
Corpus luteum	Absent	106	32.4 ± 13.08[a]	10.70 ± 3.19[a]	9.21 ± 3.19[a]	8.57 ± 4.20[a]	3.92 ± 2.50[a]	19.91 ± 5.45[a]
	Present	175	34.27 ± 15.17[b]	12.09 ± 3.82[b]	9.77 ± 4.27[a]	8.37 ± 4.46[a]	4.04 ± 2.62[a]	21.99 ± 6.61[b]
	P-value		***0.019***	***0.002***	***0.3***	***0.7***	***0.8***	***0.022***
Ovary weight (g)	<1	56	31.19 ± 13.23[a]	10.46 ± 3.34[a]	9.55 ± 3.74[ab]	7.29 ± 3.81[ab]	3.89 ± 2.34[a]	19.38 ± 5.61[ab]
	[1-2]	209	34.7 ± 14.77[a]	11.93 ± 3.75[b]	9.73 ± 3.94[b]	8.92 ± 4.43[b]	4.12 ± 2.65[a]	21.85 ± 6.41[b]
	>2	16	26.94 ± 12[a]	10.69 ± 2.61[ab]	7.25 ± 3.44[a]	6.31 ± 3.93[a]	2.69 ± 2.02[a]	19.13 ± 5.16[a]
	P-value		***0.12***	***0.004***	***0.039***	***0.004***	***0.12***	***0.004***
Ovary size (cm^3)	< 1.38 x 0.94 x 0.56	127	32.94 ± 13.86[a]	11.09 ± 3.39[a]	9.80 ± 3.75[a]	8.00 ± 4.21[a]	4.05 ± 2.51[a]	20.63 ± 5.82[a]
	≥ 1.38 x 0.94 x 0.56	154	34.08 ± 14.94[a]	11.96 ± 3.83[b]	9.36 ± 4.03[a]	8.81 ± 4.45[a]	3.95 ± 2.63[a]	21.68 ± 6.61[a]
	P-value		***0.7***	***0.045***	***0.3***	***0.11***	***0.6***	***0.13***

a,b,c : In each column, different letters indicated significant difference between groups ($p<0.05$)
N=number of goats
SD=standard deviation

Table 4 Variation in follicular population according to non-ovarian factors (mean ± SD)

Parameters	Variables	N	Number of follicles			Average number of follicles/ovary
			Small (< 3mm)	Medium (3-8 mm)	Large (> 8 mm)	
Breed	Kirdi	24	6.88 ± 2.87[a]	14.90 ± 5.73[a]	4.13 ± 1.51[a]	25.90 ± 7.79[a]
	Sahelian	257	5.78 ± 2.81[a]	15.71 ± 5.10[a]	3.84 ± 1.66[a]	25.33 ± 7.18[a]
	P-value		***0.064***	***0.6***	***0.3***	***0.5***
Age (years)	< 1	5	9.80 ± 1.60[a]	19.10 ± 7.77[a]	4.60 ± 0.89[a]	33.50 ± 9.21[a]
	[1-2[	234	5.81 ± 2.79[b]	15.57 ± 5.21[a]	3.81 ± 1.68[a]	25.20 ± 7.34[a]
	[2-3[	39	5.96 ± 2.87[b]	15.77 ± 4.51[a]	4.14 ± 1.53[a]	25.87 ± 5.65[a]
	[3-4]	3	3.00 ± 1.80[ab]	13.50 ± 2.29[a]	2.83 ± 0.76[a]	19.33 ± 3.01[a]
	P-value		***0.007***	***0.6***	***0.3***	***0.074***
BCS	Good (>3)	31	5.98 ± 2.65[ab]	15.35 ± 5.02[a]	3.69 ± 1.61[a]	25.03 ± 7.02[a]
	Medium (2.5-3)	133	5.40 ± 2.72[b]	15.93 ± 4.88[a]	4.02 ± 1.49[a]	25.35 ± 7.13[a]
	Thin (<2.5)	117	6.38 ± 2.92[a]	15.38 ± 5.50[a]	3.74 ± 1.81[a]	25.50 ± 7.43[a]
	P-value		***0.008***	***0.9***	***0.3***	***0.9***
Pregnancy status	Non pregnant	159	11.75 ± 4.48[a]	31.65 ± 8.17[a]	7.92 ± 2.62[a]	51.33 ± 11.63[a]
	Pregnant	122	11.78 ± 4.88[b]	30.66 ± 8.26[a]	7.46 ± 2.65[a]	49.90 ± 11.66[a]
	P-value		***0.90***	***0.69***	***0.48***	***0.75***
Pregnancy length in days	≤50	96	11.61 ± 4.81[a]	31.06 ± 8.36[a]	7.41 ± 2.76[a]	50.08 ± 11.54[a]
	51 – 100	26	12.19 ± 5.13[a]	29.85 ± 7.83[a]	7.69 ± 2.20[a]	49.73 ± 12.16[a]
	P-value		***0.547***	***0.296***	***0.705***	***0.285***

a,b,c : In each column, different letters indicated significant difference between group ($p<0.05$)
N=number of goats
SD=standard deviation

Table 5 Variation in oocyte yield and quality depending on non-ovarian factors (mean ± SD)

Parameters	Variables	N	Average number of oocytes/ovary	Oocyte quality				Selected oocytes for IVEP. I and II (%)
				I	II	III	IV	
Breed	Kirdi	24	35.82 ±17.37[a]	12.81 ± 3.94[a]	10.75 ± 5.14[a]	8.13 ± 4.94[a]	4.13 ± 3.35[a]	23.44 ± 7.26 (65.44)[a]
	Sahelian	257	33.34 ±14.18[a]	11.45 ± 3.62[a]	9.44 ± 3.76[a]	8.47 ± 4.30[a]	3.98 ± 2.50[a]	21.00 ± 6.15 (62.99)[b]
	P-value		***0.10***	***0.059***	***0.10***	***0.9***	***0.7***	***0.016***
Age (years)	< 1	5	40.5 ±11.23[a]	14.90 ± 1.92[a]	10.20 ± 1.30[a]	10.40 ± 4.93[a]	5.00 ± 3.08[a]	26.10 ± 3.25 (64.44)[a]
	[1-2[	234	33.47 ±14.66[a]	11.68 ± 3.72[ab]	9.52 ± 3.97[a]	8.40 ± 4.44[a]	3.87 ± 2.53[a]	21.28 ± 6.39 (63.58)[a]
	[2-3[	39	33.37 ±13.66[a]	10.50 ± 3.08[b]	9.74 ± 3.89[a]	8.49 ± 3.91[a]	4.64 ± 2.78[a]	20.22 ± 5.62 (60.59)[a]
	[3-4]	3	31.33 ±11.72[a]	11.33 ± 4.65[ab]	8.67 ± 2.08[a]	8.00 ± 3.46[a]	3.33 ± 1.53[a]	19.67 ± 6.83 (62.78)[a]
	P-value		***0.2***	***0.030***	***0.9***	***0.9***	***0.5***	***0.081***
BCS	Good (>3)	31	32.64 ±15.61[a]	10.61 ± 4.33[a]	9.16 ± 4.01[a]	8.13 ± 4.35[a]	4.74 ± 2.92[a]	19.81 ± 7.10 **(60.69)**[a]
	Medium (2.5-3)	133	33.43 ±12.77[a]	11.21 ± 3.11[a]	9.57 ± 3.37[a]	8.59 ± 3.88[a]	4.06 ± 2.41[a]	20.87 ± 4.97 (62.43)[a]
	Thin (<2.5)	117	33.96 ±15.87[b]	12.23 ± 3.95 [a]	9.64 ± 4.43[a]	8.37 ± 4.86[a]	3.72 ± 2.63[a]	21.95 ± 7.25 (64.63)[a]
	P-value		***0.009***	***0.055***	***0.8***	***0.8***	***0.2***	***0.4***
Pregnancy status	Non pregnant	159	67.02 ± 9.47[a]	23.41 ± 5.53[a]	19.24 ± 5.32[a]	16.40 ± 5.72[a]	7.96 ± 3.32[a]	42.65 ± 9.47[a]
	Pregnant	122	65.66 ± 3.14[b]	22.80 ± 5.21[b]	19.35 ± 4.80[a]	16.50 ± 5.60[a]	7.69 ± 3.06[a]	42.15 ± 8.61[b]
	P-value		***0.040***	***0.047***	***0.084***	***0.073***	***0.162***	***0.029***
Pregnancy length in days	≤50	96	67.79 ± 14.68[a]	23.03 ± 4.46[a]	19.72 ± 4.78[a]	16.79 ± 14.68[a]	8.24 ± 3.18[a]	42.75 ± 8.92[a]
	51 - 100	26	61.58 ± 12.91[b]	21.85 ± 4.34[b]	17.85 ± 4.73[a]	16.04 ± 5.04[a]	5.85 ± 2.24[b]	39.69 ± 7.66[b]
	P-value		***0.022***	***0.029***	***0.055***	***0.188***	***0.037***	***0.021***

a,b,c : In each column, different letters indicated significant difference between group ($p<0.05$)
N=number of goats
SD = standard deviation

Effect of non-ovarian factors (breed, age, BCS, pregnancy status and duration) on follicular population, oocyte yield and oocyte quality

The total number of follicles, oocyte yield and oocyte quality were higher in goats under 3 years old and with a BCS less than or equal to 3. Small follicles (< 3mm) decreased significantly with age ($P < 0.05$) (Tables 4 and 5).

DISCUSSION AND CONCLUSION

The average body condition score of slaughtered goats is similar to 2.75 reported by Ngona et al. (2012) in the Democratic Republic of Congo, but lower than 3.2 ± 0.6 obtained by Kouamo et al. (2021) in the Sudano-Guinean zone of Cameroon. Indeed, the live weight of Sahelian-type goats is higher than 23.00 ± 2.19 kg reported by Kouamo et al. (2019) in Far North of Cameroon, and that of Kirdi is higher than that reported by Djagba et al. (2019) on Djallonke goats (17.45 ± 4.33 kg) in Togo. However, even if the results of the goats studied in this work are within the ranges predicted by the previous authors, it should be noted that these values are low compared with the average for the Sahelian and Kirdi breeds used in this study. This could be explained by the uncontrolled interbreeding among the breeds, which compromises the individual potential of each breed because they are bred together in traditional systems with anarchic cross-breeding and under poor feeding and sanitary conditions; in addition to the effects of climate change, which are forcing morphological changes in response to the new conditions, with a consequent impact on reproduction (Mandonnet et al., 2011).

The pregnancy rate was higher than 38.60% observed by Manjeli et al. (1996) at the Garoua and Maroua abattoirs; however, it was lower than 49% reported by Nana et al. (2014) in the town of Dschang and 45.30% by Kouamo et al. (2019) at the Maroua municipal abattoir. The difference could be linked to the study period and breed. The slaughter of gravid goats causes huge losses and thus constitutes a shortfall for the breeders and a handicap for the country. An important factor contributing to the increased slaughter of pregnant animals in Cameroon is the poor enforcement of existing livestock legislation. Decree N° 2018/759 of 10 December, 2018 strictly prohibits livestock producers, middlemen and butchers from transporting and/or slaughtering young and pregnant animals of all breeds. No sanctions or punitive measures are imposed on those who violate the existing regulations. Poor enforcement of government regulations on livestock, therefore, perpetuates the slaughter of pregnant animals.

The average ovary weight of the goats studied was higher than 1.30 ± 0.23 g and 0.69 ± 0.01g reported by Islam et al. (2007) and Ngona et al. (2012), respectively. On the other hand, it is less than 3-5 g reported by Meyer (2008). This difference may be due to the breed effect. Specifically, the ovaries (g) of the Kirdi breed are not different (1.37 ± 0.16) than those of Sahelian-type goats (1.29 ± 0.11) ($P \geq 0.05$).

The presence of the corpus luteum increased the weight and the length, width and thickness of the ovaries. Similar results have been reported by El-Sharawy et al. (2021) in ewes, which could be explained by the fact that the corpus luteum formed from the ovulating follicle develops in all directions on the surface of the ovary. The presence of the corpus luteum on the left ovary was observed in majority of goats, indicating that ovulation is more marked on the left ovary, contrary to the results of the studies by Islam et al. (2007) and Alsafy and El-Shahat (2011). In all of domesticated ruminants, the right ovary is usually more active than the left. Local paracrine and autocrine factors and differences in lymphatic drainage of the right and left ovaries may contribute to the observed variation in their activities (Habibizad et al., 2021). The right ovaries are also more functional in ruminants because of the presence of the rumen which reduces blood supply to the left ovary, consequently GnRH. The difference is unknown and may be due to less number of ovaries processed.

The mean number of follicles was higher than 4.9 ± 0.89, 7.46 ± 0.14 and 10.38 ± 5.48 reported by

Alsafy and Shahat (2011), Wani et al. (2013) and Kouamo et al. (2019), respectively. The number of follicles with a diameter greater than 8 mm was low. This result is similar to those of Zongo et al. (2019) who had 5 to 9 pre-ovulatory follicles, and Zarrouk et al. (2001) who had mature follicles of 9 to 10 mm in goats. These proportions could be explained by the fact that the study took place during the sexual season and the females were at different stages of the estrus cycle (Lassoued and Rekik, 2005). During the estrus cycle, the ovaries undergo a series of morphological (follicular recruitment and growth), biochemical (follicle maturation) and physiological (endocrine regulation) changes, which lead to ovulation and would affect their diameter. Each weighed 0.5 to 3 grams dependent on the stage of the reproductive cycle (Osman et al., 2021). The number of follicular waves, with a new recruitment every 5 to 7 days, could explain this consistent number of follicles of different sizes. If the slicing technique is applied correctly, all the oocytes present in all the follicles can be recovered, regardless of their location on the ovarian cortex. The oocyte yield was higher than 5.87 ± 0.08 and 6.04 ± 1.01 observed by Wani et al. (2013) and Kouamo et al. (2020b), respectively. These differences could be due to the technique used (slicing), which could trap a large number of oocytes in tissues that have not been sufficiently incised.

The number of cultivable oocytes was significantly higher in the ovaries of non-pregnant goats than in the others. The same observations were made by Islam et al. (2007). This could be explained by the persistence of the corpus luteum and its production of progesterone, which inhibits follicular growth in pregnant goats. On the other hand, the negative effect of progesterone may not be effective in non-pregnant goats, which explains the role of hormonal balance on folliculogenesis in goats.

