FOLIA VETERINARIA

The scientific journal of the
UNIVERSITY OF VETERINARY MEDICINE
AND PHARMACY IN KOŠICE — Slovakia

ISSN 0015-5748
eISSN 2453-7837

2

LXIX • 2025

FOLIA VETERINARIA is a scientific journal issued by the University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia.
The journal is published quarterly in English (numbers 1–4) and distributed worldwide.

Contact: e-mail: folia.veterinaria@uvlf.sk

Electronic Publisher: De Gruyter Poland, Bogumila Zuga 32A
01-811 Warsaw, Poland

ISSN 2453-7837 on-line
ISSN 0015-5748 print
EV 3485/09

Publisher's Identification number: IČO 00397474

June 2025

FOLIA VETERINARIA

PUBLISHED BY
THE UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE
SLOVAKIA

Folia Veterinaria
Vol. 69, 2, 2025

VYDÁVA
UNIVERZITA VETERINÁRSKEHO LEKÁRSTVA A FARMÁCIE V KOŠICIACH
2025

FOLIA VETERINARIA, 69, 2, 2025

CONTENTS

sciendo

DOI: 10.2478/fv-2025-0011

FOLIA VETERINARIA, 69, 2: 1—5, 2025

ORIGINAL ARTICLE

ANTIOXIDANTS ACTIVITIES AND LIPID PROFILE IN THE HAEMOLYMPH OF BLACK AND WHITE-SKINNED GIANT AFRICAN LAND SNAIL (*Archachatina marginata*)

Kehinde Olutoyin Ademolu[1*], Funmilayo Abimbola Okeniyi[2], Beno Okechukwu Onunkwor[3], Foluke Adenike Akinnusi[4], Jolaade Agbetiloye[1], Adewunmi Babatunde Idowu[1]

[1]Department of Pure and Applied Zoology, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria; [2]Department of Animal Science, Landmark University, Omu-Aran, Kwara State, Nigeria; [3]Department of Biochemistry, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria; [4]Centre for Entrepreneurship Studies, Chrisland University, Abeokuta, Nigeria

OPEN ACCESS

***Correspondence:** kennyademolu@yahoo.com

Citation: Ademolu, K. O., Okeniyi, F. A., Onunkwor, B. O., Akinnusi, F. A., Agbetiloye, J., Idowu A. B., 2025: Antioxidants activities and lipid profile in the haemolymph of black and white-skinned giant African land snail (*Archachatina marginata*). Folia Veterinaria, 69, 2, 1–5.

Received: November 28, 2024

Accepted: March 6, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Antioxidants are compounds that eliminate oxidative stress in biological systems. The activities of antioxidants in black and white-skinned snails have not been compared in literature. This study was carried out to compare the antioxidants (glutathione peroxidase – GPx, superoxide dismutase – SOD, malondialdehyde – MDA, catalase) activities in the haemolymph of the white and black-skinned snails (*Archachatina marginata*) as well as their lipids profiles. Twenty (20) giant African land snails (10 black-skinned and 10 white-skinned snails) were used in the experiment. The lipid profile of white-skinned snails was higher than that of black-skinned snails. White-skinned snails have higher SOD activities than black-skinned snails. However, MDA, catalase and GPx activities in black-skinned snails were higher than that of white-skinned snails. The black-skinned snails were more endowed to cope with environmental disturbances than their white-skinned counterparts.

Keywords: antioxidants; *Archachatina marginata*; black-skinned snails; white-skinned snails

INTRODUCTION

Snail is a terrestrial, shell-bearing animal, belonging to the phylum *Mollusca* and class *Gastropoda*. It is normally more active after dusk and when the ground is damp [1]. The haemolymph is regarded as a blood analogue found in all arthropods and most molluscs [2]. It is blue, indicating the presence of haemocyanin, a blue copper-containing pigment in the solution. It is composed of water, inorganic salts (mostly Na, Cl, K, Mg and Ca) and organic compounds (mostly carbohydrates, proteins, and lipids) [3].

Various parts (haemolymph, flesh, shell, slime) of the giant African land snail have been used in traditional medicine in Nigeria to treat tuberculosis, measles, high blood pressure, headache and cough [1]. The increased use of snail haemolymph in native medicine is likely due to its antioxidant content. Lawal et al. [4] reported the presence of antioxidant activities in the haemolymph of land snails *Achatina achatina* and *Archachatina marginata*, referring to them as natural sources of antioxidants.

Many factors influence the activity of antioxidants in land snails. Hermes and Storey [5] observed that aestivation, anoxia, and dry and hot environments increased the activities of antioxidants while Lawal et al. [4] opined that antioxidant activities in snail haemolymph have ability to protect albino rats from potential oxidative stress from liver damage by carbon tetrachloride (CCL_4) poisoning.

One ecotype of snails that has been extensively used in traditional medicine is the albino snails popularly called the white-skinned snail. This snail, although edible, is not eaten by some people because of taboos and superstitions surrounding it [6] It is believed to be dedicated as a sacrifice to gods. Ketiku and Adeleke [7] compared the nutrient composition of the flesh of the normal snails with that of the albino (white-skinned). They observed a significantly higher ($P < 0.05$) protein and minerals in the albino snail than in the normal skinned. Albinism is a hereditary deficiency of pigmentation that may involve the entire body or parts of the body. It is believed to be caused by an enzyme deficiency involving melanin metabolism during prenatal development [8]. There are two main types of albinism in snails: albino-shelled and albino-bodied but the genes responsible for them are unknown.

Literature abounds with many studies on haemolymph antioxidant activities based on species variation, body weight, age, habitat, and feeding [9, 10] while little attention is paid to the white-skinned (albino) snail. This study aims to evaluate lipids and the antioxidant composition of the haemolymph of black-skinned and white-skinned giant African land snails, *Archachatina marginata*.

MATERIALS AND METHODS

Snail Collection

Twenty giant African land snails (10 black-skinned and 10 white-skinned snails) with the average weight of 150 ± 02g were bought from a private snail farm in Abeokuta, Ogun State, Nigeria.

Haemolymph Collection

The snails were thoroughly cleaned to remove dirt from their shells and apertures. The apex of the snails was carefully cracked open, adopting the method from Akinloye and Olorode [11]. The haemolymph from the black-skinned and white-skinned snails was drained into separate and clean sample bottles.

Determination of Antioxidant Levels

Fresh haemolymph samples collected into plain sample bottles were immediately centrifuged. The supernatant (serum) was then frozen for the antioxidant analyses. The supernatants were analyzed for lipid profile (cholesterol, triglycerides, and lipoproteins) and antioxidant activities (catalase, glutathione peroxidase, superoxide dismutase, lipid peroxidation) using Brigelius-Flohé and Maiorino [12] methods. Superoxide dismutase (SOD) activity was determined spectrophotometrically at 420 nm by measuring the inhibition of autoxidation of pyrogallol, a superoxide-reacting indicator molecule (SRIM) that competes with SOD for the reaction with superoxide in alkaline medium (pyrogallol / SOD + $O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). The specific activity of SOD was expressed as units/g haemolymph or pyrogallol 50 % oxidation auto-inhibition/min/g haemoglobin using a pyrogallol molar extinction coefficient (Σ) of $8.0 \times 10^5\ M^{-1}\ cm^{-1}$.

Catalase activity was determined spectrophotometrically at 374 nm by measuring the rate of decomposition of hydrogen peroxide ($2H_2O_2 \rightarrow 2H_2O + O_2$). The specific activity of catalase was expressed as units/g haemolymph or mmol H_2O_2 degraded/min/g haemoglobin using the H_2O_2 molar extinction coefficient (Σ) of $43.6\ M^{-1}\ cm^{-1}$.

Glutathione peroxidase (GSPx) activity was determined spectrophotometrically at 420 nm by measuring the residual GSH content during the decomposition of hydrogen peroxide using GSH as co-factor ($H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$). GSPx specific activity was expressed as units/g haemolymph or nmol of residual GSH/min/g haemoglobin using a GSH molar extinction coefficient (Σ) of 9.6 0.017 $mM^{-1}\ cm^{-1}$.

Statistical Analysis

Data collected from the experiments were analyzed using the Student T-test. Significant difference was set to $P < 0.05$.

RESULTS

The lipid profile of the haemolymph of black- and white-skinned snails is presented in Table 1. The haemolymph of the white-skinned snails had significantly ($P < 0.05$) higher total cholesterol, triglycerides, and High-Density Lipoprotein than the black-skinned snails. However, the haemolymph of black-skinned snails recorded significantly higher Very Low-Density Lipoprotein than their white-skinned counterparts.

Table 1: Lipid Profile of Black and White-Skinned Snails Haemolymph (Mean± SD)

Lipids	Black-skinned snail	White-skinned snail
Total cholesterol (mg/dl)	15.10 ± 0.05^{b}	17.30 ± 0.08^{a}
Triglycerides (mg/dl)	18.30 ± 0.10^{b}	20.20 ± 0.04^{a}
VLDL (mg/dl)	10.33 ± 5.77^{a}	4.04 ± 0.04^{b}
HDL (mg/dl)	7.55 ± 0.07^{a}	8.65 ± 0.06^{a}

Values in rows with different superscripts are statistically different ($p < 0.05$)

The black-skinned snails had significantly higher antioxidant activities in the haemolymph than the white-skinned snails (except for the SOD), however, there was no significant difference in the activities of GPx (Table 2).

Table 2. Antioxidant Activities in the Haemolymph of Black and White-Skinned Snails (Mean± SD)

Antioxidants	Black-skinned snail	White-skinned snail
SOD (U/L)	1.52 ± 0.03^{b}	2.01 ± 0.01^{a}
MDA (U/L)	2.34 ± 0.05^{a}	1.59 ± 0.04^{b}
Catalase (U/L)	2.11 ± 0.06^{a}	1.95 ± 0.03^{b}
GPx (U/L)	1.24 ± 0.02^{a}	1.03 ± 0.02^{a}

Values in rows with different superscripts are statistically different ($p < 0.05$)

DISCUSSION

White-skinned snails had been reported to exhibit slower growth and less activity than the black-skinned ones [3]. This reduced activity recorded by the white-skinned snails might be responsible for accumulating lipid derivatives in their haemolymph. Lipids are energy sources that supply more energy than carbohydrates to both snail embryos and adults [14, 15], but when not utilized, they accumulate in the tissues. Ketiku and Adeleke [7] similarly reported higher protein and mineral concentrations in the flesh of white-skinned snails. The absence of melanin pigment in white-skinned snails makes them sensitive to light and heat, thus withdrawing most times, especially during the day [13].

Black-skinned snails are usually active and carry out mating and feeding activities during the last hours of the day (18:00- 24:00 GMT) [2]. They also perform some of these tasks immediately after the rain during the day when the temperature is low and relative humidity is high [1]. These activities expose black-skinned snails to more reactive oxidative stress (ROS) than white-skinned snails that are usually restricted in their habitat [16].

Higher activity rates displayed by black-skinned snails either in the wild or in captivity expose them to more oxidative stress than the white-skinned snails, hence requiring more antioxidant defenses. The land snail, *Otala lacteal,* exhibits an increase in activities of foot muscle superoxide dismutase, catalase, and glutathione S-transferase [17] in preparation for dealing with oxidative stress during the arousal process from aestivation. Other factors like habitat, age, and feed have significant roles on the antioxidant activities of snails. For instance, pollutants (CCL_4 and AG-NP) reduced the antioxidant activities in *Archachatina marginata* and *Biomphalaria alexandrina,* respectively [4, 7]. Water snail, *Cornu aspersum aspersum* fed with different protein sources showed significant changes in their antioxidant activities and chemical composition [9].

Furthermore, due to the higher metabolic activity of black-skinned snails, they consume more oxygen than their white-skinned counterparts, which can cause ROS. The increased rate of oxygen consumption in the desert snail, *Oreohelix* spp., led to a higher rate of ROS generation which also elicits increased antioxidant defense mechanisms to protect the tissues [17].

CONCLUSION

In conclusion, black-skinned snails had more antioxidant activities and less lipid concentrations in the haemolymph than the white-skinned snails in order to pro-

tect their tissues from damage caused by ROS warranted by their higher activities.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

The study did not require any ethical approval.

Conflict of Interest

No conflict of interest.

Funding

This study was financially supported by the authors

Generative AI Statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Authors' Contributions

Conceptualization: Ademolu, K.O. and Onunkwor, B.O.

Manuscript writing: Ademolu, K.O. and Okeniyi, F.A.

Sampling and Methodology: Agbetiloye, J., Ademolu, K.O., Akinnusi, F.A,

Statistical Analysis: Idowu, A.B. and Onunkwor, B.O.

REFERENCES

1. **Akinnusi, O., 1997:** A practical approach to backyard snail farming. *Nig. J. Ani. Prod.*, 25, 2, 193–197.
2. **Abdussamad, M. A., Osinowo, O. A., Smith, O. F., Onadeko, S. A., 2010:** Some haemolymph biophysical parameters in the giant African land snail *Archachatina marginata* during a six-week aestivation period. *Glob. Vet.*, 4, 4, 400–408.
3. **Ademolu, K. O., Jayeola, O. A., Idowu, A. B., Elemide, I. O., 2011a:** Circadian variation in locomotor and feeding periods of two land snail species. *Arch. Zootec.,* 60, 232, 1323–1326.
4. **Lawal, B. K., Shittu, O. C., Ossai, P. N., Abubakar, A. M., Ibrahim, A., 2015:** Evaluation of antioxidant activity of Giant African Snail (*Archachatina marginata*) haemolymph in CCl4-induced hepatotoxixity in albino rats. *J. Pharm. Res. Int.*, 6, 3, 141–154. DOI: 10.9734/BJPR/2015/15887
5. **Hermes-Lima, M., Storey, K. B., 1995:** Antioxidant defenses and metabolic depression in a pulmonate land snail. *Am. J. Physiol*., 268, 6, 2, R1386–R1393. DOI:10.1152/ajpregu.1995.268.6.R1386
6. **Amusan, J. A., Omidiji, M. O., 1998:** Edible land snails. A technical guide to snail farming in the Tropics, Verity Printers, Ibadan, 1, 16.
7. **Ketiku, A. O., Adeleke, A. O., 1998:** Nutrient composition of black fleshed and white fleshed African giant land snails (*Archachatina marginata*). *W. Afri J. Biol. Appl. Chem.*, 44, 21–23.
8. **Allegretti, S. M., Catvalho, J. F., Magalhess, L. A., Zanotti-malgahaes, E. M., 2009:** Behavior of albino and melanic variant of *Biomphalaria glabrata* (Mollusca: Planorbidae) following infection by *Schistoma mansoni*. *Braz. J. Biol.,* 69, 8, 11.
9. **Gogas, A., Laliotis, G. P., Ladoukakis, O., Trachana, V., 2021:** Chemical composition and antioxidant profile of snails (*Cornu aspersum aspersum*) fed diets with different protein sources under intensive rearing conditions. *J. Anim. Feed Sci*., 30, 4, 391–398. DOI: 10.22358/jafs/143107/2021
10. **Khaled, M. Z., Yun-Hai, G., Shan, L. V., Yi, Z., Xiao-Nong, Z., 2022:** Molluscicidal and antioxidant activities of silver nanoparticles on the multi-species of snail intermediate hosts of schistosomiasis. *PLoS Negl. Trop. Dis.*, 16, 10, e0010667. DOI: 10.1371/journal.pntd.0010667
11. **Akinloye, O. A., Olorode, O., 2000:** Effect of different feeding condition on performance, haemolymph biochemical and mineral value of Giant African Snail (*Archachatina marginata*). *J. Agri. Envt.*, 1, 1, 143–147.
12. **Brigelius-Flohé, R., Maiorino, M., 2013:** Glutathione peroxidases. *Biochim. Biophys. Acta*, 1830, 5, 3289–3303. DOI: 10.1016/j.bbagen.2012.11.020
13. **Ademolu, K. O., Jayeola, O. A., Dedeke, G. A., Idowu, A. B., 2011b:** Comparative analysis of the growth performance and haemolymph biochemical properties of normal and albino giant land snail (*Archachatina marginata*). *Ethi. J. Environ. Stu. Mgt.,* 4, 2, 101–106.
14. **Odiete, W. O., 1999:** Basic Animal Physiology. Diversified Resources Ltd. Lagos. 257 pp.

15. **Egbebi, J. M., Ademolu, K. O., Idowu, A. B., Aladesida, A. A., Ojo, V., 2023:** Biochemical composition of eggshell and egg yolk of giant African land snail (*Archachatina marginata*) during gestation period. *Arch. Zoote.*, 72, 280, 284–287.
16. **Okon, B., Ibom, L. A., 2012:** Snail breeding and snailery management. Fresh Dew Productions, Calabar, Nigeria, 1–70.
17. **Rees, B. B., Hand, S. C., 1990:** Heat dissipation, gas exchange and acid-base status in the land snail *Oreohelix* during short-term estivation. *J. Exp. Biol.,* 152, 1, 77–92. DOI: 10.1242/jeb.152.1.77

sciendo

DOI: 10.2478/fv-2025-0012

FOLIA VETERINARIA, 69, 2: 6—11, 2025

ORIGINAL ARTICLE

THE PRESENCE OF DEOXYNIVALENOL AND ZEARALENONE IN INDUSTRIALLY PRODUCED DOG FEED

Michaela Harčárová, Lukáš Bujňák, Alena Hreško Šamudovská, Stanislav Hreško, Alica Tvrdá
Department of Animal Nutrition and Husbandry, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia

OPEN ACCESS

***Correspondence:** michaela.harcarova@uvlf.sk

Citation: Harčarová, M., Bujňák, L., Šamudovská, A. H., Hreško, S., Tvrdá, A., 2025: The presence of deoxynivalenol and zearalenone in industrially produced dog feed. Folia Veterinaria, 69, 2, 6–11.

Received: March 25, 2025

Accepted: April 23, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

In the field of pet nutrition, the administration of complete feeds has proven to be effective. Dry complete feeds are often used for dogs, which may contain cereals among other ingredients. Cereals can be a risky ingredient due to the potential presence of mycotoxins. This work was aimed at determining the concentrations of deoxynivalenol and zearalenone in 14 samples of dry feeds for young and adult dogs obtained from different vendors. Through ELISA analysis, we found that deoxynivalenol was not found in the examined feeds in concentrations higher than the detection limit of the used kit (< 0.2 mg/kg; ppm) and zearalenone was found in 10 samples (2 samples of generic dog food, 2 samples of popular dog food and 6 samples of premium dog food) in concentrations below the detection limit of the test (< 5 µm/kg; ppb). In 4 samples of premium dog food, zearalenone was present at concentrations 21.799 ppb, 69.187 ppb, 96.745 ppb, and 163.463 ppb.

Key words: animals; feeds, mycotoxins; contamination

INTRODUCTION

The dog has always been considered man's best friend. Every responsible owner should, among other things, think about providing quality nutrition for their four-legged companion. The dog is a monogastric animal, and, like other animal species, it needs nutrients to ensure all processes take place in the body. According to the physiological effect, nutrients can be divided into building blocks (proteins and some minerals), consumables (carbohydrates and fats), and replacements (water and minerals). Due to the influence of humans and the process of domestication, dogs have become omnivores, but they share some similar nutritional features with cats, such as the lack of salivary amylase, a short gastrointestinal tract, and the inability to synthesize vitamin D [1].

Unlike cats, dogs have 3 genes (AMY2B, MGAM and SGLT1) involved in starch digestion and glucose absorption, which evolved during domestication [2]. Cats do not have these genes and are generally unable to digest fiber

efficiently [1]. Another characteristic of the canine digestive system is that they can synthesize several essential nutrients, such as niacin, taurine, and arginine [3]. As for cats, they can catabolize and use amino acids as an energy source for gluconeogenesis [4].

Feed components of animal and plant origin are used in dog nutrition. Cereals such as corn, rice, sorghum, wheat, oats, barley, and others are mainly used as a source of carbohydrates in feed [5]. Plant components in food, especially the aforementioned cereals, can provide a breeding ground for the growth and development of microscopic filamentous fungi and their secondary metabolites, mycotoxins. The most common mycotoxins that can occur in cereals and other forages are deoxynivalenol and zearalenone [6].

Deoxynivalenol, also known as vomitoxin, is a type B trichothecene and epoxy-sesquiterpenoid produced primarily by the microscopic fungus *Fusarium graminearum*. It was first isolated from moldy barley grains and chemically described in 1970 [7]. It is soluble in water, ethanol, acetonitrile, and other polar solvents and is stable at high temperatures and low pH [8]. Deoxynivalenol is a natural toxicant that can negatively affect the health of animals. It has been reported that dogs with deoxynivalenol poisoning show similar clinical signs to pigs (vomiting, food refusal, and associated weight loss) [9]. Songsermsakul et al. (2007) describe, in addition to the negative impact of deoxynivalenol on the gastrointestinal tract of dogs, its immunosuppressive effects [10]. Consumption of feed contaminated with this trichothecene can lead to impaired immune resistance to pathogens in dogs, as well as to reactivation of chronic infections [11].

Zearalenone is one of the most important *Fusarium* mycotoxins. It is produced by several species, including *F. graminearum*, *F. culmorum*, *F. cereals,* and *F. equiseti*. This non-steroidal oestrogenic mycotoxin is mainly found in corn, wheat, oats, and barley and its production depends on cool temperatures and high humidity [12]. Zearalenone is a thermostable compound that does not degrade during the processing of feed materials and feed, even at high temperatures [13]. Female dogs are particularly sensitive to estrogens, and elevated concentrations of endogenous and/or exogenous hormones also may increase the risk of other systemic disorders [14]. After entering the body, zearalenone can cause acute vulvovaginitis, estrous cycle disorders, and impaired fertility [15].

According to EFSA (European Food Safety Authority) dogs are considered sensitive to zearalenone. Based on myometrium and endometrium lesions, enlargement and atrophy of uterine glands, blood haematology and biochemistry, the lowest observed adverse effect level (LOAEL) of 25 µg/kg body weight per day has been estimated for mature bitches [16]. As the presence of deoxynivalenol and zearalenone cannot be completely avoided, guidelines have been developed in the EU for maximum levels of mycotoxins in compound feed for various animals, including dogs (Table 1) [17].

The aim of this work was to determine deoxynivalenol and zearalenone in 14 samples of dry food for adult dogs using ELISA immunoassay.

MATERIALS AND METHODS

Samples

A total of 14 samples of dry dog food in the form of granules were examined. The samples were obtained from various commercial and specialized sellers and were intended for feeding young and adult dogs. The samples were divided into three categories: generic, popular, and

Table 1. Reference values of deoxynivalenol and zearalenone in products for animal animals feeding

Mycotoxins	Products intended for animal feeding	Guidance value in mg.kg^{-1} (ppm) relative to a feed with a moisture content of 12 %
Deoxynivalenol	Compound feed for:	
	pigs	0.9
	calves (< 4 months), lambs, kids and dogs	2
	other animals	5
Zearalenone	Compound feed for:	
	piglets, gilts, puppies, kittens, dogs and cats for reproduction	0.1
	adult dogs and cats other than for reproduction	0.2
	sows and fattening pigs	0.25
	calves, dairy cattle, sheep (including lambs) and goats (including kids)	0.5

Source: Commission Recommendation (2016/1319) [17]

premium. The generic feed group included samples of feed that did not bear the name of the manufacturer and were marketed locally or regionally. The second category, so-called popular feeds, included samples of feed sold in grocery store chains. The third category included premium feeds from specialized sellers.

Preparation of samples for deoxynivalenol determination

Representative samples (500 g each) were stored in a cool, dry, and dark place until analysis. Sample processing was carried out as follows: 100 ml of distilled water was added to 5 g of ground and homogenized sample. Then, a 3-minute shaking was performed, followed by filtration of the extracts through Whatman filter paper No. 1 (Cytiva, Kent, UK). 50 µl of the filtrates were used for the quantitative determination of deoxynivalenol according to the RIDASCREEN FAST DON protocol, quantitative test (R-Biopharm AG, Darmstadt, Germany).

ELISA analysis

The principle of the test is an antigen-antibody reaction. The wells in the microtiter strips are coated with capture antibodies directed against anti-aflatoxin antibodies. Standards (0 ppm; 0.222 ppm; 0.666 ppm, 2 ppm, 6 ppm), sample solutions, deoxynivalenol-enzyme conjugate, and anti-deoxynivalenol antibodies were added to the wells. Free and enzyme-conjugated aflatoxin compete for the binding sites of deoxynivalenol antibodies (competitive enzyme immunoassay). At the same time, antibodies against deoxynivalenol are bound by immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step using washing solution. Substrate/chromogen solution is added to the wells and incubated for 5 minutes in the dark. The bound enzyme conjugate converts the chromogen to a blue product. The addition of stopping solution results in a color change from blue to yellow. Measurement is performed photometrically at 450 nm, using an ELISA reader (Dynex Technologies, Inc., Chantilly, USA) where the absorbance is inversely proportional to the concentration of deoxynivalenol in the sample.

Preparation of samples for zearalenone determination

The analysis was performed using a commercial Veratox for zearalenone kit (Neogen Corporation, USA). The sample preparation procedure was as follows: 70 % methanol in amount of 25 ml was added to 5 g of ground sample. The samples were shaken for 3 minutes on an orbital shaker (Orbital Shaker – Biosan) and filtered through Whatman 1 filter paper. Filtrates were diluted with distilled water in a ratio of 1:5. 50 µl of diluted samples were used for the quantitative determination of zearalenone using an ELISA immunoassay.

ELISA analysis

The principle of ELISA analysis in the case of samples examined for the presence of zearalenone is the same as for the determination of deoxynivalenol. The standard solutions used in analysis contained zearalenone in the following concentrations: 0 ppb, 25 ppb, 75 ppb, 150 ppb, and 500 ppb. The resulting zearalenone concentrations (ppb; µg/kg) were read at the absorbance of 650 nm and were evaluated using an ELISA reader (Dynex Technologies, Inc., Virginia, USA).

RESULTS

Results of the quantitative determination of deoxynivalenol and zearalenone in samples of dry food for adult dogs are presented in Table 2. Concentrations of deoxynivalenol were below the detection limit of the test used (< 0.2 mg/kg; ppm) in all examined samples. Zearalenone was present in samples of generic and popular dry dog food samples below the detection limit of the test (< 5 ppb; µg/kg), and in 4 samples of premium dog food was zearalenone determined at concentrations of 21.799 ppb, 69.187 ppb, 96.745 ppb, and 163.463 ppb. In the remaining 6 samples of premium dog foods, the concentration was below the detection limit of the kit (< 5 ppb; µg/kg). It should be noted that none of the examined samples exceeded the recommended limits for deoxynivalenol (2 mg/kg; ppm) and zearalenone (0.2 mg/kg; ppm) in dog food as recommended by the Commission Recommendation (EU) 2016/1319 (Table 1).

DISCUSSION

A healthy and balanced diet using high-quality and safe ingredients is a modern phenomenon, not only in human nutrition but also in animal nutrition. Attention is also being paid to the nutrition of companion animals, which are

Table 2. Concentrations of deoxynivalenol (ppm; mg/kg) and zearalenone (ppb; µg/kg) in dry food for adult dogs

Type of feed for dogs	Samples (n=14)	Concentrations of deoxynivalenol	Samples (n=14)	Concentrations of zearalenone
generic	2	< 0,2 ppm	2	< 5 ppb
popular	2	< 0,2 ppm	2	< 5 pbb
premium	10	< 0,2 ppm	6 4	< 5 pbb > 5 ppb

sometimes considered family members. Dogs spend several years with us, so it is important to address the issue of the health safety of the food we offer them. Although dogs have no special requirements for carbohydrates, most feed manufacturers take advantage of dogs' ability to digest cereals and provide various types of cereals (corn, rice, wheat, barley, or sorghum, as well as cereal by-products) as a cheap source of energy. The proportion of these cereals in the feed formula can be up to 70 %, usually between 30 and 50 % [5]. The presence of cereals also brings with it the risk of various mycotoxins. Studies have shown that wheat contaminated with the mycotoxin deoxynivalenol is toxic even after 4 years of storage [18].

In our samples of dry dog food from various commercial and specialist suppliers, we did not detect deoxynivalenol in concentrations higher than the detection limit of the kit used (< 0.2 mg/kg; ppm). Concentrations of zearalenone were in 10 samples of dry dog food below the detection limit (< 5 ppb) and in 4 samples of premium dog food zearalenone was determined at concentrations ranging from 21.799 ppb to 163.463 ppb. Of the total number of 76 samples of dry dog food tested in Austria by ELISA, deoxynivalenol was present in 74 samples (97 %), and the maximum value of deoxynivalenol was > 250 µg/kg [19]. Seventy-six samples of dry dog food from 27 manufacturers were similarly purchased from retail stores, supermarkets, and specialist pet food stores. In addition to deoxynivalenol, Böhm et al. (2010) also detected the presence of zearalenone in the above-mentioned samples. Zearalenone was detected in 36 of the 76 samples examined (47 %) and maximum concentration of zearalenone was 298 µg/kg [19]. A similar study was conducted in Spain in 2020, where 60 samples of dry dog foods were examined (34 popular foods and 26 samples of premium foods), and 100 % of deoxynivalenol was detected in both cases. The authors also point out that in addition to deoxynivalenol, other mycotoxins such as aflatoxins, T2-toxin, ochratoxin A, fumonisins, and zearalenone were also found in the examined samples. Zearalenone was present in all premium foods (100 %) and in 88.2 % of supermarket foods in Spain in the above-mentioned samples. However, they point out that the examined feeds should not pose a risk of acute health complications in the form of mycotoxicoses for dogs [20]. In the past, studies have confirmed that dogs fed feed containing deoxynivalenol at 4.5 mg/kg of feed refuse food due to the reaction of the digestive tract to the mycotoxin [9, 10]. However, according to Songsermsakul et al., (2007), the aforementioned concentration of deoxynivalenol is not high enough to induce vomiting [10]. Zearalenone is toxic to both males and females [21]. According to a study by Boermans and Leung (2007), a dose of ~1 mg/kg zearalenone in pet food can cause infertility and negatively affect ovulation, implantation, pregnancy, and viability of the newborn [22]. In addition, daily intake of low doses of zearalenone could gradually damage the health of the dog [23] and may induce changes in metabolic processes in dogs that may cause hypoestrogenism [14, 24]. Given the presence of deoxynivalenol and zearalenone in our samples and in samples from foreign research, regular monitoring of mycotoxins in dry dog food would be necessary.

CONCLUSIONS

Mycotoxins can be a source of spoilage at any stage of feed production, processing, transport, storage, and consumption. Since there is relatively little information on the toxicological effects of mycotoxins on dogs, further studies are needed to determine the sources of mycotoxins that could contaminate feed intended for this type of companion animal. Future research primarily focuses on the issue of long-term administration of feed containing deoxynivalenol/zearalenone, particularly in relation to its immunosuppressive effects on the body and chronic diseases. In conclusion, it is necessary to state that the dog food samples examined in our study by us do not pose a risk of acute toxicosis in dogs, and it would be necessary to

determine the concentrations of other mycotoxins that may be the cause of impaired animal health.

Data Availability Statement

Data are contained within the article.

Conflict of Interest

The authors declare no conflicts of interest.

Funding

The study was supported by VEGA project No. 1/0698/24.

Generative AI Statement

No generative AI and AI-assisted technologies were used in writing the manuscript,

Authors' Contributions

Conceptualization, M.H; methodology, M.H.; investigation, M.H., A.T.; data curation, M.H., A.H.Š.; writing—original draft preparation, M.H.; writing—review and editing, S.H., L.B.; funding acquisition, L.B.

All authors have read and agreed to the published version of the manuscript.