In conclusion, this study has indicated that the ovaries of local Cameroonian goats have a fairly good potential to produce fertilizable oocytes for *in vitro* embryo production. Factors such as the age, body condition, pregnancy status and stage, ovary weight and corpora lutea should be take into account to maximise the success of *in vitro* embryo production.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHORS CONTRIBUTION

JK designed and planned the study; JBM collected the data; JK and JBM analyzed the data and wrote the first version of the manuscript; all authors have revised the article and authorize submission of the final version for publication.

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OVARIJALNI POTENCIJAL LOKALNIH KOZA ZA *IN VITRO* PROIZVODNJU EMBRIJA NA DALEKOM SJEVERU KAMERUNA

SAŽETAK

Istraživanje je provedeno na 281 lokalnoj kozi (257 Sahel i 24 Kirdi) u klaonici za male preživače u općini Maroua, u regiji Daleki Sjever u Kamerunu. Prikupljena su ukupno 562 jajnika koja su potom prevezena u laboratorij u 9% NaCl otopini. Nakon što je odstranjeno okolno tkivo, na svakom jajniku su prebrojani i izmjereni folikuli, čiji je dijametar (Φ) klasificiran kao mali (Φ< 3 mm), srednji (3 ≤ Φ ≤ 8 mm) i veliki (8 ≤ Φ< 10 mm). Nakon toga su korištenjem tehnike rezanja prikupljene oocite koje su uskladištene u Dulbeko fosfatni slani pufer i analizirane svjetlosnim mikroskopom s povećanjem 40X. Oocite su klasificirane u četiri grupe na osnovu stupnja razvoja kumulusnih stanica i transparentnosti ooplazme. Srednji broj folikula po jajniku je iznosio 25.38 ± 6.84. Dijametar malih, srednjih i velikih folikula je iznosio 5.87 ± 2.90, 15.64 ± 4.71 i 3.86 ± 1.69. Broj oocita po jajniku je iznosio 33.39 ± 11.71. Oociti su prema kvaliteti I, II, III, IV klasificirani kao 11.57 ± 3.33 (34.65%), 9.64 ± 3.05 (28.87%), 8.25 ± 3.26 (24.71%) i 3.93 ± 2.01 (11.77%). Indeks kvalitete oocita je iznosio 2.11. Kvalitetni oociti (grupa I i II) koji su prihvatljivi za *in vitro* proizvodnju embrija (IVEP) su činili 63.52% prikupljenih oocita. Ovi rezultati pokazuju da određeni faktori kao što su dob, tjelesna kondicija, stanje i stadij trudnoće, težina ovarija i korpus luteum moraju biti uzeti u obzir kako bi se povećao potencijal jajnika za *in vitro* proizvodnju embrija.

Ključne riječi: Kamerun, koze, kvalitet oocita, ovariji, populacija folikula

SHORT COMMUNICATION

SERUM PRODUCTION OF ANDROCTONUS CRASSICAUDA (SCORPION) VENOM: EVALUATING THE IMPACT OF HIGH-QUALITY PROTEIN DIETS

Tuba Aydın[1*],Mehmet Ali Kanat[2], Ali Çalık[3], Adnan Şehu[3]

[1]Ankara University Graduate of Health Science, Ankara, Türkiye
[2]Ministry of Health, General Directorate of Public Health, Ankara, Türkiye
[3]Ankara University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Disease Ankara, Türkiye

Corresponding author:
Dr. Tuba Aydın
Ankara University Graduate Of Health Science.Ankara,Türkiye

Address:
06110 Dışkapı, Ankara/Türkiye
Phone:
+90 312 316 66 71 – 72 - 43
ORCID: 0000-0002-1872-6980
E-mail: tuba.aydin2007@gmail.com

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ABSTRACT

In this study, serum horses were utilized to evaluate the effect of feeding high-quality, protein-enriched diets on antibody production. The horses were divided into two groups, each containing three animals (two females and one male per group). Two feeding periods were implemented, with one group serving as the control and fed a diet containing 13.65% crude protein, while the other group received a trial diet containing 18.05% crude protein. To increase accuracy, the control and experimental groups were alternated in a crossover design.

No statistically significant differences were observed between the trial and control groups ($p \geq 0.05$). Similarly, no significant differences were found when the data from the trial and control groups were analyzed separately by sex. However, a statistically significant difference was found between sexes ($p=0.004$), with antibody titers increasing by 40.2% in the trial groups overall and by 42.6% in male horses.

No statistically significant changes were found for serum total protein values, albumin, and globulin levels ($p>0.05$).

It was observed that individual antibody levels varied considerably, which limited the ability to achieve statistical significance. However, it can be inferred that the quantity and quality of dietary proteins significantly enhance antibody production in quantitative terms.

Keywords: Antibody titer, horse, lysine, methionine

INTRODUCTION

Scorpion stings are frequently encountered in tropical and subtropical climates. This poses a significant public health concern, with some patients experiencing severe clinical manifestations and life-threatening complications (Abroug et al., 2020). Scorpion envenomation may present clinically with a wide array of symptoms, from localized dermal reactions to potentially fatal neurologic, respiratory, or cardiovascular collapse (Reckziegel et.al, 2014). Globally, among the 1.753 known scorpion species, approximately 50 are venomous, with 20-25 considered potentially fatal (Khatony et al., 2015). The annual global incidence of scorpion stings is estimated at 1.200.000 cases, with an associated mortality rate of approximately 3,000 (Isbister, 2014).

In Turkey, *Androctonus crassicauda* is the most common and highly venomous scorpion species, known for its potentially lethal stings. These scorpions are predominantly found in the Southeastern Anatolia Region and certain provinces in the Eastern Anatolia Region (Özkan et al., 2003).

The use of antivenom is currently regarded as the most effective treatment for envenomed patients. Consequently, further research is necessary to develop more specific and purified antivenoms to improve the treatment of scorpion sting victims. According to Russell et al. (1964), antivenom remains the sole definitive treatment for such cases.

In Turkey, the venom of *Androctonus crassicauda* is the preferred antigen for antivenom production. This venom induces immunity more effectively in animals, and fatalities among animals used in antivenom production are rare. Additionally, the broad paraspecific efficacy of antivenom prepared against *A. crassicauda* suggests its potential utility in treating stings from various scorpion species both within Turkey and globally (Filazi et al., 2020).

In the treatment of scorpionism cases, antivenoms are produced by injecting venom into animals such as sheep, goats, and horses, followed by the periodic collection of antibodies. Horses are often used as hyperimmunized animals in the antivenom production process due to their ability to produce large volumes of antiserum, providing a significant advantage (Theakston et al., 2003). However, their use may result in higher costs compared to other animals (Pratanaphon et al., 1997).

Horses belong to the group of monogastric herbivores adapted to a specialized diet.It is recommended to add high-quality protein to meet the daily crude protein requirements of horses. Methionine, lysine, and threonine can be included in the diet as sources of high-quality protein. These added proteins are primarily used to increase metabolic energy, particularly in horses with a negative energy balance (Laurie 2008; NRC 2007).

However, studies investigating the relationship between nutrition and immune function in horses are limited (Maggini et al., 2007). The dynamic interaction between nutrient availability and nutritional status significantly influences immune health and disease resistance (Scrimshaw et al., 1968; Calder and Jackson, 2000). Recent studies highlight that amino acids play significant roles in the development of cytotoxic immune cells such as T cells, B cells, and macrophages; in facilitating cellular chemical reactions, lymphocyte proliferation, and gene expression; and in promoting cytokine production, antibody synthesis, and the regulation of immune responses (Peng et al., 2007)

The aim of this study is to assess the effects of high-quality proteins feeding on the immune system of horses that have been acquired with scorpion serum.

MATERIAL AND METHODS

Approval of the Ethics Committee

This study was approved by Ankara Universıty Animal Experiment Ethical Commitee (date and permit no: 18/03/2020, 2020-6-48).

Animals and diet

The study sample consisted of six native horses aged 4 to 8 years, with a body weight ranging from 350 to 400 kg. This experiment was conducted with two groups of horses, one experimental and one control, each consisting of one male and two females (n=3). Horses were randomly assigned into two groups.

In our study, we investigated the efficacy of scorpion venom serum under two different dietary conditions. Due to the small sample size (N=3 horses/treatment), a repeated study design was implemented to increase statistical power and account for individual variability. Initially, the horses were divided into two groups: control and experimental, with three horses in each group.

To ensure robust results, after the first phase of the experiment, the group assignments were switched. Horses initially in the control group were moved to the experimental group, and vice versa. This crossover design allowed all horses to serve as their own controls at different stages of the study, effectively doubling the number of observations (N=6 horses/treatment) and reducing variability caused by individual differences in response to treatments.

The horses us ed for the production of scorpion serum were housed individually in compartments and fed with either control or experimental diets for a period of four months. The control and experimental groups were alternated to account for potential individual differences and ensure the reliability of the findings.

The concentrate feed used in the experiment was prepared at the Faculty of Veterinary Medicine Education, Research and Application Center (Ankara University), utilizing supplied raw materials. Barley straw and dried alfalfa grass were provided as roughage for both groups. Additionally, the feed for the experimental group was supplemented with lysine-rich soybean meal and methionine to enhance protein quality.

Three horses were randomly assigned to each of a control and an experimental group. To account for individual variability, a repeated study design was implemented, with the grouping rearranged accordingly. Each horse was fed daily with 4 kg of concentrated feed, 3 kg of alfalfa hay, and 2 kg of barley straw. The control group received a diet composed of 80.5% barley straw and alfalfa hay, and 15.7% concentrate mix. In contrast, the experimental group was fed a diet consisting of 66% barley straw and alfalfa hay, and 30.2% concentrate mix.Water was provided *ad libitum* to all horses.

Table 1 Composition and chemical analysis of concentrated diet

Composition		
	Control Group	**Experimental Group**
Barley Straw (g/kg)	400.00	400.00
Alfalfa Grass (g/kg)	405.00	260.00
Corn (g/kg)	100.00	100.00
DDGS (g/kg)	57.00	57.00
Soybean Meal,CP 48 % (g/kg)	0	143.00
Limestone (g/kg)	3000	30.00
Salt (g/kg)	7.00	7.00
Vitamin-mineral Premix*(g/kg)	1.00	1.00
DL-Methionine **(g/kg)	0.00	2.00
Total	1000	1000

Composition		
	Control Group	**Experimental Group**
Chemical Composition		
Dry matter (%)	90.94	91.60
Crude protein (%)	13.65	18.05
Crude fiber (%)	6.26	5.71
Crude oil (%)	2.97	2.60
Crude ash (%)	6.18	6.35
Calcium (%)	1.10	1.37
Available Phosphorus (%)	0.45	0.46

***Vitamin-mineral Premix:** Vitamin D3, Vitamin A, Vitamin E, Selenium, Manganese, Cobalt, Iodine, Iron, Zinc, Copper, Calcium Carbonat. ****DL-Methionine origin:** France.

Experiment design

Scorpion antiserum was produced in horses housed at the Ankara University Faculty of Veterinary Education Research and Practice; an institution affiliated with the General Directorate of Public Health under the Ministry of Health. To enhance antibody production for scorpion antiserum, high-quality, protein-rich diets were prepared for the horses. One week prior to venom administration and throughout the entire study, the experimental group was fed a protein-enriched diet.The antigenized scorpion venom was administered intramuscularly with adjuvants. During the first feeding period, horses received their initial venom injections, which were repeated seven times at one-week intervals. In the second feeding period, venom injections were administered for one week,with a total of four applications.

This approach ensured that the small sample size did not compromise the validity of the results, as each animal's response under both dietary conditions was evaluated.

Blood sampling

Antibody titers were assessed 1–3 days after the final injection. Blood samples were collected via venipuncture of the external jugular vein from each horse, using 10 ml vacuum tubes without anticoagulant. After centrifugation, serum samples were stored in tubes at +4°C for 24 hours prior to conducting laboratory analyses. A complete blood count was conducted to measure the size of red blood cells, hemoglobin levels, white blood cell counts, platelet counts, and blood protein concentrations, including albumin and globulin, utilizing a photometric method for analysis..

A two-phase feeding program was implemented in this study, with the second phase involving the reassignment of horses between control and experimental groups to enhance the accuracy of the findings. We determined higher concentrations of total protein and globulin, while albumin level decreased. However, these changes were not statistically significant ($p>0.05$).

Scorpion neutralizing antibody titer

For each venom type provided by manufacturers, the LD_{50} of the scorpion venom contained within the antiserum requires verification.The manufacturer's LD_{50} value serves as the median dilution for this purpose; four additional dilutions, two lower and two higher, are prepared using a dilution factor of 1,2. Each of these dilutions is intravenously injected at a volume of 0.25 ml into four mice weighing 18-20 grams. The same gender of mice used in the LD_{50} determination must also be used in the potency test. After a 48-hour observation period, the data obtained is analyzed using the Sperman-Kärber method.

The venom of *Androctonus crassicauda* scorpions, collected by means of electrical stimulation, is utilized as an antigen following the determination of its LD_{50} in mice weighing 18–20 grams. For each horse, the antigen and adjuvant (aluminum potassium sulfate extra pure crystalline) were prepared in a fixed volume of five milliliters for injection.

Table 2 Steps for Using Venom

First-period feeding		Second-period feeding	
Time (week)	Venom dose (LD_{50})	Time (week)	Venom dose (LD_{50})
1	5	1	50
2	10	2	100
3	25	3	250
4	50	4	400
5	100	5	-
6	250	6	-
7	400	7	-

Statistical analysis

The data were analyzed using the Mann-Whitney U test, with statistical significance set at $p<0.05$.

RESULTS

Each horse was injected with venom subcutaneously to measure blood serum parameters and antibody titer.

Table 3 Blood serum parameters and antibody titer across different experimental groups

Parameters	**n**	**Control Group** $\bar{x}\pm S\bar{x}$	**Experimental Group** $\bar{x}\pm S\bar{x}$	**p-value**
Antibody Titer (NU/ml)*	6	46.87± 40.31	61.14 ± 65.76	0.660
Total protein (g/dl)**	6	8.51± 0.62	8.63± 0.73	0.767
Albumin (g/dl)	6	5.23 ± 0.44	5.13± 0.33	0.690
Globulin (g/dl)	6	3.28± 0.32	3.49 ± 0.54	0.428

Difference between groups is statistically insignificant (p>0,05).

*NU/ml: Antiserum potency per milliliter that neutralizes the LD_{50} of *A. Crassicauda* venom.**g/dl: grams per deciliter.

Table 4 Blood serum parameters and antibody titers in horses of different genders during the experiment

Female				
Parameters	**n**	**Control Group** $\bar{x}\pm S\bar{x}$	**Experimental Group** $\bar{x}\pm S\bar{x}$	**p-value**
Antibody Titer (NU/ml)*	4	22.63 ± 16.01	23.72 ± 15.22	0.773
Total protein (g/dl)**	4	8.54± 0.59	8.68± 0.92	0.988
Albumin (g/dl)	4	5.33 ± 0.51	5.19± 0.42	0.564
Globulin (g/dl)	4	3.21± 0.09	3.49 ± 0.34	0.555
Male				
Antibody Titer (NU/ml)	2	8.44± 0.94	8.53± 0.28	0.980
Total protein (g/dl)	2	5.03 ± 0.26	5.03± 0.98	0.891
Albumin (g/dl)	2	3.41± 0.67	3.50 ± 0.18	0.986
Globulin (g/dl)	2	95.37 ± 17.39	136.00 ± 64.20	0.439

Difference between groups is statistically insignificant (p>0,05).

*NU/ml: Antiserum potency per milliliter that neutralizes the $LD_{50}$of *A. Crassicauda* venom.**g/dl: grams per deciliter.

Table 5 Blood serum parameters and antibody titers in male and female horses involved in the experiment

Parameters	Gender		p-value
	Male(n=2) $\bar{x}\pm S\bar{x}$	Female(n=4) $\bar{x}\pm S\bar{x}$	
Antibody Titer (NU/ml)*	115.68^{a}**± 45**.00	23.178^{b}**± 14**.47	0.004
Total protein(g/dl)**	8.48 ± 0.56	8.6 ± 0.72	0.979
Albumin(g/dl)	5.08 ± 0.21	5.26 ± 0.44	0.885
Globulin (g/dl)	3.40 ± 0.39	3.35 ± 0.47	0.806

The difference between the antibody titers indicated by different letters (a, b) is statistically significant (p<0.05). Difference between groups is statistically insignificant (p>0,05).

*NU/ml: Antiserum potency per milliliter that neutralizes the LD_{50} of *A. Crassicauda* venom.**g/dl: grams per deciliter.

Hematological analysis

At the end of the two study periods, blood samples were collected from the jugular vein of horses into purple-capped tubes containing EDTA. Hematological analyses were conducted at the Central Research Center of the Faculty of Veterinary Medicine, Ankara University.

Table 6 Hematological parameters of control and experimental groups

	n	Control Group	Experimental Group	p-value
WBC (10^9/L)	6	10.76± 2.50	11.06 ±1.68	0.813
LYM (10^9/L)	6	3.03 ± 0.93	2.76 ± 1.05	0.652
MONO (10^9/L)	6	0.78 ± 0.29	0.81 ± 0.23	0.831
NEUT (10^9/L)	6	6.85 ± 2.60	6.15 ± 3.87	0.721
EOS (10^9/L)	6	0.10 ± 0.15	0.01 ± 0.04	0.231
LYM (%)	6	29.26 ± 9.42	26.48 ± 11.84	0.662
MONO (%)	6	6.85 ± 2.33	6.91 ± 2.36	0.962
NEU(%)	6	62.76 ± 12.02	53.70 ± 29.46	0.501
EOS (%)	6	1.11 ± 1.75	0.23 ± 0.52	0.264
RBC (10^{-2}/L)	6	8.35 ± 1.35	7.94 ± 1.39	0.615
HGB (g/dL)	6	15.38 ± 2.08	14.73 ± 2.13	0.605
HCT(%)	6	42.00 ± 5.77	39.45 ± 5.90	0.467
MCV (fL)	6	50.53 ± 3.86	49.91 ± 3.71	0.784
MCH (pg)	6	18.50 ± 1.14	18.70 ± 1.27	0.781
MCHC (g/dL)	6	36.66 ± 0.89	37.48 ± 1.19	0.211
RDWa (fL)	6	27.03 ± 2.01	26.21 ± 2.32	0.530
RDW (%)	6	15.93 ± 0.71	16.03 ± 0.73	0.817
PLT (10^9/L)	6	142.33 ± 26.50	160.83 ± 53.43	0.465
MPV (fL)	6	5.66 ± 0.37	5.68 ± 0.44	0.945

Difference between groups is statistically insignificant (p>0.05). WBC - white blood cell . NEUT - neutrophil. LYM - lymphocyte . MONO - monocyte . NEU – neutrophil. EOS - eosinophi . RBC – red blood cells. HGB –haemoglobin. HCT - haematocrite. MCV – mean cell volume. PLT – platelets. MPV - mean platelet volume . MCH- mean corpuscular hemoglobin. MCHC- mean corpuscular hemoglobin concentration. RDW- red blood cell distribution width. RDWa- red cell distribution width (absolute)

Table 7 Hematological parameters of female horses in control and experimental groups

	N	Control Group $\bar{x}\pm S\bar{x}$	Experimental Group $\bar{x}\pm S\bar{x}$	p-value
WBC (10^9/L)	4	11.07±2.58	10.87± 1.96	0.564
LYM (10^9/L)	4	3.55 ± 0.62	3.35± 0.64	0.564
MONO (10^9/L)	4	0.92 ± 0.25	0.95± 0.12	0.963
NEUT (10^9/L)	4	6.52 ± 2.65	6.55± 2.29	0.773
EOS (10^9/L)	4	0.07 ± 0.15	0.02± 0.05	0.850
LYM (%)	4	33.37 ± 8.12	32.27± 9.44	0.960
MONO (%)	4	8.00 ± 1.71	8.20± 1.49	0.564
NEU(%)	4	57.90 ± 10.33	59.15± 10.32	0.564
EOS (%)	4	0.72 ± 1.45	0.35± 0.63	0.741
RBC (10^{-2}/L)	4	9.02 ± 1.00	8.55± 1.31	0.882
HGB (g/dL)	4	16.42 ± 1.26	15.85± 1.60	0.772
HCT(%)	4	45.05± 3.38	42.82± 3.46	0.386

	N	Control Group x̄±Sx̄	Experimental Group x̄±Sx̄	p-value
MCV (fL)	4	50.22± 4.95	50.55±4.62	0.077
MCH (pg)	4	18.30± 1.40	18.70± 1.60	0.772
MCHC (g/dL)	4	36.50± 0.94	37.00± 0.85	0.248
RDWa (fL)	4	27.27± 2.53	27.25± 2.12	0.773
RDW (%)	4	16.22± 0.65	16.17± 0.79	0.773
PLT (10^9/L)	4	131.75± 26.51	144.00± 50.83	0.564
MPV (fL)	4	5.65± 0.26	5.75± 0.41	0.561

Difference between groups is statistically insignificant (p>0,05). WBC - white blood cell . NEUT - neutrophil. LYM - lymphocyte. MONO - monocyte . NEU – neutrophil. EOS - eosinophil. RBC – red blood cells. HGB –haemoglobin. HCT - haematocrite. MCV – mean cell volume. PLT – platelets. MPV - mean platelet volume. MCH- mean corpuscular hemoglobin. MCHC- mean corpuscular hemoglobin concentration. RDW- red blood cell distribution width. RDWa - red cell distribution width (absolute)

Table 8 Hematological parameters of male horses in control and experimental groups

	N	Control Group x̄±Sx̄	Experimental Group x̄±Sx̄	p- value
WBC (10^9/L)	2	10.15 ±3.18	11.45 ± 1.48	0.439
LYM (10^9/L)	2	2.00 ± 0.00	1.60 ± 0.42	0.102
MONO (10^9/L)	2	0.50 ± 0.00	0.55 ± 0.07	0.317
NEUT (10^9/L)	2	7.50 ± 3.39	5.35 ± 7.56	0.948
EOS (10^9/L)	2	0.15 ± 0.21	0.00 ± 0.00	0.317
LYM (%)	2	21.05 ± 6.57	14.90 ± 5.65	0.439
MONO (%)	2	4.55 ± 1.62	4.35 ± 1.20	0.992
NEU(%)	2	72.50 ± 10.88	42.80 ± 60.52	0.969
EOS (%)	2	1.90 ± 2.68	0.00 ± 0.00.	0.317
RBC (10^{-2}/L)	2	7.02 ± 0.91	6.71 ±0.20	0.987
HGB (g/dL)	2	13.30 ± 1.97	12.50 ± 0.00	0.889
HCT(%)	2	35.90 ± 4.52	32.70 ± 1.27	0.439
MCV (fL)	2	51.15 ± 0.21	48.65 ± 0.49	0.121
MCH (pg)	2	18.90 ± 0.42	18.70 ± 0.56	0.439
MCHC (g/dL)	2	37.00 ± 0.98	38.45 ± 1.48	0.439
RDWa (fL)	2	26.55 ± 0.49	24.15 ± 0.77	0.121
RDW (%)	2	15.35 ± 0.49	15.75 ± 0.77	0.439
PLT (10^9/L)	2	163.50 ± 7.77	194.50 ± 55.86	0.896
MPV (fL)	2	5.70 ± 0.70	5.55 ± 0.63	0.439

Difference between groups is statistically insignificant (p>0,05). WBC - white blood cell . NEUT - neutrophil. LYM - lymphocyte. MONO - monocyte. NEU – neutrophil. EOS - eosinophil. RBC – red blood cells. HGB –haemoglobin. HCT - haematocrite. MCV – mean cell volume. PLT – platelets. MPV - mean platelet volume. MCH- mean corpuscular hemoglobin. MCHC- mean corpuscular hemoglobin concentration. RDW- red blood cell distribution width. RDWa- red cell distribution width (absolute)

Table 9 Hematological parameters of male and female horses in control and experimental groups

	Female (n=4) $\bar{x}\pm S\bar{x}$	Male (n=2) $\bar{x}\pm S\bar{x}$	p-value
WBC (10^9/L)	10.97 ±2.12	10.80 ± 2.16	0.80
LYM (10^9/L)	**3.45^a ± 0.59**	**1.80^b ± 0.33**	**0.004**
MONO (10^9/L)	**0.93^a ± 0.18**	**0.52^b ± 0.05**	**0.004**
NEUT (10^9/L)	6.53 ± 2.29	6.42 ± 4.94	0.93
EOS (10^9/L)	0.05 ± 0.10	0.07 ± 0.15	0.93
LYM (%)	32.82 ± 8.17	17.97 ± 6.13	0.01
MONO (%)	**8.10^a ± 1.49**	**4.45^b ± 1.17**	**0.004**
NEU(%)	58.52 ± 9.58	76.62 ± 8.82	0.01
EOS (%)	0.53 ± 1.05	0.95 ± 1.90	0.93
RBC (10^{-2}/L)	**8.78^a± 1.11**	**6.86^b ±0.57**	**0.008**
HGB (g/dL)	**16.13^a± 1.37**	**12.90^b ± 1.23**	**0.008**
HCT(%)	**43.93^a ± 3.38**	**34.30^b ± 3.28**	**0.008**
MCV (fL)	50.38 ± 4.43	49.90 ± 1.47	0.57
MCH (pg)	18.50 ± 1.41	18.80 ± 0.42	0.80
MCHC (g/dL)	36.75 ± 0.87	37.72 ± 1.32	0.21
RDWa (fL)	27.26 ± 2.16	25.35 ± 1.48	0.10
RDW (%)	16.20 ± 0.67	15.55± 0.58	0.15
PLT (10^9/L)	137.87 ± 38.103	179 ± 37.15	0.21
MPV (fL)	5.70 ± 0,32	5.62 ± 0.55	0.80

Difference between groups is statistically insignificant (p>0,05). WBC- white blood cell . NEUT- neutrophil. LYM- lymphocyte . MONO- monocyte. NEU – neutrophil. EOS- eosinophil. RBC – red blood cells. HGB –haemoglobin. HCT- haematocrite. MCV – mean cell volume. PLT – platelets. MPV- mean platelet volume. MCH- mean corpuscular hemoglobin. MCHC- mean corpuscular hemoglobin concentration. RDW- red blood cell distribution width. RDWa - red cell distribution width (absolute)

DISCUSSION AND CONCLUSION

In this study, variations in gamma-globulin, total protein, globulin, and other blood parameters were observed in the horses. However, no significant differences were found in antibody titer, total protein, albumin, and globulin levels between horses fed high-quality protein diets and those fed standard diets. Although statistical significance was not achieved, the 30.45% increase in antibody titer observed in the experimental group is noteworthy. Furthermore, male horses exhibited significantly higher antibody titers (p=0.004) compared to females.

Previous studies have assessed the impact of high-quality protein diets on muscle density and energy metabolism in horses. Waghmarea et al. (2014) investigated the immune response of 33 horses randomly assigned to four groups, receiving subcutaneous injections of cobra venom at two-week intervals. Over the immunization period, biochemical and hematological parameters were monitored at baseline and on weeks 14, 21, 30, and 42. Although mean hemoglobin values initially declined, they later normalized. Serum total protein and globulin levels increased across all groups, while albumin levels showed a slight decrease. No significant changes were noted in

serum creatinine, bilirubin, alkaline phosphatase, or blood urea nitrogen levels. Similarly, in our study, while total protein, albumin, and globulin concentrations differed between groups after exposure to scorpion venom, these differences were not statistically significant.

In a study on rats, diets supplemented with wheat gluten, soy protein, lysine, and methionine increased hepatic nitrogen levels and weight gain. However, supplementation with only soy protein and methionine led to a reduction in antibody titer, whereas lysine and wheat gluten supplementation had no effect (Smith and Kenney, 1969). Consistently, our experimental group, provided with a lysine- and methionine-enriched diet, exhibited no significant difference in antibody titers compared to controls.