REFERENCES

1. **Di Cerbo, A., Morales-Medina, J. C., Palmieri, B., Pezzuto, F., Cocco, R., Flores, G., Iannitti, T., 2017:** Functional foods in pet nutrition: Focus on dogs and cats. *Res. Vet. Sci.,* 112, 161–166. DOI: 10.1016/j.rvsc.2017.03.020
2. **Axelsson, E., Ratnakumar, A., Arendt, M. L., Maqbool, K., Webster, M. T., Perloski, M., Liberg, O., Arnemo, J. M., Hedhammar, A., Lindblad-Toh, K., 2013:** The genomic signature of dog domestication reveals adaptation to a starch-rich diet. *Nature*, 495, 360–364. DOI: 10.1038/nature11837
3. **Bosch, G., Hagen-Plantinga, E. A., Hendriks, W. H., 2015:** Dietary nutrient profiles of wild wolves: insights for optimal dog nutrition? *Br. J. Nutr.*, 113, S40–S54. DOI: 10.1017/S0007114514002311
4. **Morris, J. G., 2002:** Idiosyncratic nutrient requirements of cats appear to be diet-induced evolutionary adaptations. *Nutr. Res. Res.*, 15, 153–168. DOI: 10.1079/NRR200238
5. **Kempe, R., Saastamoinen, M., Hyyppä, S., 2004:** Composition, digestibility and nutritive value of cereals for dogs. *Agric. Food Sci.,* 13, 5–17. DOI: 10.2137/1239099041838067
6. **Yu, J, Pedroso, I. R, 2023:** Mycotoxins in cereal-based products and their impacts on the health of humans, livestock animals and pets. *Toxins*, 15, 8, 480. DOI: 10.3390/toxins15080480
7. **Han, X., Chen, L., Li, W., Zhang, L., Dong, H. J. F. G., 2020:** Endocytic FgEde1 regulates virulence and autophagy in Fusarium graminearum. *Fungal Genet. Biol.*, 141, 103400. DOI: 10.1016/j.fgb.2020.103400
8. **Li, Y., Gao, H., Wang, R., Xu, Q., 2023:** Deoxynivalenol in food and feed: Recent advances in decontamination strategies. *Front. Microbiol.*, 14, 1141378. DOI: 10.3389/fmicb.2023.1141378
9. **Hughes, D. M., Gahl, M. J., Graham, C. H., Grieb, S. L., 1999:** Overt signs of toxicity to dogs and cats of dietary deoxynivalenol. *J. Anim. Sci.,* 1999, 77, 693–700. DOI: 10.2527/1999.773693x
10. **Songsermsakul, P., Razzazi-Fazeli, E., Böhm, J., Zentek, J., 2007:** Occurrence of deoxynivalenol (DON) and ochratoxin A (OTA) in dog foods. *Mycotoxin Res.*, 23, 65–67. DOI: 10.1007/BF02946027
11. **Oswald, I. P., Marin, D. E., Bouhet, S., Pinton, P., Taranu, I., Accensi, F., 2005:** Immunotoxicological risk of mycotoxins for domestic animals. *Food Addit. Contam.*, 22, 4, 354–360. DOI: 10.1080/02652030500058320
12. **Yazar, S., Omurtag, G. Z., 2008:** Fumonisins, trichothecenes and zearalenone in cereals. *Int. J. Mol. Sci.,* 9, 2062–2090. DOI: 10.3390/ijms9112062
13. **Thapa, A., Horgan, K. A., White, B., Walls, D., 2021:** Deoxynivalenol and zearalenone—synergistic or antagonistic agri-food chain co-contaminants? *Toxins*, 13, 8, 561. DOI: 10.3390/toxins13080561
14. **Gajęcka, M., Zielonka, Ł., Gajęcki, M., 2015:** The effect of low monotonic doses of zearalenone on selected reproductive tissues in pre-pubertal female dogs —A Review. *Molecules*, 20, 11, 20669–20687. DOI: 10.3390/molecules201119726
15. **Svoboda, M., Senior, D. F., Doubek, J., Klimeš, J., 2001:** *Nemoci psa a kočky II. díl.* Brno, Noviko, pp.1022. ISBN 8090259537.
16. **EFSA panel on contaminants in the food chain (contam), 2017:** Risks for animal health related to the presence of

zearalenone and its modified forms in feed. *EFSA J.,* 15, 7, e04851. DOI: 10.2903/j.efsa.2017.4851

17. **Commission Recommendation (Eu) 2016/1319 Of 29 July 2016 amending Recommendation 2006/576/EC as regards deoxynivalenol, zearalenone and ochratoxin A in pet food.** Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016H1319. Accessed March 14, 2024.
18. **Cao, H., Wu, S., Sun, C., 2013:** Research advancement on biosynthesis and biodegradation of deoxynivalenol (Don). *J. Chin. Cereals Oils Assoc*., 28, 116–123.
19. **Böhm, J., Koinig, L., Razzazi-Fazeli, E., Blajet-Kosicka, A., Twaruzek, M., Grajewski, J., Lang, C., 2010:** Survey and risk assessment of the mycotoxins deoxynivalenol, zearalenone, fumonisins, ochratoxin A, and aflatoxins in commercial dry dog food. *Mycotoxin Res*., 26, 3, 147–153. DOI: 10.1007/s12550-010-0049-4
20. **Macías-Montes, A., Rial-Berriel, C., Acosta-Dacal, A., Henríquez-Hernández, L. A., Almeida-González, M., Rodríguez-Hernández, Á., Luzardo, O. P., 2020:** Risk assessment of the exposure to mycotoxins in dogs and cats through the consumption of commercial dry food. *Sci. Total Environ*., 708, 134592. DOI: 10.1016/j.scitotenv.2019.134592
21. **Stopa, E., Gajęcka, M., Babińska, I., Zielonka, Ł., Gajęcki, M., 2014:** The effect of experimental exposure to low doses of zearalenone on uterine histology and morphometry in prepubertal bitches. *Theriogenology,* 82, 537–545. DOI: 10.1016/j.theriogenology.2014.05.002
22. **Boermans, H. J., Leung, M. C., 2007:** Mycotoxins and the pet food industry: Toxicological evidence and risk assessment. *Int. J. Food Microbiol.,* 119, 95–102. DOI: 10.1016/j.ijfoodmicro.2007.07.063
23. **Witaszak, N., Waśkiewicz, A., Bocianowski, J., Stępień, Ł., 2020:** Contamination of pet food with mycobiota and *Fusarium* mycotoxins—Focus on dogs and cats. *Toxins*, 12, 2, 130. DOI: 10.3390/toxins12020130
24. **Gajęcka, M., 2012:** The effect of low-dose experimental zearalenone intoxication on the immunoexpression of oestrogen receptors in the ovaries of pre-pubertal bitches. *Pol. J. Vet. Sci.,* 15, 685–691. DOI: 10.2478/v10181-012-0106-3

sciendo

DOI: 10.2478/fv-2025-0013

FOLIA VETERINARIA, 69, 2: 12—19, 2025

ORIGINAL ARTICLE

PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF *SALMONELLA* SPP. ISOLATED FROM VEGETABLES IN FARMS AND MARKETS WITHIN ZARIA METROPOLIS, KADUNA STATE, NIGERIA

Victoria Oluwaseyi Caleb, Daniel Obinna Esonu*, Anas Isa Ahmad

Department of Veterinary Public Health and Preventive medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Kaduna State, Nigeria

OPEN ACCESS

***Correspondence:** esonu25@gmail.com

Citation: Caleb V. O., Esonu, D. O., Ahmad, A. I., 2025: Prevalence and antimicrobial susceptibility profile of *Salmonella* spp. isolated from vegetables in farms and markets within Zaria metropolis, Kaduna state, Nigeria. Folia Veterinaria, 69, 2, 12–19.

Received: January 18, 2025

Accepted: April 24, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

***Salmonella* is a significant zoonotic pathogen that causes diseases in humans and animals, often transmitted through contaminated foods, including vegetables. This study investigated the occurrence and antimicrobial resistance profile of *Salmonella* in vegetables obtained from farms and markets in Zaria metropolis. A total of 120 vegetable samples was processed following Clinical and Laboratory Standards Institute (CLSI) standards. An overall *Salmonella* prevalence of 15.8 % was observed from this study. Lettuce was the most contaminated vegetable (36.0 %), followed by onions (16.7 %), cabbage (12.0 %), cucumber (9.1 %), and carrots (4.2 %). Leafy vegetables had the highest contamination rate (23.1 %), compared to root (12.2 %) and fruit vegetables (5.3 %). Market samples showed a higher prevalence (18.3 %) than farm samples (13.3 %). Antimicrobial-resistance patterns of the positive isolates of *Salmonella* showed the highest percentage of resistance to erythromycin (100 %). Only 5.3 % of the positive isolates were resistant to ciprofloxacin, while none of the *Salmonella* isolates showed resistance to imipenem. Antibiotic susceptibility testing revealed that all isolates were resistant to at least one antibiotic, with 94.7 % exhibiting multidrug resistance. This study highlights the contamination of vegetables with *Salmonella* and its antimicrobial resistance patterns. It underscores the need for improved agricultural and retail practices to mitigate public health risks.**

Keywords: antimicrobial resistance; farm; market; Salmonella; vegetable; Zaria

INTRODUCTION

Fresh vegetables play a critical role in human nutrition, providing essential vitamins, minerals, and dietary fiber [1]. However, their consumption, particularly when raw or inadequately washed, poses significant public health risks due to their potential to act as vectors for pathogenic microorganisms linked to foodborne illnesses [2]. Epidemiological data have shown an increasing number of outbreaks associated with contaminated vegetables, often linked to the presence of coliforms, including *Escherichia coli* and *Salmonella*, which are indicators of fecal contamination [3]. These microorganisms are introduced through various contamination routes, such as the application of organic fertilizers, irrigation with contaminated water, direct contact with livestock, and poor post-harvest practices, including inadequate hygiene during handling and transportation [4]. *Salmonella* is one of the most prevalent causes of sporadic and outbreak-related gastroenteritis worldwide, with children under five, the elderly, and immunocompromised individuals being particularly vulnerable [5]. Mortality rates from *Salmonella* infections vary depending on serotype but can reach up to 21% in severe cases.

In Nigeria, the increasing consumption of ready-to-eat vegetables, driven by their established health and nutritional benefits, has not been matched by adequate attention to the potential microbiological hazards associated with these foods [6]. Studies have highlighted the contamination of raw vegetables with microorganisms, including *Salmonella*, due to poor hygiene practices and environmental factors [7]. Multidrug-resistant (MDR) *Salmonella* serotypes pose significant challenges to treatment efficacy and may lead to increased morbidity and mortality [8]. Factors such as limited food hygiene awareness among farmers, retailers, and consumers, as well as the growing prevalence of typhoid fever caused by *Salmonella* spp., further exacerbate the public health risks [9].

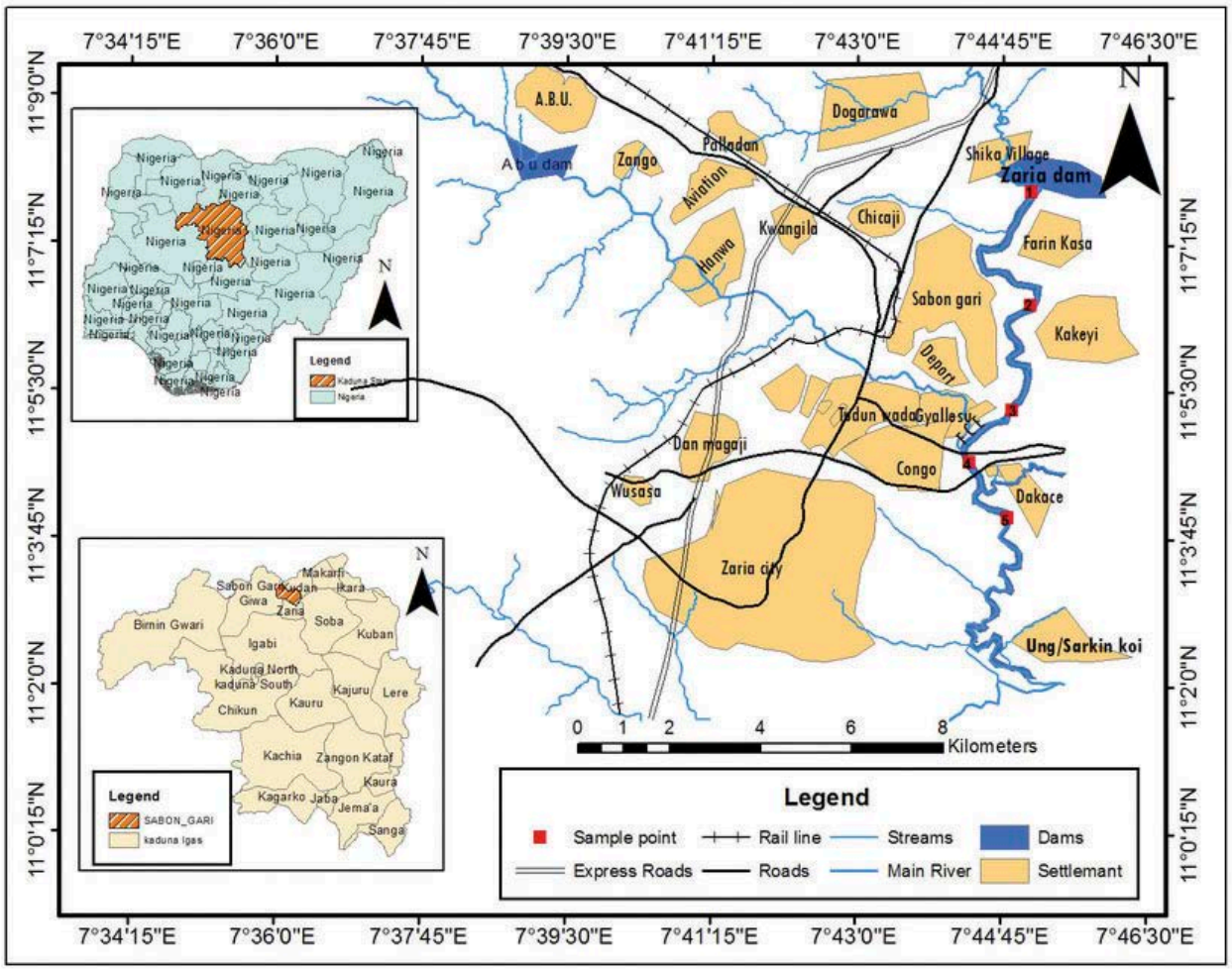

Fig. 1. Map of Zaria Metropolis (study area). Source: Satellite Image (2013)

This study aimed to determine the prevalence and antimicrobial susceptibility profile of *Salmonella* isolated from vegetables at the point of harvest and in markets within Zaria metropolis, Kaduna State, Nigeria. The findings will contribute to understanding contamination trends and inform public health interventions to mitigate the risks associated with vegetable consumption.

MATERIALS AND METHODS

Study area

The study was conducted in Zaria Local Government Area, located in Kaduna State in the northern region of Nigeria (Fig. 1). Agriculture is the primary occupation of the inhabitants, with a wide variety of vegetables grown and marketed within the area [10].

Study design

A cross-sectional study was conducted in July 2023 to investigate vegetable samples from farms and retail points in Zaria and Sabon-Gari local government areas, Kaduna State, Nigeria. Samples were collected based on availability, with prior consent obtained from participating farmers and retailers. A total of 120 vegetable samples—comprising carrot, cucumber, cabbage, onion, and lettuce—were systematically collected. Of these, 60 samples were obtained from five selected farms, while the remaining 60 were sourced from five selected retail markets within the study area.

Sample collection

Approximately 200 grams of each vegetable sample were collected in clean, sterile bags to prevent contamination and were appropriately labeled. The samples were stored and transported under chilled conditions (4 °C) using ice packs to the laboratory. All samples were processed within two hours of collection to ensure sample integrity.

Laboratory procedures

1) Pre-enrichment

Each vegetable sample was aseptically cut into smaller pieces using a sterilized knife, and 10 grams were weighed. The sample was transferred into a sterile stomacher bag containing 90 mL of peptone water, mixed for 1–2 minutes, and incubated at 37 °C for 24 hours to facilitate pre-enrichment.

2) Enrichment

For selective enrichment, Rappaport Vassiliadis medium was added to pre-enriched samples at a 9:1 ratio. The mixtures were appropriately labeled and incubated at 37 °C for 24 hours to promote the growth of *Salmonella* species.

3) Selective Plating

Using a sterile Pasteur loop, inoculated Rappaport Vassiliadis samples were streaked onto Bismuth Sulfite Agar (BSA) plates. The plates were labeled and incubated at 37 °C for 24 hours. Colony morphology was evaluated, with suspected *Salmonella* colonies appearing as black or brown colonies. These isolates were stored on nutrient agar slants in sterile sample bottles, incubated at 37 °C for 24 hours, and subsequently refrigerated for further analysis.

4) Biochemical Tests

Suspected *Salmonella* isolates were subjected to standard biochemical tests for confirmation. These tests included: Indole Production: Using SIM medium, Hydrogen Sulfide (H_2S) Production and Motility: Using SIM medium, Citrate Utilization: Using Simmons Citrate medium, Methyl Red (MR) and Voges-Proskauer (VP): Using MR-VP medium, Urease Production: Using urea broth. Additionally, isolates were inoculated into Triple Sugar Iron (TSI) agar and incubated at 37 °C for 24 hours. Isolates with typical Salmonella biochemical profiles (e.g., indole-negative, MR-positive, VP-negative, citrate-positive, motile, and H_2S production) were considered positive and stored on nutrient agar slants at 4 °C for further analysis.

5) Sugar Fermentation Test

Andrade's peptone water was used as a pH indicator to test sugar fermentation. Sugars (sucrose, mannitol, glucose, lactose, and maltose) were prepared according to the manufacturer's specifications and combined with Andrade's peptone water. Positive fermentation, indicative of Salmonella spp., was confirmed by a pink coloration due to pH changes, with lactose being an exception as it is typically not fermented by *Salmonella* spp.

6) Antibiotic Susceptibility Testing

Antibiotic susceptibility was evaluated using standard methods. The antibiotics tested included tetracycline,

gentamicin, imipenem, ciprofloxacin, trimethoprim/sulfamethoxazole, and erythromycin. Inoculated plates were incubated at 37°C for 24 hours, and zones of inhibition were measured according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Results were categorized as sensitive (S), intermediate (I), or resistant (R) based on CLSI standards.

Data analysis

The collected data were analyzed using SPSS software (version 26). Descriptive statistics, including frequencies and percentages, were employed to determine the prevalence of *Salmonella* in the sampled vegetables. Chi-square tests were conducted to evaluate associations, with statistical significance set at a p-value ≤ 0.05.

RESULTS

The overall prevalence of *Salmonella* spp. in 120 fresh vegetables collected from selected markets and farms within Sabon-Gari and Zaria Local Government Areas was 15.8 % (19/120). Higher contamination rates were observed in samples from Zaria Local Government Area (21.7 %) compared to Sabon-Gari Local Government Area (10.0 %). However, no significant association was found between *Salmonella* prevalence and the source of the sample (p = 0.509). Market samples exhibited a higher prevalence of contamination at 18.3 % (11/60) compared to farm samples, which had a prevalence of 13.3 % (8/60) (Table 1).

Table 2 details the prevalence within the four markets. In descending order, *Salmonella* prevalence was highest in Tudun-Wada (26.7 %), followed by Galma Market (20.0 %), Samaru Market (13.3 %) and Sabo Market (13.3 %). Similarly, Table 2 also shows that farms in Tudun-Wada had the highest prevalence (26.7 %), followed by Gyellesu (13.3 %), Bomo (6.7 %), and Shika Farm (6.7 %).

Salmonella contamination was most prevalent in leafy vegetables (lettuce and cabbage) at 23.1%, followed by root vegetables (carrot and onion) at 12.2 %, with the lowest prevalence observed in fruit vegetables (cucumber) at 5.3 %. A statistically significant association was observed between *Salmonella* prevalence and vegetable type (p = 0.025) (Table 3).

Antimicrobial-resistance patterns of the positive isolates of *Salmonella* in Table 4, showed the highest percentage of resistance to erythromycin (100 %), followed by tetracycline (94.7 %), gentamicin (73.7 %), and trimethoprim/sulfamethoxazole (57.9 %). Only 5.3 % of the positive isolates were resistant to ciprofloxacin, while none of the *Salmonella* isolates showed resistance to imipenem.

According to Table 5, 18 (94.7 %) of the isolates exhibited multidrug resistance during the antibiotic sensitivity test, thus resistant to three (3) different classes of antimicrobials. Only 1 (5.3 %) isolate was resistant to two different classes of antimicrobials (tetracycline and erythromycin).

DISCUSSION

According to the World Health Organization [11], the presence of Salmonella in foods such as vegetables is a public health concern, as these foods can act as potential vehicles for transmitting the organism to humans and animals. The findings from this study reveal an overall Salmonella prevalence of 15.8 % in vegetables. This prevalence is higher than those reported by Yang et al. [12] in China (3.4 %), Raufu et al. [13] in Maiduguri, Nigeria (6.3 %), and Nair et al. [14] (4 %). However, it is lower than the prevalence reported by Azimirad et al. [15] in Iran (19.44 %) and Maikaji et al. [16] in Zaria (50 %). Variations in prevalence among studies may be attributed to differences in geographic and environmental factors, seasonal sampling, hygiene practices, and isolation techniques employed by researchers.

A higher *Salmonella* prevalence was recorded in Zaria Local Government Area (21.7 %) compared to Sabon-Gari Local Government Area (10.0 %). This disparity could be linked to Zaria's larger population, estimated at approximately 601,300 in 2021 compared to 430,000 in Sabon-Gari, leading to greater contamination risks through human and animal activities such as open grazing and defecation near farms. Additionally, questionnaire data revealed that most farmers in Zaria rely on the Kubani River for irrigation. This river has been reported as highly contaminated, partly due to effluents from an abattoir, which may contribute to the higher prevalence of Salmonella in this area [17].

Salmonella contamination of vegetables can occur at multiple stages of production, including cultivation, farm

Table 1. Prevalence of *Salmonella* isolates on vegetables grown and sold within Zaria metropolis, Kaduna State, Nigeria

Variables	Categories	Number Examined	Number positive	Specific Rate (%)	χ2	P-Value
LGA	Sabon Gari	60	6	10.0	0.436	0.509
	Zaria	60	13	21.7		
	Total	**120**	**19**	**15.8**		
Source	Market	60	11	18.3	0.563	0.453
	Farm	60	8	13.3		
	Total	**120**	**19**	**15.8**		

Table 2. Prevalence of *Salmonella* isolates on vegetables in selected markets and farms within Zaria metropolis, Kaduna state, Nigeria

Variables	Categories	Number sampled	Number Positive	Specific rate (%)	χ2	P-Value
Market	Sabo	15	2	13.3	1.939	0.747
	Samaru	15	2	13.3		
	T/Wada	15	4	26.7		
	Galma	15	3	20.0		
	Total	**60**	**11**	**18.3**		
Farm	Shika	15	1	6.7	3.564	0.468
	Bomo	15	1	6.7		
	T/Wada	15	4	26.7		
	Gyellesu	15	2	13.3		
	Total	**60**	**8**	**13.3**		

Table 3. Prevalence of *Salmonella* in different classes and types of vegetables from selected farms and markets within Zaria metropolis, Kaduna state, Nigeria

Variables	Categories	Number Examined	Number Positive	Specific rate (%)	χ2	P-Value
Class of Vegetable	Fruit Vegetable	19	1	5.3	4.114	0.128
	Root Vegetable	49	6	12.2		
	Leafy Vegetable	52	12	23.1		
	Total	**120**	**19**	**15.8**		
Type of Vegetable	Cabbage	25	3	12.0	11.119	0.025
	Carrot	24	1	4.2		
	Lettuce	25	9	36.0		
	Cucumber	22	2	9.1		
	Onions	24	4	16.7		
	Total	**120**	**19**	**15.8**		

Table 4. Antibiotic susceptibility profile of positive isolates of *Salmonella* on vegetables in farms and markets from Zaria metropolis, Kaduna State, Nigeria

Antibiotics	Susceptible (%)	Intermediate (%)	Resistance (%)
Tetracycline	1 (5.3)	0 (0)	18 (94.7)
Gentamicin	3 (15.8)	2 (10.5)	14 (73.7)
Imipenem	19 (100)	0 (0)	0 (0)
Ciprofloxacin	17 (89.5)	1 (5.3)	1 (5.3)
Trimethoprim/sulfamethoxazole	7 (36.9)	1 (5.3)	11 (57.9)
Erythromycin	0 (0)	0 (0)	19 (100)

Table 5. Antimicrobial resistance patterns of positive isolates of *Salmonella* on vegetables in farms and markets from Zaria metropolis, Kaduna State, Nigeria

Number of Antibiotics	Resistance Pattern	Number of Isolates (%)
2	TET, E	1 (5.3)
3	TET, E, CN	5 (26.3)
3	E, CN, SXT	1 (5.3)
3	TET, E, SXT	4 (21.1)
4	TET, E, CN, SXT	6 (31.6)
4	TET, E, CN, Cip	2 (10.5)

TET = Tetracycline, E = Erythromicin, SXT = Trimethoprim/sulphamethoxazole, CN = Gentamicin, Cip = Ciprofloxacin

practices, harvesting, transportation, and post-harvest handling [4]. Market samples in this study exhibited a higher contamination rate (18.3 %) compared to farm samples (13.3 %), indicating additional contamination during post-harvest processes. This may result from factors such as cross-contamination between mixed vegetables, unhygienic transportation methods, the use of contaminated water for washing, open display on tables or floors, poor storage practices, and contact with infected individuals or fomites.

Among the five types of vegetables sampled, lettuce showed the highest prevalence of contamination, followed by onion, cabbage, cucumber, and carrot. The high contamination in lettuce can be attributed to its proximity to the ground, where soil-borne bacteria may be introduced through manure fertilization, and its large surface area, which increases susceptibility to contamination [18, 19]. Inadequate post-harvest washing by retailers further exacerbates this issue [20]. Across vegetable classes, leafy vegetables were the most contaminated, followed by root and fruit vegetables, consistent with findings by Franz et al. [21].

Antimicrobial resistance among the isolates is a growing global concern. The isolates from this study exhibited varying degrees of resistance to tetracycline, erythromycin, trimethoprim/sulfamethoxazole, gentamicin, and, to a lesser extent, ciprofloxacin. However, all isolates demonstrated high susceptibility to imipenem. The resistance trend for tetracycline aligns with findings by Lerttworapreecha et al. [22], attributing high resistance to the widespread use of tetracycline in livestock, its affordability, broad-spectrum activity, and easy accessibility. Similar results were reported by Toe et al. [23], highlighting multidrug resistance among *Salmonella* isolates. The observed multidrug resistance could be linked to human, animal, and environmental contamination. Contributing factors include the indiscriminate use of antibiotics in livestock farming and the misuse of antibiotics in human medicine. These practices emphasize the need for stricter antibiotic stewardship to mitigate the emergence and spread of multidrug-resistant *Salmonella* strains.

CONCLUSIONS

The findings in this study are of veterinary and public health importance, and they include: firstly, the study revealed that 15.8 % of vegetables grown and sold in selected farms and markets across Sabon-Gari and Zaria Local Government Areas in Zaria, Kaduna State, Nigeria, were contaminated with *Salmonella*. Secondly, *Salmonella* contamination was higher in market samples compared to those from farms, and almost all *Salmonella* isolates (94.7 %) exhibited multidrug resistance, highlighting a significant public health concern for vegetable consumers. Lastly, the two most effective antibiotics observed in this study were imipenem (100 % susceptibility) and ciprofloxacin (89.4 %), underscoring the pressing need to address the growing issue of antibiotic resistance.

We therefore recommend that regulatory bodies establish and enforce microbiological standards for farmers and marketers to ensure proper handling of vegetables. Public education campaigns should promote the responsible use of antibiotics in both livestock management and human medicine to mitigate resistance. Lastly, consumers should be advised to thoroughly wash vegetables, particularly those intended to be consumed raw, to reduce the risk of contamination.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

Ethical approval was not necessary for this work.

Conflict of Interest

No conflict of interest.

Funding

No funding.

Generative AI Statement

Authors disclosed that no form of generative AI and AI-assisted technologies were used in the writing process.

Authors' Contributions

Caleb V. O., Esonu D. O. and Ahnad A. I. designed the research, Caleb V. O. and Ahnad A. I. carried it out while

Esonu D. O. analyzed the data. Caleb V. O., Esonu D. O. and Ahnad A. I. were involved in writing and proof reading of the manuscript.

Acknowledgements

We sincerely appreciate the efforts of the laboratory staff of the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, who assisted in processing the samples.

REFERENCES

1. **Saha, M., Debnath, C., Biswas, M. K., Pramanik, A. K., Murmu, D., 2016:** Studies on the prevalence of *Salmonella* spp. in meat shop premises intended to sell meat for human consumption in North Kolkata, India. *Int. J. Curr. Microbiol. Appl. Sci.*, 5, 4, 297–302. DOI: 10.20546/ijcmas.2016.504.034
2. **Garg, N., Garg, K. L., Mukerji, K. G., 2010:** *Laboratory Manual of Food Microbiology*. I.K. International Publishing House, New Delhi, India.
3. **Hanson, L., Zahn, E. A., Wild, S. R., Dopfer, D., Scott, J., Stein, C., 2012:** Estimating global mortality from potentially foodborne diseases; an analysis using vital registration data. *Pop. Hlth Metropol.*, 10, 1–7.
4. **Heaton, J. C., Jones, K., 2008:** Microbial contamination of fruit and vegetables and the behavior of enteropathogens in the phyllosphere: A review. *J. Appl. Microbiol.*, 104, 3, 613–626. DOI: 10.1111/j.1365-2672.2007.03587.x
5. **Food and Drug Administration, 2018:** *"Bad Bug Book: Foodborne Pathogenic Microorganisms and Natural Toxins Handbook."* 2nd Edition. U.S. Food and Drug Administration.
6. **Adeshina, G. O., Samuel, E. A., Victor, E. A., 2012:** Antibacterial susceptibility pattern of pathogenic bacteria isolates from vegetable salad sold in restaurants in Zaria, Nigeria. *J. Microbiol. Res.*, 2, 2, 5–11. DOI: 10.5923/j.microbiology.20120202.02
7. **Szabo, E. A., Coventry, M. J., 2001:** Vegetables and vegetable products. In C.J. Moir et al. (Eds.), *Spoilage of processed foods; causes and diagnosis.* Food Microbiol. Grp. Waterloo, 1, 2, 1–3.
8. **Eng, S.-K., Pusparajah, P., Mutalib, N.-S. A., Ser, H.-L., Chan, K.-G., Lee, L.-H., 2015:** Salmonella: A review on pathogenesis, epidemiology and antibiotic resistance. *Front. Life Sci.*, 8, 3, 284–293. DOI: 10.1080/21553769.2015.1051243
9. **Forstinus, N. O., 2015:** Epidemiology of Salmonella and Salmonellosis. *Int. Letters of Nat. Sci.*, 47, 54. DOI: 10.18052/www.scipress.com/ILNS.47.54
10. **Food and Agriculture Organization of the United Nations, 2019:** Kaduna State Agricultural Profile. Available at: http://www.fao.org/3/i9353en/i9353en.pdf
11. **World Health Organization (WHO), 2002:** WHO Global Strategy for Food Safety: Safer Food for Better Health. World Health Organization, Geneva, Switzerland, pp. 10–20.
12. **Yang, X., Huang, J., Zhang, Y., Liu, S., Chen, L., Xiao, C., et al., 2020:** Prevalence, abundance, serovars, and antimicrobial resistance of *Salmonella* isolated from retail raw poultry meat in China. *Sci. Total Environ.*, 713, 136385.
13. **Raufu, I. A., Zongur, L., Lawan, F. A., Bello, H. S., Adamu, M. S., Ameh, J. A., Ambali, A. G., 2014:** Prevalence and antimicrobial profiles of *Salmonella* serovars from vegetables in Maiduguri, Northeastern Nigeria. *Sokoto J. Vet. Sci.*, 12, 1, 23–28. DOI: 10.4314/sokjvs.v12i1.4
14. **Nair, A., Balasaravanan, T., Malik, S. V., Mohan, V., Kumar, M., Vergis, J., et al., 2015:** Isolation and identification of *Salmonella* from diarrheagenic infants and young animals, sewage waste, and fresh vegetables. *Vet. World*, 8, 5, 669–673. DOI: 10.14202/vetworld.2015.669-673
15. **Azimirad, M., Nadalian, B., Alavifard, H., Negahdar Panirani, S., Mahdigholi Vand Bonab, S., Azimirad, F., et al., 2021:** Microbiological survey and occurrence of bacterial foodborne pathogens in raw and ready-to-eat green leafy vegetables marketed in Tehran, Iran. *Int. J. Hyg. Environ. Health*, 237, 113824. DOI: 10.1016/j.ijheh.2021.113824
16. **Maikaji, F., Chiezey, N., Samuel, F., Bello, T., 2015:** Efficacy of some sanitizing agents on *Salmonella* species detected in rinsed water of vegetables eaten raw in Samaru, Zaria. *J. Anim. Prod. Res.*, 27, 99–107.
17. **Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., Jiang, X., 2004:** Persistence of *Salmonella enterica* serovar Typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. *Foodborne Pathog. Dis.*, 1, 1, 27–35. DOI: 10.1089/153531404772914437
18. **Bello, O., Bello, T., Fashola, M., Oluwadun, A., 2014:** Microbiological quality of some locally produced fruit juices in Ogun State, South Western Nigeria. *E3 J. Microbiol. Res.*, 2, 1, 1–8.