In a study conducted by Pablack et al. (2017), three dietary regimens varying in protein quality (low, medium, and high) were administered to 10 adult cats over six weeks in a randomized crossover design. Blood analyses revealed that lower protein quality was associated with increased phagocytic activity of blood monocytes and reduced eosinophil counts. However, our study found no significant differences in WBC, lymphocyte, or monocyte values between groups. Similarly, Sahlu et al. (1992) demonstrated that plasma total protein levels significantly increased with higher protein diets in female Angora goats. In contrast, our study did not show a significant increase in total protein levels in horses fed diets with varying protein content.

This study evaluated the effects of dietary protein and amino acid levels on antibody titer, serum total protein, albumin, globulin, and hematological parameters in horses used for scorpion serum production. The results confirmed that serum albumin and total protein levels remained within physiological limits. However, individual variability in serum production was substantial. This suggests that identifying and utilizing horses with high serum yields could enable more efficient production, reducing the number of animals required.

The large individual variation among horses limited the statistical significance of the positive effects of protein quantity and quality on antibody production. Future studies should focus on well-defined, homogeneous groups with sufficient sample sizes to better evaluate changes in antibody titers and their additive effects.

One of the key findings of this study is the considerable variability in antibody production among horses. It was concluded that production costs could be significantly reduced by selecting horses on proper diets with high antivenom production capacity.

The results of our research are somewhat limited due to the small sample size used in the experiment. Further studies involving a larger number of horses are needed to identify any potential differences between the experimental and control groups.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Conception: TA, MAK, AŞ; Design: TA, AÇ; Supervision: TA, MAK, AŞ; Materials: TA, MAK, AŞ, AÇ; Data Collection: TA; Analysis of Data: TA, AÇ; Literature Review: TA, AÇ, AŞ; Writing: TA; Critical Review: MAK, AÇ, AŞ

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PROIZVODNJA SERUMA IZ VENOMA *ANDROCTONUS CRASSICAUDE* (ŠKORPION): PROCJENA UTJECAJA VISOKOKVALITETNE PROTEINSKE PREHRANE

SAŽETAK

U ovom istraživanju smo na konjskom serumu procjenjivali učinak visokokvalitetne, proteinski obogaćene prehrane na proizvodnju antitijela. Konji su podijeljeni u dvije grupe sa po tri životinje (dvije ženke i jedan mužjak). Životinje su prehranjivane dvaput, pri čemu je jedna grupa korištena kao kontrolna i prehranjivana je sa 13.65% sirovih proteina, dok je ispitivana grupa prehranjivana sa 18,05% sirovih proteina. Da bi se povećala preciznost, kontrolna i ispitivana grupa su se smjenjivale u ukriženom dizajnu studije.

Između ispitivane i kontrolne grupe nisu uočene statistički signifikantne razlike ($p \geq 0.05$). Također, nisu uočene signifikantne razlike kada su podaci iz ispitivane i kontrolne grupe odvojeno analizirani po spolu. Međutim, uočena je statistički signifikantna razlika među spolovima ($p=0.004$), pri čemu su titrovi antitijela ukupno porasli za 40.2% u ispitivanoj grupi, a za 42.6% kod mužjaka.

Nisu uočene statistički sigifikantne razlike za vrijednosti ukupnih serumskih proteina, albumina i globulina ($p>0.05$).

Uočeno je da su pojedinačni titrovi antitijela znatno varirali, što je ograničilo mogućnost postizanja statističke signifikantnosti. Međutim, možemo zaključiti da količina i kvalitet proteina u prehrani značajno povećavaju proizvodnju antitijela u kvantitativnom smislu.

Ključne riječi: Konj, lizin, metionin, titar antitijela

SHORT COMMUNICATION

COMPARISON OF LARVAE MORPHOLOGY AND DNA PRESERVATION IN PLASTINATION USING DIFFERENT FIXATIVES

Dilan Biroğul[1*], Betül Baygeldi[2], Duygu Neval Sayin Ipek[3]

[1]Department of Internal Medicine, Faculty of Dicle University, Dicle University, Diyarbakır, Türkiye
[2]Department of Anatomy, Faculty of Veterinary Medicine, Fırat University, Elazığ,Türkiye
[3]Department of Parasitology, Faculty of Veterinary Medicine, Dicle University, Diyarbakır, Türkiye

***Corresponding author:**
Dilan Biroğul
Address: Department of Internal Medicine, Faculty of Dicle University, Dicle University, Diyarbakır, Türkiye
ORCID: 0009-0000-2390-6321
E-mail: dilanbirogul@gmail.com

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ABSTRACT

This study was conducted to determine the extent to which the structures of parasite larvae treated with different fixatives were preserved through the silicone plastination procedure and to investigate the degree of DNA preservation in plastinated larvae. For morphometric evaluation, the length (anterior-posterior) and maximum width (widest trunk width) of the larvae were measured to record changes before and after the plastination process. The silicone plastination protocol was applied at room temperature using three different fixatives. Subsequently, the larvae were deplastinated. Total genomic DNA (gDNA) from third-instar plastinated and deplastinated larval samples was extracted using the GeneJET Genomic DNA Purification Kit. DNA concentrations were measured at 260 nm using a UV-Vis spectrophotometer. The PCR protocol was applied, and photographs were taken using a UV transilluminator. Following plastination, the larvae were completely dry, and the process rendered them harmless. They could be stored for an extended period and, most importantly, their morphological characteristics remained suitable for morphological analysis by graduate students. No visible morphological issues were observed during the dehydration and impregnation stages of plastination; however, after curing, some specimens exhibited significant structural collapse, resulting in alterations of their normal anatomical structure. Additionally, DNA concentrations in the deplastinated groups were found to be higher than in the plastinated groups. In the second step of PCR, a ~300 bp band was obtained in all plastinated and deplastinated groups. In conclusion, the silicone plastination method enables the long-term preservation of larval morphological structures with minimal alterations, while causing a partial reduction in DNA integrity.

Keywords: Anatomy, DNA, larvae, morphometry, PCR, preservation, plastination

INTRODUCTION

From the past to the present, the protection of parasite samples using various preservatives has been necessary for teaching or research. Most parasites used as teaching and research tools in schools, universities, research centers, and other institutions are usually protected with a 4-10% formaldehyde solution (formalin) and/or 70% alcohol (Rosilawati et al., 2014). However, besides causing shrinkage and color change in the samples, these chemicals have significant disadvantages such as toxicity, carcinogenicity, odor, the wetness of the samples, maintenance of liquid levels, storage capacity, limited time, and storage space (McLaughlin, 1994; Swemberg et al., 2013; Gonzálvez et al., 2017). The plastination technique (von Hagens et al., 1987) is a well-established and innovative technique that has been an alternative option in conserving biological organisms in recent years. The procedure occurs as a gradual exchange of fluids and adipose tissue in the tissues with polymers under certain conditions (Lattore et al., 2007; Sundar et al., 2019). This technique allows for the production of clean, dry, durable models that can be examined without gloves or other protective equipment, and does not require any special storage conditions. In addition, since they do not contain toxic substances, students and instructors are not exposed to formaldehyde, phenol, alcohol, etc., thereby preventing exposure to harmful substances. Plastination models are durable and resistant to microbial decomposition and degradation. They can be easily stored, even in humid environments, as the plastination solution prevents water absorption (Chaturvedi et al., 2014). At the same time, this method allows samples to maintain their original form, size, and enhanced cellular-level appearance (Kocevski et al., 2010).

Despite significant progress made in molecular biology, there are few studies in the literature that examine nucleic acids in post-plastination tissues and investigate the preservation, extraction, and detection of DNA in plastinated samples (DeJong and Henry, 2007; Sagoo and Adds, 2013; Nagaraj et al, 2018). Although the plastination technique is an excellent alternative for educational materials, further studies on its suitability for various examination techniques are needed to demonstrate its potential as a museum or research material.

This study was conducted to determine the extent to which the structures of parasite larvae treated with different fixatives were preserved through the silicone plastination procedure and to investigate the preservation of DNA in plastinated larvae.

MATERIAL AND METHODS

The larvae used in this study were collected from the wounds of an injured dog that was brought to the Animal Hospital of the Faculty of Veterinary Medicine.

Plastination protocol

Firstly, for morphometric evaluation, length (from front to back) and maximum width (widest trunk width) were measured to record changes before and after the plastination process. The standard silicone plastination method (Biodur® S10) was used (von Hagens et al., 1987). The collected larvae were divided into three groups according to the fixatives used before plastination: Group A was fixed in 70% alcohol, Group B in pure acetone, and Group C in 10% formaldehyde. After fixation, the first step in the plastination process was the dehydration stage. Larvae were placed in cold (-25 ºC) acetone baths. The second step involved impregnating the samples with the silicone mixture (S10 + S3) at room temperature by reducing the pressure (increasing the vacuum). During impregnation, the acetone in the tissue evaporates from the cells and is replaced with the silicon mixture. Finally, the silicone in the tissue was cured (polymerized) using the hardener S6.A plastinated image of larvae fixed with different fixatives is shown in Figure 1.

Deplastination procedure

The stages of the deplastination procedure have been previously described (Ripani et al., 1996). For deplastination, larvae samples were sequentially incubated in 99% alcohol (Merck, #107017) for 24

hours, followed by incubation in methylbenzene (Merck, #108327) for 24 hours. To monitor the effectiveness of methylbenzene deplastination tissue, softening was checked regularly until the pin could easily be inserted into the tissue. After the methylbenzene application, the larvae were placed in 70% alcohol.

gDNA Extraction protocol

Total genomic DNA (gDNA) from third-instar plastinated and deplastinated larval samples was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA). For gDNA isolation, all larval samples were washed five times with PBS and cut into small pieces. 50 mg of each sample was placed in 1.5 ml falcon tubes and 20 µl of Proteinase K (20 mg/ml) and 180 µl of lysis buffer and incubated at 56°C for 24 hours to 72 hours. At the end of the incubation period, gDNA was extracted by following the kit's protocol, and the samples were stored at -20 °C until use.

Quantitative analysis of extracted DNA

The samples DNA concentrations were measured at 260 nm using a UV-Vis Spectrophotometer (NanoDrop™ 2000, Thermo Scientific).

Semi Nested PCR

In the first step, two primers designed to amplify DNA from a wide range of insects, including those belonging to the Oestridae family (Otranto et al., 2003), were used: UEA7 and UEA10. PCRs were carried out in a total volume of 50µl containing 5µl 10× PCR buffer, 5µl 25mM $MgCl_2$, 250µM each of dNTP, 20 pmol of each primer, 10 µl of template DNA, and 1.25U of Taq DNA polymerase (Thermo Scientific). The PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min.

The second step was performed using the primers UEA9 and UEA10. In this step, the first-round PCR products were diluted at a 1:10 ratio. The annealing temperature for the second step was set to 58 °C, while all other conditions remained the same.

Positive and negative samples were included in all PCR reactions. All PCR amplicons were electrophoresed in a 2% agarose gel stained with ethidium bromide. The gel was then visualized under a UV transilluminator, and photographs were taken.

RESULTS

The final state of the larvae after plastination: all the samples in the three groups (A, B, C) were dry, and plastination rendered them harmless. They could be stored for a long time and, most importantly, their morphological characteristics could be studied by postgraduate students. They were able to make precise measurements for both length and width. A significant darkening of the color of the larvae in group A and group B was observed, while the larvae in group C retained their original color (Figure 1). No visible morphological problems were observed in all three groups, but, after curing, significant collapse occurred in some samples in groups A and B, which led to changes in their normal anatomy.

Quantitative analysis of extracted DNA

Table 1 shows the results of the DNA quantification obtained using the UV-vis spectrophotometer. The DNA concentrations in the deplastinated groups were higher than in the plastinated groups.

Semi-nested PCR

In the first step of semi-nested PCR, the gel results of PCR products obtained from all groups are shown in Figure 2. As a result of the first step PCR, □680 bp bands could not be obtained from the groups fixed with 10% formaldehyde and the freshly plastinated and deplastinated groups. However, after alcohol fixation, plastinated and deplastinated bands were obtained from larvae. In the second step of PCR, □a 300bp band was obtained in all plastinated and deplastinated groups.

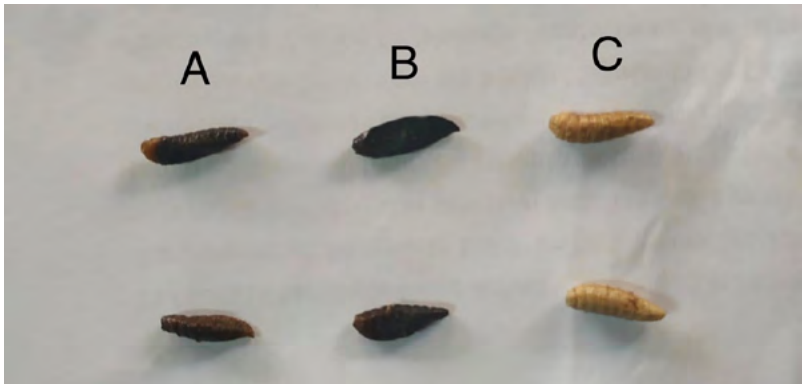

Figure 1 Classification of plastinated larvae (Groups A,B,C)

Table 1 Genetic material obtained from three groups' plastined and deplastined samples (Group A: %70 Alcohol; Group B: Pure Acetone; Group C: %10 formaldehyde)

Sample name	Amount of DNA
Group A	19.2 ug/ml
Deplastinated Group A	20.2 ug/ml
Group B	12.8 ug/ml
Deplastinated Group B	39 ug/ml
Group C	7 ug/ml
Deplastinated Group C	17.7 ug/ml

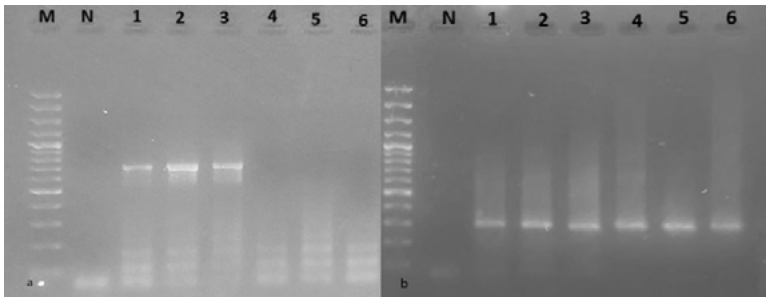

Figure 2 a, b. Agarose gel electrophoresis of amplification products of first and second PCR. Lane M, 100 bp marker; N, negative control; lane 1–6, samples (1, A; 2, Deplastine Group A; 3, B; 4, Deplastine Group B; 5, C; 6, Deplastine Group C).

DISCUSSION AND CONCLUSION

The plastination technique, which quickly gained popularity in the technical and medical fields, especially among anatomists, was developed by Gunther von Hagens in 1977 to preserve biological specimens (Lattore et al., 2007). Studies focusing mainly on anatomy support using biological samples obtained by using the plastination technique as educational material for students (Kumar et al., 2017). Studies investigating the use of the plastination technique as a suitable tool for protecting and preserving various endo- and ectoparasites have increased (Ramakrishna et al., 2010a; 2010b; Menaka et al., 2010). For the first time, Asadi and Mahmodzaeh (Ramakrishna et al., 2010a) used the S10 plastination technique to plastinate *Ascaris lumbricoides*. Since then, scientists have made necessary changes to the plastination protocol to develop ideally plastinated parasites based on size and morphology (Kocevski et al., 2010; Menaka et al., 2015; Gonzálvez et

al., 2017). To develop a more helpful plastination technique for *Toxocara* (*Neoascaris*) *vitulorum*, the impregnation of the melamine polymer was carried out at normal ambient pressure, in contrast to the low-pressure requirement (below five mmHg) of the traditional silicone plastination technique (Kocevski et al., 2010; Menaka et al., 2015). The temperature during plastination was kept at -20°C. This ultra-low temperature causes fixation and expansion in the samples and prevents their separation (Sagoo and Adds, 2013). Plastinated parasites were generally dry, non-sticky, shiny, odorless, chemical-free, and harmless, with some degree of flexibility and detectable morphological structure, and retained their natural form but lost their natural color. The results indicate that plastination is an effective method for preserving parasites and is widely used as an educational tool in parasitology. It is evident that the required times for each step of the plastination protocol vary depending on size and morphology of the different parasite species tested. This is consistent with reports of other specimens requiring modifications to the protocol to achieve optimal morphological and morphometric results (Gonzálvez et al., 2017). These plastination protocol modifications have also been described in other fields, such as embryology, herpetology, and zoology. For example, a sample's cuticle, tissue, or skin acts as a barrier that can interfere with acetone and polymer exchange during the dehydration and impregnation phases. (Asadi, 1998; Schaap, 1998; Wendel et al., 2008; Tiwari et al., 2012; October et al., 2017). Nematodes, acanthocephalans, and small trematodes often collapse and change their morphology and morphometry at a macroscopic level. No morphological or morphometric differences were observed between the cut and uncut specimens, and no correlation was found between the level of slump and the present cuticle cuts (Gonzálvez et al., 2017). Samples with a strong, thick surface layer (such as cuticle or membrane) should be cut to prevent the collapse of the entire sample. However, this protocol modification was found to be unnecessary for parasites without a body cavity (e.g., *Fasciola hepatica*) or with a very thin cuticle (e.g., *Dirofilaria immitis*) (Cheng, 1978). Ramakrishna et al. also used the plastination technique to preserve ecto- and endo-parasites (Ramakrishna et al., 2010b; Menaka et al., 2015). In this study, all samples fixed with formaldehyde and plastinated with silicone preserved their color.

Menaka et al. (2015) reported that plastinated larvae both retained their color and did not cause any respiratory irritation or allergies, as observed. Based on this study, silicone plastination can be effectively used to preserve fly larvae.

Shrinkage and flexibility are the two main limitations of this technique, especially for biological samples composed of soft tissues. These limitations were also noted in macroparasites plastinated using the Biodur TM S10/S3 polymer by Sagoo and Adds (2013) and the melamine polymer by Kumar et al. (2017). According to Latorre et al. (2007), shrinkage and color changes are the main causes of failures in the plastination technique. However, plastinated products remain intact in environmental conditions, and we share this opinion.

Although the number of studies on preserving DNA in plastinated and deplastinated tissues is limited, no study has yet been found focusing on arthropods with chitinous tissue. Our study observed that the amount of DNA extracted from the deplastinated groups was higher than from the plastinated groups. We believe this is due to the fact that DNA extraction was more successful in the deplastinated group, as the methylbenzene used in the deplastination process disrupts the cell membrane structure. Although the amount of DNA extracted from deplastinated larvae was similar to the amount of DNA extracted from deplastinated dog liver tissue in Ottone et al. (2020), it was higher than the amount of DNA extracted from deplastinated rat muscle tissue in the same study.

Nagaraj et al. (2018) reported that the amount extracted from plastinated tissue samples was 878 ng/µl, which is considerably higher than the amount obtained in our study. This difference is likely due to variations in the extraction methods used and differences in the plastinated tissues themselves.

In our study, following commercial mass DNA extraction, the first step of semi-nested PCR yielded an amplicon only in alcohol-fixed groups, while in the second step, the amplicons were obtained from all groups. Ottone et al. (2020) reported successfully amplifying the beta-actin gene from DNA extracted from deplastinated dog liver and rat muscle tissues using real-time PCR, though amplification occurred in the final cycles. Similarly, while not all samples produced amplicons in the first PCR step in our study, successful amplification in the second step supports these findings. This issue is likely caused by the presence of PCR inhibitors. However, the successful amplification of DNA in the first PCR step from alcohol-fixed tissues suggests that these inhibitors are either present in low amounts or that alcohol fixation better preserves the DNA structure.

Consequently, the results obtained in this study validate the use of plastinated parasites as an alternative tool for teaching, learning and scientific research in applied parasitology courses. The traditional method of fixing samples with formaldehyde poses significant health risks. However, the cost of plastination equipment and chemicals is considerably higher. Moreover, a thorough understanding of the plastination technique is required to produce high-quality samples. Plastination provides a dry, odorless, non-irritating, non-carcinogenic, and non-toxic material, ensuring safe procedures in higher education. Additionally, easy handling and storage of plastinated material offer an added advantage, as it is a durable teaching tool in educational institutions. When plastinating arthropods with chitinous tissue, the DNA in alcohol-fixed groups was better preserved, while no degradation was observed in any of the three methods.

These findings indicate that the larvae preserved using this plastination technique can be utilized for educational purposes as well as in future genetic studies, including forensic medicine, taxonomy, and evolutionary research. Additionally, the limitations of plastination can be overcome by using formaldehyde fixation prior to plastination, which better preserves morphological features. High-quality DNA can still be obtained from these larvae using alternative extraction methods.

AUTHOR CONTRIBUTIONS

Conceptualization, writing—original draft preparation, resources, DB and BB; technical assistance, DB and DNSI; data validation, review and editing, DB, BB, DNSI; supervision, writing—review and editing, DB.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding this article..

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KOMPARACIJA MORFOLOGIJE LARVI I PREZERVACIJA DNA KOD PLASTINACIJE UPOTREBOM RAZLIČITIH FIKSATIVA

SAŽETAK

Istraživanje je provedeno sa ciljem određivanja opsega prezervacije strukture larvi parazita tretiranih sa različitim fiksativima u procesu silikonske plastinacije, kao i stepen prezervacije DNA u plastiniranim larvama. U morfometrijskoj evaluaciji su izmjereni dužina (anterior-posterior) i maksimalna širina (najveća širina trupa) larvi kako bi se zabilježile promjene prije i poslije plastinacije. Na sobnoj temperaturi je primijenjen protokol silikonske plastinacije korištenjem tri različita fiksativa. Larve su na kraju deplastinirane. Iz plastiniranih i neplastiniranih larvalnih uzoraka larvi trećeg stadija je ekstrahirana ukupna genomska DNA (gDNA) korištenjem GeneJET kita za purifikaciju genomske DNA. Na 260 nm suizmjerene koncentracije DNA korištenjem UV-Vis spektrofotometra. Primijenjen je PCR protokol, pri čemu su korištenjem UV transiluminatora napravljene fotografije. Larve su nakon plastinacije bile potpuno suhe i bezopasne. Mogle su biti uskladištene duži vremenski period i, što je najvažnije, njihove morfološke karakteristike su ostale pogodne za morfološku analizu od strane studenata. U dehidracijskoj i impregnacijskoj fazi plastinacije nisu uočeni morfološki problemi, međutim, nakon očvršćivanja neki uzorci su pokazali signifikantni strukturni kolaps, koji je imao za posljedicu promjene u nihovoj normalnoj anatomskoj strukturi. Osim toga, koncentracije DNA u deplastiniranim grupama su bile više od onih u plastiniranim. U drugom koraku PCR-a dobivena je traka od ~300 bp za sve plastinirane I neplastinirane grupe. U zaključku, metoda silikonske plastinacije omogućava dugotrajnu prezervaciju morfoloških struktura larvi uz minimalne promjene, uzrokujući djelomično smanjenje integriteta DNA.

Ključne riječi: Anatomija, larve, morfometrija, PCR, plastinacija, prezervacija DNA

CASE REPORT

CONGENITAL AND ACQUIRED OCULAR ABNORMALITIES IN A DOG: CLINICAL DESCRIPTION AND MANAGEMENT

Aslı Uygur[1], Digdem Uygur[1,2], Irem Ergin[2*]

[1]Ankara University, Graduate School of Health Sciences, Ankara, Türkiye
[2] Department of Surgery Faculty of Veterinary Medicine, Ankara University, Ankara, Türkiye

[*]**Corresponding author:**
Assoc. Prof. Dr. Irem Ergin
Address: Ankara University, Faculty of Veterinary Medicine, 06110 Dışkapı, Ankara/ Türkiye
Phone: 0535 467 6879
ORCID: 0000-0003-2373-5133
E-mail: iremerg@gmail.com

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ABSTRACT

Congenital ocular malformations, though rare, are clinically significant anomalies in veterinary ophthalmology. The most commonly observed congenital ocular anomalies in cats and dogs include microphthalmia, colobomatous defects, palpebral agenesis, and dermoids, which may occur either independently or in combination. This case report details the clinical evaluation, diagnosis, and surgical management of a dog presenting with two congenital ocular anomalies, concurrently with an acquired disorder. A one-year-old male Anatolian Shepherd Dog was referred to the Ankara University, Faculty of Veterinary Medicine, Animal Hospital Ophthalmology Clinic with bilateral conjunctivitis and prolapse of the third eyelid gland. Ophthalmic examination revealed a well-defined dermoid lesion at the lateral canthus of the upper eyelid in the left eye, accompanied by palpebral agenesis located posterior to the dermoid tissue. The dermoid involved the bulbar and palpebral conjunctiva as well as the dorsal corneal surface. Surgical intervention was performed to excise the dermoid tissue and correct both the third eyelid gland prolapse and palpebral agenesis. Additionally, the prolapsed third eyelid gland in the right eye was surgically repositioned. The postoperative period was uneventful, and the patient was monitored for three months without complications. This report highlights the successful simultaneous surgical correction of ocular dermoid, palpebral agenesis, and third eyelid gland prolapse in a single procedure, demonstrating an effective approach for managing complex congenital ocular anomalies in dogs.

Keywords: Eyelid coloboma, ocular dermoid, ocular malformation, third eyelid gland prolapse

INTRODUCTION

Congenital ocular malformations are rare but clinically significant anomalies. These malformations are thought to result from abnormal embryonic development, with genetic and environmental factors-such as exposure to toxic substances, nutritional deficiencies, and hypo- or hypervitaminosis A-also contributing to their formation (Cognard et al., 2023; Saraiva and Delgado, 2020). The most frequently observed ocular anomalies in cats and dogs include congenital microphthalmia, colobomatous defects, palpebral agenesis, and dermoid formations. Many of these structural abnormalities require surgical intervention and, in some cases, may even lead to permanent blindness in affected animals (Ofri, 2017).

Dermoids are congenital anomalies defined as the presence of histologically normal cutaneous tissue in an ectopic location due to aberrant tissue development during the embryonic period (Cathelin et al., 2022). Ocular dermoids are characterized by the presence of ectopic tissue-such as skin, hair follicles, blood vessels, nerves, fibrous tissue, fat, and occasionally sweat glands-on the cornea, conjunctiva, or eyelids (Balland et al., 2015). These abnormal structures, though rare across different animal species, have been reported in dogs (Badanes and Ledbetter, 2019), cats (Cathelin et al., 2022), horses (Makra and Jakab, 2018), cattle (Kumar et al., 2020), and guinea pigs (Wappler et al., 2002).

Third eyelid gland hyperplasia, also known as nictitating membrane gland prolapse or cherry eye, is an acquired condition most commonly observed in dogs, but has also been reported in cats, rabbits, various bird species, and wild animals. It occurs due to the displacement of the lacrimal gland of the third eyelid from its anatomical position and is clinically characterized by a visible red mass at the medial canthus. Although its exact etiology remains unclear, breed predisposition and environmental irritants are thought to contribute to its development (Cook, 2021; Oguntoye et al., 2022).

This case report describes the concurrent occurrence of a rare ocular dermoid, coloboma, and third eyelid gland hyperplasia in a dog, detailing the clinical presentation, diagnosis, and treatment. It aims to emphasize the coexistence of congenital and acquired ocular disorders in veterinary ophthalmology, offering insights into their clinical management and therapeutic approaches.

CASE DESCRIPTION

A one-year-old male Anatolian Shepherd Dog was presented to Ankara University Faculty of Veterinary Medicine Animal Hospital Ophthalmology Clinic with complaints of bilateral conjunctivitis and third eyelid gland prolapse. According to the history obtained from the owner, the dog had experienced persistent eye problems since puppyhood, exhibited reluctance to open its eyes, and had continuous ocular discharge despite regular cleaning. The dog was owned and lived in a rural area with a garden. It weighed approximately 40 kg and was fed a homemade diet.

An “Informed Consent Form” was obtained from the animal’s owner prior to the examination and surgical procedure. All procedures in this case report were conducted in accordance with national animal welfare regulations, and no additional experimental interventions were performed.