19. **Itohan, A. M., Peters, O., Kolo, I., 2011:** Bacterial contaminants of salad vegetables in Abuja Municipal Area Council, Nigeria. *Malaysian Journal of Microbiology*, 7, 111–114. DOI: 10.21161/mjm.24610

20. **Jawetz, E., Melnick, J. L., Adelberg, E. A., 2007:** Medical Microbiology. 24th Edition. McGraw-Hill Education, pp. 227–245.

21. **Franz, E., van Hoek, A. H., Bouw, E., Aarts, H. J., 2015:** Variability of *Escherichia coli* O157 contamination in leafy green produce grown in the open field and under controlled conditions. *Food Microbiol.*, 51, 91–100.

22. **Lerttworapreecha, M., 2012:** Antimicrobial resistance in *Salmonella enterica* isolated from pork, chicken, and vegetables in Southern Thailand. *Jundishapur J. Microbiol.*, 6, 1, 36–41. DOI: 10.5812/jjm.4312

23. **Toe, E., Attien, P., Moroh, A. J.-L., Sina, H., Kouame, D. N., Kambire, O., et al., 2022:** Prevalence and characterization of *Salmonella* isolated from vegetable salads and ready-to-eat raw mixed vegetable salads in Abidjan, Côte d'Ivoire. *J. Microbiol. Antimicrob.*, 14, 1, 15–25. DOI: 10.5897/jma2021.0449

sciendo

DOI: 10.2478/fv-2025-0014

FOLIA VETERINARIA, 69, 2: 20—27, 2025

CASE REPORT

UNCOMPLICATED CANINE BABESIOSIS WITHOUT PROPHYLAXIS: CASE REPORT AND RETROSPECTIVE ANALYSIS

Veronika Vinclérová[1]*, Csilla Tóthová[2], Viera Karaffová[1]

[1]Department of Morphological Disciplines, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia; [2]Clinic of Ruminants, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia

OPEN ACCESS

***Correspondence:** veronika.vinclerova@uvlf.student.sk

Citation: Vinclérová, V., Tóthová, C., Karaffová, V., 2025: Uncomplicated canine babesiosis without prophylaxis: Case report and retrospective analysis. Folia Veterinaria, 69, 2, 20–27.

Received: January 2, 2025

Accepted: May 1, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Canine babesiosis is a serious disease caused by intraerythrocytic protozoa of the genus *Babesia*, most commonly *Babesia canis* and *Babesia gibsoni*. A four-year-old male Yorkshire Terrier was presented to a private veterinary clinic with anorexia, fever, weakness, vomiting, and hematuria. Microscopic examination of peripheral blood revealed the presence of *Babesia canis*. Further laboratory findings indicated marked thrombocytopenia and elevated total bilirubin levels, suggesting a severe clinical course potentially associated with hematopoietic dysfunction. The aim of this study was to describe a clinical case of uncomplicated babesiosis in a dog without a history of prophylactic antiparasitic treatment and to compare the findings with data from a retrospective group of ten dogs that had been diagnosed with babesiosis. Based on our findings, we strongly recommend the regular administration of antiparasitic supplements to prevent *Babesia* spp. infections, thereby minimizing animal suffering and reducing treatment costs.

Key words: babesiosis; canine; tick-borne disease

INTRODUCTION

The increase in global temperatures in Slovakia was reflected in an increase in the average annual air temperature by 1.1 °C over the last hundred years. In many regions of the country, temperature records that have been valid for more than two decades have already been exceeded. This fact represents the risk of the spread of tick-borne diseases [1]. The first confirmed cases of canine babesiosis in Slovakia were diagnosed in May 2000, when a male Siberian Husky was treated at the Internal-Clinic of the University of Veterinary Medicine in Košice [2]. Babesiosis vectors are ticks, most often from the *Ixodidae* family and in the temperate zone *Dermacentor reticulatus*. In the tropics and subtropics, the vector is *Rhipicephalus sanguineus* and *Haemaphysalis leachi*, making the disease endemic in areas with a high prevalence of these ectoparasites [1].

It is well known that *Babesia* organisms reproduce in the definitive host through multiple fission producing merozoites. Ticks become infected after ingesting parasitized erythrocytes from the host. During this process, multiple fission of merozoites results in the production of sporozoites (infective undeveloped cells) within the salivary glands of the arthropod. These sporozoites are then transmitted into the host's bloodstream via tick saliva. An attachment period of 2–3 days is required for *Babesia canis* transmission to occur [3].

The dog tick *Dermacentor reticulatus*, a vector of *Babesia canis*, is showing a significant expansion of its range, especially in European countries [4]. Similarly, *B. canis* is the most commonly diagnosed species of *Babesia* in erythrocytes of affected dogs in Slovakia. Clinically, babesiosis is characterized mainly by high fever, pale mucous membranes, loss of appetite, anemia, jaundice, enlarged lymph nodes, and splenomegaly. However, the most typical clinical presentation is the sudden onset of high fever with anemia [5]. The severity of babesiosis varies from subclinical infection to extensive organ failure and death. The diagnosis of babesiosis involves the microscopic identification of the parasite in blood smears, serological methods, and particularly PCR tests, which allow for species-specific detection of the parasite. The prognosis depends on the host's immune response, age, clinical condition, and the speed of treatment initiation. Effective prevention is based on targeted tick control and the application of antiparasitic measures [6]. This study presents a case of uncomplicated canine babesiosis and aims to contextualize it within a broader framework by retrospectively comparing hematological data from a cohort of other infected dogs.

CASE PRESENTATION

A 4-year-old, intact male Yorkshire Terrier was presented to a private veterinary clinic with a 2-day history of lethargy and anorexia. The owner reported that five ticks had been removed from the dog approximately five days prior to presentation. The dog was not receiving any antiparasitic treatment and had no previous history of relevant illness.

Clinical examination revealed an apathetic and unresponsive dog with pale mucous membranes and icteric discoloration of the conjunctivae. Dehydration was evident (reduced skin turgor, capillary refill time >3 seconds), the body temperature was elevated (39.0 °C), and hemoglobinuria was observed.

Blood samples were collected for hematological and biochemical analyses. In addition, a rapid CaniV-4 test (Bionote Co., Ltd., Republic of Korea) was used to screen for vector-borne pathogens (*Ehrlichia* spp*., Anaplasma* spp*., Borrelia burgdorferi,* and *Dirofilaria immitis*). Blood was also obtained from a peripheral vessel in the claw area for cytological examination. Blood smears were air-dried, fixed with methanol, and stained using the Diff-Quik staining kit (Siemens Healthineers). Microscopic examination was performed under light microscopy at 40× and 100× magnification.

MANAGEMENT AND OUTCOMES

The result of the CANIV-4 test was negative, which means that the presence of these pathogens was not detected at the time of the examination. On the other hand, the presence of *Babesia* spp. was finally confirmed cytologically in blood smears, where the typical finding of basophilic staining ring-shaped formations inside erythrocytes was observed (Fig. 1). This finding was considered sufficient to establish the diagnosis. In addition, the dog's hematological examination revealed significant abnormalities, most notably thrombocytopenia, which is commonly associated with *Babesia* infections. The clinical finding was accompanied by a lower neutrophil count within the normal reference range and an increase in the number of lymphocytes (lymphocytosis). It may indicate an inflammatory or immune response of the body to the presence of an infection. Other laboratory findings include elevations in liver enzymes such as Alkaline Phosphatase (ALP) and Aspartate Aminotransferase (AST), which in this case reflect concomitant hepatopathy. Bilirubin concentrations are elevated, commensurate with the degree and rapidity of onset of the anemia and the severity of the accompanying hepatopathy (Table 1).

Treatment for babesiosis is aimed at eliminating the parasite, controlling inflammation, and supporting systemic recovery. Due to the owner's limited financial resources, an affordable yet clinically effective protocol was selected. Imidocarb dipropionate (Imizol; MSD Animal Health,

Table 1. Result of hematological and biochemical examination in a dog with babesiosis

Parameter	Result	Units	Reference Range	Conclusion
White Blood Cells (WBC)	**4.22**	**10^9/l**	**6.00 – 12.00**	**Low**
Red Blood Cells (RBC)	5.81	10^{12}/l	5.50 – 8.50	Normal
Hemoglobin (HGB)	140.0	g/l	120.0 – 180.0	Normal
Hematocrit (HCT)	0.38	ratio	0.37 – 0.55	Normal
Mean Corpuscular Volume (MCV)	65.80	fl	60.00 – 77.00	Normal
Mean Corpuscular Hemoglobin (MCH)	**24.00**	**pg**	**17.00 – 23.00**	**High**
Mean Corpuscular Hemoglobin Concentration (MCHC)	**366.0**	**g/l**	**310.0 – 340.0**	**High**
Platelets (PLT)	**14.00**	**10^9/l**	**150.0 – 500.0**	**Low**
Neutrophils (NE)	58.0	%	55.0 - 75.0	Normal
Lymphocytes (LY)	**42.0**	**%**	**12.0 – 30.0**	**High**
Eosinophils (EO)	0	%	0.0 – 6.0	Normal
Monocytes (MO)	0	%	0.0 – 4.0	Normal
Basophils (BA)	0	%	0.0 – 1.0	Normal
Reticulocytes	0.79	%	0.50 – 1.50	Normal
Reticulocytes (absolute count)	47.70	10^9/l	–	Normal
Calcium (Ca)	**1.95**	**mmol/l**	**2.10 – 3.00**	**Low**
Phosphorus (P)	1.16	mmol/l	0.90 – 1.90	Normal
Glucose (Glc)	5.66	mmol/l	3.60 – 6.70	Normal
Urea	**14.64**	**mmol/l**	**3.97 – 8.05**	**High**
Creatinine	55.97	µmol/l	35.0 – 105.0	Normal
Total Protein	**50.63**	**g/l**	**54.0 – 75.0**	**Low**
Total Bilirubin	**23.34**	**µmol/l**	**0.00 – 3.40**	**High**
Aspartate Aminotransferase (AST)	**2.09**	**µkat/l**	**0.00 – 0.60**	**High**
Alanine Aminotransferase (ALT)	0.42	µkat/l	0.00 – 0.95	Normal
Gamma-Glutamyl Transferase (GGT)	<0.12	µkat/l	0.00 – 0.14	Normal
Alkaline Phosphatase (ALP)	**3.38**	**µkat/l**	**0.00 – 1.24**	**High**

Table 2. Result of hematological and biochemical examination in a dog with babesiosis after treatment

Parameter	Result	Units	Reference Range	Conclusion
White Blood Cells (WBC)	6.63	10^9/l	6.00 – 12.00	Normal
Red Blood Cells (RBC)	6.57	10^{12}/l	5.50 – 8.50	Normal
Hemoglobin (HGB)	159.0	g/l	120.00 – 180	Normal
Hematocrit (HCT)	0.43	ratio	0.37 – 0.55	Normal
Mean Corpuscular Volume (MCV)	65.10	fl	60.00 – 77.0	Normal
Platelets (PLT)	391.0	10^9/l	150.0 – 500.0	Normal
Urea	**2.91**	**mmol/l**	**3.97 – 8.05**	**Low**
Total Proteins	68.15	g/l	54.0 – 75.0	Normal
Total Bilirubin	<3	µmol/l	0.00 – 3.40	Normal
AST	0.28	µkat/l	0.00 – 0.60	Normal
ALT	0.35	µkat/l	0.00 – 0.95	Normal
ALP	0.52	µkat/l	0.00 – 1.24	Normal

Table 3. Mean hematological parameters in a group of 10 dogs with confirmed babesiosis (retrospective analysis)

Parameter	Result	Units	ReferenceRange	Conclusion
White Blood Cells (WBC)	**3.03 ± 3.22**	**10^9/l**	**5.05 – 16.76**	**Low**
Red Blood Cells (RBC)	**5.07 ± 0.63**	**10^{12}/l**	**5.65 – 8.87**	**Low**
Hemoglobin (HGB)	13.43 ± 1.46	g/dl	13.1 – 20.5	Normal
Hematocrit (HCT)	**33.8 ± 3.97**	**%**	**37.3 – 61.7**	**Low**
Mean Corpuscular Volume (MCV)	63.21 ± 3.65	fL	61.6 – 73.5	Normal
Mean Corpuscular Hemoglobin (MCH)	24.2 ± 1.35	pg	21.2 – 25.9	Normal
Mean Corpuscular Hemoglobin Concentration (MCHC)	**39.1 ± 1.11**	**g/dl**	**32.0 – 37.9**	**High**
Platelets (PLT)	**15.6 ± 20.03**	**10^9/l**	**148 – 484**	**Low**
Neutrophils (NEU)	**2.145 ± 1.46**	**10^9/l**	**2.95 – 11.64**	**Low**
Lymphocytes (LYM)	2.82 ± 0.76	10^9/l	1.05 – 5.10	Normal
Monocytes (MONO)	1.1 ± 1.11	10^9/l	0.16 – 1.12	Normal
Eosinophils (EOS)	**0.007 ± 0.009**	**10^9/l**	**0.06 – 1.23**	**Low**
Basophils (BASO)	0.013 ± 0.02	10^9/l	0.00 – 0.10	Normal
Reticulocytes (RETIC)	39.42 ± 26.77	K/ml	10.0 – 110.0	Normal
Reticulocyte Hemoglobin Content (RETIC-HGB)	**20.05 ± 1.71**	**pg**	**22.3 – 29.6**	**Low**
Mean Platelet Volume (MPV)	**16.9 ± 2.45**	**fL**	**8.7 – 13.2**	**High**
Plateletcrit (PCT)	**0.02 ± 0.03**	**%**	**0.14 – 0.46**	**Low**

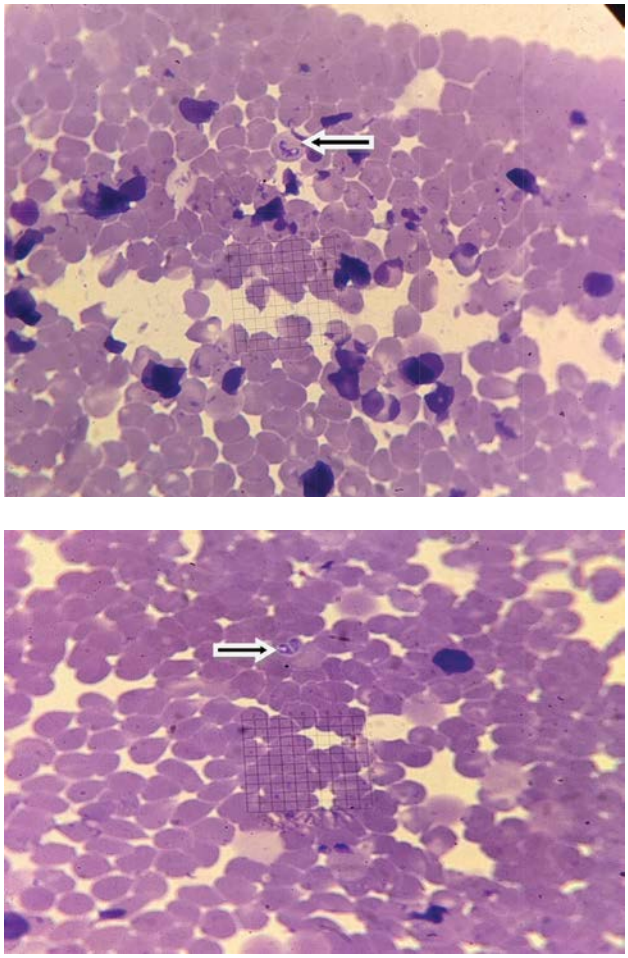

Fig 1. Microscopic finding (40x) *Babesia* spp. with typical basophilic ring shape *(arrow)*

UK) was administered intramuscularly at a dose of 5 mg/kg, with a repeated dose given two weeks later. Rehydration was initiated through intravenous fluid therapy using isotonic crystalloids (Ringer's lactate; e.g., B. Braun, Germany), administered as part of a 24-hour fluid replacement plan, adjusted to ambulatory conditions. To reduce inflammation and fever, meloxicam was used as a non-steroidal anti-inflammatory drug (NSAID). A single subcutaneous dose of 0.2 mg/kg body weight was administered on the first day (Meloxidolor; Le Vet Beheer B.V., Netherlands), followed by oral administration of meloxicam (Meloxoral; Cymedica, Czech Republic) at a dose of 0.1 mg/kg once daily for five consecutive days. Supportive therapy included Catosal (Bayer, Germany), administered at a dose of 0.5 ml twice a week to stimulate metabolism and recovery. Despite the constrained budget, the chosen protocol led to clinical improvement and hematological stabilization, as confirmed by follow-up laboratory testing (Table 2).

For comparative purposes, a retrospective group consisting of 10 dogs with confirmed babesiosis was included. The data was obtained from our student's diploma thesis carried out at the University Veterinary Hospital, where dogs of different breeds, sexes and ages (9 months to 2 years) were carefully documented. The owners of the patients were informed about the use of the results for research purposes. Hematological parameters of this group are summarized in Table 3.

DISCUSSION

The most common hematological abnormalities associated with *Babesia canis* infections are anemia and thrombocytopenia. This was confirmed in our case, where the patient exhibited significant thrombocytopenia in the hematological profile. Although thrombocytopenia is a characteristic feature of the disease, petechiae or epistaxis are rarely observed, except in cases with concurrent *Ehrlichia* infections [7]. Similar findings were observed in a clinical study conducted on 248 dogs positive for *Babesia canis*, where thrombocytopenia was detected in 247 dogs (99.5 %) . We hypothesize that the decrease in platelet count was caused by immune-mediated destruction of platelets and subsequent sequestration in the spleen [8].

These findings are consistent with the study by Kettner et al. (2003), which reports that up to 99 % of dogs with babesiosis exhibit thrombocytopenia, with 62 % having platelet counts below 25 × 10^9/L. This finding is considered characteristic of the disease and holds high diagnostic value [9]. Compared to a retrospective group of 10 dogs with confirmed babesiosis (Table 3), the described patient exhibited a significantly lower platelet count (14 × 10^9/L vs. an average of 15.6 ± 20.03 × 10^9/L), placing him at the lowest end of the observed range. This data supports the hypothesis of pronounced immune-mediated thrombocytopenia. Thrombocytopenia is a consistent finding in canine babesiosis and is usually severe in the acute phase of infection. Platelet counts typically rise within a week after initiating treatment, suggesting that immune-mediated mechanisms are involved in its pathogenesis [10].

In addition to thrombocytopenia, another common hematological abnormality in babesiosis is anemia, which can significantly affect the clinical picture of the patient [11]. Anemia is a frequent finding in canine babesiosis, with its prevalence ranging from 20 % to over 90 % of infected dogs, depending on various studies. The mechanisms leading to the development of anemia are multifactorial, including intravascular hemolysis caused by the rupture of parasitized erythrocytes, extravascular hemolysis mediated by phagocytosis in the spleen and liver, immune-mediated damage to erythrocytes, and in some cases, inhibition of erythropoiesis [12]. In our patient's case, however, anemia was not present, which aligns with normal hematocrit and hemoglobin values upon admission. The average hematocrit value in the retrospective group of dogs was 33.8 ± 3.97 %, while our patient's value was 38 %, corresponding to the reference range. This deviation may be due to early diagnosis and treatment, which prevented the development of the hemolytic phase of the infection.

The leukocyte response in canine babesiosis is variable, with leukopenia, neutropenia, and lymphocytosis being the most commonly observed changes [13]. These alterations reflect the dynamic response of the immune system to parasitic infection, as well as the disease phase. In the acute phase, a decrease in white blood cell count may occur due to their migration into tissues or increased destruction resulting from the inflammatory response [14]. In the retrospective group of 10 dogs, the average WBC count was 3.03 ± 3.22 × 10^9/L, indicating mild to moderate leukopenia (Table 3). In our patient's case, leukopenia with a value of 4,22 × 10^9/L was recorded, confirming the presence of an inflammatory response and aligning with findings described in the literature.

The patient in this case exhibited a normal neutrophil count and mild lymphocytosis. These findings align with the typical progression of *Babesia canis* infection, where neutropenia—commonly reported in other cases—may result from the redistribution of neutrophils into tissues or their increased consumption during the acute inflammatory response. Conversely, lymphocytosis may reflect activation of the adaptive immune system and is more frequently observed in the later stages or in less severe forms of the disease [15]. In contrast, analysis of a retrospective group of ten dogs with confirmed babesiosis revealed a trend toward lower neutrophil counts and normal lymphocyte levels, highlighting the variability in leukocyte responses associated with canine babesiosis.

Among the retrospective group of 10 dogs, an increased average MPV value (16.9 ± 2.45 fL) was observed, which may indicate compensatory production of young platelets in response to thrombocytopenia. This finding is consistent with the study by Goddard et al. (2015), which states that elevated MPV values may reflect platelet activation in canine babesiosis [16]. The concordance between the retrospective group's results and data from the literature confirms that increased MPV is a common finding in babesiosis.

Biochemical analysis revealed elevated AST, ALP, and total bilirubin levels in our patient. These changes are common in *Babesia canis* infection and are primarily as-

sociated with hemolytic damage and secondary liver involvement. Elevated AST activity may result from hepatocellular or muscle damage [17]. ALP elevation is typical in cholestasis or inflammatory processes in the hepatobiliary system. Hyperbilirubinemia in our patient corresponded with the clinical finding of jaundice and indicates the breakdown of infected erythrocytes during the hemolytic phase of babesiosis [18]. Azotemia occurs in many cases of dehydration, which was also demonstrated in our case [19].

It is important to note that the hematological and biochemical analyses of the individual patient were performed by an external diagnostic laboratory (UNILABS, Slovakia) using their standard reference intervals, while the retrospective group data were obtained using the IDEXX ProCyte Dx analyzer (IDEXX Laboratories, Kobe, Japan). Therefore, minor discrepancies in reference ranges were anticipated. Nevertheless, the comparative interpretation was based on relative deviations from each laboratory's standards, allowing for an accurate evaluation of the results.

For the treatment of canine babesiosis, Imizol containing the active substance imidocarb dipropionate was used. It is an aromatic diamidine for which several mechanisms of action against *Babesia canis* have been proposed. The most likely include damage to nucleic acids, inhibition of cellular repair and replication, or direct action on the parasite by targeting its nucleus and cytoplasmic structures. Additionally, hypoglycemia in the parasite and inhibition of the division of its unicellular structure are also suggested mechanisms [20].

During treatment, some dogs may develop parasympathetic symptoms such as excessive salivation, vomiting, general weakness, tremors, and muscle contractions. These symptoms can be mitigated by administering atropine as premedication at a dose of 0.05 mg/kg. Other, less frequent adverse effects include dyspnea, restlessness, diarrhea, renal tubular or hepatic necrosis, and local inflammation at the injection site. Ulceration occurs rarely and usually heals within a few days to weeks. Fatal anaphylactic reactions have also been reported [21]. None of these adverse effects were clinically observed in our case.

Prevention of babesiosis is essential for safeguarding canine health, as the disease can have severe and potentially life-threatening consequences. Basic preventive measures include the regular use of antiparasitic agents, thorough inspection of the coat after outdoor activity, and prompt removal of any detected ticks. These measures are especially important in regions with a high incidence of ticks. Many pet owners mistakenly believe that ticks pose a threat only during the summer months, as was the case with the presented patient. However, ticks can remain active in the autumn and during mild winters, which significantly increases the risk of *Babesia* infection [22].

Our case was successfully managed through early diagnosis and appropriate treatment. In this context, it is important to note that babesiosis is often fatal in cases of delayed diagnosis or inadequate therapy. This highlights the importance of timely veterinary care and the responsibility of pet owners to respond promptly to clinical signs such as fever, lethargy, reduced appetite, and pale mucous membranes. Effective protection against parasites can significantly help prevent serious health complications associated with babesiosis.

CONCLUSION

This study described a clinical case of babesiosis in a four-year-old Yorkshire Terrier with no history of prophylactic antiparasitic treatment and retrospectively compared hematological findings with a group of ten dogs infected with *Babesia* spp., also without preventive antiparasitic care. Characteristic hematological alterations, such as anemia, neutropenia, and thrombocytopenia, were observed in the affected animals. The presence of Babesia spp. was confirmed by cytological examination. Based on our findings, we strongly recommend the regular administration of antiparasitic supplements to prevent *Babesia* spp. infections, thereby reducing animal suffering and treatment costs. Furthermore, long-term monitoring of the seasonal occurrence of babesiosis in relation to climatic conditions may contribute to a better understanding of the disease's epidemiology and support more effective implementation of preventive and therapeutic measures in veterinary practice.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Ethical Statement

All owners of dogs used in this study consented to data collection, and all dogs underwent routine clinical examinations.

Conflict of Interest

The authors have no pertinent financial or non-financial conflicts of interest to declare.

Generative AI Statement

No generative artificial intelligence or artificial intelligence-enabled technologies were used in writing the manuscript.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Veronika Vinclérová], [Csilla Tóthová] and [Viera Karaffová]. The first draft of the manuscript was written by [Veronika Vinclérová] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Statement

This work was funded by the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic VEGA 1/0569/24.

REFERENCES

1. **Goldová, M.** (n.d.): Babezióza a hepatozoonóza psov [Babesiosis and hepatozoonosis in dogs]. Univerzita veterinárskeho lekárstva a farmácie v Košiciach, Katedra epizootológie a parazitológie, Ústav parazitológie. Retrieved from https://infovet.sk/babezioza-a-hepatozoonoza-psov/.
2. **Chandoga, P., Goldová, M., Baranová, D., Kozák, M., 2002:** First cases of canine babesiosis in the Slovak Republic. *Vet. Rec.*, 150, 3, 82–84. DOI: 10.1136/vr.150.3.82
3. **Ayoob, A. L., Hackner, S. G., Prittie, J., 2010:** Clinical management of caninebabesiosis. *J. Vet. Emerg. Crit. Care*, 20, 1, 77–89. DOI: 10.1111/j.1476-4431.2009.00489.x
4. **Springer, A., Lindau, A., Probst, J., Fachet, K., Schäfer, I., Dobler, G., Mackenstedt, U., Strube, C., 2024:** Germany-widecitizenscience study reveals spread of Babesia canis-infected Dermacentor reticulatus ticks by dogstravelling within the country. *Curr. Res. Parasitol. Vector-Borne Dis.*, 6, 100187. DOI: 10.1016/j.crpvbd.2024.100187
5. **Köster, L. S., Lobetti, R. G., Kelly, P., 2015:** Canine babesiosis: A perspective on clinical complications, biomarkers, and treatment. *Vet. Med.: Res. Rep.*, 6, 119–128. DOI: 10.2147/VMRR.S60431
6. **Salem, N. Y., Farag, H. S., 2014:** Clinical, hematologic, and molecular findings in naturally occurring Babesia canis vogeli in Egyptian dogs. *Vet. Med. Int.*, 2014, Article 270345. DOI: 10.1155/2014/270345
7. **Petrucelli, J. V., Bermúdez, S., 2017:** Clinical and Serological Evidence of Canine Anaplasmosis and Ehrlichiosis in Urban and Rural Panama. *Ann. of Clin. Cytol. and Pathol.*, 3, 1, 1050. Retrieved from https://www.jscimedcentral.com/public/assets/articles/clinicalcytology-3-1050.pdf
8. **Furlanello, T., Fiorio, F., Caldin, M., Lubas, G., Solano-Gallego, L., 2005:** Clinico pathological findings in naturally occurring cases of babesiosis caused by large form Babesia from dogs of northeastern Italy. *Vet. Parasitol.*, 134, 1–2, 77–85. DOI: 10.1016/j.vetpar.2005.07.016
9. **Kettner, F., Reyers, F., Miller, D., 2003:** Thrombocytopaenia in canine babesiosis and its clinical usefulness. *J. S. Afr. Vet. Assoc.*, 74, 3, 63–68.
10. **Narurkar, R., Mamorska-Dyga, A., Agarwal, A., Nelson, J. C., Liu, D., 2017:** Babesiosis-associated immune thrombocytopenia. *Stem Cell Investig.*, 4, 1. DOI: 10.21037/sci.2017.01.02
11. **Abakpa, S. A. V., Mustapha, E. I., Mshelbwala, F. M., Idowu, K. R., Olasoju, M. I., Akintunde, O. G., et al., 2023:** Prevalence and role of anemia in mortalities of dogs with babesiosis. *World Vet. J.,*, 13, 3, 373–378. DOI: 10.54203/scil.2023.wvj41
12. **Zygner, W., Gójska-Zygner, O., Norbury, L. J., 2023:** Pathogenesis of anemia in caninebabesiosis: Possible-contribution of pro-inflammatorycytokines and chemokines—A review. *Pathogens*, 12, 2, 166. DOI: 10.3390/pathogens12020166
13. **Kučer, N., Matijatko, V., Kiš, I., Grden, D., Brkljačić, M., Foršek, J., et al., 2008:** White blood cell count and neutrophil to lymphocyte ratio in uncomplicated and complicated canine babesiosis caused by *Babesia canis canis*. *Veterinarski Arhiv*, 78, 4, 321–330.

14. **Jacobson, L. S., 2006:** The South African form of severe and complicated canine babesiosis: clinical advances 1994–2004. *Vet. Parasitol.*, 138, 1–2, 126–139. DOI: 10.1016/j.vetpar.2006.01.047
15. **Schetters, T. P. M., Kleuskens, J. A. G. M., van de Crommert, J., de Leeuw, P. W. J., Finizio, A. L., Gorenflot, A., 2009:** Systemicin flammatory responses in dogs experimentally infected with *Babesia canis*; a haematological study. *Vet. Parasitol.*, 162, 1–2, 7–15. DOI: 10.1016/j.vetpar.2009.02.012
16. **Goddard, A., Leisewitz, A. L., Kristensen, A. T., Schoeman, J. P., 2015:** Platele tactivation and platelet–leukocyte interaction in dogs naturally infected with *Babesia rossi*. *Vet. J.,* 205, 3, 387–392. DOI: 10.1016/j.tvjl.2015.05.008
17. **Solano-Gallego, L., Baneth, G., 2011:** Babesiosis in dogs and cats – expanding parasitological and clinical spectra. *Vet. Parasitol.*, 181, 1, 48–60. DOI: 10.1016/j.vetpar.2011.04.023
18. **Bilwal, A. K., Mandali, G. C., Tandel, F. B., 2018:** Liver Enzyme Activity in Dogs Infected with *Babesia canis*. *Intas Polivet*, 19, 2, 97–100.
19. **Schoeman, J. P., 2008:** Canine Babesiosis: An Update. In *World Small Animal Veterinary Association World Congress Proceedings.* Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa.
20. **Baneth, G., 2018:** Antiprotozoal treatment of canine babesiosis. *Vet. Parasitol.*, 254, 58–63. DOI: 10.1016/j.vetpar.2018.03.001
21. **Malinovská, Z., 2024:** Canine Babesiosis and Therapy Options – A Review. *Folia Veterinaria*, 68, 2, 49–56. DOI: 10.2478/fv-2024-0017
22. **Pawełczyk, O., Kotela, D., Asman, M., Witecka, J., Wilhelmsson, P., Bubel, P., Solarz, K., 2022:** The first records of canine babesiosis in dogs from Dermacentor reticulatus-freezone in Poland. *Pathogens*, 11, 11, 1329. DOI: 10.3390/pathogens11111329

sciendo

DOI: 10.2478/fv-2025-0015

FOLIA VETERINARIA, 69, 2: 28—36, 2025

ORIGINAL ARTICLE

EVALUATION OF LYOPHILIZED PCR MIXES STABLE AT ROOM TEMPERATURE FOR DETECTION OF VIRUSES

Božena Kočíková, Alica Pavlová, Anna Jacková, Štefan Vilček

Department of Epizootiology, Parasitology and Protection of One Health, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia

OPEN ACCESS

***Correspondence:** bozena.kocikova@uvlf.sk

Citation: Kočíková, B., Pavlová, A., Jacková, A., Vilček, Š., 2025: Evaluation of lyophilized PCR mixes stable at room temperature for detection of viruses. Folia Veterinaria, 69, 2, 28–36.

Received: April 8, 2025

Accepted: May 9, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Polymerase chain reaction is the most common laboratory method used in diagnosis of infectious diseases. It still requires cold-chain transportation and storage of reaction components, adequate instrumentation, as well as a skilled specialist during the preparation of reaction mixes. This study was focused on the evaluation of pre-prepared freeze-dried PCR mixes for virus detection in ready-to-use form. PCR mixes with new commercial glycerol-free DNA polymerase, cryoprotectants as trehalose, dextran or mannitol and other PCR components were lyophilized and stored at room temperature for several months. Our study revealed that 10 % trehalose, 5 % dextran and their combination stabilized PCR mixes for the detection of porcine circovirus 2 (PCV2) and hepatitis E virus (HEV) for 3 months. The freeze-dried PCR mixes detected those viruses in concentrated and 10 to 100-fold diluted clinical samples. The lyophilized PCR mixes containing commercially available glycerol-free DNA polymerase stabilized with sugar cryoprotectants were suitable for the preparation of diagnostic PCR kits.

Key words: cryoprotectant; dextran; HEV; lyophilization; PCR; PCV2; trehalose

INTRODUCTION

Polymerase chain reaction represents a gold standard in molecular biology for nucleic acid amplification and detection, and it is widely used in the diagnosis of human and animal infectious diseases [1]. Over recent decades, various modifications of PCR methods for the detection of different viruses have been developed, ranging from single PCR, reverse transcriptase PCR, nested PCR, multiplex PCR [2], quantitative PCR [3], through to digital droplet PCR [4]. However, all of these methods require cold-chain transportation and storage, adequate instrumentation, as well as skilled specialists conducting the laboratory process. Moreover, multiple pipetting steps of small volumes (1-10 µl) may lead to errors during the preparation of PCR mixes.

The pre-prepared lyophilized PCR mixes (instant PCR) present a way to simplify PCR assays for the detection of microorganisms. Such mixes may be stored at room temperature and are ready to use. Instant PCR minimizes the handling of PCR reagents, including pipetting small liquid volumes with possible errors, requires less skilled personnel, and minimizes the risk of contamination. Therefore, the preparation of dry PCR mixes for the detection of bacterial infections [5, 6, 7, 8] is not surprising. In addition, the approach has also been used for the detection of viruses such as hepatitis B virus [9] and other viruses [8, 10, 11, 12].

However, experience in different laboratories has shown that the preparation of mixes for instant PCR using lyophilization can meet problems. Glycerol, which is used for the protection of DNA polymerase during low-temperature storage (-20 °C), cannot be completely removed by sublimation because of its super hygroscopicity. To stabilize DNA polymerase activity during lyophilization, additional cryoprotectants have also been used in freeze-dried mixes, most commonly sugars, including disaccharides and polymeric saccharides [13].

Recently, commercial glycerol-free DNA polymerase has become available. This form of enzyme opens up new possibilities for the development of instant PCR assays using lyophilized reagents. The aim of this study was to evaluate the stability of freeze-dried, easy-to-use PCR mixes containing glycerol-free DNA polymerase for instant PCR to detect viruses in clinical samples. To keep the enzyme stable, three different types of cryoprotectants (trehalose, dextran, and mannitol) and a combination of trehalose with dextran were added to our PCR reaction mixes. The lyophilized mixtures were tested for the detection of the DNA porcine circovirus 2 (PCV2), which causes post-weaning multiple systemic wasting syndrome in pigs, and hepatitis E virus (HEV), a zoonotic RNA virus that infects pigs and can cause hepatitis E in humans.

MATERIALS AND METHODS

Experimental strategy and viruses

In the development stage of this project, the testing of glycerol-free lyophilized mixes was carried out with two positive clinical samples confirmed by classic PCR (without cryoprotectant) found in our previous works. One sample originated from a naturally PCV2-infected pig on a Slovakian farm [14], and the other one from liver tissue of a naturally HEV infected hunted wild boar [15]. Subsequently, optimal lyophilized PCR mixes were used for the detection of both viruses on a small collection of clinical samples which were previously positive by classic PCR.

DNA/RNA isolation

Pooled organ suspensions (tonsils, spleen, and kidney) from PCV2-infected pigs were prepared as 20 % w/v (weight/volume) tissue homogenates in PBS (Calbiochem, Darmstadt, Germany) or nuclease-free water (SERVA Electrophoresis GmbH, Heidelberg, Germany). Total DNA was extracted from 200 µl of tissue homogenate using Chelex resins (Bio-Rad Laboratories, Inc., Hercules, California, USA) according to manufacturer's recommendations.

Liver samples from wild boars and stool samples collected from human patients suspected of infection with HEV were homogenized in a 1 ml saline solution (Merck Millipore Corp., Rahway, New Jersey, USA). RNA was isolated from 400 µl of homogenate using the MagMAX™ Viral RNA Isolation Kit (Thermo Fisher Scientific, Inc., Vilnius, Lithuania) according to the manufacturer´s protocol and then stored at -80 °C.

cDNA synthesis

For HEV detection, the isolated RNA was transcribed to cDNA to be used with the lyophilized PCR mix. cDNA was synthesized using random hexamers and Premium reverse transcriptase with 1xRT buffer (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA), as described by Jacková et al. [15].

Cryoprotectants

Three different types of cryoprotectants added to the PCR mix were tested individually, namely D-(+)-Trehalose dihydrate (Merck, GmbH, Darmstadt, Germany), Dextran 100 (SERVA Electrophoresis GmbH, Heidelberg, Germany), and D-mannitol (Sigma-Aldrich, Burlington, Massachusetts, USA). They were used in different concentrations: 1, 3, and 5 % final concentration (w/v) for dextran and mannitol; 5, 10, and 15 % final concentration (w/v) for trehalose. Thereafter, stabilizers were evaluated at the optimal combination 10 % trehalose + 5 % dextran.

Preparation of lyophilized PCR mixtures

The reaction mixture was composed of 1x Standard Buffer (Ampliqon, Odense, Denmark), 0.2 mM dNTPs (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA), 300 nM of forward/reverse primers, and cryoprotectants in various concentrations (w/v). Finally, 1 U Taq DNA polymerase, glycerol-free (Ampliqon, Odense, Denmark), and molecular biology grade water (Merck, GmbH, Darmstadt, Germany) were added to give a final 23 µl volume. The published PCR primers flanking a 263 bp fragment of ORF2 for detection of PCV2 [16] and a 514 bp fragment from ORF1 for detection of HEV [17] were used in PCR assays.

The PCR mixes were frozen at -20 °C overnight, and before lyophilization they were placed at -70 °C for an hour. The lyophilization was performed according to protocol, which is generally used for the stabilization of drugs and vaccines at our university. The freeze-drying process was carried out in a CoolSafe Freeze Dryer (LaboGene, Allerød, Denmark) at -54°C and a starting pressure of 8,423 kPa for 24 h. After 24 h the pressure dropped to approximately 0,061 kPa, and then PCR mixes were lyophilized. The PCR tubes containing the dried reagent mixes were enclosed with parafilm (Sigma-Aldrich, Burlington, Massachusetts, USA) and stored at room temperature (20 °C – 23 °C).

PCR with lyophilized mixes

To the lyophilized PCR mix, 23 µl of molecular biology grade water were added, then vortexed briefly to dissolve the dried pellet. Subsequently, 2 µl of DNA (PCV2 detection) or cDNA (HEV detection) was added to the reaction. PCR was carried out under the following thermal profile: 1 cycle at 95 °C for 5 min, and 35 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C (HEV) and 62 °C (PCV2) for 40 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min using Thermocycler C1000 (Bio-Rad Laboratories, Inc., Hercules, California, USA). The PCR amplification was performed periodically on the day of lyophilization (Day 0) and on Day 30, Day 60 and Day 90 after lyophilization. Electrophoresis was carried out in 2 % agarose gels, stained with GelRed™ (Biotium, Inc., San Francisco, California, USA) and visualized by Gel Doc EZ imager (Bio-Rad Laboratories, Inc., Hercules, California, USA).

Detection of viruses in clinical samples

The lyophilized glycerol-free PCR mixes with optimal chemical composition were used for detection of viruses in different clinical samples. PCV2 was detected in four organ suspensions from naturally infected pigs. To detect HEV, four liver samples from wild boars and four human stool samples from patients suspected of having hepatitis E were chosen.

RESULTS

Evaluation of physical properties of lyophilized PCR mixtures

In preliminary experiments (data not shown), the optimal concentration of cryoprotectants was found. Finally, three various cryoprotectants at the optimal concentration were used for lyophilization in subsequent experiments.

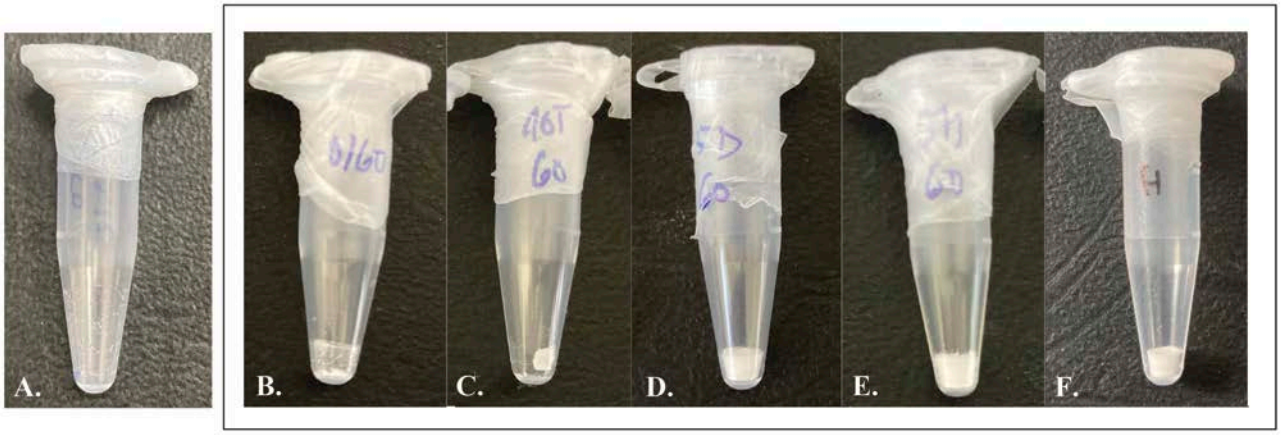

Fig. 1. Physical appearance of lyophilized PCR mixes. A. PCR mix containing glycerol. In frame (B.-F.) are glycerol-free-PCR mixes, B. PCR mix without protectants, C. PCR mix with 10 % trehalose, D. PCR mix with 5 % dextran, E. PCR mix with 5 % D-mannitol, F. PCR mix with 10 % trehalose + 5 % dextran

We observed various physical appearances of dried PCR mixes. While PCR mixes containing glycerol protected DNA polymerase seemed liquid without creating a powder (Fig. 1A), cryoprotectant-glycerol-free mixes appeared as a gentle dust (Fig. 1B). Mixes containing 10 % trehalose appeared as a light sticky, cake (Fig. 1C). Mixes with 5 % dextran, 5 % D-mannitol, and 10 % trehalose + 5 % dextran were observed after lyophilization as solid powder cakes with good appearance (Fig. 1 D – F). The dried mixes in the reaction tubes were stored at room temperature for analysis immediately after lyophilization and after 30, 60, and 90 days of storage.

The use of dried PCR mixes for detection of viruses

The PCR amplification was verified by the intensity of electrophoretic bands for DNA amplicons. To show results as clearly as possible, the electrophoretic bands for Day 30 and Day 60 have not been presented in Fig. 2 and Fig. 3, because the intensity of the bands gradually decreased to Day 90 or the bands were stable throughout 90 days.

Detection of PCV2 (Fig. 2A)

The lyophilized glycerol-free PCR mixes without cryoprotectants could detect the virus at Day 0 only but not after 30–90 days of storage. Similar intensity of electrophoretic bands was seen with concentrated as well as 10-fold and 100-fold diluted samples at Day 0 and Day 90 with PCR mixes containing 10 % trehalose or 5 % dextran. The combination of both cryoprotectants produced bands with stronger intensities than other mixes. The mixes stabilized with all concentrations of mannitol stop to work after 60 days of storage.

Detection of HEV (Fig. 2B)

The lyophilized glycerol-free reaction mixes with no cryoprotectant could detect virus in concentrated samples on Day 0 only. The presence of cryoprotectants significantly improved results of PCR amplification. Virus could be detected with similar intensity of bands at Day 0 and Day 90 in the presence of 10 % trehalose, 5 % dextran, as well as with a combination of 10 % trehalose + 5 % dextran in concentrated samples. Glycerol-free mixes with 5 % dextran were also able to detect virus in a 10-fold diluted sample. Mannitol at all concentrations did not protect PCR mixes even after 30 days of storage.

Detection of viruses in clinical samples

Following the above results, viruses in other clinical samples were detected with lyophilized solutions containing glycerol-free DNA polymerase protected with 10 % trehalose or a mixture of 10 % trehalose + 5 % dextran. Results with 5 % dextran did not confirm positive detection of viruses presented in Fig. 2 and they are not presented in Fig. 3.

The detection of PCV2 was successful up to Day 90 in almost all four clinical samples (Fig. 3A, lanes 1 – 4). PCR mixes stabilized by a mixture of 10 % trehalose + 5 % dextran provided more intensive electrophoretic bands than were observed with 10 % trehalose only.

Overall, four stool samples collected from human patients suspected of having hepatitis E (Fig. 3B, lanes 5 – 8) as well as four liver samples from wild boars (Fig. 3B, lanes 9 – 12) were used for HEV detection. On Day 0 and Day 90, virus could be detected in all four stool samples but only in one liver sample (lane 10).

DISCUSSION

This work has been initially inspired by McGoldrick et al. [18] who lyophilized in the lid of a PCR tube Taq DNA polymerase stored in glycerol together with trehalose and other reaction components for nested (second) PCR to detect pestiviruses. When the first PCR was finished, the tube was inverted, and the lyophilized reaction components were dissolved by shaking a tube to start the nested PCR with fresh chemicals. Renewed impetus for this project came when glycerol-free DNA polymerase recently appeared on the market. Here we present results that show that this form of enzyme is better for the preparation of lyophilized PCR mixes containing the cryoprotectants trehalose and dextran or their mixture. The glycerol-free lyophilized mixes stayed stable for at least 3 months at room temperature and could be reliably used for the detection of viruses in clinical samples, as demonstrated using PCV2 and HEV.

Generally, the freeze-drying method is utilized for various applications, such as stabilization of biological samples (proteins, peptides) and drug products at ambient temperature [19]. To stabilize samples during lyophilization, additives like polyethylen glycol, dextran, mannitol [20], collagen [21], bovine serum albumin [10], sorbitol,

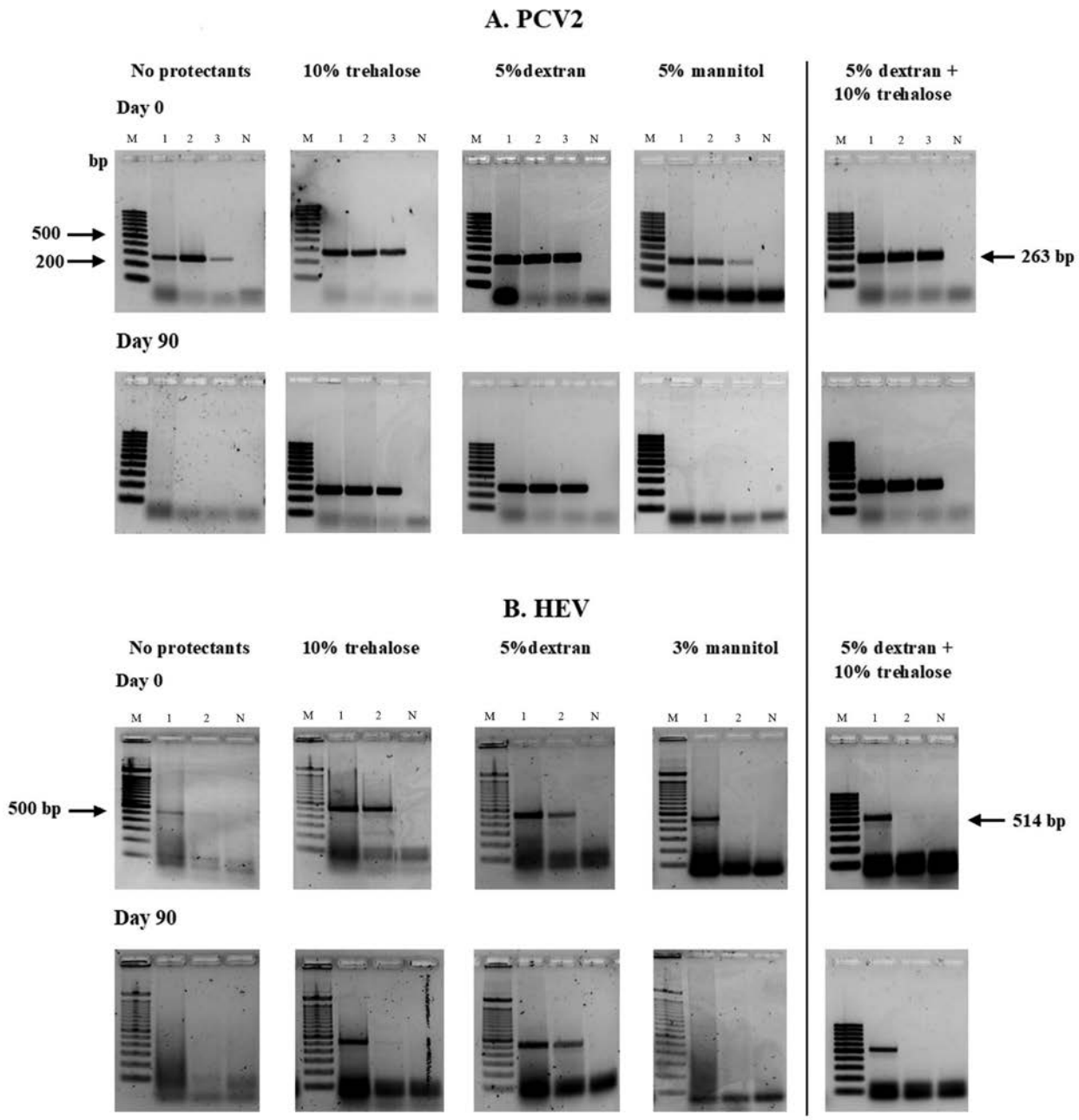

Fig. 2. Results of different cryoprotectants used on Day 0 (the day of lyophilization) and up to Day 90. A. PCV2 virus (total DNA in concentrated sample: 230 ng/µl), B. Hepatitis E virus (total RNA in concentrated sample: 138 ng/µl). Lanes: M, 100 bp ladder (Gene Ruler 100 bp, ThermoFisherScientific for PCV2 and combination of cryoprotectants for HEV; Invitrogen 100 bp, ThermoFisherScientific for HEV with individual cryoprotectant); 1, concentrated sample; 2, 10-fold diluted sample; 3, 100-fold diluted sample; N, negative control

gelatin, raffinose, Ficoll 400, and polyvinylpyrrolidone [9] have been used. At present, trehalose usually represents the most effective stabilizer during lyophilization.

In early experiments, trehalose found application for lyophilization of PCR mixes with glycerol-containing DNA polymerase for detection of various bacteria, e.g. *Mycobacteria* spp. [6], *Vibrio cholerae* [22], *Entamoeba* spp. [23], and others [5, 24, 25]. In addition, lyophilized real-time one-step RT-qPCR assays composed of glycerol DNA polymerase with sugar additives were developed to detect a few types of viruses, including foot-and-mouth disease virus [26], chikungunya virus, Rift Valley fever phlebovirus [27], as well as SARS-CoV-2 [10, 11].

Difficulty with sublimation of glycerol can be minimized by using glycerol-free PCR enzymes. There have been few reports where glycerol-free DNA polymerase has been used for the preparation of instant PCR. Khazani et al. [5] used such a form of polymerase for lyophilization in a

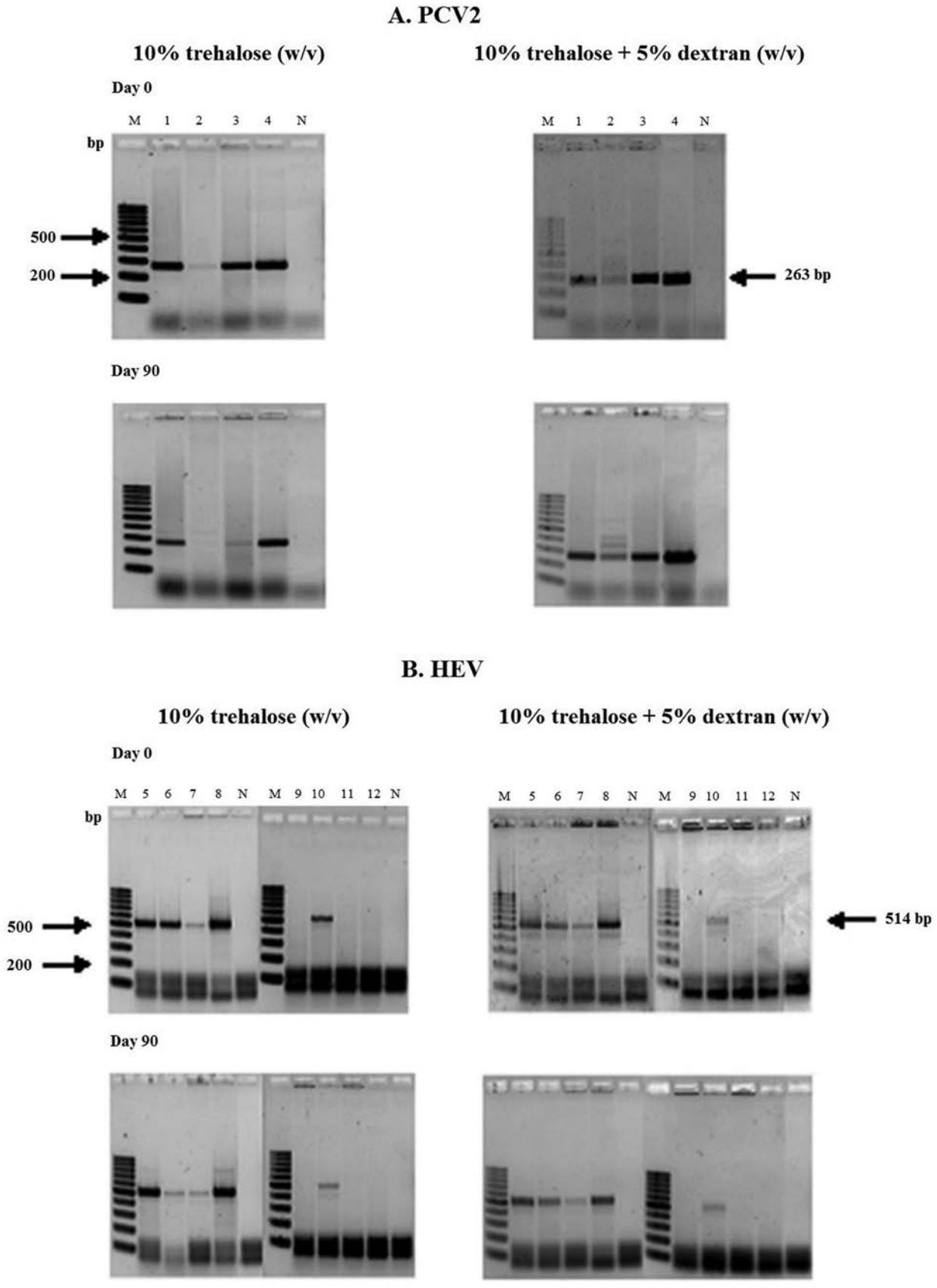

Fig. 3. Results of the presence of two types of cryoprotectants on clinical samples at Day 0 and up to Day 90. A. PCV2 virus, B. Hepatitis E virus. Lanes: M, 100 bp ladder (Gene Ruler 100 bp, ThermoFisherScientific); 1-4, organ suspensions from pigs; 5-8, human stool samples; 9-12, liver samples from wild boars; N, negative control

one-step PCR assay for detection of *Klebsiella pneumoniae* and *Haemophilus influenzae.* For virus detection, Yang and Wen [9] used self-prepared glycerol-free components for lyophilization of real-time PCR mixes for detection of the hepatitis B virus. Now commercially available glycerol-free DNA polymerase simplifies the whole process of instant PCR preparation.

Systematic analysis focused on the stability of lyophilized PCR mixes at -86 °C, -20 °C, 4 °C, 37 °C, and 56 °C showed that higher storage temperatures lead to reduced PCR efficiency [6, 8, 11, 25-27]. Since lower temperatures require refrigeration, we stored our reaction mixes at room temperature. In our hands, glycerol-free TaqDNA polymerase protected with sugar stabilizers in lyophilized mixes was stable for at least 3 months. In contrast, Agel and Sagcan [7] published only 2 weeks of stabilization of lyophilized mixes containing glycerol DNA polymerase and sugar additive for the diagnosis of tuberculosis using instant PCR assay. The lyophilized real-time one-step RT-qPCR mixes utilizing glycerol DNA polymerase and sugar stabilizer for detection of various viruses maintained activity at room temperature up to 30 days [10, 11, 12].

We have to point out that detection of HEV, which is RNA virus, was not carried out with lyophilized PCR mix in one-step due to problems with sublimation of glycerol protected reverse transcriptase. Therefore, we prepared cDNA by classical reverse transcription of HEV RNA to cDNA, which was used in the next instant PCR step. Meanwhile, glycerol free reverse transcriptase becomes available on the market. This form of enzyme will simplify its application in the freeze-drying process.

The combination of different cryoprotectants has synergic action [28, 29]. In our study, we also observed a synergic protective effect using trehalose with dextran in comparison to mixes with a single stabilizer.

We also tested pre-prepared glycerol-free PCR mixes on a limited collection of clinical samples. In all cases, viruses were detected in lymphoid organs (PCV2), liver homogenate, and human patient stool samples (HEV). These encouraging results show that preparation of diagnostic kits using lyophilized PCR mixes in the future is possible.

We believe that freeze-dried glycerol-free PCR components protected by sugar cryoprotectants can be used soon in the field where conditions for preservation of classical reaction mixes are minimal. Application of pre-prepared mixes is possible with portable PCR machines for the rapid diagnosis of various diseases directly under field conditions. In addition, such mixes can find application in countries where refrigeration capacities are limited.

CONCLUSIONS

In summary, our work demonstrated that the preparation and long-time storage at room temperature of lyophilized PCR mixes with glycerol-free DNA polymerase is a promising direction for the development of instant PCR assays. The cryoprotectants such as trehalose, dextran, and their combination can effectively protect enzymes and pre-prepared lyophilized glycerol-free PCR mixes and make them suitable for the detection of viruses in clinical samples.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Statement

The protocol for collection of animal clinical samples followed the guidelines stated in the Guide for the Care and Use of Animals (protocol number 3323/16–221/3) which was approved by the State Veterinary and Food Administration of the Slovak Republic and by Ethics Commission of the University of Veterinary Medicine and Pharmacy in Kosice, Slovakia. The collection of human stool samples was carried out in accordance with the International Code of Medical Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Ethics Committee of the Louis Pasteur University Hospital in Kosice, Slovakia (No. 104/2011).

Data Availability

The preprint of this manuscript is openly available in the online collaborative writing tool, Authorea, at http://doi.org/10.22541/au.172959374.48638715/v1.

Funding

This work was supported by VEGA project No. 1/0220/24 and by the project OPENMED

(ITMS2014+:313011V455) supported by the Operational Programme Integrated Infrastructure, funded by European Regional Development Fund.

Generative AI Statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Authors' Contributions

Božena Kočíková (Investigation – Equal, Methodology – Equal, Validation – Equal, Visualisation – Lead, Writing – original draft – Equal, Writing – review and editing – Equal), Alica Pavlová (Writing – review and editing – Equal), Anna Jacková (Funding acquisition – Equal, Supervision – Equal, Methodology – Supporting, Validation – equal, Writing – review and editing – Equal), Štefan Vilček (Conceptualization – Lead, Funding acquisition – Lead, Investigation – Lead, Methodology – Lead, Project administration – Lead, Supervision – Lead, Validation – Lead, Visualisation – Equal, Writing – review and editing – Lead).

Acknowledgements

Authors thank Dr. Sona Gancarcikova for help with lyophilization procedure, and to Dr. Peter Nettleton from Edinburgh for critical reading and English grammar correction of the manuscript.