Ophthalmic examination revealed severe purulent discharge and diffuse keratitis and scar tissue affecting the entire corneal surface of the left eye. Closer inspection identified a conjunctival dermoid measuring approximately 3 cm in length, originating from the lateral conjunctival fornix of the upper eyelid. This dermoid was covered with numerous hairs that irritated the corneal surface. Upon careful removal of the hairs to assess the cutaneous structure, the dermoid was found to extend toward the bulbar conjunctiva in the temporal canthal region, further progressing beneath the upper eyelid to involve the limbal area and a 4 mm-diameter region of the corneal surface. Additionally, a small dermoid fragment, a few millimeters in size, was detected on the palpebral surface at the lateral canthus of the upper eyelid,

with long hairs protruding from it. Further detailed examination revealed the presence of palpebral agenesis, as the upper eyelid was incompletely developed up to the lateral canthus (Figure 1). Despite severe keratitis in the left eye, the visual response was positive, and the direct pupillary light reflex was normal in both eyes.

Surgical intervention was planned for the animal, which did not have any abnormalities in complete blood count (performed using a Mindray BC-5000 Vet hematology analyzer), serum biochemistry (analyzed with a Randox RX Monaco biochemistry analyzer) (Table 1), and thorax radiographs, except for mild tracheitis observed on the radiograph, with no other pathological findings noted (Figure 2). The reference intervals for the evaluated blood parameters were obtained from the respective devices.

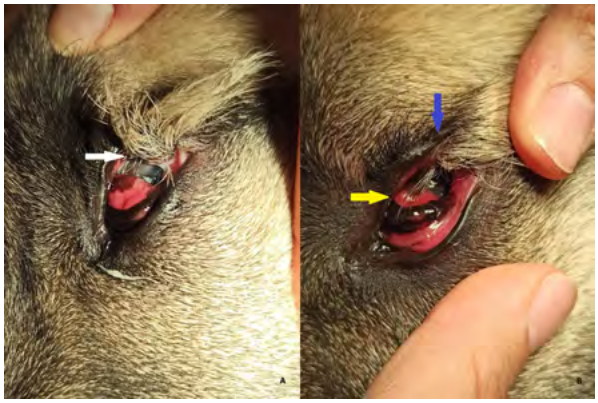

Figure 1 (A) A corneal dermoid located near the lateral canthus of the upper eyelid. The dermoid tissue, characterized by long hairs emerging from its surface, extensively involves the bulbar conjunctiva. Additionally, its margin extends into the dorsal quadrant of the cornea (white arrow). **(B)** Retraction of the long hairs laterally reveals palpebral agenesis toward the lateral canthus of the upper eyelid (blue arrow). Concurrently, third eyelid gland hyperplasia is observed (yellow arrow)

Table 1 Hematological and serum biochemistry parameters of the dog

Parameters	Unit	Values	Reference Interval (min - max)
Serum Biochemistry			
Blood urea nitrogen	mg/dL	19.50	15.00-59.00
Creatinine	mg/dL	1.17	0.50-1.50
Alanine aminotransferase (ALT)	IU/L	34.50	0.00-50.00
Alkaline phosphatase (ALP)	IU/L	46.00	0.00-130.00
Aspartat aminotransferase (AST)	IU/L	23.00	0.00-40.00
Creatin kinase (CK)	IU/L	85.10	0.00-200.00
Glucose	mg/dL	104.00	65.00-118.00
Cholesterol	mg/dL	321.00	125.00-250.00
Hematological Parameters			
White blood cell count (WBC)	10^9/L	6.03	6.00-17.00
Neutrophils	10^9/L	3.90	3.60-12.30
Lymphocytes	10^9/L	1.26	0.83-4.91
Monocytes	10^9/L	0.59	0.30-2.50
Eosinophils	10^9/L	0.28	0.10-19.00

Parameters	Unit	Values	Reference Interval (min - max)
Basophils	10^9/L	0.00	0.00-0.12
Neutrophils	%	64.60	52.00-81.00
Red blood cells (RBC)	10^{12}/L	6.97	5.10-8.50
Haemoglobin (HGB)	g/dL	17.50	11.00-19.00
Hematocrit (HCT)	%	47.50	33.00-56.00
Mean corpuscular volume (MCV)	fL	68.20	60.00-76.00
Mean corpuscular hemoglobin (MCH)	pg	25.20	20.00-27.00
Mean corpuscular hemoglobin concentration (MCHC)	g/L	36.90	30.00-38.00
Red cell distribution width – standard deviation (RDW-SD)	fL	34.70	33.20-46.30
Platelets (PLT)	10^9/L	150.00	117.00-490.00
Mean platelet volume (MPV)	fL	12.70	8.00-14.10

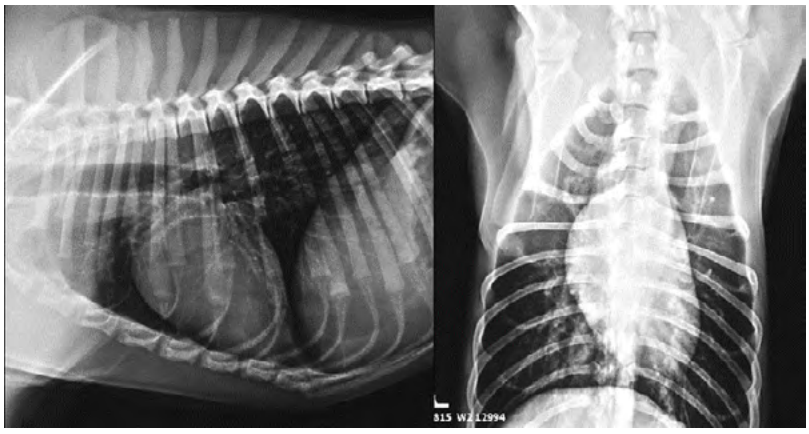

Figure 2 Thoracic radiographs of the dog in ventrodorsal (V/D) and laterolateral (L/L) views. The images illustrate the thoracic cavity, including cardiac silhouette and pulmonary fields

General anesthesia was performed. Induction was achieved with propofol (4 mg/kg, IV, Polifarma, Turkiye), followed by maintenance through orotracheal intubation and administration of isoflurane via a circle breathing system. Analgesia was provided with butorphanol (0.2 mg/kg, SC, Richter Pharma, Austria). During surgery, the animal was perfused with Ringer's lactate solution and cephalosporin (25 mg/kg, IV, Tumekip Med, Turkiye) was administered.

Surgical intervention for the ocular dermoid was performed first. Under an operating microscope, the affected tissue was excised from the palpebral conjunctiva, ensuring complete removal of all hair follicles. To achieve this, the conjunctival dermoid was initially incised along its perimeter while preserving the integrity of the palpebral and bulbar conjunctiva. Throughout the procedure, anatomical structures at the lateral conjunctival junction and bulbar conjunctiva were carefully maintained. Subsequently, the fine dermoid tissue on the corneal surface was excised up to the limbal region.

Following complete tissue removal, the exposed conjunctival area was closed with simple continuous sutures using absorbable multifilament 6-0 polyglycolic acid suture (Dogsan, Turkiye). After dermoid excision, the upper eyelid agenesis

Figure 3 Preoperative **(A)** and postoperative 0. day **(C)** and 15. day **(D)** view of ocular malformations. **(B)** The removal of the corneal dermoid reveals a more distinct corneal scar tissue (white arrow). Third eyelid gland hyperplasia is prominently visible on the bulbar surface of the eyelid (blue arrow). Palpebral agenesis is clearly noticeable (yellow arrow)

became more pronounced in the affected eye. The defect was repaired by creating a new margin from the intact upper eyelid border using simple continuous subcuticular sutures. Care was taken to prevent inward rotation of the skin toward the eye, minimizing the risk of irritation from future hair growth (Figure 3). Histopathological examination confirmed the excised tissue as a dermoid.

Finally, the prolapsed third eyelid gland was repositioned using the modified Morgan pocket technique. Two parallel incisions were first made dorsally and ventrally on the conjunctival tissue overlying the prolapsed gland. A sub-conjunctival pocket was then created by dissecting beneath the incision line using Stevens tenotomy scissors. The conjunctival tissue between the incision lines was excised, and the gland was secured within the pocket using monofilament absorbable 5-0 polydioxanone suture material (Katsan, Turkiye) with the Schmieden suturing technique. Care was taken to position the starting and ending knots on the palpebral surface of the nictitating membrane, leaving a 3–4 mm gap at both ends of the suture line. At the end of the procedure, a sterile swab moistened with physiological saline was gently pressed against the third eyelid for one minute to reduce postoperative swelling.

During the postoperative period, amoxicillin (25 mg/kg, PO, Deva, Turkiye) was administered for seven days. Additionally, topical tobramycin and steroidal anti-inflammatory eye drops were applied twice daily, while topical sodium hyaluronate and artificial tear lubricant gel were used three times daily. An Elizabethan collar was placed to protect the eye throughout the 15-day follow-up period.

Postoperative monitoring was conducted via phone communication and photographs, as the owner did not return for in-person evaluations. At the second- and fourth-week follow-ups, as well as at the three-month postoperative assessment, no signs of discomfort were observed, and no complications such as ocular pain or irritation were reported.

DISCUSSION AND CONCLUSION

Although the etiology and pathophysiology of ocular abnormalities have not yet been fully elucidated, it is believed that many of these conditions result from developmental abnormalities occurring during the embryonic period (Cook,

2021). In cats and dogs, abnormalities such as microphthalmia, dermoids, and agenesis typically manifest individually, although it is rarely the case that multiple anomalies may occur together (Saraiva and Delgado, 2020; Berkowski et al., 2018).

Eyelid agenesis, also referred to as eyelid coloboma, most commonly affects the upper eyelid in dogs. Although its pathophysiology remains unclear, it is widely regarded as a result of abnormal differentiation during the embryonic period (Cathelin et al., 2022). Palpebral agenesis is a high-risk anomaly primarily due to the eyelids' inability to fully protect the ocular surface. This defect leads to rapid evaporation of the aqueous layer of the tear film and inadequate distribution of the lipid layer, resulting in corneal and conjunctival dryness. Consequently, severe keratoconjunctivitis and serious complications, such as corneal opacity and vision loss, may develop (Ofri, 2017; Demir and Karagozoglu, 2019). Therefore, timely medical and surgical intervention is crucial for the management of eyelid agenesis (Whittaker et al., 2010). In the present case, given the dog's age, the intervention was delayed. Chronic, thick scar tissue had developed on the cornea of the left eye due to persistent irritation from the hairs associated with the dermoid. Although the animal retained visual function, the extensive corneal opacity in this eye resulted in significant vision impairment. Despite postoperative treatment aimed at improving corneal health, the scar tissue remained unchanged, indicating permanent damage.

In the surgical management of eyelid agenesis, it is essential to restore the region in alignment with its anatomical structure. This approach not only ensures the success of the procedure but also mitigates potential postoperative complications. The choice of surgical method is dictated by the defect size. Small defects, constituting less than 25% of the eyelid, are amenable to direct repair, whereas most lesions are unsuitable for primary repair. Larger defects necessitate more intricate reconstructive techniques, such as advancement and rotational flaps (Demir and Karagozoglu, 2019;Whittaker et al., 2010). In the presented case, given that the defect size was less than one-third of the eyelid length, the dermoid was meticulously delineated from the surrounding tissue, excised, and removed. The area was subsequently closed in accordance with its anatomical position. Due to the absence of the eyelid margin, particular attention was given during the procedure to prevent the eyelid from turning towards the bulbar surface.

Ocular dermoids are referred to as choristomas by some researchers due to their occurrence on the ocular surface, whereas those affecting the eyelids are termed hamartomas (Dubielzig et al., 2010). However, some authors have proposed that, given the lack of clear distinction in most dermoids, such as conjunctiva-palpebral dermoids, they should be described as choristo-hamartomas. In dogs, dermoids can be observed on the conjunctiva and/or corneal surface, particularly in the temporal region. This congenital anomaly is believed to be associated with genetic factors, and certain dog breeds, including the German Shepherd, Dalmatian, Saint Bernard, Basset Hound, and Dachshund, are predisposed to this condition. Ocular dermoids have been anatomically classified in various ways. The most commonly encountered form is conjunctiva-corneal dermoids, which affect both the conjunctiva and cornea, whereas conjunctiva-palpebral dermoids, affecting both the conjunctiva and eyelid, are rarer. Dermoids that exclusively affect the corneal surface have been classified into three distinct types. The first type, the most common and least severe form, comprises dermoids covering the limbal or epibulbar surface. The second type includes dermoids that cover nearly the entire cornea, extending into the stroma but not affecting the Descemet's membrane or corneal endothelium. The third type consists of dermoids that cover the entire corneal surface and layers and may even extend into the eye. When dermoid tissue affects only the conjunctiva, the prognosis is favorable; however, involvement of the cornea renders the prognosis uncertain, depending on the depth and extent of the lesion. If dermoids are not removed, the long hairs on the tissue may irritate the cornea and conjunctiva,

leading to clinical symptoms such as corneal ulceration, pigmentation, epiphora, keratitis, and blepharospasm (Balland et al., 2015). In the presented case, the dermoid tissue was observed to form laterally to the eyelids, as noted by many researchers. The involvement of the palpebral, conjunctival, and corneal tissues indicated that the case should be more accurately described as a corneo-conjunctival-palpebral dermoid.

While the conjunctiva of the upper eyelid was most prominently affected, the absence of hair on the dermoid tissue affecting the limbal portion of the cornea was considered fortunate, given the animal's age. This absence resulted in less irritation to the cornea over an extended period. Another notable observation in the dermoid structure was the clear demarcation between the boundary of the tissue on the upper eyelid and the boundary of the agenesis, with only a few millimeters of separation. The fact that the dermoid had not progressed into the deeper layers of the cornea facilitated the easy removal of the lesion.

Although the exact etiology of third eyelid gland prolapse is not fully understood, it is believed to be influenced by genetic and congenital factors in dogs. Large breeds, particularly Cane Corso, Neapolitan Mastiff, and Lhasa Apso, are known to be predisposed to gland prolapse (Mazzucchelli et al., 2012). Deformation of the fascia retinaculum, which holds the gland in its anatomical position, is also considered a potential contributing factor in the etiology (Oguntoye et al., 2022). Additionally, eye irritants may trigger an irritative process in the third eyelid gland, which is covered by lymphoid tissue-rich conjunctiva (Mazzucchelli et al., 2012). In the present case, the animal's breed, the Anatolian Shepherd Dog, a giant breed, could have predisposed it to the bilateral development of third eyelid gland hyperplasia. More importantly, the animal's living environment, particularly the dirt floor causing significant dust exposure, likely contributed to severe eye irritation throughout the day.When these two factors are combined, the development of gland prolapse becomes inevitable. The occurrence of prolapse in an eye without ocular anomalies weakens its connection to these anomalies. However, further research is required to explore the genetic predisposition for this condition.