REFERENCES

1. **Louten, J. 2016:** Detection and Diagnosis of Viral Infections. *Essential Human Virology,* 6, 111-132. DOI: 10.1016/B978-0-12-800947-5.00007-7
2. **Elnifro, E. M., Ashshi, A. M., Cooper, R. J., Klapper, P. E., 2000:** Multiplex PCR: optimization and application in diagnostic virology. *Clin. Microbiol. Rev.*, 13, 4, 559–570. DOI: 10.1128/CMR.13.4.559
3. **Mackay, I. M., Arden, K. E., Nitsche, A., 2002:** Real-time PCR in virology. *Nucleic Acids Res.*, 30, 6, 1292–1305. DOI: 10.1093/nar/30.6.1292
4. **Kojabad, A. A., Farzanehpour, M., Galeh, H. E. G., Dorostkar, R., Jafarpour, A., Bolandian, M., Nodooshan, M. M., 2021:** Droplet digital PCR of viral DNA/RNA, current progress, challenges, and future perspectives. *J. Med. Virol.*, 93, 7, 4182–4197. DOI: 10.1002/jmv.26846
5. **Khazani, N. A., Noor, N. Z., Yean Yean, C., Hasan, H., Suraiya, S., Mohamad, S. A., 2017:** Thermostabilized, One-Step PCR Assay for Simultaneous Detection of *Klebsiella pneumoniae* and *Haemophilus influenzae*. *J. Trop. Med.*, 2017, 7210849. DOI: 10.1155/2017/7210849
6. **Klatser, P. R., Kuijper, S., van Ingen, C. W., Kolk, A. H., 1998:** Stabilized, freeze-dried PCR mix for detection of mycobacteria. *J. Clin. Microbiol.*, 36, 6, 1798–1800. DOI: 10.1128/JCM.36.6.1798-1800.1998
7. **Agel, E. and Sagcan, H., 2020:** Optimization of Lyophilized LAMP and RT-PCR Reaction Mixes For Detection of Tuberculosis. *EuroBiotech J.*, 4, 4. DOI: 10.2478/ebtj-2020-0027
8. **Xu, J., Wang, J., Su, X., Qiu, G., Zhong, Q., Li, T., Zhang, D., Zhang, S., He, S., Ge, S., Zhang, J., Xia, N., 2021:** Transferable, easy-to-use and room-temperature-storable PCR mixes for microfluidic molecular diagnostics. *Talanta*, 235, 122797. DOI: 10.1016/j.talanta.2021.122797
9. **Yang, S. and Wen, W., 2021:** Lyophilized Ready-to-Use Mix for the Real-Time Polymerase Chain Reaction Diagnosis. *ACS Appl. Bio Mater.*, 4, 5, 4354–4360. DOI: 10.1021/acsabm.1c00131
10. **Xu, J., Wang, J., Zhong, Z., Su, X., Yang, K., Chen, Z., Zhang, D., Li, T., Wang, Y., Zhang, S., Ge, S., Zhang, J., Xia, N., 2020:** Room-temperature-storable PCR mixes for SARS-CoV-2 detection. *Clin. Biochem.*, 84, 73–78. DOI: 10.1016/j.clinbiochem.2020.06.013
11. **Hammerling, M. J., Warfel, K. F., Jewett, M. C., 2021:** Lyophilization of premixed COVID-19 diagnostic RT-qPCR reactions enables stable long-term storage at elevated temperature. *Biotechnol. J.*, 16, 7, e2000572. DOI: 10.1002/biot.202000572
12. **Thirion, L., Dubot-Peres, A., Pezzi, L., Corcostegui, I., Touinssi, M., de Lamballerie, X., Charrel, R. N., 2020:** Lyophilized Matrix Containing Ready-to-Use Primers and Probe Solution for Standardization of Real-Time PCR and RT-qPCR Diagnostics in Virology. *Viruses*, 12, 2, 159. DOI: 10.3390/v12020159
13. **Wilding, K. M., Zhao, E. L., Earl, C. C., Bundy, B. C., 2019:** Thermostable lyoprotectant-enhanced cell-free protein synthesis for on-demand endotoxin-free therapeutic production. *N. Biotechnol.*, 53, 73-80. DOI: 10.1016/j.nbt.2019.07.004
14. **Vlasakova, M., Leskova, V., Sliz, I., Jackova, A., Vilcek, S., 2014:** The presence of six potentially pathogenic viruses

in pigs suffering from post-weaning multisystemic wasting syndrome. *BMC Vet. Res.*, 10, 221. DOI: 10.1186/s12917-014-0221-8

15. **Jackova, A., Dudasova, K., Salamunova, S., Mandelik, R., Novotny, J., Vilcek, S., 2021:** Identification and genetic diversity of hepatitis E virus in domestic swine from Slovakia. *BMC Vet. Res.*, 17, 1, 232. DOI: 10.1186/s12917-021-02936-4
16. **Larochelle, R., Antaya, M., Morin, M., Magar, R., 1999:** Typing of porcine circovirus in clinical specimens by multiplex PCR. *J. Virol. Methods.*, 80, 1, 69–75. DOI: 10.1016/s0166-0934(99)00032-4
17. **Xia, H., Liu, L., Linde, A. M., Belák, S., Norder, H., Widén, F., 2008:** Molecular characterization and phylogenetic analysis of the complete genome of a hepatitis E virus from European swine. *Virus Genes.*, 37, 1, 39–48. DOI: 10.1007/s11262-008-0246-9
18. **McGoldrick, A., Bensaude, E., Ibata, G., Sharp, G., Paton, D. J., 1999:** Closed one-tube reverse transcription nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. *J. Virol. Methods.*, 79, 1, 85–95. DOI: 10.1016/s0166-0934(99)00010-5
19. **Nail, S. L., Jiang, S., Chongprasert, S., Knopp, S. A., 2002:** Fundamentals of freeze-drying. *Pharm. Biotechnol.*, 14, 281–360. DOI: 10.1007/978-1-4615-0549-5_6
20. **Ahlford, A., Kjeldsen, B., Reimers, J., Lundmark, A., Romani, M., Wolff, A., Syvänen, A. C., Brivio, M., 2010:** Dried reagents for multiplex genotyping by tag-array minisequencing to be used in microfluidic devices. *Analyst*, 135, 9, 2377–2385. DOI: 10.1039/c0an00321b
21. **Babonneau, J., Bernard, C., Marion, E., Chauty, A., Kempf, M., Robert, R., Marsollier, L., Franco-Beninese Buruli Research Group, 2015:** Development of a dry-reagent-based qPCR to facilitate the diagnosis of *Mycobacterium ulcerans* infection in endemic countries. *PLoS Negl. Trop. Dis*., 9, 4, e0003606. DOI: 10.1371/journal.pntd.0003606
22. **Chua, A. L., Elina, H. T., Lim, B. H., Yean, C. Y., Ravichandran, M., Lalitha, P., 2011:** Development of a dry reagent-based triplex PCR for the detection of toxigenic and non-toxigenic *Vibrio cholerae*. *J. Med. Microbiol.*, 60, 4, 481–485. DOI: 10.1099/jmm.0.027433-0
23. **Foo, P. C., Chan, Y. Y., See Too, W. C., Tan, Z. N., Wong, W. K., Lalitha, P., Lim, B. H., 2012:** Development of a thermostabilized, one-step, nested, tetraplex PCR assay for simultaneous identification and differentiation of *Entamoeba species*, *Entamoeba histolytica* and *Entamoeba dispar* from stool samples. *J. Med. Microbiol.*, 61, 9, 1219–1225. DOI: 10.1099/jmm.0.044552-0
24. **Al-Talib, H., Yean, C. Y., Al-Khateeb, A., Hasan, H., Ravichandran, M., 2014:** Rapid detection of methicillin-resistant *Staphylococcus aureus* by a newly developed dry reagent-based polymerase chain reaction assay. *J. Microbiol. Immunol. Infect.*, 47, 6, 484–490. DOI: 10.1016/j.jmii.2013.06.004
25. **Arunrut, N., Kiatpathomchai, W., Ananchaipattana, C., 2018:** Multiplex PCR assay and lyophilization for detection of *Salmonella* spp., *Staphylococcus aureus* and *Bacillus cereus* in pork products. *Food Sci. Biotechnol.*, 27, 3, 867–875. DOI: 10.1007/s10068-017-0286-9
26. **Sharma, G. K., Mahajan, S., Das, B., Ranjan, R., Kanani, A., Sanyal, A., Pattnaik, B., 2014**: Comparison of stabilisers for development of a lyophilised multiplex reverse-transcription PCR mixture for rapid detection of foot and mouth disease virus serotypes. *Rev. Sci. Tech.*, 33, 3, 859–867. DOI: 10.20506/rst.33.3.2323
27. **Thirion, L., Dubot-Peres, A., Pezzi, L., Corcostegui, I., Touinssi, M., de Lamballerie, X., Charrel, R. N., 2020:** Lyophilized Matrix Containing Ready-to-Use Primers and Probe Solution for Standardization of Real-Time PCR and RT-qPCR Diagnostics in Virology. *Viruses*, 12, 2, 159. DOI: 10.3390/v12020159
28. **Tonnis, W. F., Mensink, M. A., de Jager, A., van der Voort Maarschalk, K., Frijlink, H. W., Hinrichs, W. L., 2015:** Size and molecular flexibility of sugars determine the storage stability of freeze-dried proteins. *Mol. Pharm. Mar.*, 12, 3, 684- 694. DOI: 10.1021/mp500423z
29. **Gregorio, N. E., Kao, W. Y., Williams, L. C., Hight, C. M., Patel, P., Watts, K. R., Oza, J. P., 2020:** Unlocking Applications of Cell-Free Biotechnology through Enhanced Shelf Life and Productivity of E. coli Extracts. *ACS Synth. Biol.*, 9, 4, 766–778. DOI: 10.1021/acssynbio.9b00433

sciendo

DOI: 10.2478/fv-2025-0016

FOLIA VETERINARIA, 69, 2: 37—42, 2025

CASE REPORT

AN UNUSUAL FINDING OF A GUNSHOT WOUND CAUSED BY A DIABOLO PELLET IN THE LIVER OF A CAT: A CASE REPORT

Mária Makovická[1,2], Peter Bolgáč[3], Pavol Makovický[4], Klaudia Kráľová[5], Peter Makovický[1,2*]

[1]Cancer Research Institute, Biomedical Research Centre of the Slovak Academy of Sciences, Bratislava, Slovak Republic; [2]Institution of Histology and Embryology, Faculty of Medicine, University of Ostrava, Czech Republic; [3]Department of Pathology, Veterinary and Food Institute, Bratislava, Slovak Republic; [4]Department of Biology, Faculty of Education, J. Selye University, Komárno, Slovak Republic; [5]Department of Zoology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovak republic

OPEN ACCESS

***Correspondence:** pmakovicky@email.cz

Citation: Makovická, M., Bolgáč, P., Makovický, P., Kráľová, K., Makovický, P., 2025: An unusual finding of a gunshot wound caused by a diabolo pellet in the liver of a cat: A case report. Folia Veterinaria, 69, 2, 37–42.

Received: December 19, 2024

Accepted: May 6, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Here we report the accidental discovery of a gunshot wound involving a diabolo pellet in the liver of a cat. Toxicology results investigating the possibility of poisoning by anticoagulant rodenticide-type agents (brodifacoum, bromadiolone, difenacoum, diphethialone, flocoumafen, and warfarin) and for cholinesterase inhibitor pesticides (carbofuran) were negative. Internal examination discovered minor amounts of free blood in the chest cavity and uncoagulated blood in the heart cavities. The cardiac musculature of the ventricles is hypertrophied, and cavities of the ventricles are narrowed. The lungs exhibit an edema, and there are extensive confluent diffuse hemorrhages. The kidneys are light brown in color, soft in consistency, with dotted or diffuse hemorrhages in the cortex and marrow. There is dark red fluid in the bladder. There is a slightly protruding structure in the center of the right lateral lobe of the liver in view of the diaphragmatic surface, in which the diabolo pellet, with a wound, has been encased. Autopsy documents reveal renal failure. In relation to the autopsy findings, attention is paid to the impact of the release of lead from the diabolo pellet into the body, impairing filtration, leading to renal failure and death. In the puzzle of the cause of death based on autopsy and toxicological findings, the work is a good example of the importance of considering several factors in renal failure even without further investigation.

Key words: entrapped diabolo pellet; forensic medicine; gunshot wounds; renal impairment; veterinary pathology

INTRODUCTION

Injuries caused by firearms are usually life-threatening and often fatal. In this respect, air guns are generally considered less dangerous compared to, for example, professional hunters' weapons (rifles, shotguns). However, one cannot ignore the fact that airguns, as designed, have the potential ability to harm vital organs. Injuries, as well as deaths, of dogs and cats because of gunshot wounds have been reported in the literature [1, 2]. For example, in one study in a set of 171 animals that had been shot, a species ranking was compiled based on radiological and forensic examination, with dogs having the highest casualty rate (78.36 %), followed by cats (15.20 %), then rabbits (2.34 %), roe deer (1.17 %), and finally birds (2.92 %) [3]. Other studies also consider other forms of traumatic deaths. One reports that deaths of dogs and cats due to gunshot wounds were recorded at a lower rate than poisonings, but at a higher rate than deaths caused by blunt or sharp objects and occurred more frequently than deaths due to neglect [4]. In a study on traumatic deaths in cats, collisions with automobiles dominated, followed by attacks by other animals, followed by gunshot wounds [5]. In terms of therapeutic approaches, successfully resolved cases [6, 7, 8] as well as cases that were terminated by euthanasia [9, 10] have been documented in the literature. In doing so, it has been documented that up to 80 % of all gunshot wounds in cats were carried out with air guns [11]. Here, we report a case of an older airgun gunshot wound with an accidental finding of a diabolo pellet lodged in the liver. As such a case has not yet been described in the literature reviewed by us, we report it as unusual in relation to the primary cause of death in the form of renal failure. This work is a good example for veterinary forensic doctors and veterinary pathologists, where the causal relationships and causes of death of domestic cats are discussed based on the accidental finding of an encased diabolo pellet and renal failure.

CASE PRESENTATION

Case description

This is a domestic cat (♂, 9 years old) that was kept in home care with occasional outdoor activity. Cat was found lying unresponsive, breathing deeply and heavily. Despite receiving priority admission at the veterinary clinic, the doctor's repeated attempts to revive the cat were unsuccessful. As the owner claimed, she had seen the neighbor shooting an air gun from his backyard earlier and, moreover, throwing unknown chemicals around. The case was investigated as a suspected criminal offense of cruelty to an animal, the intentional and unjustified killing of an animal by an unknown perpetrator, in an unknown manner. An autopsy has been ordered with toxicology screening for evidence of possible poisoning. Toxicological examination was carried out on a liver sample using the accredited high-performance liquid chromatography (HPLC) method.

Necropsy findings

The body of a 5.5 kg male domestic cat was received in a red plastic bag, covered in soil and with green clover leaves present (Fig. 1A). Otherwise, the coat is predominantly white with alternating patches of light brown and grey to completely black coloration at the tail. A cannula is inserted on the left forearm. External inspection of the body is without definite pathology. No signs of exogenous trauma after skin removal (Fig. 1B-1C). After opening the abdominal cavity, increased visceral fat is seen (Fig. 2A). After opening the thoracic cavity, a small amount of free, uncoagulated blood was present. Increased functional fat of the heart and uncoagulated blood in the pericardium and in the heart. Hypertrophied cardiac ventricular musculature with narrowing of the ventricular space. Mucosa of the trachea is smeared with blood. Mild pulmonary edema with extensive superficial confluent hemorrhages on the lung surface and in the lung parenchyma. The kidneys are light brown in color, soft in consistency, with dotted or diffuse hemorrhages in the cortex and marrow (Fig. 2B). A dark red fluid is present in the bladder (Fig. 2C). The spleen is without pathological characteristics (Fig. 2B). The liver is macroscopically dark brown in color, but a slightly protruding structure of pale color is present in the imaginary center of the right lateral lobe in view of the diaphragmatic surface, in which a diabolo pellet from an air gun is embedded (Fig. 3A). The area surrounding the embedded diabolo pellet is free of hemorrhage, with no other changes with granulomatous tissue, which is in contact with liver tissue, but the base is in contact with the liver parenchyma (Fig. 3B, 3C). The entire intestinal tract is more or less anemic. The stomach is empty, and there is

Fig. 1. View of the cat's body from the left (A), and after skinning in both individual and close-up positions (B-C) with no clear visibility of exogenous trauma

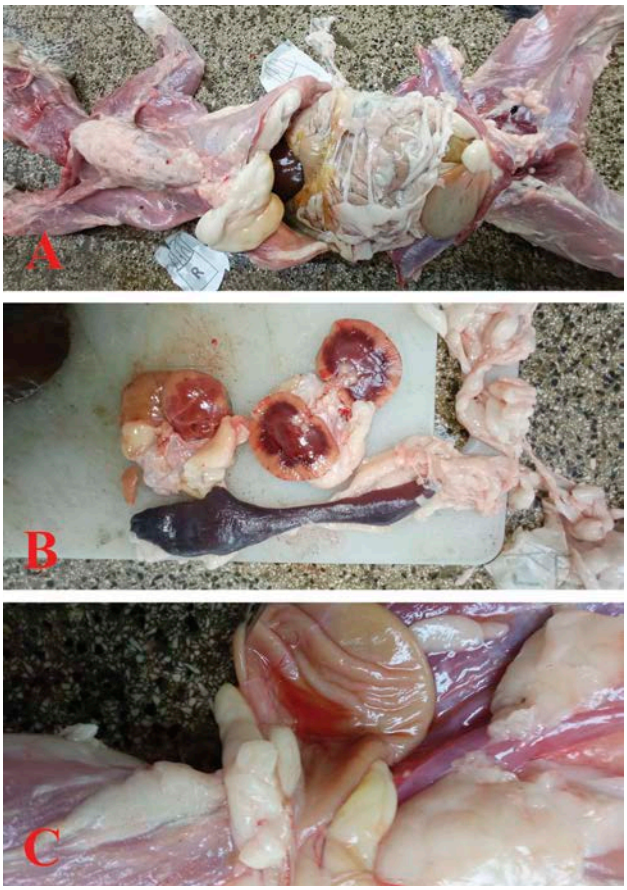

Fig. 2. View of individual internal organs with pale colored visceral fat (A). Kidneys tending to merge into a homogeneous dark red hue (B) with dark fluid in the bladder (C)

some slimy light brown coating on its mucosa. The intestines contain more pastosus than liquid contents of a light brown color, while the section near the rectum has firmer contents of a dark green color.

Toxicology results

The results of accredited screening tests for evidence of poisoning by anticoagulant rodenticides (brodifacoum, bromadiolone, difenacoum, difethialone, flocoumafen, warfarin) and for evidence of a cholinesterase inhibitor pesticide (carbofuran) are shown in Table 1. Within the range of the set of seven chemically relevant substances monitored, the presence of positivity was not documented in any case.

DISCUSSION

In our necropsy practice, we routinely encounter animal deaths due to gunshot wounds, and it is not always

Table 1. The results of toxicological examinations

Indicator	Measured values (mg/kg)	Limits of positivity determination (mg/kg)
Brodifacoum	<0.005	0.005
Bromadiolone	<0.005	0.005
Carbofuran	<0.005	0.005
Difenacoum	<0.005	0.005
Difethialone	<0.005	0.005
Flocoumafen	<0.005	0.005
Warfarin	<0.005	0.005

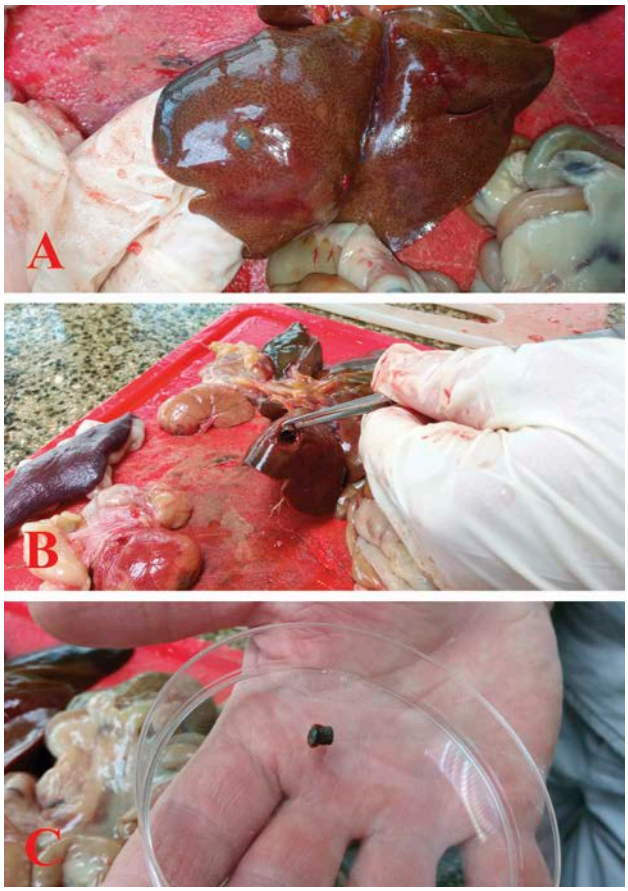

Fig. 3. View of the liver with parenchyma-fixed diabolo pellet (A) in contact with the liver parenchyma from the inside (B) and the diabolo pellet itself after dissection (C)

possible to trace the projectile. However, it is frequently possible to identify a gunshot trajectory with an entrance, a canal, and, possibly, an exit through which the projectile leaves the body and is unaccounted for. In our documented case, it was an older gunshot wound. Here, in our opinion, several alternatives can be considered in relation to the entry of the projectile, the gunshot canal, and the entrapment of the projectile in the liver. In the first case, the diabolo pellet passed cranially behind the ribs through the haired skin, the subcutaneous tissue, the space between the subcutaneous tissue and the peritoneum, then the peritoneum itself, then the space between the peritoneum and the liver, then became lodged between the diaphragm and the liver, being resorbed by the liver parenchyma over time. In fact, it is more likely that, along this pathway, it became trapped in the fatty tissue beneath the peritoneum, and in the process of gradual reparation of the wound, it was passively displaced into the space between the diaphragm and the liver, and only subsequently was it reabsorbed over time by the liver parenchyma itself. The second alternative is based on the assumption of a shot with the above trajectory, but with the intervention of the liver parenchyma, where it became stuck, while its localization on the diaphragmatic surface can be explained by the temporal passage through the liver parenchyma even with the complete regeneration of the liver. A third alternative is a penetrating shot elsewhere in the body, with the diabolo pellet entering the bloodstream and subsequently becoming trapped in the liver. We favor the first possibility because the others, although feasible, are less likely. Often, and especially in cases of human firearm injuries, the possible movements of the projectile are discussed. The blood vessels in the liver are comparatively thin, and it is less likely that by arterial or venous circulation the entire diabolo pellet would reach the end of the lobe. This is documented in the case of the shot cat also with transport of the diabolo pellet through the arterial circulation, which ended up as embolization of the abdominal aorta [12]. In our case, the superficial part of the liver covering the diabolo pellet was surrounded in granulomatous tissue with organization, but the inner and surrounding part of the wound may not have closed, while there may have been preserved contact with the liver parenchyma. Although we have closed the case as being an instance of renal failure, it is hypothetically possible that over the years lead may have been released from the diabolo pellet into the host's system. In practice, acute lead poisoning is particularly manifested by gastrointestinal distress, whereas chronic poisoning is presented with a broader symptomatology with damage to the reproductive organs, kidneys, and nervous system [13]. In doing so, a relationship between kidney damage and lead has been demonstrated [14]. This could be suggested by the autopsy findings of the kidneys with loosening of the parenchyma and hemorrhages. These facts could also be attributed to progressive autolytic changes, which would then be present in other organs as well. These, however, appeared vital in their macroscopic appearance. Evidence of damage to the kidneys is also provided by the dark-colored fluid in the bladder, which corresponds to impaired filtration. Here, however, it is controversial whether lead toxicity is manifested at low levels or only with repeated exposure to high levels. It is less likely that a single diabolo pellet is the cause of poisoning, but on the other hand, the results of studies that have confirmed the relationship between fragments of residual projectile parts in the body of shot individuals with lead admixture and elevated lead

levels in their blood cannot be ignored [15]. As long as lead compounds remain in contact with stomach acid, near the joint, or synovial fluid, lead poisoning can be possible, and deaths due to lead poisoning have been reported in this way [16]. Although we have not been able to verify blood lead levels in the setting of basic necropsy equipment, we can at least theoretically concede that the stuck diabolo pellet may have contributed to the development of pathological changes that ultimately resulted in renal failure. A basic set of toxicological investigations looking for evidence for anticoagulant rodenticide poisoning and for the pesticide cholinesterase inhibitor were negative in this case. Other types of poisoning are less likely, although this was a domestic cat that had been kept in a household, went out into the garden, and was accustomed to meat and meat products. He may have taken these from other people or accidentally eaten them. Based on our experience with the types of poisoning from this area, this reduces the likelihood of poisoning by another active substance. Despite his age and being overweight with increased stores of depot tissue, cardiovascular failure is a less likely cause of death as well. Here, we rely on our autopsy findings of free blood in the thoracic cavity and free uncoagulated blood in the cardiac cavities. Such findings are more common in poisonings. In cases with direct evidence of the presence of metabolites of anticoagulant rodenticides alongside cholinesterase inhibitor pesticides, the findings are accompanied by dark-colored, bloody viscera. Further, with the presence of more or less uncoagulated blood in the abdominal cavity and the thoracic cavity, hematomas in the musculature, discharge of uncoagulated blood from body orifices, and uncoagulated blood in the heart. In our case, the gastrointestinal tract was pale colored, and there was renal damage with failure of filtration and blood passing into the urine. These facts are commonly reported in adult cats, and our case could also, ultimately, be concluded as chronic kidney disease resulting in renal failure [17, 18]. However, our case was a domestic cat, and in addition, we documented the aforementioned diffuse hemorrhages in the lungs in the autopsy findings, but they were not clearly present in other organs. Therefore, without the benefit of other examinations, we concluded the case as being an instance of renal failure, which could have had multiple causes, including the possibility of the role of lead compounds from the trapped diabolo pellet in the liver.

CONCLUSION

We discuss the causes of death based on the accidental diagnostic finding of a gunshot wound with an entrapped diabolo pellet in the liver of a cat. Autopsy documented that it was renal failure, which, combined with the above finding, opens up a discussion of the possible role of lead with progressive filtration impairment and renal failure that ultimately led to death. These facts provide a good example for veterinary forensic doctors and veterinary pathologists to consider combinations of causes of renal failure in the setting of basic necropsy equipment without further special investigations with suspected and subsequently unproven poisoning.

Conflict of Interest

The authors declare no conflict of interest.

Funding

This article received no specific grant from any funding agency in the public, commercial and any other private profit sectors.

Generative AI Statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Authors' Contributions

Conceptualization: MaM and KK. Methodology: PB and PaM. Investigation: PB and PaM. Data curation and supervision: PB and PeM. Writing–original draft preparation: MaM. Writing–review and editing: KK and PeM.

Acknowledgement

Thanks to Simon Charlesworth for proof-reading and English grammar corrections.

REFERENCES

1. **Munro, H. M., Thrusfield, M. V., 2001:** Battered pets: non-accidental physicical injuries found in dogs and cats. J. *Small Anim. Pract.,* 42, 6, 279–290. DOI: 10.1111/j.1748-5827.2001.tb02041.x
2. **Olsen, E. L., Streeter, E. M., DeCook, R. R., 2014:** Review of gunshot injuries in cats and dogs and utility of a triage scoring system to predict short-term outcome: 37 cases (2003-2008). *J. Am. Vet. Med. Assoc.,* 245, 8, 923–929. DOI: 10.2460/javma.245.8.923
3. **Listos, P., Komsta, R., Lopuszynski, W., Gryzinka, M., Teresinski, G., Chagowski, W., Buszewicz, G., Dylewska, M., 2016:** Radiological and forensic veterinary analysis of gunshot cases in eastern Poland. *Med. Wet-Vet. Med. Sci. Pract.,* 72, 7, 453–457. DOI: 10.21521/mw.5531
4. **Radojkovic, J. A., Nesic, V., Bozovic, A. I., Davitkov, D., Djuric, M., Krstic, V., Davitkov, D., 2024:** Cruelty towards dogs and cats in the republic of Serbia during a 10 – year period. *Animals (Basel).,* 14, 13, 1926. DOI: 10.3390/ani14131926
5. **Olsen, T. F., Allen, A. L., 2001:** Causes of sudden and unexpected death in cats: A 10-year retrospective study. *Can. Vet. J.,* 42, 1, 61–62.
6. **Job, Ch., Bureau, S., Faucher, M., 2024:** Video-asisted ventral bulla osteotomy to remove a bullet foreign body in a cat. *J. Am. Anim. Hosp. Assoc.,* 60, 5, 219–222. DOI: 10.5326/JAAHA-MS-7417
7. **Pavletic, M. N., Trout, N. J., 2006:** Bullet, bite, and burn wounds in dogs and cats. *Vet. Clin. North Am-Small. Anim. Pract.,* 36, 4, 873. DOI: 10.1016/j.cvsm.2006.02.005
8. **Sunghan, J., Khantaprab, N., Thongtharb, A., 2020:** Bullet-induced chronic cystitis in cat. *Turk. J. Vet. Anim. Sci.,* 44, 2, 469–472. DOI: 10.3906/vet-1912-1
9. **Ahn, S. Y., Yoon, H. Y., Jeong, S. W., 2015:** A Case of Gunshot Injury to the Spinal Cord in a Cat: Clinical, Surgical, and Computed Tomographic Features. *J. Vet. Clin.,* 32, 2, 187.
10. **Withoeft, J. A., Cristo, T. G., Biezus, G., Costa, L. S., Dal Pont, T. P., Freitas, A. C., Traverso, S. D., Casagrande, R. A., 2019:** Small Animal Diseases Causes of death and euthanasia in domestic cats in the Santa Catarina plateau (1995-2015). *Pesq. Vet. Bras.,* 39, 3, 192–200. DOI: 10.1590/1678-5150-pvb-5814
11. **Vnuk, D., Capak, H., Gusak, V., Maticic, D., Popovic, M., Bottegaro, N. B., 2016:** Metal projectile injuries in cats: review of 65 cases (2012–2014). *J. Feline Med. Surg.,* 18, 8, 626–631. DOI: 10.1177/1098612X15590869
12. **Kettner, F., Kirberger, R. M., 2006:** Aortic foreign body (airgun pellet) embolism in a cat. *J. Small Anim. Pract.,* 47, 4, 221–225. DOI: 10.1111/j.1748-5827.2006.00077.x
13. **Goyer, R. A., 1990:** Lead toxicity: from overt to subclinical to subtle health effects. *Environ. Health Perspect.,* 86, 6, 177–181. DOI: 10.1289/ehp.9086177
14. **Flora, G., Gupta, D., Tiwari, A., 2012:** Toxicity of lead: A review with recent updates. *Interdiscip. Toxicol.,* 5, 2, 47–58. DOI: 10.2478/v10102-012-0009-2
15. **Apte, A., Bradford, K., Dente, Ch., Smith, R. N., 2019:** Lead toxicity from retained bullet fragments: A systematic review and meta-analysis. *J. Trauma Acute Care Surg.,* 87, 3, 707–716. DOI: 10.1097/TA.0000000000002287
16. **DiMaio, V. J., DiMaio, S. M., Garriott, J. C., Simpson, P., 1983:** A fatal case of lead poisoning due to a retained bullet. *Am. J. Forensic Med. Pathol.,* 4, 2, 165–169. DOI: 10.1097/00000433-198306000-00013
17. **Lawson, J. S., Jepson, R. E., 2021:** Feline comorbidities: The intermingled relationship between chronic kidney disease and hypertension. *J. Feline Med. Surg.,* 23, 9, 812–822. DOI: 10.1177/1098612X211037872
18. **Xavier, F. A. F., Almeida, A. D., Souza, R. M., Araujo, S. L., Pereira, T. H. D., Paiva, D. D. D., Morais, G. B., Evangelista, J. S. A. M., 2020:** Ultrasonography in cats with chronic kidney disease – Literature review. *Braz. J. Hyg. Anim. Sanity,* 14, 4, 360.

sciendo

DOI: 10.2478/fv-2025-0017

FOLIA VETERINARIA, 69, 2: 43—55, 2025

ORIGINAL ARTICLE

SERO-EPIDEMIOLOGY OF *COXIELLA BURNETII* INFECTION IN DOMESTIC RUMINANTS IN THE PREFECTURES OF BEYLA AND MAMOU, GUINEA

Mama Agnès Tea[1,2,3*], Dieudonné Tialla[2,3,4], Lanan Wassy Soromou[1,6], Albert Sourou Salako[1], Lanceï Kaba[1,7], William Tchabo[1], Martin Bienvenu Somda[4,5]

[1]Institute of Veterinary Sciences and Medicine/Dalaba, Dalaba, Guinea; [2]Microbiology-Epidemiology, Zoonosis and One Health Laboratory (LMEZOH), Laboratory of Research on Infectious and Parasitic Diseases (LRMIP), Unit of Epidemic-Prone Diseases, Emerging Diseases and Zoonosis (UMEMEZ), National Reference Laboratory for Influenza (LNR-G), Department of Biomedical and Public Health, Health Science Research Institute (IRSS), Ouagadougou, Burkina Faso; [3]National Center for Scientific and Technological Research (CNRST), Ouagadougou, Burkina Faso; [4]Institute of Rural Development (IDR), Nazi BONI University (UNB), Bobo-Dioulasso, Burkina Faso; [5]International Centre for Research and Development on Subhumid Livestock (CIRDES), Bobo-Dioulasso, Burkina Faso; [6]University of Labé, Labe, Guinea; [7]Guinean Centre for Research and Training in Infectious Diseases, Conakry, Guinea

OPEN ACCESS

***Correspondence:** teaagnesmama@gmail.com

Citation: Tea, M. A., Tialla, D., Soromou, L. W., Salako, A. S., Kaba, L., Tchabo, W., Somda, M. B., 2025: Sero-epidemiology of *Coxiella burnetii* infection in domestic ruminants in the prefectures of Beyla and Mamou, Guinea. Folia Veterinaria, 69, 2, 43–55.

Received: April 28, 2025

Accepted: May 19, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

This study determined the seroprevalence and identified risk factors for exposure to *Coxiella burnetii* in domestic ruminants in the prefectures of Mamou and Beyla in Guinea. A structured qualitative and quantitative questionnaire was administered by trained interviewers to 48 people, mainly livestock farmers. Besides, 216 serum samples from 75 cattle, 69 sheep, and 72 goats were analysed by indirect enzyme-linked immunosorbent assay (iELISA) for *Coxiella burnetii* IgG and IgM antibodies. The results of the survey showed that farmers had little information about Q fever. Of 216 serum samples analysed from 48 different herds, 101 (46.76 %) animals and 18 (37.5 %) herds were positive for *Coxiella burnetii*. The seroprevalence was significantly higher in Beyla (54.63 %) than in Mamou (38.89 %) (p = 0.029). It was 52.78 % in goats, 40.58 % in sheep and 46.67 % in cattle (p = 0.34). Binary logistic regression analysis showed that tick infestation, age, sex and contact with other animals had a significant impact on the prevalence of Q fever. Given its zoonotic and economic impact, it is important to establish a disease surveillance system in all regions of the country. Further research, particularly in molecular diagnosis, is needed to confirm the involvement of Q fever in cases of reproductive disorders in domestic ruminants in Guinea.

Key words: *Coxiella burnetii*; domestic ruminants; Guinea; risk factors; seroprevalence

INTRODUCTION

Coxiella burnetii is an obligate intracellular Gram-negative bacterium responsible for Q fever [1]. It is a zoonotic disease for which domestic ruminants: cattle, sheep and goats are the reservoirs [2, 3]. Slaughterhouse workers, veterinary officers, and livestock farmers are most exposed to this infection [4]. Animal-to-animal transmissions are important in the sense that they contribute to the persistence and circulation of the bacteria in nature [5, 6]. However, the vast majority of human epidemics of Q fever are epidemiologically related to small ruminants, highlighting their critical role in the zoonotic transmission of *Coxiella burnetii* [7]. Humans mostly contract *Coxiella burnetii* infections mainly through zoonotic route by inhaling contaminated dust with birth debris, abortion, urine, or faeces from infected animals [8]. In addition, infections from contaminated food or unpasteurized milk and transmission by ticks can occur [9, 10]. For this reason, consequently, this disease represents a significant economic burden, by causing, production losses due to reproductive disorders such as infertility, stillbirths, abortions, and the weakness of the offspring, as well as the costs of implementing vaccination and control programmes [11].

Q fever is cosmopolitan with the exception of New Zealand [12]. Information on the epidemiology of this disease has been reported in humans and domestic ruminants in 24 of 54 African countries over the last two decades. Mean seroprevalence estimates were 16 % (95 % CI [11 – 23 %]) in humans, 14 % (95 % CI [10 – 20 %]) in cattle, 13 % (95 % CI [9 – 18%]) in sheep, and 21 % (95 % CI [15 – 29 %]) in goats [13]. In West Africa, several surveys have reported variable prevalences of Q fever in ruminants and humans. Bovine prevalence was estimated at 4 % in Senegal [14], 18 % (30/166) in Ghana [15], 2 % in Togo [16], 10.44 % in northern Nigeria [17], 26.2 % in Burkina Faso [18], and 55.3 % in Mali [19]. In humans, estimated seroprevalences of 10 % and 17 % were recorded in children in Niger [20] and Ghana [21], respectively. As for small ruminants, respective prevalences of 3.16 % and 3.8 % in northern Nigeria [17], 22.6 % and 16.9 % in Mali [19], and 38.2 % and 30.3 % in Burkina Faso [6] were detected in sheep and goats, respectively. Another study reported a prevalence of 21.59 % in small ruminants in Mali [22].

In Guinea, very few data are available on Q fever [23]. While this pathology was previously considered a rare tropical disease restricted to Africa, the findings of some studies show that it circulates in many prefectures of Guinea. In a study carried out in the four regions of Guinea, respective seroprevalences of 0.4 %, 6.9 %, 9,3 %, and 9.2 % were found in Forest Guinea, Upper Guinea, Middle Guinea, and Maritime Guinea, respectively [24]. A few years later, Troupin et al. [25] reported overall prevalence of 20.5 %, 4.4 %, and 2.3 % respectively in cattle, goats and sheep, in the country. This study detected Q fever in cattle in 14 (93 %) out of the 15 prefectures visited, in goats in 11 (69 %), and in sheep in 9 (56 %) out of 16 prefectures [25]. Recently, Naidenova et al. [26] reported the first official case of human Q fever in Mamou (Middle Guinea), admitted to the infectious diseases department of the regional hospital. This suggests that Q fever could be considered a public health problem in Guinea, but due to lack of vigilance on the part of hospital health staff, this infectious disease is underdiagnosed.

Beyla and Mamou are two prefectures located in two different eco-geographical regions of Guinea, where livestock farming represents the second major economic activity after agriculture. However, Q fever prevalences above 20 % were reported between 2017 and 2019 in Dalaba, Dabola, Faranah, and Kouroussa, four sub-prefectures of Mamou. In Belya, located in Forest Guinea, a higher prevalence estimated at 25.9 % was reported by Barry et al. [27]. According to the authors of these studies, climatic conditions with high relative humidity favor the maintenance of the bacteria and a high prevalence of the disease. Indeed, the prefecture of Beyla borders northern Côte d'Ivoire, where seroprevalences of 13.9 %, 9.4 %, and 12.4 % were reported, respectively in cattle, sheep, and goats [28]. Livestock, often transhumant, congregates along this border, sharing watering points and grazing areas.

The aim of this study was to determine the seroprevalence of Q fever in domestic ruminants in the prefectures of Mamou and Beyla and the risk factors associated with transmission among livestock keepers in these localities. The results will provide a better understanding of the epidemiological aspects of this disease, leading to the definition of appropriate prevention and control strategies.

MATERIALS AND METHODS

Study area

Epidemiological data were collected in two prefectures belonging to two different eco-geographical regions: the prefecture of Mamou, located in Middle Guinea, and the prefecture of Beyla, in Forest Guinea (Figure 1). Mamou covers an area of 8,000 km^2 with an estimated population of 318,981 inhabitants, while Beyla has an area of 15,500 km^2 with an estimated population of 379,337 people [29]. The prefectures of Mamou (Middle Guinea) and Beyla (Forest Guinea) are two distinct areas from each other in terms of climate and relief. Mamou is characterized by a tropical climate with the alternation of two seasons: a dry season (from November to May) and a rainy season (from June to October). As for the climate of Beyla, it's tropical wet and dry with two seasons: a rainy season from April to mid-November and a dry season from mid-November to March. Average annual rainfall can be up to 2,400 mm in Mamou but varies between 1,700 mm and 3,000 mm in Beyla. The temperatures vary from 10 ºC to 33 ºC, with an average of 22 ºC in Mamou, and vary between 19 ºC and 29 ºC, with an annual average of 24 ºC in Beyla [30]. The average aridity index is approximately 1.30 in Mamou compared to 1.97 in Beyla. The Mamou region is dominated by tropical savannahs, mountain forests, gallery forests, shrub and grass savannahs, plateaus and plains. As for Beyla, this area is characterised by a transition zone between the savannahs of Upper Guinea and the forest, although the forest is little developed in this region.

Both areas are crossed by numerous rivers and streams that irrigate neighboring countries, making Guinea the water tower of the sub-region. The main economic activity is agriculture, followed by livestock farming. Livestock farming is essentially family-based, with most livestock migrating throughout the year except during the cropping season in both zones in both areas. Common livestock consists of cattle, goats and sheep. However, the grouping of different livestock species, the extensive mode of breeding, and poor breeding management practices are the main factors in the appearance and spread of animal diseases and zoonosis.

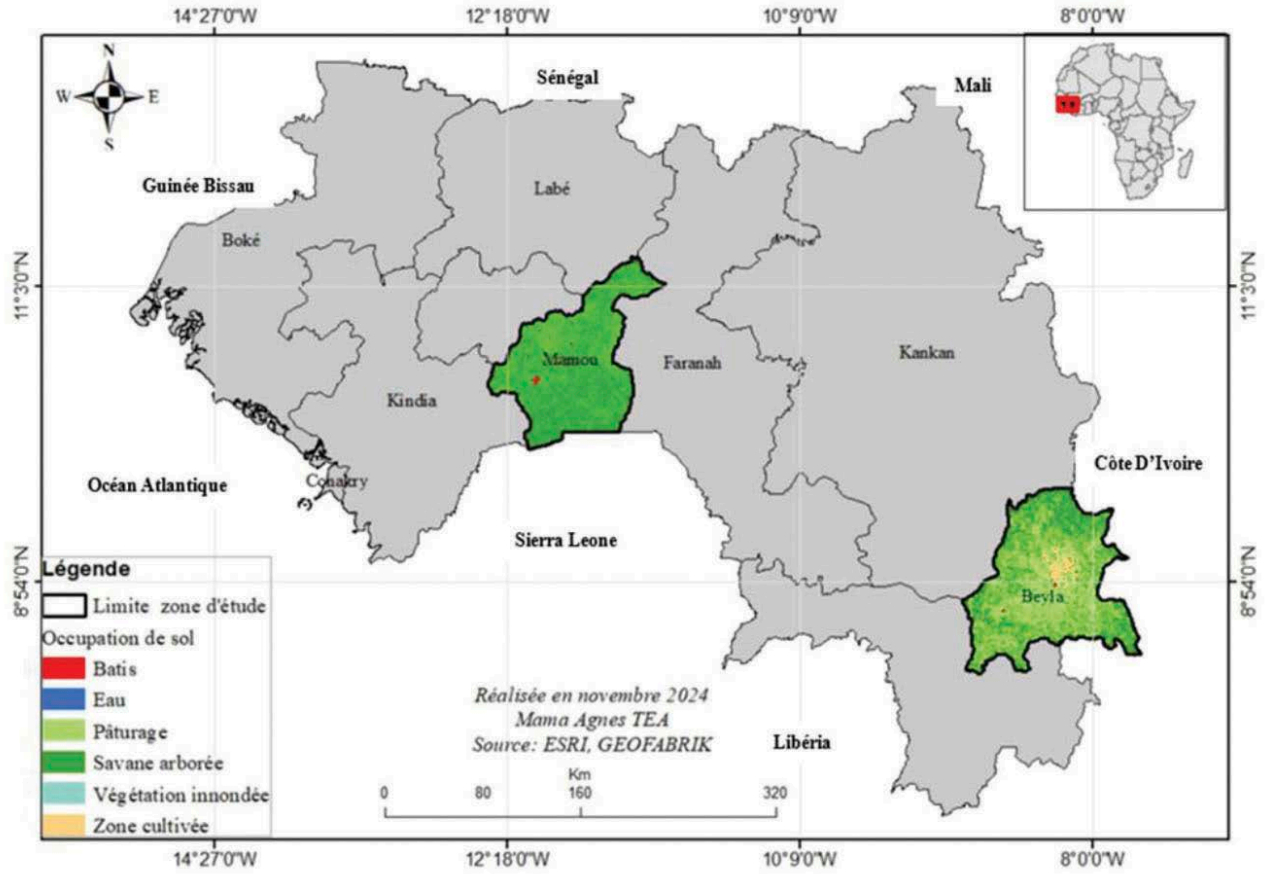

Fig.1. Map of a study area

Epidemiological data collection

The demographic data of the prefectures are presented in Table 1. Before sampling, a predefined data sheet was completed to collect demographic and epidemiological data to assess risk factors for Q fever. Owners/breeders of cattle, goats, and sheep were interviewed in their native language. The geographical coordinates of each sub-prefecture were recorded using GPS. Information on tick infestation was collected by asking owners/breeders whether they had taken precautions against ticks and by inspecting the animals and their habitats. The simple random sampling method was used for the selection of herds, animals, and blood samples from farms scattered throughout the study areas.

Blood sampling and serum separation

A thorough review of the literature revealed that no information was available on the prevalence of Q fever in domestic ruminants (cattle, sheep, and goats) in our targeted study areas. To calculate the sample size, a 95 % confidence interval with an expected prevalence of 15 % and a preferred absolute precision of 5 % was applied using the Thrusfield et al. [31] formula:

$$Nt = \frac{Z^2 . Pexp(1-Pexp)}{d^2}$$

Where *Nt* is the sample size, *Z* is the statistic corresponding to the level of confidence, *Pexp* is the expected prevalence (that can be obtained from the same studies or a pilot study conducted by the researchers), and *d* is precision (corresponding to effect size).

A total of 216 serum samples from all farms were required for this procedure. Blood samples were collected aseptically from the jugular vein of 75 cattle, 72 goats, and 69 sheep. The blood samples were transported to the laboratory and stored at 4 °C. Serum was separated by centrifugation at 3920 rpm for 5 min. Supernatants were pipetted into 1.5 ml Eppendorf tubes and stored at -20 °C.

Serological diagnosis by indirect enzyme-linked immunosorbent assay (iELISA)

The indirect enzyme-linked immunosorbent assay (i-ELISA) was used for *Coxiella burnetii* IgG and IgM antibodies according to the protocol described by Sakamoto et al. [32]. This test was used due to its sensitivity ≥ 95 % (CI 95 %: 89.28 % – 100 %) and specificity ≥ 95 % (CI 95 %: 97.75 % – 100 %).

Each serum sample was tested for the presence of anti-*Coxiella* antibodies using a commercial (indirect) multispecies kit (ID Screen® Q Fever Indirect Multispecies, catalog number FQS-MS-5P). Serum was diluted (1:400) as recommended. Specific antibodies were detected using a peroxidase labeled anti-ruminant conjugate. An ELISA reader was used to measure the optical density (OD) of positive and negative controls, as well as the tested samples, at 450 nm. The results were expressed as a percentage of the optical density (OD) of the tested sample and interpreted according to the manufacturer's recommendations, with S/p values ≤ 30 % being negative and values ≥ 40 % being positive. The test is validated if the mean optical density of the positive control (DOCP) is greater than 0.350 (DOCP > 0.350), and when the ratio between the mean optical density of the positive control (DOCP) and the mean optical density of the negative control (DOCN) is greater than 3 (DOCP / DOCN > 3).

Statistical analysis

Survey data were collected using the KoboCollect mobile application, then transferred to Kobotoolbox and processed. Microsoft Excel spreadsheet software was used for data storage, and R statistical software, version 4.1.2 [33], was used for analysis. The seroprevalence of Q fever was calculated by dividing the number of positive animals by the total number of animal sera tested. The chi-square test for comparison of proportions was used to compare proportions of breeders and seroprevalence in ruminants. Binary logistic regression analysis was applied to identify risk factors associated with seropositive Q fever results, odds ratios, and 95 % confidence intervals. Factors take into account domestic ruminant species, age, sex, contact with other animals, cleaning of premises, rearing method, tick infestation, urbanization of area, metritis and abortion management. A p-value of less than or equal to 0.05 was considered statistically significant.

RESULTS

Extensive livestock farming is the main type of livestock farming practiced in Mamou and Beyla. Indeed, 81.3 % ([66.9 – 90.5]; n = 39) of the 48 farmers surveyed practiced it, with the majority of them (75 % [60.1 – 85.9]; n = 36) being illiterate (Table 1). Overall, a very small

proportion (6.3 % [1.6 – 18.2]; n=3) of livestock farmers were aware of the existence of Q fever in ruminants. The proportion of farmers who were aware of brucellosis (33.3 % [20.8 – 48.5]; n = 16) is significantly higher than that of those who report being aware of Q fever (6.3 % [1.6 – 18.2]) and chlamydia (6.3 % [1.6 – 18.2]). However, this percentage is similar to that of those who are aware of abortive salmonellosis (18.8 % [9.4 – 33.1]), murrain of small ruminants (25.0 % [14.1 – 39.8]) and trichomonas (10.4 % [3.9 – 23.4]) (Table 1).

Regarding the type of livestock farming, out of a total of 48 breeders visited, 22.9 % [12.5 – 37.6] practiced cattle breeding, against 18.8 % [9.4 – 33.1] for sheep breeding, 20.8 % [10.9 – 35.4] for goats and 37.5 % [24.3 – 52.6] for mixed farming (cattle-sheep-goats). For that of hygiene measures on the farm, 83.3 % [69.2 – 92.0] of the breeders did not apply any hygiene measures, 10.4 % [3.9 – 23.4] carried out regular cleaning, 6.3 % [1.6 – 18.2] disinfected the premises. Overall, 87.5 % ([74.0 – 94.8], n = 42) of farmers did not apply any sanitary measures before introducing a new animal to the herd compared to 12.5 % ([5.2 – 25.9], n = 6) who quarantined the animals (Table 1). Furthermore, 64.6 % [49.4 – 77.4] of the farmers surveyed threw the fetus and placenta into the wild, compared to

Table 1. Breeding practices and breeders' knowledge of Q fever

Modality	Designation	Number of breeders surveyed	Percentage (%)	95% CI
Educational level	Literate	12	25.0	[14.1 – 39.8]
	Illiterate	36	75.0	[60.1 – 85.9]
Breeders' knowledge of Q Fever	Knowledgeable	3	6.3	[1.6 – 18.2]
	Unaware	45	93.8	[81.8 – 98.4]
Type of farming	Extensive	39	81.3	[66.9 – 90.5]
	Semi-extensive	9	18.8	[9.4 – 33.1]
	Intensive	0	0.0	[0.0 – 9.2]
Common diseases in livestock	Brucellosis	16	33.3	[20.8 – 48.5]
	Chlamydiosis	3	6.3	[1.6 – 18.2]
	Q-fever	3	6.3	[1.6 – 18.2]
	Abortive Salmonellosis	9	18.8	[9.4 – 33.1]
	Murrain of Small ruminants	12	25.0	[14.1 – 39.8]
	Trichomonas	5	10.4	[3.9 – 23.4]
Livestock species	Cattle	11	22.9	[12.5 – 37.6]
	Sheep	9	18.8	[9.4 – 33.1]
	Goats	10	20.8	[10.9 – 35.4]
	Mixed (Cattle-Sheep-Goats)	18	37.5	[24.3 – 52.6]
Sanitary measures applied before introducing a new animal into the herd	Quarantine	6	12.5	[5.2 – 25.9]
	No measures	42	87.5	[74.0 – 94.8]
Livestock management	Regular cleaning	5	10.4	[3.9 – 23.4]
	Disinfection	3	6.3	[1.6 – 18.2]
	No measures	40	83.3	[69.2 – 92.0]
Management of aborted animals	Burial	7	14.6	[6.5 – 28.4]
	Incineration	2	4.2	[0.7 – 15.4]
	Disposal in the environment	31	64.6	[49.4 – 77.4]
Socio-economic impacts of the disease on farms	No measures	8	16.7	[7.9 – 30.7]
	Abortions	24	50.0	[36.4 – 63.6]
	Treatment costs	6	12.5	[5.2 – 25.9]
	Stillbirths	18	37.5	[24.3 – 52.6]

16.7 % [7.9 – 30.7] who took no measures to manage abortions cases (Table 1). In addition, some minor measures, including burial (14.6 % [6.5 – 28.4]) and incineration (4.2 % [0.7 – 15.4]) of the foetus and placenta, were used in the management of abortion cases.

The socioeconomic impact of Q fever on farms is diverse. According to the farmers interviewed, Q fever abortions and neonatal deaths resulted in economic losses of 50.0 % [36.4 – 63.6] and 37.5 % [24.3 – 52.6], respectively. Other economic losses were due to treatment costs (12.5 % [5.2 – 25.9], n = 6).

Prevalence of anti-*Coxiella burnetii* antibodies in relation to demographic variables of cattle, sheep, and goat populations in Mamou and Beyla

Of 216 serum samples obtained from 48 different herds analyzed, a total of 101 animals and 18 (37.5 %) herds were positive for *C. burnetii*, giving an overall prevalence of Q fever estimated at 46.75 % [39.9 – 53.6]. It was significantly higher in Beyla (54.63 %) compared to Mamou (38.89 %) (p = 0.02912). This prevalence varied significantly from one sub-prefectures to another one in Beyla unlike in Mamou. In the localities of Mamou, the seroprevalence of Q fever was 37.5 % in Dounet, 50 % in Gongoret, and 30.56 % in Poredakar (p = 0.2533). As for Beyla localities, it was 41.18 %, 44.4 % and 76.32 % in Boola, Fouala and Sinko, respectively (p = 0.003). However, the highest seropositivity for *C. burnetii* was observed in the district of Sinko (p = 0.003) (Table 2). The seropositivity of goats for Q fever antibodies (52.78 %) was similar to that of sheep (40.58 %) and cattle (46.67 %) (p = 0.3488). However, it was higher in females (58.02 %) than in males (29.41 %) (p < 0.001). In rural areas, 23.96 % of positive sera were detected compared to 65 % in peri-urban areas. The prevalence was found to be significantly (p < 0.044)

Table 2. Prevalence of anti-*Coxiella burnetii* antibodies in relation to demographic variables in cattle, sheep, and goat populations in Mamou and Beyla

Categories	Variables	Number of samples	Number of positives	Seroprevalence n (%)	Chi-Square	p -Value
Sub-prefectures	Boola	34	14	41.18	11.28	0.003698
	Fouala	36	16	44.44		
	Sinko	38	29	76.32		
	Total Beyla	108	59	54.63	-	-
	Dounet	40	15	37.50	2.7468	0.2533
	Gongoret	32	16	50.00		
	Poredakar	36	11	30.56		
	Total Mamou	108	42	38.89	-	-
Species	Cattle	75	35	46.67	2.1063	0.3488
	Sheep	69	28	40.58		
	Goats	72	38	52.78		
	Total animals	216	101	46.76	-	-
Gender	Female	131	76	58.02	15.812	< 0.001
	Male	85	25	29.41		
	Total Gender	216	101	46.76		
Urbanization	Rural	96	23	23.96	34.45	< 0.001
	Peri-urban	120	78	65		
	Total	216	101	46.76		
Age	Youth [12 – 42 months]	118	63	53.39	4.0248	0.04484
	Adult [43 – 92 months]	98	38	38.78		
	Total	216	101	46.75		

Table 3. Prevalence by age group

Characteristics	0. N =158		1. N = 48		2. N = 10	
Age Group (months)	n (%)	95 % CI	n (%)	95 % CI	n (%)	95 % CI
Youth [12 - 42]	112 (71)	[63.0 – 77.7]	32 (66.6)	[51.4 – 79.1]	10 (100)	[65.5 – 1.00]
Adult [43 - 92]	46 (29,1)	[22.3 – 36.9]	16 (33.3)	[20.8 – 48.5]	0 (0.0)	[0.00 – 34.4]

N = total number; 0 = no farrowing; 1 = primiparous; 2 = multiparous, n = number; % = percentage; CI: Confidence Interval

Table 4. Risk factors associated with the prevalence of anti-*Coxiella burnetii* antibodies in domestic ruminants

Categories	Variable	Nb of positives/ Nb analyzed	Prevalence	Odds ratio	95% CI	p-value
Animal species	Cattle	35/75	46.67	-	-	-
	Sheep	28/69	40.58	1.4	[0.7 – 2.8]	0.29
	Goats	38/72	52.78	0.9	[0.5 – 1.7]	0.17
Gender	Female	76/131	58.02	3.3	[1.9 – 5.9]	0.00004
	Male	25/85	29.41			
Ages	Youth [12 – 42 months]	63/118	53.39	1.8	[1.0 – 3.1]	0.03
	Adult [43 – 92 months]	38/98	38.78			
Introducing a new animal to the herd	Quarantine	18/48	37.50	0.9	[0.4 – 1.95]	0.77
	No measures	23/57	40.35			
Reproductive disorder	Abortion	35/131	26.72	-	-	-
	Placental retention	29/131	22.14	1.3	[0.7 – 2.3]	0.39
	Metritis	12/131	9.16	3.6	[1.8 – 7.3]	0.0002
Breeding method	Extensive	86/185	46.49	0.9	[0.4 – 1.98]	0.84
	Semi-intensive	15/31	48.39			
Urbanicity	Rural	23/96	23.96	0.2	[0.1 – 0.3]	< 0.001
	Peri-urban	78/120	65.00			
Cleaning of the Corrals	Yes	12/25	48.00	1.1	[0.5 – 2.4]	0.89
	No	89/191	46.60			
Contact with other Species	Yes	101/216	46.76	NA	NA	NA
	No	0	0.00			
Ticks Infestation	Yes	44/83	53.01	2.6	[1.5 – 6.4]	0.003
	No	12/133	9.02			

CI: Confidence Interval; Nb: number: p-value of the statistically significant odds ratio; NA: not applicable

impacted by age, with a high prevalence in young animals (53.39 %).

Comparative analysis of prevalence between age groups showed that 71 % [63.0 – 77.7], 66.66 % [51.4 – 79.1], and 100 % [65.5 – 1.00] of young females aged 12 to 42 months who had not given birth, primiparous and multiparous were receptive to *Coxiella burnetii* infection unlike adult females aged 43 to 92 months (Table 3).

Correlation between the prevalence of Q fever and certain risk factors

Table 4 shows the correlation between the prevalence of Q fever and certain risk factors. The seroprevalence of Q fever was higher (53.01 %) in tick-infested animals than in tick-free animals (9.02 %). The presence of ticks was a potentially significant risk factor (p = 0.003). Animals with abortion and retained placenta had high prevalences of 26.72 % and 22.14 %, respectively, compared with animals with metritis (9.16 %). Unlike the history of abortion and retained placenta, which had no significant impact on the prevalence of Q fever, metritis was a significant risk factor (p = 0.0002). Animals that had been in contact with other animals had the highest seroprevalence (46.76 %) compared with zero that had not been exposed. Animals that had been in direct contact with other farm animal species were seropositive at 46.76 %, compared with zero that had not been exposed to other animal species. Higher seropositivity was detected in females (58.02 %) compared

with males (29.41 %) and in young animals aged between 12 and 42 months (53.39 %) compared with adults aged between 43 and 92 months (38.78 %). These two factors, such as sex ($p = 0.00004$) and age ($p = 0.03$), were statistically significant for Q fever seroprevalence.

It is remarkable that animals found in peri-urban areas were significantly more seropositive (65 %) than those in rural areas (23.96 %). Remarkably, animals kept in cleaned corals were significantly less likely to be seropositive (48 %) than others (46.6 %). Furthermore, factors such as animal species (OR = 1.4 [0.7 – 2.8] in sheep vs. cattle; OR = 0.9 [0.5 – 1.7] in goats vs. cattle ($p > 0.05$), the introduction of a new animal into the herd (OR = 0.9 [0.4 – 1.95]; $p = 0.77$), rearing method (OR = 0.9 [0.4 – 1.98]; $p = 0.84$), and cleaning of sheep pens (OR = 1.1 [0.5 – 2.4]; $p = 0.89$) were not significantly associated with the distribution of *C. burnetii* in domestic ruminants in Beyla and Mamou.

DISCUSSION

This cross-sectional study was conducted in the prefectures of Mamou and Beyla in Guinea to determine the prevalence of antibodies to *Coxiella burnetii* in domestic ruminants and to assess associated risk factors to aid in the development of surveillance and control plans. The results obtained in this study confirm that domestic ruminant populations are exposed to *C. burnetii*, which may represent a public health problem. The overall prevalence of antibodies against *Coxiella burnetii* was 46.75 %. It was 54.63 % in Beyla compared to 38.89 % in Mamou. This overall seroprevalence observed in this study is significantly higher than that reported by a previous national study conducted in 2013 in four regions of Guinea, where respective seroprevalences of 0.4 %, 6.9 %, 9.3 % and 9.2 % are recorded in Forest Guinea, Upper Guinea, Middle Guinea, and in Maritime Guinea [24]. Our results are also contrary to those of [25], who found a lower prevalence of 9.1 % in small ruminants seropositive for *C. burnetii* between 2017 and 2019 in Guinea. This increase in *C. burnetii* infectivity in domestic ruminants could be due to a more active circulation of the bacteria in Guinea during the last decade.

Given that extensive livestock farming is extensive in both prefectures, this rapid spread of Q fever in ruminants could be due to the inhalation or ingestion of particles contaminated by farrowing and abortion products, feces, and urine of animals infected with bacteria [34, 35]. This risk factor for *C. burnetii* infection is particularly high in arid areas [36]. Due to a shortage of pasture in arid areas, ruminants are put to pasture on large areas, which indicates an uncontrolled bovine-goat-sheep interaction with a potential risk of transmission. This hypothesis is confirmed with 46.76 % of animals having had contact with other animals detected seropositive in our study. Similarly, the climatic conditions with high relative humidity that prevail in Beyla and Mamou at certain periods of the year could influence the distribution and maintenance of aerosolized bacteria, leading to a higher prevalence of the disease [26, 25]. This hypothesis seems to confirm the results of Troupin et al. [25], who reported a seroprevalence greater than 50 % in cattle in Dalaba, one of the prefectures close to Mamou. In addition to these environmental factors, this potential spread of *C. burnetii* could be favored by the lack of information on the disease and the practices of farmers that would increase the chances of introducing infected ruminants into the herds. As confirmed by the results of our survey, farmers had little knowledge about the pathology (93.8 %), which explains why they did not follow the instructions that would allow them to manage herds free from the disease. It is therefore important to recommend to breeders quarantine and mandatory testing of new animals for *Coxiella* infection before introducing them into the herds. However, it would be interesting to re-evaluate in the coming year the seroprevalence of ruminants in the same areas to verify if this trend of spread remains, in order to recommend appropriate mitigation measures. Besides, the high seroprevalence observed in Beyla compared to Mamou could be due to a higher circulation of the bacteria in this locality, where gatherings of transhumant livestock are observed. Indeed, the prefecture of Beyla borders the west of Côte d'Ivoire, where animals share the same watering points as well as the same grazing areas. Our work corroborates that of [22], who reported higher prevalence rates in the localities of Nioro and Kéniébougouwéré, two localities bordering Beyla in the west of Côte d'Ivoire. Similar observation had already been made in the Volta region of Ghana [37].