In conclusion, this study exemplifies the simultaneous management of complex congenital ocular anomalies, demonstrating the successful surgical treatment of an ocular dermoid, palpebral coloboma, and third eyelid gland hyperplasia in a single surgical procedure.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

A.U.: Data collection and processing, literature review, writing. D.U.: Conception, design, writing. I.E.: Supervision, writing, critical review.

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KONGENITALNE I STEČENE OČNE ABNORMALNOSTI KOD PSA: KLINIČKI OPIS I TRETMAN

SAŽETAK

Kongenitalne očne malformacije, iako rijetke, predstavljaju klinički značajne anomalije u veterinarskoj oftalmologiji. Najčešće kongenitalne očne anomalije kod mačaka i pasa su mikroftalmija, kolobomatozni defekti, palpebralna agenezija i dermoidi, koji mogu nastati neovisno ili u kombinaciji. Ovaj prikaz slučaja opisuje kliničku evaluaciju, dijagnosticiranje i operativni tretman psa sa dvije kongenitalne očne anomalije, zajedno sa stečenim poremećajem kod jednogodišnjeg mužjaka anatolskog ovčarskog psa koji je upućen na Kliniku za oftalmologiju Bolnice za životinje Veterinarskog fakulteta Univerziteta u Ankari zbog bilateralnog konjunktivitisa i prolapsa žlijezde trećeg kapka. Oftalmološki pregled pokazao je dobro definiranu dermoidnu leziju na lateralnom kantusu gornjeg kapka lijevog oka, zajedno s palpebralnom agenezijom posteriorno od dermoidnog tkiva. Dermoid je uključivao bulbarnu i palpebralnu konjunktivu, kao i dorzalnu kornealnu površinu. Proveden je operativni tretman u svrhu ekscizije dermoidnog tkiva i korekcije prolapsa žlijezde trećeg kapka i palpebralne agenezije. Osim toga, prolaps žlijezde trećeg kapka u desnom oku je operativno repozicioniran. Postoperativni period je protekao mirno, a pacijent je praćen tri mjeseca, pri čemu nisu uočene nikakve komplikacije. Ovaj prikaz slučaja prikazuje uspješnu istovremenu operativnu korekciju očnog dermoida, palpebralne agenezije i prolapsa žlijezde trećeg kapka u samo jednoj proceduri, demonstrirajući učinkovit pristup tretmanu kompleksnih kongenitalnih anomalija kod pasa.

Ključne riječi: Kolobom očnog kapka, očni dermoid, očna malformacija, prolaps žlijezde trećeg kapka

CASE REPORT

PERFORMANCE AND FINANCIAL ANALYSIS IN BROILER (COBB 430 Y STRAIN): A CASE REVIEW ON THE FEED CONVERSATION RATIO AND MORTALITY RATE

Vanlalhmangaihsanga[1*], Nanda Kumar Roy[1], Lalrinkima[2], Nabakrushna Praharaj[1], Samarendra Mohanty[1], Surita Majumder[3]

[1]Department of Livestock Production Management, IVSH, SOA, Bhubaneswar, Odisha, India
[2]Department of Veterinary Pathology, IVSH, SOA, Bhubaneswar, Odisha, India
[3]Department of Animal Genetic and Breeding Institute of Veterinary Sciences and Animal Husbandry, Siksha 'O' Anusandhan University, Odisha, India

***Corresponding Author:**
Asst. Prof. Dr. sc. Vanlalhmangaihsanga

Address:
IVSH, SOA, Bhubaneswar, India – 751030
Phone: + 9612542135
ORCID: 0009-0001-0635-0621
Email:
hmangaihatochhong@gmail.com

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ABSTRACT

This study investigates the operational, production, and financial performance of broiler farming using the COBB 430 Y strain at the Instructional Livestock Complex (ILFC). A total of 1,439 chicks were reared over 40 days, achieving an average Feed Conversion Ratio (FCR) of 1.52 and an average market weight of 2.12 kg by the final selling phase. The farm's mortality rate of 4.65%, which was mostly caused by enteritis (6%), and colibacillosis (44.8%), surpassed the aim of 3.18% even though the farm consistently increased its weight. Financially, the farm made a total expenditure of Rs 2,55,427, including feed, chick procurement, and health management, while generating an income of Rs 2,67,098.95 through broiler sales. This resulted in a modest net income of Rs 11,671.95 and a benefit-cost ratio of 1.05, confirming the economic feasibility of the operation despite the challenges faced. The findings emphasise optimizing health management, biosecurity measures, and environmental conditions to minimize losses and enhance profitability. This report provides valuable insights into raising broiler farming and shows how strategic management techniques can lead to sustainable production. In order to further increase productivity and financial returns, future initiatives should focus on health issues including colibacillosis, bettering feed efficiency, and lowering mortality rates.

Keywords: Biosecurity, feed conversion ratio, mortality, production metrics, profitability

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INTRODUCTION

This report sets out the results of the first rearing report on the broiler that was conducted on 1,439 chicks on January 3, 2024. This report aimed to obtain information from the first rearing of the broiler about the broiler's growth, profitability, and mortality. Broilers are one kind of poultry that are reared for mainly meat purposes. It is kept for the commercial production of meat in our country. Compared to other agricultural sub-sectors, in the situation of enhancing the agriculture industry meat farming sub-sector is with the high potential (Ali et al., 2013). In poultry farms focused on meat production, broiler breeds are raised mainly in environmentally controlled poultry houses. Broiler farming has become popular both in urban and rural areas. It encouraged the people of different sections such as small farmers, landless labourers and educated unemployed as well as industrialists to establish broiler farms on small and large scales (Chatterjee and Rajkumar, 2015). The growth performance of broiler birds might simply be a function of higher feed conversion. Feed consumption followed a similar trend to that of weight gain.

In addition to providing food to human beings, the poultry industry concentrates on providing employment not only to those engaged in production directly, but also for the hatchery operations, feed dealers, manufacturers of incubators, building materials, processors of egg and poultry products, and all dealers engaged in the marketing process (Nkukwana, 2018). Many factors may affect the production process in broiler production. The type of the production system is the major one. The intensive production system is a largely adopted production system which decreases prorates inputs per bird during the production period (Castellini et al., 2006).

In Indian conditions, the production of poultry in the backyard was far more prevalent in comparison to ten years ago. As it's evident from the 20th Livestock Census, backyard poultry has touched new highs with an additional of almost 45.78% increase in population time from 217.49 million in 2012 to 317.07 million in 2019 (20th Livestock Census). This exceptional rate of population increase rate might be attributed to different cases like bringing in the administration's incentives, information programs, and backyard farms' intrinsic merits.

At present, the proper distribution of poultry among various areas all across the country is reported as being at a high level. The role of the mentioned three states, among others, in the commercial poultry sector is of immense significance. States such as Tamil Nadu, Andhra Pradesh, and Telangana are among the states that have the largest share of the poultry population and the most developed poultry farming in India. The share of poultry production in the total poultry livestock in India varies and ranges from a low of 0.3% to even full coverage in some states (Chatterjee & Rajkumar, 2015). In practical terms, this means that poultry flocks have a wider dispersal within states influenced, however, by geographic factors and the type of livestock raised.

Broiler meat is a simple and cheap source of protein. Poultry meat accounts for a significant percentage of the total meat production in India. India ranks 8th in chicken meat production in the world. Poultry meat production in India has emerged as a vital component of the country's meat industry, contributing significantly to overall meat production. In the year 2022-23, poultry meat production continued to dominate, reflecting the sector's growth, technological advancements, and increasing consumer demand for poultry products. This section provides a comprehensive overview of poultry meat production in India, highlighting its contribution, growth trends, and factors influencing its success (USDA, 2019). The meat production from poultry is 4.995 million tonnes, contributing about 51.14% of total meat production (BAHS, 2023). The contribution of poultry to GDP and foreign exchange is essential and increasing day by day. The per capita availability was 5.72 kg per annum during the period 2016-17. There has been a steady increase in per capita availability of meat. The per capita availability reached at 7.10

kg/annum in the year 2022-23, a 0.28-point jump from 6.82 g/annum in the previous year 2021-22 (Singh, 2023). High and fluctuating feed prices are other important obstacles in the development of the broiler industry. Increasing the broiler farm capacity to 25,000 broiler chickens will make farming financially feasible (Al-Sharafat and Al-Fawwaz, 2013). Bird stocks, operating costs, and other costs were important factors to broiler output. Age, education, family size, training and access to credit, were found to be technical inefficiencies of farmers' abilities that largely affected production (Binam et al., 2004). Factors such as feeding system, water quality and the type of ration among the managerial factors have noticed the effect on broiler rearing operations (Manning et al., 2007). Therefore, this aims to assess the operational, production, and financial performance of broiler farming using the COBB 430 Y strain under controlled conditions at the Instructional Livestock Farm Complex (ILFC).

MATERIALS AND METHOD

Operational Highlight

To determine the feasibility of poultry production, this study used a small rental farm located in Instructional Livestock Farm Complex (ILFC) in Binjhagiri, Khordha, Bhubaneswar. On January 3 2024, 1,439-day-old chicks were introduced into a poultry house furnished with sawdust and supplied with heaters, feeders, and drinkers. The chicks were bought at a price of Rs. 22/- each from a local supplier.

Upon arrival, the broiler chicks of COBB 430 Y strain were placed in a carefully prepared brooding area, where the temperature was maintained at a consistent 25 degrees Celsius. This initial stage was critical for the health and well-being of the chicks, as it provided the necessary warmth and environment to support their early development. However, the importance of maintaining optimal conditions was highlighted early in the rearing period when the first-week mortality was attributed to cold shock. This incident underscored the challenges of managing the delicate balance required in the early stages of chick rearing.

The management of the brooding area was meticulous. The newspaper that lined the brooding area was removed on the second day and the temperature continued to be closely monitored and maintained at 25 degrees Celsius. After eight days, the brooder guard, which had provided the chicks with protection and warmth, was removed. This allowed the chicks more space to move around and adapt to their environment, an important step in their development.

Feeding

Since feed consumption has a direct impact on growth rates, health, and overall performance, feeding was monitored closely throughout the rearing period (Hamra, 2010). There were three stages to the feeding schedule: pre-starter, starter, and finisher. The chicks were fed 500 kg of feed during the pre-starter period, which was intended to give them the vital nutrients they needed for their first development surge. A total of 1,200 kg was consumed by the chicks as they grew and switched to the starter feed. The purpose of the starter feed was to promote quick growth and get the chicks ready for the last stage of rearing. Finally, during the finisher phase, a total of 3,000 kg of feed was allocated, but 67 kg was left over, making the actual consumption 2,933 kg. This phase was crucial as it determined the final weight and market readiness of the birds. In total, 4,733 kg of feed was consumed during the 40-day rearing period.

Feed management was also a critical component of the operation's success. The phased approach to feeding, starting with the pre-starter feed and progressing through to the finisher feed, ensured that the nutritional needs of the chicks were met at each stage of their development. The careful calculation and monitoring of feed consumption allowed the operations team to optimize growth rates and achieve the target FCR. For this reason, managing feed formulas for accuracy is an important step in poultry farm management to safeguard the environment, and reduce operating costs (Karcher, 2009).

Vaccination

Vaccination was another crucial aspect of the rearing process. Viral diseases can be reduced by proper sanitation on the farm, biosecurity measures, and vaccination of the chicks and chickens (Hamra, 2010). The chicks were vaccinated on four occasions during the 40 days to ensure their health and immunity against common poultry diseases. The first vaccination was administered on the 4th day, followed by subsequent vaccinations on the 14th, 21st, and 30th day. The vaccines used were Lasota, IBD (Infectious Bursal Disease), Lasota Booster, and IBD Booster, respectively. Each of these vaccines played a critical role in preventing outbreaks of diseases that could severely impact the flock.

The use of vaccines such as Lasota and IBD, along with their boosters, was particularly important in safeguarding the flock against respiratory diseases and infectious bursal disease, both of which can have devastating effects on poultry, if not properly managed. The strategic timing of these vaccinations helped to build immunity within the flock, ensuring that the birds remained healthy and productive throughout the rearing period.

Throughout the rearing period, careful implementation of all rearing parameters was paid to every aspect of the chicks' environment, health, and nutrition. The operations team worked diligently to maintain the brooding area at the correct temperature, manage feed distribution, and administer vaccinations at the appropriate times. Despite the initial setback of mortality due to cold shock, the overall operation adhered closely to the planned schedule and performance targets.

Data Collection

Data on dead chicks, feed, and body weight used, were collected to monitor the flock's performance. The scope of the study only focused on broiler production performance in the Instructional Livestock Farm Complex (ILFC). The data were gathered from a sample of 150 poultry birds through observations of the weekly performance of the birds. An economic assessment for this research was carried out to study the profitability of the broiler farm. The different calculations for the performance of the farm are as shown below:

Weekly Body Weight

The weekly body weight gain of the birds and daily feed intake were recorded to assess the growth performance and feeding efficiency of the birds. Body weight was recorded using a digital weighing balance. The body weight and daily feed intake data determined the following growth performance.

Feed Conversion Ratio (FCR)

Feed conversion ratio was calculated as a gram of feed consumed per gram of body weight gain with correction for mortality, if any.

$$\text{FCR} = \frac{\text{Feed Intake(g)}}{\text{Body Weight Gain (g)}}$$

Percent Morbidity and Mortality

A record of mortality (if any) was maintained daily. The necropsy examination was done to evaluate any gross pathological lesion and cause of death of each bird. Total mortality in each treatment was then calculated and expressed on a percentage basis.

$$\text{Mortality \%} = \frac{\text{Total number of animals die during the experimental period}}{\text{Total number of animals atthe beginning of experiment}} \times 100$$

Economic Analysis (cost of feed/kg weight gain)

The economic viability of the broilers was evaluated based on the total expenditure incurred on these products and the return from the sale of live birds.