Coxiella burnetii seroprevalence rates were similar in sheep (40.58 %), goats (52.78 %) and cattle (46.67 %) in Mamou and Beyla. This result confirms the correlation between infection and risk factors which showed that there is not a significant association between prevalence and ani-

mal species (OR = 1.4 [0.7 – 2.8]; p = 0.29 in sheep versus cattle; OR = 0.9 [0.5 – 1.7]; p = 0.17 in goats versus cattle). These observed seroprevalences are higher than those reported by Troupin et al. [25] in a previous study conducted between 2017 and 2019 in cattle (20.5 %), goats (4.4 %), and sheep (2.3 %) in the Guinea. Prevalence below those found in our study, ranging from 13 % to 32 %, had also been documented in previous studies in Africa [15, 17, 14, 38, 16, 39, 6]. However, our results are lower than those obtained in Mali and Quebec, where seroprevalences of 55.3 % and 68.75 %, respectively, were detected in ruminants [19, 40]. Higher seropositivity rates for *C. burnetii* were reported in areas with higher livestock density (> 100 per 100 animals) in some African countries, including Guinea [41]. In these areas, the probable cause would be the wind-borne distribution of *C. burnetii* spores from agricultural land to more populated areas. Other possible reasons for these variations could be differences in sample size, sampling methods, diagnostic tests used and the geographical locations.

The prevalence was significantly higher in females (58.02 %) than in males (29.41 %). The correlation revealed that gender is a potential statistically significant risk factor for Q fever (p = 0.00004). This result is consistent with previous reports indicating a high affinity of *C. burnetii* with female animals (26.65 %) compared with male animals (17.21 %) [42]. The authors mentioned that males are used for breeding purposes and are therefore transferred from one area to another and may be in contact with many different herds. This method may put them at higher risk of contracting and spreading diseases, which is not the case in our study area. This method may present them with a higher risk of contracting and spreading diseases, which is not the case in our study area. Moreover, this seroprevalence is significantly higher in young non-pregnant females (71 %), primiparous (66.66 %) and multiparous (100) animals aged 12 – 42 months, unlike in adult females aged 43-92 months. This trend could be explained by the fact that young animals (calves, kids, lambs) born to infected mothers are very susceptible to the disease. Similarly, a previous study showed that older animals shedding *C. burnetii* apparently showed no clear signs or symptoms of the disease and had no history of abortion. Our results are in contrast to those of several other studies, which reported that *C. burnetii* seropositivity in domestic ruminants increases with age [13, 43, 44].

In the present study, most of the Q fever-positive samples were collected from animals in peri-urban areas (n = 78; 65 %) where breeding is extensive, without hygiene measures in the facilities, such as cleaning the animals and their premises. However, no correlation was established in this study between infection and lack of cleaning of animal premises. However, these facts may contribute to higher prevalences of the disease. Likewise, contaminated birthing products are thrown into the environment; this justifies a higher risk of Q fever (OR = 0.2 [0.1 – 0.3]; p < 0.001) observed in animals in rural and peri-urban areas.

Ticks are considered an important risk factor and have a significant association with *C. burnetii* seropositivity. In the present study, 53.01 % of seropositive animals were found infested with ticks. These arthropods have a significant link in this infection (OR = 2.6 [1.5 – 6.4]; p = 0.003). This result is consistent with the findings of one of two previous studies conducted in Pakistan that found prevalences of 50 % and 97.9 %, respectively, in animals that harbored ticks [45, 46]. These studies found that ticks can carry the Q fever pathogen and contaminate the environment or the host. Several species of ticks can transfer *Coxiella* spp. by transstadial route as well as transovarial route and can transmit Q fever from a sick animal to a healthy animal via the blood meal [47].

No statistically significant association of infection was associated with abortion product management and retained placenta in this study. This result is contrary to that of Beaudeau et al. [48], who reported that the placentas and aborts of infected females induce a very high bacterial load and are sources of environmental pollution. On the other hand, a significant link was observed with a history of metritis (OR= 3.6 [1.8 – 7.3]; p = 0.0002) in our study and confirms the work of Agag et al. [49], who observed a similar result.

In these different cases, certain preventive measures could be of interest in terms of public health: vector control against ticks, serological tests and vaccination of animals, parturition in buildings, and appropriate disposal of placentas and litter. However, the feasibility of such measures in Guinea, and particularly in the Mamou and Beyla regions, is low since Q fever is not a notifiable disease; tests (in case of abortion) are carried out on request and are the responsibility of the breeder. An effective phase I animal vaccine is not yet available in Guinea. Although indoor parturition is not possible in an extensive livestock

area due to the lack of barns, recommendations have been made regarding the appropriate disposal of placentas. They should not be left on the ground to dry but collected and cremated appropriately, but this is only possible when parturition takes place in the presence of breeders.

Many other personal and behavioral factors could be involved and could be preventable. Further studies are needed to identify and confirm these preventable risk factors.

CONCLUSION

This study demonstrated the presence of Q fever with a higher seroprevalence in cattle, sheep, and goats in the prefectures of Mamou and Beyla in Guinea. These results highlight the need to implement surveillance of this bacterial infection in ruminants in all prefectures of the country. Given the zoonotic nature of the disease, we recommend screening at-risk human populations (veterinarians, breeders, slaughterers) using a One Health approach. Infestation of animals by ticks, metritis, degree of urbanization, management of parturition products, age and sex of animals, as well as climatic variability are risk factors to take into account in the management of this pathology in the study area. Livestock farmers' knowledge of the disease and its effects on animals as well as humans is still limited, as the disease is not considered a priority among zoonoses in Guinea. Therefore, research and awareness are recommended, especially in areas where large numbers of animals and people are at risk. Animals excrete the pathogen during parturition or abortion, which leads to environmental contamination and high exposure of other animals and humans. Hence, separation of animals at parturition and appropriate disposal of placentas and/or aborted fetuses are recommended. We also recommend the inclusion of Q fever in the differential diagnosis of abortion in domestic ruminants using molecular diagnostics to confirm the involvement of Q fever in cases of reproductive disorders in domestic ruminants in Guinea.

Availability of Data and Materials

The datasets that were analyzed in this study are available from the corresponding author and the lead author.

Ethical Approval and Consent to Participate

Prior approval was obtained from the Mamou and Beyla Prefectoral Livestock Directorates. After contacting the farmers, with the agreement of the local chiefs, the objectives and framework of the study were clearly explained. In this way, we were able to obtain the herders' consent to participate in the study without any negative consequences for their activities. Only after their verbal consent was obtained were the questionnaires administered and serum samples taken. The privacy of the participating farmers was respected, as other farmers prefer to hide what is happening in their herds to avoid being judged by their peers. To ensure confidentiality, the questionnaires did not include the farmers' names but rather the names of the villages where they were administered and numbered.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

This study was not financially supported by any grant.

Generative AI Statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Authors' Contributions

The original study was conducted by MAT, DT and LWS who also supplied the data. The idea for the study was conceptualized and generated by MAT, DT and LWS. Data were collected by MAT, DT, MBS, ASS and WT. MAT and ASS drafted the manuscript. Statistical data analysis by LK and ASS. WT, MBS, LK, DT and MAT provided intellectual criticism on the content of the manuscript. All authors have read and approved the final submitted manuscript.

Acknowledgements

This research was made possible thanks to the financial support of the Ministère de l'Enseignement supérieur,

de la Recherche scientifique et de l'Innovation (MESRSI) of Guinea, to whom we express our gratitude. We would also like to thank the General Management of the Institut Supérieur des Sciences et de Médecine Vétérinaire (ISSMV) in Dalaba for their support in carrying out this study.

To all the staff of the Central Veterinary Diagnostic Laboratory Conakry-Guinea of the Serology section, and in particular to Dr. Kouramoudou BERETE for facilitating the work.

REFERENCES

1. **Musso, D., Broult, J., Parola, P., Raoult, D., Fournier, P.-E., 2014:** Absence of antibodies to *Rickettsia* spp., *Bartonella* spp., *Ehrlichia* spp. and *Coxiella burnetii* in Tahiti, French Polynesia. *BMC Infect. Dis.,* 14, 255. DOI: 10.1186/1471-2334-14-255
2. **Eldin, C., Mélenotte, C., Mediannikov, O., Ghigo, E., Million, M., Edouard, S., et al., 2017:** From Q Fever to *Coxiella burnetii* infection: a paradigm change. *Clin. Microbiol. Rev.,* 30, 1. DOI: 10.1128/CMR.00045-16
3. **Van den Brom, R., van Engelen, E., Roest, H. I. J., van der Hoek, W., Vellema, P., 2015:** *Coxiella burnetii* infections in sheep or goats: an opinionated review. *Vet. Microbiol.*, 181, 1–2, 119–129. DOI: 10.1016/j.vetmic.2015.07.011
4. **Vanderburg, S., Rubach, M. P., Halliday, J. E. B., Cleaveland, S., Reddy, E. A., Crump, J. A., 2014:** Epidemiology of *Coxiella burnetii* infection in Africa: A OneHealth systematic review. *PLoS Negl. Trop. Dis.*, 8, e2787. DOI: 10.1371/journal.pntd.0002787
5. **Gardon, J., Héraud, J. M., Laventure, S., Ladam, A., Capot, P., Fouquet, E., et al., 2001:** Suburban transmission of Q fever in French Guiana: evidence of a wild reservoir. *J. Infect. Dis.*, 184, 3, 278–284. DOI: 10.1086/322034
6. **Tialla, D., Téa, M. A., Tialla, A. L., Dah, J.-B. S., Kiendrébéogo, A., Kaboré, J. W., et al., 2024:** First study on Q Fever in the rural commune of Bama in Burkina Faso: Seroprevalence among small ruminants, knowledge, skills, practices and behaviours at risk of zoonotic transmission of this pathology among breeders. *EC Microbiol.,* 20, 01–11.
7. **Eldin, C., Mélenotte, C., Mediannikov, O., Ghigo, E., Million, M., Edouard, S., et al., 2017:** From Q Fever to *Coxiella burnetii* infection: a paradigm change. *Clin. Microbiol. Rev.,* 30, 1. DOI: 10.1128/CMR.00045-16
8. **European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), 2023**: The European Union One Health 2022 Zoonoses Report. *EFSA J.,* 21, 12. DOI: 10.2903/j.efsa.2023.8442
9. **Gale, P., Kelly, L., Mearns, R., Duggan, J., Snary, E. L., 2015:** Q fever through consumption of unpasteurised milk and milk products - a risk profile and exposure assessment. *J. Appl. Microbiol.*, 118, 5, 1083–1095. DOI: 10.1111/jam.12778
10. **Pexara, A., Solomakos, N., Govaris, A., 2018:** Q fever and seroprevalence of *Coxiella burnetii* in domestic ruminants. *Vet. Ital.*, 54, 4, 265–279. DOI: 10.12834/VetIt.1113.6046.3
11. **Van Asseldonk, M. A. P. M., Bontje, D. M., Backer, J. A., van Roermund, H. J. W., Bergevoet, R. H. M., 2015:** Economic aspects of Q fever control in dairy goats. *Prev. Vet. Med.,* 121, 1–2, 115–122. DOI: 10.1016/j.prevetmed.2015.06.010
12. **Fournier, P.-E., Marrie, T. J., Raoult, D., 1998:** Diagnosis of Q fever. *J. Clin. Microbiol*., 36, 7, 1823–1834. DOI: 10.1128/jcm.36.7.1823-1834.1998
13. **Bwatota, S. F., Shirima, G. M., Hernandez-Castro, L. E., Bronsvoort, B. M. C., Wheelhouse, N., Mengele, I. J., et al., 2022:** Seroprevalence and risk factors for Q fever (*Coxiella burnetii*) exposure in smallholder dairy cattle in Tanzania. *Vet. Sci.*, 9, 12, 662. DOI: 10.3390/vetsci9120662
14. **Kamga-Waladjo, A. R., Gbati, O. B., Kone, P., Lapo, R. A., Chatagnon, G., Bakou, S. N., et al., 2010:** Seroprevalence of *Neospora caninum* antibodies and its consequences for reproductive parameters in dairy cows from Dakar-Senegal, West Africa. *Trop. Anim. Health Prod.,* 42, 953–959. DOI: 10.1007/s11250-009-9513-6
15. **Adu-Addai, B., Koney, E. B., Addo, P., Kaneene, J., Mackenzie, C., Agnew, D. W., 2012:** Importance of infectious bovine reproductive diseases: an example from Ghana. *Vet. Rec.*, 171, 2, 47. DOI: 10.1136/vr.100789
16. **Kponmassi, T., Akakpo, J. A., 1991:** Epidémiologie des affections abortives des bovins au Togo: enquête sérologique sur la brucellose, la chlamydiose et la fièvre Q. Thèse Dr. Vet., Université Cheikh Anta Diop de Dakar, Senegal, 88p.
17. **Elelu, N., Adefolake, A. B., Ramat, J. M., Odetokun, I. A., Musa, R., Khalid, T. B., et al., 2020:** Serospatial epidemiology of zoonotic *Coxiella burnetii* in a cross section of cattle and small ruminants in northern Nigeria. *PLoS ONE,* 15, 10, e0240249. DOI: 10.1371/journal.pone.0240249
18. **Tialla, D., Sondo, I., Sebou Dah, J.-B., 2022:** Fièvre Q dans la zone périurbaine de Ouagadougou au Burkina Faso : séro-

prévalence chez les vaches laitières, connaissance et comportements à risque de transmission zoonotique de cette zoonose chez les bouviers. *Épidémiol. Santé Anim.*, 79, 95–102.

19. **Dione, M. M., Séry, A., Sidibé, C. A. K., Wieland, B., Fall, A., 2022:** Exposure to multiple pathogens - serological evidence for Rift Valley fever virus, *Coxiella burnetii*, Bluetongue virus and *Brucella spp*. in cattle, sheep and goat in Mali. *PLoS Negl. Trop. Dis.*, 16, 4, e0010342. DOI: 10.1371/journal.pntd.0010342
20. **Julvez, J., Michault, A., Kerdelhue, C., 1997:** [Serological study of rickettsia infections in Niamey, Niger]. *Med. Trop. Rev.*, Corps Sante Colon, 57, 2, 153–156.
21. **Kobbe, R., Kramme, S., Kreuels, B., Adjei, S., Kreuzberg, C., Panning, M., et al., 2008:** Q fever in young children, Ghana. *Emerg. Infect. Dis.*, 14, 2, 344–346. DOI: 10.3201/eid1402.070971
22. **Sidibe, S. S., Coulibaly, K. W., Dakouo, M., Tarnagda, Z., Sery, A., Niang, M., et al., 2013:** Fièvre Q chez les petits ruminants au Mali: Résultats d'une enquête sérologique. *Artic. Rev. Délevage Médecine Vét. Pays Trop.,* 66, 1. DOI: 10.19182/remvt.10144
23. **Tea, M. A., Tialla, D., Somda, M. B., Soromou, L. W., 2025:** Systematic review of reproductive disorders in ruminants linked to bacteria *Coxiella burnetii* in Africa during the last ten years (2014-2023). *EC Microbiol.*, 21, 1, 16.
24. **Buaro, M. I., Simonova, E. G., Pokrovsky, V. I., 2019:** Comprehensive assessment of the epidemic situation in the Republic of Guinea. *Epidemiol. Vaccinal Prev.,* 18, 5, 56–62. DOI: 10.31631/2073-3046-2019-18-5-56-62
25. **Troupin, C., Ellis, I., Doukouré, B., Camara, A., Keita, M., Kagbadouno, M., et al., 2022:** Seroprevalence of brucellosis, Q fever and Rift Valley fever in domestic ruminants in Guinea in 2017–2019. *BMC Vet. Res.*, 18, 64. DOI: 10.1186/s12917-022-03159-x
26. **Naidenova, E. V., Zakharov, K. S., Agafonov, D. A., Kartashov, M. Y., Senichkina, A. M., Khalilov, E. S., et al., 2024:** Current data on the circulation of the Q fever pathogen in the Republic of Guinea. *J. Microbiol. Epidemiol. Immunobiol.*, 101, 5, 606–618. DOI: 10.36233/0372-9311-557
27. **Barry, A. A., Soumah, L. I., Balde, T. A. L., Souare, M. L., 2022:** Demonstration of the DNA carriage rate of *Coxiella burnetii* (Q fever agent) in ticks in the prefectures of Kindia, Beyla, Lola and N'Zérékoré in the Republic of Guinea. *IJAHMR*, 6, 7, 1–4.
28. **Kanouté, Y. B., Gragnon, B. G., Schindler, C., Bonfoh, B., Schelling, E., 2017:** Epidemiology of brucellosis, Q Fever and Rift Valley Fever at the human and livestock interface in northern Côte d'Ivoire. *Acta Trop.,* 165, 66–75. DOI: 10.1016/j.actatropica.2016.02.012
29. **I. N. S. G., 2014:** Recensement General de la Population et de l'Habitation (RGPH3) 125
30. **Bureau, S. D., 2020:** Annuaire des statistiques de l'élevage 2015-2019
31. **Thrusfield, M., Mansley, L., Dunlop, P., Pawson, A., Taylor, J., 2005:** The foot-and-mouth disease epidemic in Dumfries and Galloway, 2001. 2: Serosurveillance, and efficiency and effectiveness of control procedures after the national ban on animal movements. *Vet. Rec.*, 156, 9, 269–278. DOI: 10.1136/vr.156.9.269
32. **Sakamoto, S., Putalun, W., Vimolmangkang, S., Phoolcharoen, W., Shoyama, Y., Tanaka, H., et al., 2018:** Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. *J. Nat. Med.*, 72, 32–42. DOI: 10.1007/s11418-017-1144-z
33. **Lüdecke, D., Ben-Shachar, M. S., Patil, I., Waggoner, P., Makowski, D., 2021:** Performance: An R package for assessment, comparison and testing of statistical models. *J. Open Source Softw.,* 6, 60, 3139. DOI: 10.21105/joss.03139
34. **Dordain-Bouesnard, C., 2001:** Revue bibliographique et contribution à l'étude épidémiologique de la fièvre Q en région Rhône-Alpes. Thèse Dr. Vet., Université Claude Bernard, Lyon, 208p.
35. **Zeeshan, M. A., Sarmad, A., Ishtiaq, A., Rehman, A. U., Rafique, M. K., Nasir, A., et al., 2023:** Sero-epidemiological study of zoonotic bacterial abortifacient agents in small ruminants. *Front. Vet. Sci.*, 10, 1195274. DOI: 10.3389/fvets.2023.1195274
36. **Wolf, A., Prüfer, T. L., Schoneberg, C., Campe, A., Runge, M., Ganter, M., et al, 2020:** Risk factors for an infection with Coxiella burnetii in German sheep flocks. *Epidemiol. Infect.*, 148, e260. DOI: 10.1017/S0950268820002447
37. **Johnson, S. A. M., Kaneene, J. B., Asare-Dompreh, K., Tasiame, W., Mensah, I. G., Afakye, K., et al., 2019:** Seroprevalence of Q fever in cattle, sheep and goats in the Volta region of Ghana. *Vet. Med. Sci.*, 5, 3, 402–411. DOI: 10.1002/vms3.160
38. **Knobel, D. L., Maina, A. N., Cutler, S. J., Ogola, E., Feikin, D. R., Halliday, J. E. B., et al., 2013:** *Coxiella burnetii* in humans, domestic ruminants, and ticks in rural western Kenya. *Am. J. Trop. Med. Hyg.,* 88, 3, 513–518. DOI: 10.4269/ajtmh.12-0169

39. **Mangena, M. L., Gcebe, N., Thompson, P. N., Adesiyun, A. A., 2023:** Q fever and toxoplasmosis in South African livestock and wildlife: a retrospective study on seropositivity, sporadic abortion, and stillbirth cases in livestock caused by *Coxiella burnetii*. *BMC Vet. Res.,* 19, 168. DOI: 10.1186/s12917-023-03645-w
40. **Turcotte, M.-E., 2015:** Prévalence et Facteurs de Risque de L'infection Par *Coxiella Burnetii* Chez Les Ruminants D'élevage au Québec [Prévalence et facteurs de risque de l'infection à Coxiella Burnetii chez les ruminants d'élevage au Québec] (Thèse de maîtrise). Faculté de médecine vétérinaire, Université de Montréal, Québec, Canada, 220 p.
41. **Tissot, D.H., Brouqui, P., Faugere, B., Raoult, D., 1995:** Prevalence of antibodies to *Coxiella burnetii*, *Rickettsia conorii*, and *Rickettsia typhi* in seven African countries. *Clin. Infect. Dis*., 21, 5, 1126–1133. DOI: 10.1093/clinids/21.5.1126
42. **Milkesa, A., Rufael, T., Kinfe, G., Belaineh, R., Bulbula, A., Cho, D., et al., 2024:** A sero-epidemiological analysis of *Coxiella burnetii* infection and its risk factors in livestock from Addis Ababa, Adama, and Modjo abattoirs and pastoral areas of Oromia, Ethiopia. *PLoS Negl. Trop. Dis*., 18, 7, e0012287. DOI: 10.1371/journal.pntd.0012287
43. **Rahman, M. A., Alam, M. M., Islam, M. A., Bhuiyan, A. K. F. H., Rahman, A. K. M. A., 2016:** Serological and molecular evidence of Q Fever in domestic ruminants in Bangladesh. *Vet. Med. Int.*, 2016, 9098416. DOI: 10.1155/2016/9098416
44. **Ezatkhah, M., Alimolaei, M., Khalili, M., Sharifi, H., 2015:** Seroepidemiological study of Q fever in small ruminants from Southeast Iran. *J. Infect. Public Health*, 8, 2, 170–176. DOI: 10.1016/j.jiph.2014.08.009
45. **Ruiz-Fons, F., Astobiza, I., Barandika, J. F., Hurtado, A., Atxaerandio, R., Juste, R. A., et al., 2010:** Seroepidemiological study of Q fever in domestic ruminants in semi-extensive grazing systems. *BMC Vet. Res.*, 6, 3. DOI: 10.1186/1746-6148-6-3
46. **Amin, F., Ali, S., Javid, A., Imran, M., Rashid, M. I., Mertens-Scholz, K., et al., 2022:** Sero-Epidemiology of *Coxiella burnetii* infection in small ruminants in the eastern region of Punjab, Pakistan. *Pathogens,* 11, 6, 664. DOI: 10.3390/pathogens11060664
47. **Ullah, Q., Jamil, H., Qureshi, Z. I., Saqib, M., Neubauer, H., 2019:** Sero-epidemiology of Q Fever (Coxiellosis) in small ruminants kept at government livestock farms of Punjab, Pakistan. *J. Zool.*, 51, 1, 135–140. DOI: 10.17582/journal.pjz/2019.51.1.135.140
48. **Beaudeau, F., Guatteo, R., Seegers, H., 2006:** Voies d'excrétion de *Coxiella burnetii* par la vache laitière: implication pour le dépistage et la maîtrise de l'infection en élevage. *Epidémiol. Santé Anim.,* 49, 1–4.
49. **Agag, S., Kaidi, R., Khelef, D., 2016:** Séroprévalence de la fièv re Q chez les bovins de la région de Bejaïa (Algérie). *Rev. D'élevage Médecine Vét. Pays Trop.*, 69, 4, 155–159. DOI: 10.19182/remvt.31200

sciendo

DOI: 10.2478/fv-2025-0018

FOLIA VETERINARIA, 69, 2: 56—71, 2025

ORIGINAL ARTICLE

CONCENTRATIONS OF SOME HEAVY AND TRACE METALS IN SERUM AND BRAIN OF FIVE SPECIES OF WILD VERTEBRATES FROM NIGERIA

Olumayowa Olawumi Igado[1], Funmilayo Eniola Olopade[2], Taidinda Tashara Gilbert[1], Adedunsola Adewunmi Obasa[1], Irene Chizubelu Omile[1], Iheanyi Kemdirim Peter-Ajuzie[1,3], Kehinde Iyanuoluwa Adedokun[1], James Olukayode Olopade[1*]

[1]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria; [2]Department of Anatomy, Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, Nigeria; [3]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu, Nigeria

OPEN ACCESS

***Correspondence:** jkayodeolopade@yahoo.com

Citation: Igado, O. O., Olopade, F. E., Gilbert, T. T., Obasa, A. A., Omile, I. C., Peter-Ajuzie, I. K., Adedokun, K. I., Olopade, J. O., 2025: Concentrations of some heavy and trace metals in serum and brain of five species of wild vertebrates from Nigeria. Folia Veterinaria, 69, 2, 56–71.

Received: April 26, 2025

Accepted: May 26, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Environmental pollution from heavy metals is a growing concern, largely driven by increased industrial activity. This study measured the concentrations of seven heavy metals—lead, cadmium, chromium, manganese, cobalt, copper, and iron—and two trace metals—magnesium and nickel—in the serum, cerebrum, cerebellum, and brainstem of the hedgehog (Eulipotyphla), pigeon (Columbiformes), cattle egret (Pelecaniformes), and two species of squirrel (Sciuromorpha), using Atomic Absorption Spectrophotometry. Among the metals tested, magnesium showed the highest concentrations across all sample types and brain regions in all studied animals. Magnesium levels reached as high as 817,598 µg/mL in the serum of female pigeons. Metal levels were generally higher than those reported in previous studies from other regions, except for cobalt, which was undetectable in the tested tissues. The highest concentrations for all metals were found in the serum. No consistent pattern was observed in metal concentrations across the brain regions. These findings reveal complex patterns of metal accumulation and distribution, potentially reflecting interspecies differences in physiology, diet, and environmental exposure. The data offers insights into metal presence in wildlife serum and brains. It will also provide crucial baseline dataset with information that can be invaluable for future ecological and environmental studies.

Keywords: blood; brain regions; heavy metals; wildlife

INTRODUCTION

The increasing human population and accelerating industrialization have contributed to the rising levels of heavy metals in the environment. This is particularly concerning due to the accumulation of these metals in air, water resources, and food consumed by humans and animals. [1, 2]. Heavy metals such as arsenic (As), manganese (Mn), mercury (Hg), lead (Pb), copper (Cu), iron (Fe), cadmium (Cd), nickel (Ni), zinc (Zn), and cobalt (Co) are pollutants that may occur naturally in the environment or result from anthropogenic activities [3]. Metals play principal roles in biological processes such as nucleic acid configurations, serving as transporters or second messengers during neurotransmission as in zinc [4], or vanadium's protective functions on the pyramidal cells of hydrocephalic mice as recently reported [5]. The human body requires essential trace metals like iron, zinc, copper, manganese, cobalt, and molybdenum for proper functioning [6]. In the brain, zinc and copper are represented in ample amounts, while iron has the highest concentration [7, 8]. Previous studies have indicated the role of trace elements in neurodevelopment with early stages of deficiency resulting in foetal anomalies. Iron deficiency during the early stages of foetal development could lead to acute brain dysfunction which is less likely to reverse after iron repletion [9].

Excessive accumulation of metals is known to be neurotoxic and increases the risk for certain neurodegenerative disorders like Parkinson's disease and Alzheimer's disease [10] as well as inducing cytogenotoxicity and hepato-renal toxicity in rats [11, 12]. Although some of these metals are essential due to their biological functions, they are required only in trace amounts and can become toxic when accumulated excessively in the body. These effects could lead to gastrointestinal and kidney dysfunctions, cognitive impairments, vascular damage and immune dysfunctions [13]. Some metals like cadmium, arsenic and chromium exerts disruptive actions on DNA synthesis and repair due to their carcinogenic abilities [14, 15]. Animals get exposed to heavy and trace metals through direct exposure, polluted water, and crops grown on irrigated sewage, industrial effluents as well as through anthropogenic activities [16]. An example of anthropogenic metal exposure was observed in a study carried out in Zamfara State, where indiscriminate small-scale artisanal gold mining led to high levels of lead and cadmium in the liver, kidney, muscle, intestine, cerebrum, cerebellum, and midbrain of goats [17, 18]. It can be quite challenging to get human samples for assessment of metal concentration in serum and tissues especially the brain. Using animals that are readily available as sentinels provides an indicator for the level of metals in the environment. Examples of sentinel indicators of metal exposure are the cases of lead poisoning in horses and cattle living close to a smelter [19] and the ataxic dancing cats of Minamata alerting local residents of methyl mercury poisoning from contaminated sea food [20].

Ibadan, the site for this study, is the capital city of Oyo State in southwestern Nigeria, and it has experienced significant urbanization over the past few decades. Originally established in the early 1800s, Ibadan has grown from a small settlement to one of Nigeria's largest cities [21]. The rapid urbanization of Ibadan has brought about significant environmental pollution challenges. Studies have shown that particulate matter (PM) levels in various locations within Ibadan can vary widely, with some areas experiencing exceptionally high concentrations. This variability is indicative of localized pollution sources, such as traffic emissions, industrial activities, and open burning of waste.

This study investigates the concentrations of heavy metals in the serum and three brain regions (cerebrum, cerebellum, and brainstem) of five sentinel animal species – squirrel (*Heliosciurus gambianus* – Gambian sun squirrel (GSS) and *Funisciurus anerythrus* – Thomas's rope squirrel (TRS)), speckled pigeon (*Columba guinea*), western cattle egret (CE) (*Bubulcus ibis*), and hedgehog (*Atelerix albiventris*) in Ibadan, Oyo State, southwest Nigeria. This study aims to provide insights on the metal presence in serum and brain tissue of specific wildlife, taking into consideration the interspecies differences in the serum, cerebrum, cerebellum, and brainstem. The study will address how the various levels of heavy metal concentrations vary across these different species, thus offering a valuable baseline dataset for future ecological and environmental research.

MATERIALS AND METHODS

Ethical Approval

Ethical approval was obtained from the National Veterinary Research Institute, Vom, Plateau State (Nigeria). All

animals were handled humanely, with the utmost care, and to avoid causing undue pain, according to the NIH guidelines for handling research and experimental animals.

Study Site

All the animals were obtained from Ibadan, a tropical savannah town (7°23'47"N 3°55'0"E). The squirrels and pigeons were captured from within the campus of the University of Ibadan, while the cattle egrets and hedgehogs were captured from the forests in Ibadan with the assistance of local hunters.

Animals

A total of 50 animals were captured from their natural environments by using food baits in metal cage traps and local traps and transported to the laboratory in individual cages. They were certified to be healthy by a qualified veterinarian. The animals (five males and five females for each animal species, 5 species in total) were euthanised with ketamine (100 mg/kg) and xylazine (10 mg/kg) combination through intramuscular injection [22]. Blood (0.5 – 1.5 mL) was collected into plain tubes from all animals via the intracardiac route once there was no breathing and there was cessation of response to tactile stimuli. Blood collected was centrifuged at 10,000 rpm for 10 mins to obtain the sera, which were stored at -20 °C until analysed. These animals were selected because they are quite abundant in Ibadan, which is a suitable habitat for them. The hedgehog and squirrels were sexed by the presence or absence of a scrotal sac, while birds were sexed after sacrifice.

Brain Collection

All the brains (n = 50) were harvested from the skull and subsequently examined *in situ* to rule out any gross morphological pathology. The brains were further carefully dissected to expose the cerebrum, cerebellum, and brainstem as previously described [23]. This was followed by immediate cryopreservation at -20 °C till analysed.

Atomic Absorption Spectrophotometry

Metal concentrations in serum and brain regions were analysed using Atomic Absorption Spectrophotometry (AAS), following the methods described [24, 25, 26]. The concentration of nine (9) metals were analysed – lead (Pb), magnesium (Mg), cadmium (Cd), chromium (Cr), iron (Fe), nickel (Ni), copper (Cu), manganese (Mn), and cobalt (Co). For the quality assurance check, deionized water was used to establish a baseline, while calibration standards with known concentrations of the analyte were used to create a calibration curve. Linearity checks were also carried out to ensure the accuracy and reliability of the measurements. This was done using known standard concentrations after every 10 samples and periodically during the analysis to detect drift while all samples were analysed in duplicates to ensure precision. The detection limit for each of the metals are Pb – 0.08 µg/l; Ni – 0.05µg/l; Mg – 0.005µg/l; Fe – 0.05µg/l; Cr – 0.04µg/l; Cd – 0.01µg/l; Mn – 0.03µg/l; Co – 0.05µg/l.