Economic efficiency involves evaluating the costs of feed, medication, energy, and other resources of revenue generated from meat production. This article presents the key production indicators of broiler chickens that affect rearing economic efficiency, such as final BW, mortality, and FCR. FCR was calculated by summing the amount of feed used to produce one kilogram of body

weight, which is the main factor in reducing production costs (Marcu et al., 2013). EPEF is an indicator of the production efficiency of a given flock (Allison et al., 2000; Perić et al., 2009)a calf disease. However, it is likely that the vitamin E requirement of the modern dairy cow is very different from that of a calf. This review of the literature investigates the effect of vitamin E supplementation on the health and fertility of the dairy cow. Supplementation of high levels of vitamin E (at least 1000 iu per day. The index was calculated according to the following formula:

$$\text{EPEF} = \frac{Mean\ BW\ (kg) X\ Survival\ rate\ (\%)}{Number\ of\ rearing\ days\ X\ \square\ CR} \text{ X } 100$$

All calculations and analysis were done using Microsoft Excel®.

RESULTS AND DISCUSSION

Production Analysis

The 40-day rearing period provided detailed insights into the growth and weight distribution of 150 poultry birds from a sample total population of 1,439 as shown in Table 1.

Table 1 Average body weight distribution of birds

First-week average body weight distribution	
Weight (g)	**Count of Body Weight (g)**
100-150	101
150-200	48
>200	1
Second-week average body weight distribution	
200-300	20
300-400	81
400-500	49
Third-week average body weight distribution	
500-699	30
700-899	113
900-1099	7
Fourth-week average body weight distribution	
1.19-1.34	45
1.34-1.49	95
1.49-1.64	10
Fifth-week average body weight distribution	
1.7-2	72
2-2.3	77
2.3-2.6	1

This data highlights trends in weight gain and influencing factors, such as diet, and management. During the first week, the average body weight was 142.92 grams, with most chicks weighing between 100 and 150 grams. A few chicks exceeded 200 grams, reflecting variability in early growth. Research by Khalid et al. (2021) on Cobb-500 and Ross-308 broiler strains showed slightly higher average weights of 207.40±14 grams and 196.00±16 grams, respectively.

By the second week, the chicks' average weight increased significantly to 360.48 grams, with most weighing between 300 and 500 grams. This indicated a more uniform growth pattern as the chicks developed. In the third week, the average weight rose to 761.33 grams, with a majority weighing between 700 and 899 grams. Growth became more uniform, with many nearing the 1-kilogram mark. By the third week, the average weight nearly doubled to 761.33 grams, with most chicks (113) weighing between 700-899 grams.

In the fourth week, the average weight reached 1.38 kilograms, with most birds weighing between 1.34 and 1.49 kilograms. In the fifth week, the average weight peaked at 2 kilograms, with most birds weighing between 2 and 2.3 kilograms. These figures reflect the effectiveness of the management practices in achieving substantial growth. The flock's performance culminated in two selling phases (Table. 2).

Table 2 Average Selling Live weight distribution of birds

Weight (kg)	Count of Body Weight (kg)
First Live Weight (750 birds)	
1.5-1.8	20
1.8-2.1	600
2.1-2.4	130
Second Live Weight (609 birds)	
1-1.6	9
1.6-2.2	220
2.2-2.8	380

In the first phase, 750 birds were sold, with the majority weighing between 1.8 and 2.1 kilograms. In the second phase, 609 birds were sold, with most weighing between 2.2 and 2.8 kilograms. By the fifth week, the majority of birds (77) reached 2-2.3 kilograms, nearing market weight. Overall, the production analysis demonstrated a successful growth trajectory, with most birds reaching or exceeding target weights, contributing to profitability.

Production Parameters

The flock's growth was closely linked to variations in the Feed Conversion Ratio (FCR), a critical metric in poultry production (Table 3). A lower FCR indicates better feed efficiency, with the industry standard for broiler performance is the achievement of 2.5 kg live weight with a feed conversion ratio of 1.72 at 42 days of age in recent times (Creswell, 2005). During the first week, the chicks had an average body weight of 142.92 grams and an FCR of 1.36, indicating efficient feed conversion. By the second week, the average weight increased to 360.48 grams, but the FCR rose to 1.69, reflecting increased energy demands as the chicks entered a rapid growth phase.

Table 3 Production and selling parameters of broiler

Average Weekly Body Weight	
Week	**Average Body Weight (g)**
1	142.92
2	360.48
3	761.33
4	1380.78
5	2000
Average Weekly FCR	
Week	**FCR**
1	1.36
2	1.69
3	1.33
4	1.28
5	1.87
Average FCR	1.52
Selling Parameter	
First Selling (35 days)	2.00
Second Selling (40 days)	2.24

In the third week, the average weight jumped to 761.33 grams, with the FCR improving to 1.33, indicating enhanced feed efficiency. By the fourth week, the average weight reached 1,380.78 grams, and the FCR dropped to 1.28, the lowest recorded. This peak feed efficiency suggested optimal growth conditions. However, by the fifth week, the average weight reached 2,000 grams, and the FCR increased to 1.87, reflecting a slight decline in feed efficiency as the birds approached maturity.

The overall average FCR across the five weeks was 1.52, slightly above the ideal threshold but still within the optimal range. This indicated that the flock efficiently converted feed into body mass, resulting in satisfactory growth rates and economic feed use. Studies by Farhadi and Hosseini (2014) and Premavalli et al. (2020) corroborate similar FCR trends in controlled environments. Adegbenro et al. (2020) from their study revealed the highest final weight, highest weight gain, highest eviscerated weight, lowest feed intake (2388.00 ± 1.44g, 1716.25 ± 0.32g, 1890.00 ± 27.00g, 3475.75 ± 36.57g, respectively), and best feed conversion ratio (2.03 ± 0.07) were recorded in birds on fold unit system.

Mortality

Mortality analysis revealed a 4.65% rate (Table 4 and Figure 1), exceeding the target of 3.18% and the industry benchmark of 3-5% (Viban & Mfondo, 2021). Early chick mortality is associated with disease, poor management, inadequate brooding temperatures and heat stress in hot climates (Chou et al., 2004). Colibacillosis, the leading cause of mortality, accounted for 30 of 67 deaths, highlighting the need for enhanced biosecurity and environmental controls. Apart from viral diseases, coccidiosis, necrotic enteritis, colibacillosis and salmonellosis are considered to be the common infectious enteric diseases of birds responsible for decreased feed intake, nutrient absorption, increased feed conversion ratio, reduced body weight gain, high morbidity and mortality thus leading to a huge economic loss (Dahiya et al., 2006; Shirzad et al., 2011; Abera et al., 2017). Enteritis caused four deaths, indicating potential feed or water

quality issues. Nephritis and pneumonia caused three and two deaths, respectively, underscoring the importance of optimal health management. Putrefaction accounted for 12 deaths, emphasizing the need for better monitoring and timely removal of dead birds. Unconfirmed causes contributed to 14 deaths, necessitating improved diagnostic efforts. According to Jacob et al., 1998 the viral diseases which cause major mortality in birds include: Marek's disease, Newcastle disease, infectious bronchitis, laryngotracheitis, fowl pox, fowl cholera, and avian encephalomyelitis. Comparisons to studies by Farhadi & Hosseini, (2014) underscore the importance of addressing these challenges to reduce mortality rates. Premavalli et al. (2020) have found the average livability of broiler birds up to 42 days was 91 % and 92 % in Nandanam broiler-2 and Nandanam broiler-3, respectively

Table 4 Cause of mortality

Cause of Death	Number of Birds
Colibacillosis	30
Enteritis	4
Internal Haemorrhage	2
Nephritis	3
Pneumonia	2
Putrified	12
Unconfirmed	14
Mortality Percentage	**4.28%**

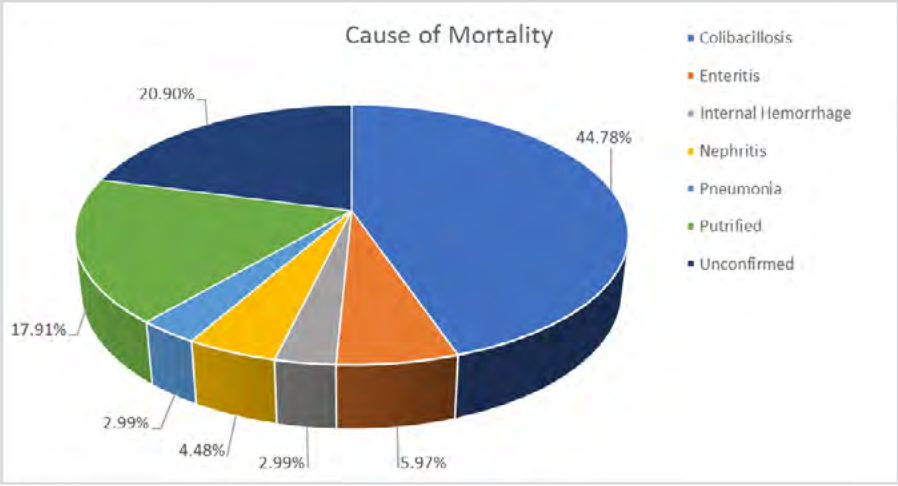

Figure 1
Percentage of cause of mortality in broiler during the 40 days rearing

Economic Impact

The financial overview of the poultry farm over the 40-day rearing period provides a comprehensive picture of the costs incurred and the income generated, ultimately leading to a modest net profit, as shown in Table 5.

Table 5 Economics of the farm

Particulars	Amount (Rs.)
Total Expenditure from purchasing of chicks (@ Rs. 20/- per chick)	34,500/-
Total Expenditure from feeds	1,68,705/-
Total Expenditure on medicine and miscellaneous	52,222/-
Total Expenditure: (1+2+3)	2,55,427/-
Total income from selling (2863.15kg * Rs. 93+11.8kg * Rs. 70)	2,67,098.95/-
Net income amount (5 - 4)	11,671.95/-
Benefit-cost Ratio	1.05
EPEF	333.76

The financial overview of the poultry farm revealed a total expenditure of Rs. 2,55,427, with feed costs constituting 66% of the total. The farm generated Rs. 2,67,098.95 in revenue from selling broilers, resulting in a net income of Rs. 11,671.95 and a benefit-cost ratio of 1.05. This showed that the farm was making Rs. 1.05 for every rupee invested, indicating a favorable return on investment. Despite obstacles including a higher-than-anticipated death rate, the farm turned a profit, underscoring the significance of ongoing observation and modification for sustained success. Similar findings in the financial analysis of broiler farms are highlighted by studies by Adegbenro et al. (2020) and Premavalli et al. (2020).

In the current study, the average mortality rate was 4.28%, the average BW was 2.12 kg and average FCR was 1.52. Data on the production efficiency of broiler chickens are used to calculate the EPEF. The higher the EPEF value, the more favourable the production result of broiler chickens, and production with an EPEF above 220 is considered effective (Perić et al., 2009). The level of the EPEF index during the rearing period was 333.76, as shown in Table 5. According to Karaman et al. 2023, an EPEF value exceeding 190 can be considered satisfactory. However, many authors believe that the EPEF value should not fall below 220 (Perić et al., 2009; Van Limbergen et al., 2020). Poultry companies are improving breeds used in breeding programs, resulting in a significant improvement in performance traits compared to previously used chicks, which in turn translates into improved production indicators (Alves et al., 2024; Neeteson et al., 2023; Van Limbergen et al., 2020).

CONCLUSIONS

The obtained results are affirmative for the project conducted with the aim of investigating the impact of Feed Conversion Ratio (FCR) optimization. The farm's ability to generate a net income of Rs. 11.671.95 and a benefit-cost ratio of 1.05 indicate that poultry farming can be a viable business, though it requires attention to detail and effective cost management. The thin profit margins highlight the importance of regular supervision of disease outbreaks, the farm observed a higher-than-expected mortality rate of 4.65% in which 44.8% of the cause of death during the rearing period is due to colibacillosis, followed by enteritis, which is 6%. The main cause of mortality during the first week is mainly due to improper maintenance of the brooding temperature. Therefore, efforts are needed to address health issues, such as colibacillosis and enteritis, to optimize performance and financial outcomes further.

This report is a valuable reference for assessing the poultry farm's performance and guiding future improvements and strategic decisions.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest

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PERFORMANSE I FINANSIJSKA ANALIZA BROJLERA (SOJ COBB 430 Y): PRIKAZ SLUČAJA STOPE KONVERZIJE HRANE I STOPE MORTALITETA

SAŽETAK

Ova studija ispituje operativne, proizvodne i finansijske performanse uzgoja brojlera korištenjem COBB 430 Y soja u Instructional Livestock Complex-u (ILFC). Ukupno 1439 pilića je uzgajano duže od 40 dana, pri čemu su dosegli prosječnu stopu konverzije hrane (FCR) od 1,52 i prosječnu tržišnu masu od 2,12 kg do konačne prodaje. Stopa mortaliteta od 4,65% na farmi, uzrokovana uglavnom enteritisom (6%) i kolibacilozom (44.8%), premašila je granicu od 3.18%, iako je farma neprekidno povećavala težinu. Finansijski su ukupni troškovi farme iznosili Rs 2,55,427, uključujući hranu, nabavku pilića i zdravstvenu zaštitu, dok je prodajom brojlera generiran prihod od Rs 2,67,098.95. Ovo je rezultiralo skromnim neto prihodom od Rs 11,671.95, a odnos korist-troškovi od 1.05 je potvrdio ekonomsku izvodljvost operacije uprkos izazovima. Rezultati naglašavaju važnost optimiziranja zdravstvene zaštite, biosigurnosnih mjera i okolišnih uvjeta u svrhu minimiziranja gubitaka i povećanja profitabilnosti. Naše istraživanje pruža značajan uvid u upravljanje farmom brojlera i pokazuje kako tehnike strateškog upravljanja mogu voditi održivoj proizvodnji. Kako bi se dalje povećala produktivnost i povrat finansijskih sredstava, buduće inicijative bi se trebale usredotočiti na zdravstvene probleme uključujući kolibacilozu, poboljšanje kvalitete hrane i smanjivanje stopa mortaliteta.

Ključne riječi: Biosigurnost, mortalitet, obim proizvodnje, profitabilnost, stopa konverzije hrane

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Watts AJR. 2012. Nutritional status and trophic dynamics of the Norway lobster Nephrops norvegicus (L.). PhD, University of Glasgow, Glasgow, UK.

Tables and Figures

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- ✓ Materials and Methods,
- ✓ Results
- ✓ Discussion and conclusion;
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