Statistical Analysis

The collected data were analysed using 2-way ANOVA with Tukey post hoc test and descriptive statistics. The data were expressed as means ± SEM and as bar diagrams. The statistical package used was GraphPad Prism version 8.0.0 (GraphPad Software, San Diego).

RESULTS

Values obtained are presented as mean ± standard deviation and presented in µg/mL (sera) or µg/g (tissue) in bar diagrams Figures 1 – 8.

Cadmium

The highest observable concentration was in the serum of the male pigeon (3,015 ± 2,382.95), and there was a statistically significant difference ($p > 0.05$) when compared to the level in male GSS, which had the lowest level (690 ± 500.30) (Figure 1). Non-detectable levels were observed in the cerebrum of the female hedgehog and the cerebellum and brainstem of both male and female pigeons.

Chromium

The sera of the female TRS (1,635 ± 332.34) and female GSS (1,600 ± 1,032.38) had the highest concentration values, while the cerebellum of the male hedgehog had the highest values in the brain region (635.38 ± 31.71), and the cerebrum of the female GSS (0.955 ± 0.26) had the lowest value. However, there was no statistically significant differences (Figure 2).

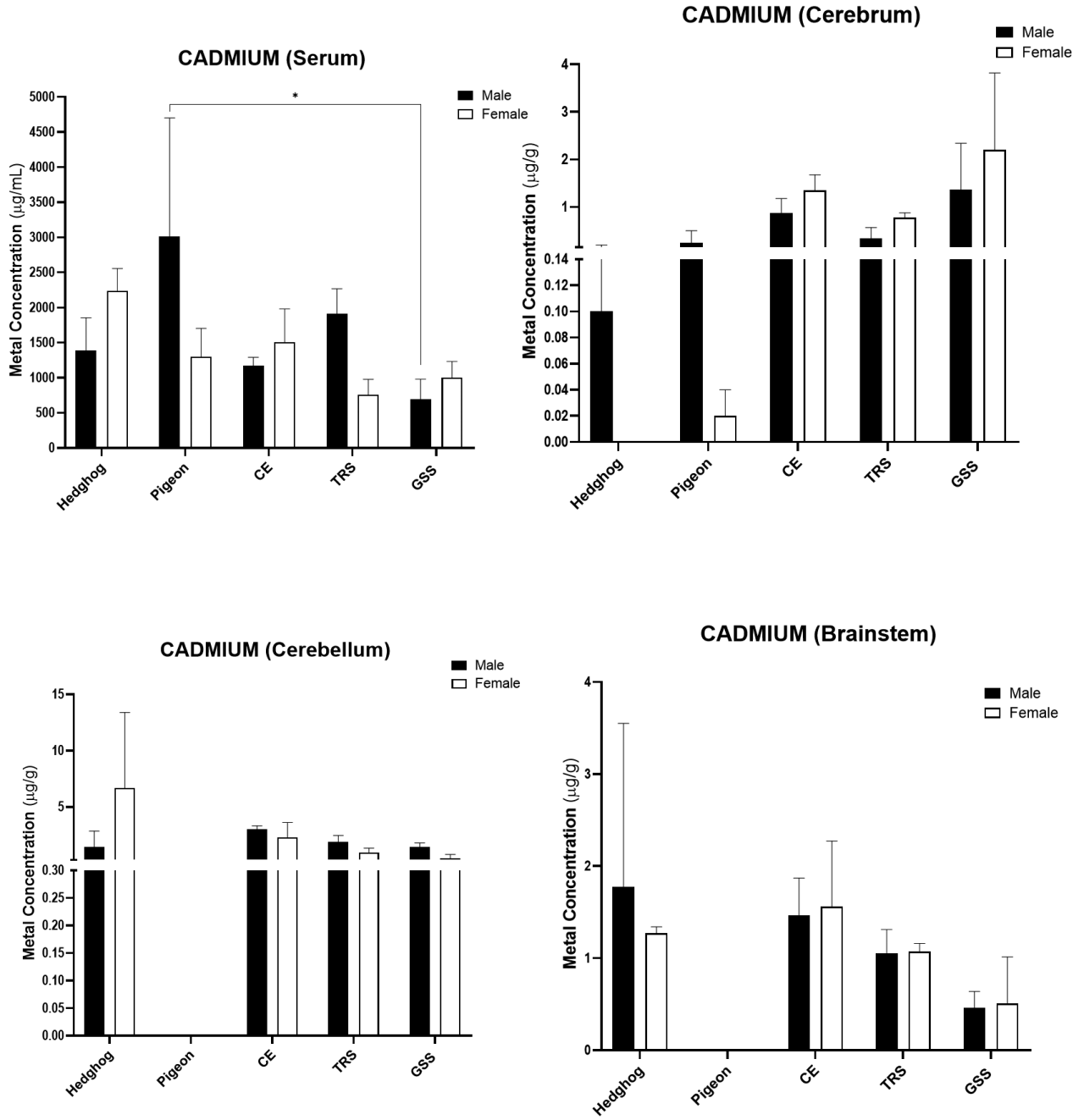

Fig. 1. Bar charts showing concentration of cadmium in serum, cerebrum, cerebellum, and brainstem of male and female hedgehog, pigeon, cattle egret (CE), Thomas's rope squirrel (TRS), and Gambian sun squirrel (GSS). (*- P ≤ 0.05)

Cobalt

There were no detectable levels of cobalt in all the sera and brain regions analysed.

Copper

The sera, cerebrum and brainstem of all 5 species analysed, as well as the cerebellum of female CE and TRS, had no detectable level of copper. The detectable levels observed in the cerebellum ranged from 0.18 ± 0.31 (male GSS) to 5.29 ± 2.23 (male pigeon) (Figure 3).

Iron

The highest values were observed in sera of male hedgehog (14,988.3 ± 8,302.57) and female CE (11,668 ± 7,819.30), while the lowest value was observed in the cerebellum of the female GSS (23.48 ± 29.30). Iron concentration in the brainstem as well as the cerebrum showed statistically significant differences between either males or females across species (Figure 4).

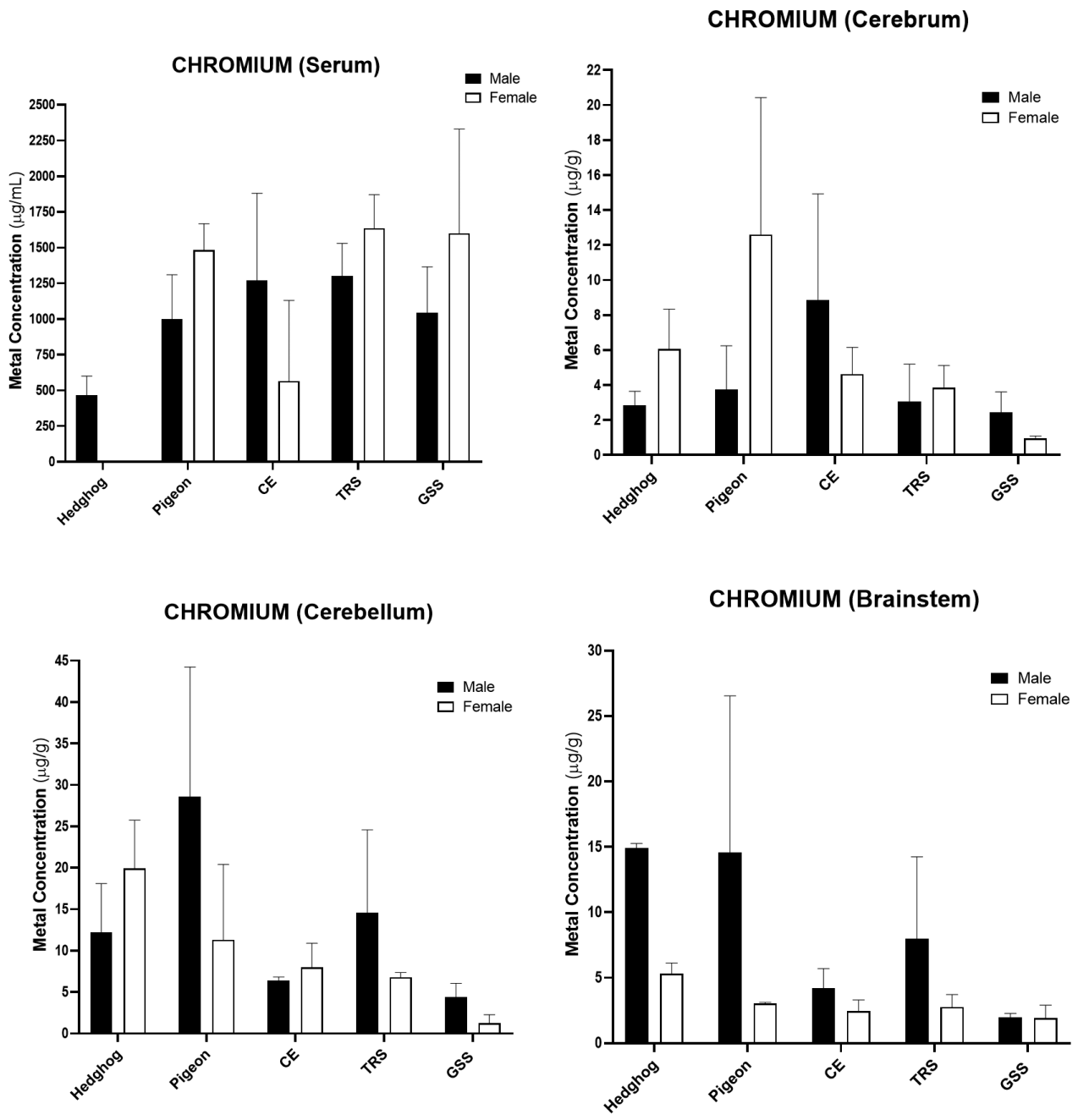

Fig. 2. Bar charts showing concentration of chromium in serum, cerebrum, cerebellum, and brainstem of male and female hedgehog, pigeon, cattle egret (CE), Thomas's rope squirrel (TRS), and Gambian sun squirrel (GSS).

Lead

The highest values were observed in the serum of both male and female of all the species. CE (male 15,330 ± 2,828.43; female 5,000 ± 1,414.21) and TSS (male 5,110 ± 2,33.77; female 14,000 ± 1,880.90) showed the highest variations between genders in serum concentrations as seen in Figure 5. Some regions of the brain however had values below detection limit.

Magnesium

The highest values were observed in the serum of the male hedgehog (617,540 ± 77,5407.4) and female TRS (736,668 ± 409,859.8). Of all the metals assessed, Mg showed the highest values across all the brain regions and in serum (Figure 6). The cerebrum of the pigeon had the highest concentration of Mg across all the brain regions and was statistically significantly different from the levels in the cerebrum of the other males.

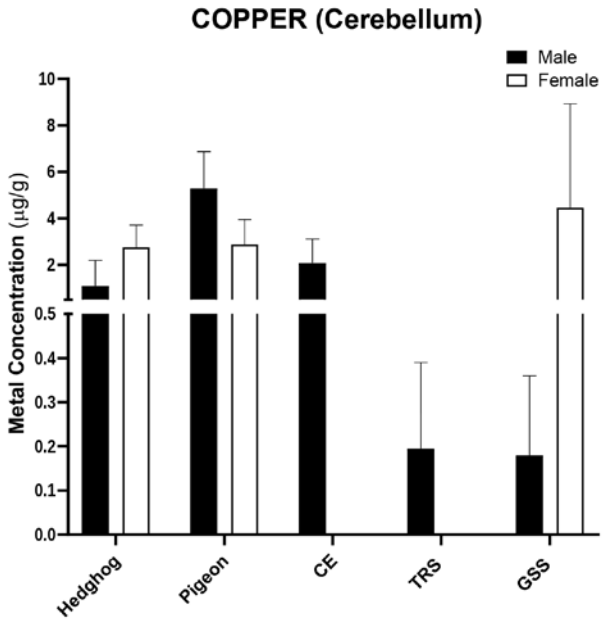

Fig. 3. Bar charts showing concentration of copper in the cerebellum of male and female hedgehog, pigeon, cattle egret (CE), Thomas's rope squirrel (TRS), and Gambian sun squirrel (GSS).

Manganese

The only detectable levels were observed in the sera of the female CE (266 ± 594.79) and that of the male TRS (168 ± 335) (Figure 7).

Nickel

The highest values were observed in the serum of the male CE (3,235 ± 3,061.77) and female TRS (3,300 ± 3,776.32). (Figure 8). There was no nickel detected in the cerebrum of all the animals.

DISCUSSION

Environmental pollution by metals is becoming a global health concern as it threatens the ecosystem due to bioaccumulation. These metals last a long time in the environment and cause various health effects when there is an exposure to them. Heavy and trace metals are important environmental pollutants, with their toxicity being an increasingly significant challenge for ecological, nutritional, environmental and evolutionary reasons [18]. Entry of these metals into the environment is notably through natural means (like soil erosion and natural weathering of earth's crust) and human activities (like industrial effluents, mining, sewage discharge, use of insecticides and use of disease control agents on crops) [27]. Metal pollution is a universal environmental problem affecting all living organisms through their bioaccumulation and biomagnification along the food chain [28]. Bioaccumulation of heavy metals in the brain has been documented in wildlife like the polar bears [29–31], beluga whales [31, 32], bottlenose dolphins [33], river otters [34], and rodents [35]. Using animals as bioindicators helps to determine the extent of environmental contamination and also the exposure to certain compounds [33].

Blood serum serves as a short-term indicator of the extent of exposure to certain elements, thereby enabling their current intake to be evaluated [33]. Accumulations of heavy metals in different body tissues have been documented, but the blood-brain barrier serves as a semi-permeable membrane to limit the entrance of noxious substances into the brain [36]. This could be the reason why the highest concentrations of metals in this study were found in the serum.

Cadmium

Reports have shown that exposure to cadmium in the human population primarily occurs through the use of rechargeable nickel-cadmium batteries and cigarettes, while exposure through food is the main route in non-smokers. Cadmium affects the nervous system by the inhibition of acetylcholinesterase activity [37]. Brain concentration levels obtained from this report were considerably higher than those reported in mallards, 0.006 ± 0.012 µg/g wet weight (adults) and 0.013 ± 0.012 µg/g wet weight (immature) [38] and slightly higher than those reported in various carnivorous mammals from northwest Poland [39]. A study from Argentina, in three different species of birds (Great Kiskadee – *Pitangus sulphuratus*; Neotropic Cormorant – *Nannopterum brasilianum*; Great Grebe – *Podiceps major*) reportedly had varied values of cadmium in the whole brain [40]. In a similar study in Nigeria, in the African giant rat from three ecological zones, brain cadmium levels ranged from 0.03 to 0.05 mg/L [22], values that are generally lower than that obtained in different brain regions in animals in this current study.

Cobalt

Cobalt is categorised as one of the less common heavy metals and is essential for the synthesis of vitamin B_{12} [41]. Cobalt serum levels obtained from grazing cattle from different reserves in Kenya were on the average 0.017 and

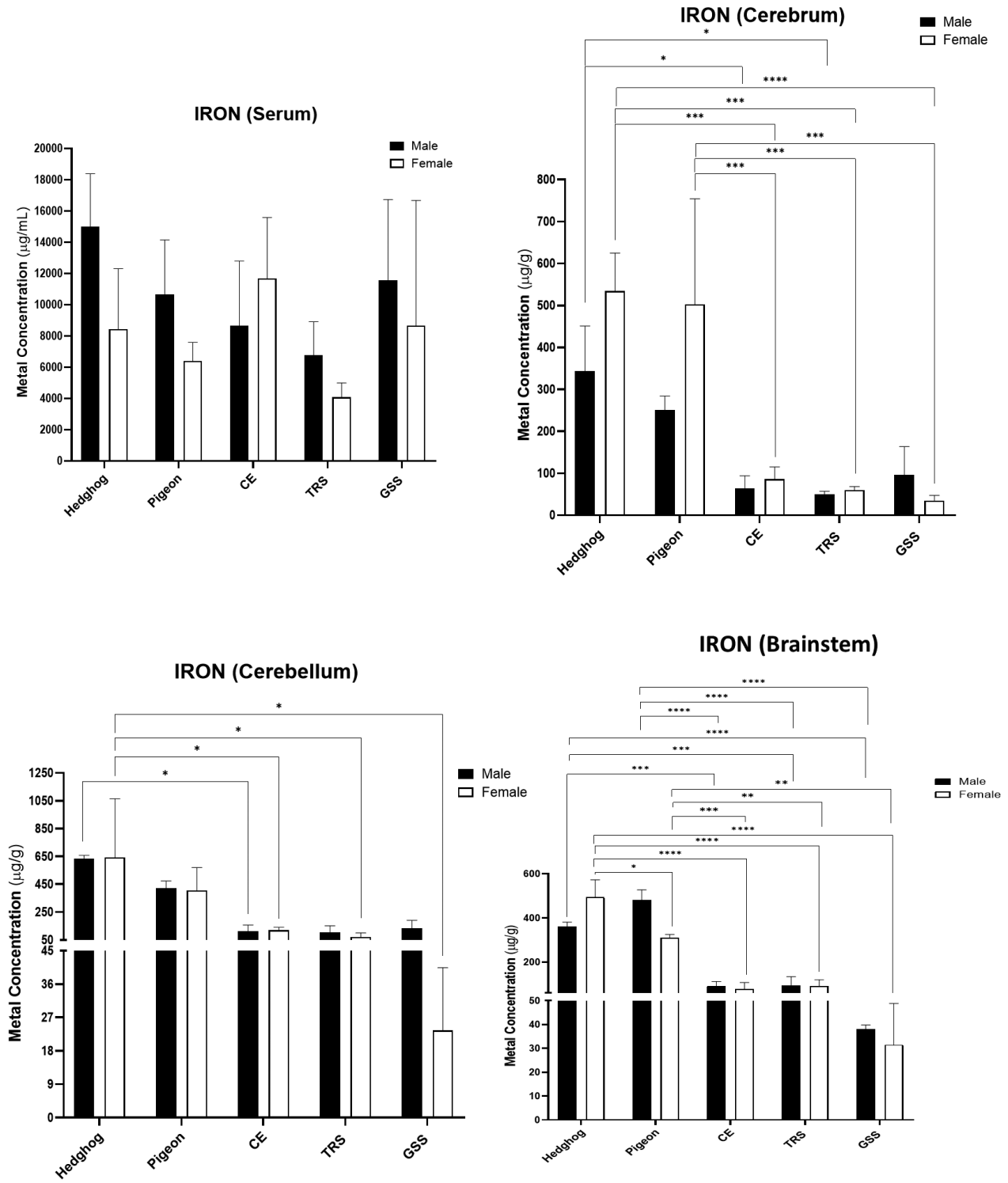

Fig. 4. Bar charts showing concentration of iron in the sera, cerebrum, cerebellum, and brainstem of male and female hedgehog, pigeon, cattle egret (CE), Thomas's rope squirrel (TRS), and Gambian sun squirrel (GSS). (*- P ≤ 0.05; **-P ≤ 0.01; *- P ≤ 0.001; ****-P ≤ 0.0001)**

0.018 mg/L [42], unlike the non-detectable levels in this study.

Chromium

Chromium is a heavy metal found in plants, rock and soil. Chromium exposure is usually by consuming contaminated food and water and breathing contaminated air. Theses can cause ulcer formation which may take months to heal, lead to cancer, anaemia and also cause damage to the stomach and intestines [43, 44]. Along with arsenic, cadmium, mercury, copper, and lead, chromium has been listed by the United States Environmental Protection

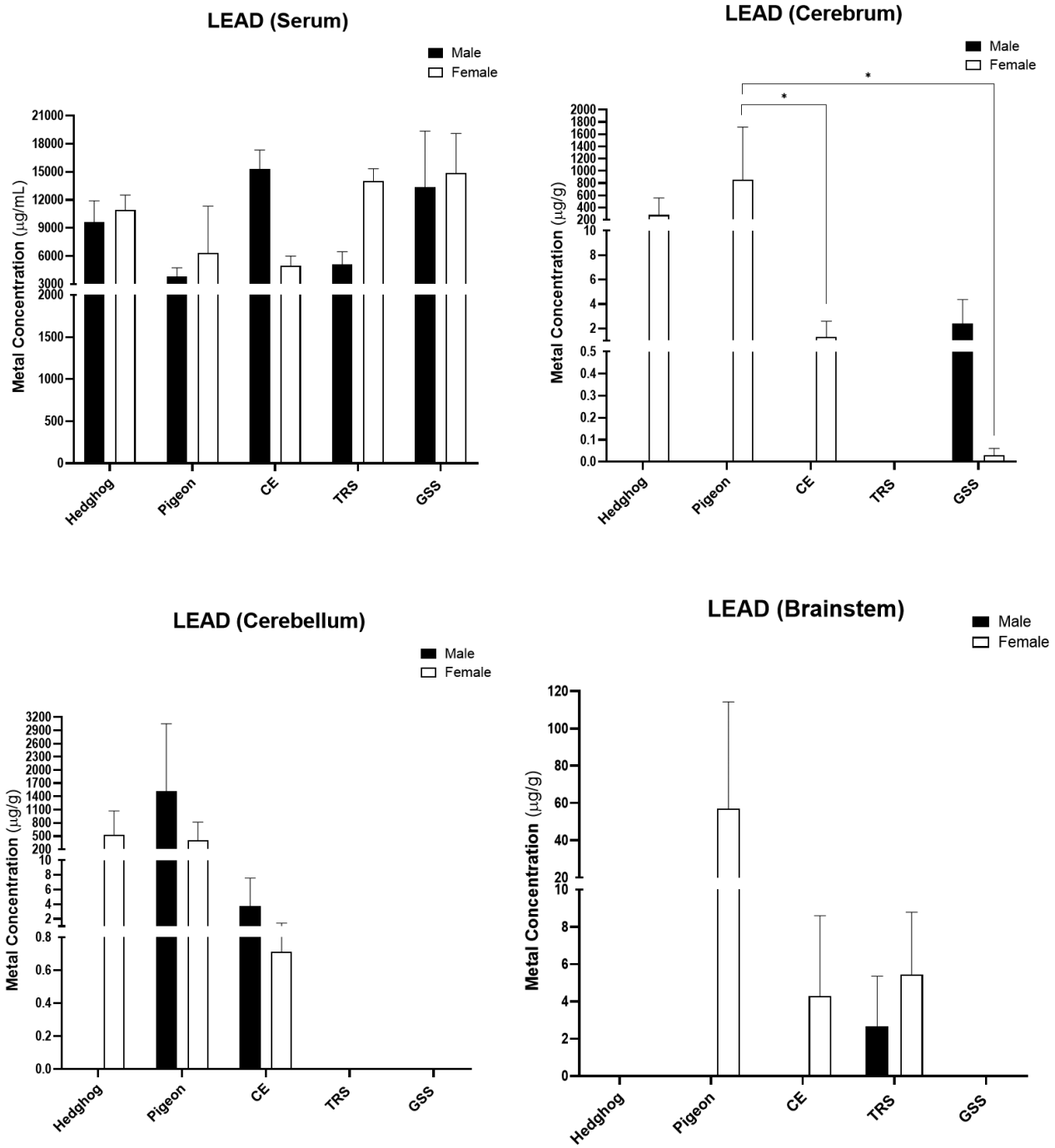

Fig. 5: Bar charts showing concentration of lead in the sera, cerebrum, cerebellum, and brainstem of male and female hedgehog, pigeon, cattle egret (CE), Thomas's rope squirrel (TRS), and Gambian sun squirrel (GSS). (*- P≤0.05)

Agency (USEPA) as priority control pollutant because of its persistence and its irreversible toxic characteristics [44]. Concentration of this metal in tissues from wildlife from two regions in Slovakia were reported not to be dangerous [45], unlike reports from this current study. Concentrations levels observed in the brains of different waterfowls from Evros Delta, Greece, were all below 0.5 mg/kg of metal/tissue [46], which is considerably lower than results obtained from this study. Maximum concentration in drinking water should not exceed 0.1 mg/L [44].

Copper

Copper remains one of the most commonly found heavy metals in wastewater [47]. Its pollution is caused majorly by manufacturing operations, farming, mining, industrial and municipal wastewater. Signs of toxicity in-

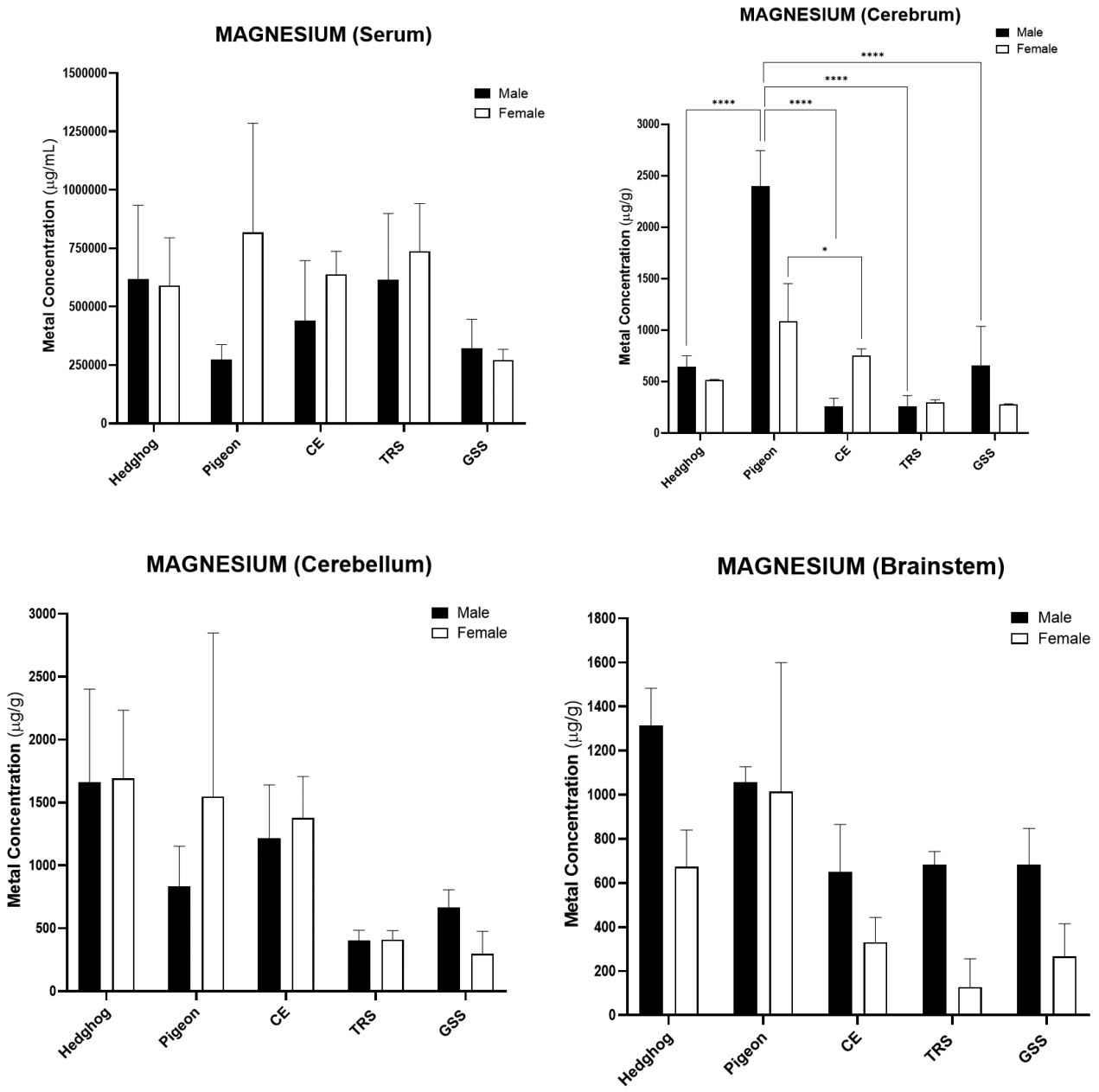

Fig. 6. Bar charts showing concentration of magnesium in the sera, cerebrum, cerebellum and brainstem of male and female hedgehog, pigeon, cattle egret (CE), Thomas's rope squirrel (TRS) and Gambian sun squirrel (GSS). (*- P ≤ 0.05; **-P ≤ 0.0001)**

clude vomiting, jaundice, abdominal pain, gastrointestinal distress and hypotension. Long-term exposure results in damage to the brain, liver, and kidney [48, 49]. Unlike the report in goats [50], where there were detectable levels in all regions analysed, only the cerebellum had detectable levels. However, concentration levels from the cerebellum in this study were similar to that obtained from the brains of mallards [38]. Copper serum levels from cattle in three game reserves in Kenya were 0.652, 0.659, and 0.785 mg/L [42], which were all considerably higher than the non-detectable levels observed in this study.

Iron

This is the second most abundant metal on the earth's crust. It is a very important element for the survival and growth of practically all living organisms, being a very important component of enzymes and also found in organisms [43]. Iron is one of the heavy metals required for proper function of various enzymes and proteins [37]. In a study in goats, the olfactory bulb was seen to have the highest concentration (98.66 ± 23.02 ppm) relative to other regions tested (cerebral cortex, cerebellum, thalamus, and hippocampus) [50]. The increase in mining activities has

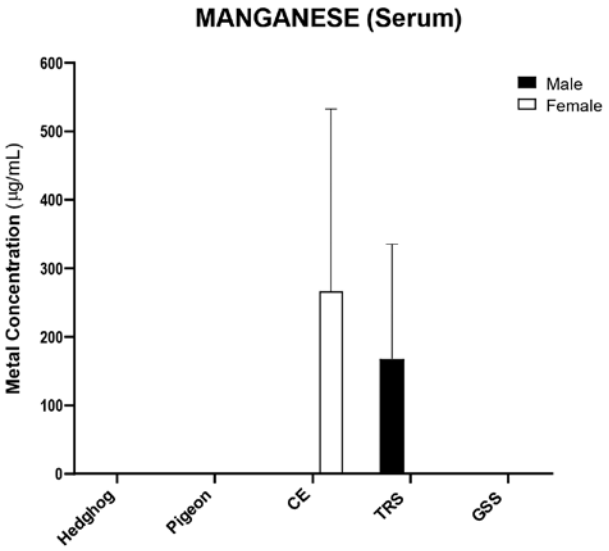

Fig. 7. Bar charts showing concentration of manganese in the serum of female cattle egret (CE) and male Thomas's rope squirrel (TRS).

been reported to increase the incidence or presence of iron in surface water [51], resulting in human exposure to iron by drinking water [43]. Values obtained from the brains of mallards in Poland, without anatomical region specificity, ranged from 20.6 to 25.5 µg/g [38], values far lower than what was obtained from any animal in this current study, which may be because the location is heavily urbanized and industrialized.

Lead

Bioaccumulation of lead in the striatum and nucleus accumbens of the Mexican spiny pocket mouse (*Liomys irroratus*) has been documented to result in variations in the dopaminergic neurons in those areas and cause changes in behavioural tests, showing modifications in learning and memory [26]. In a previous study on mallards, concentrations of Pb, expressed as µg/g, in the whole brain from two different locations in Poland were 0.294 ± 0.603 and 0.672 ± 1.128 (adults) and 0.564 ± 0.600 and 0.401 ± 0.425 (immature subjects) [38]. No distinction was made as to gender in the Polish study. These values are generally lower than the values obtained in the brain regions of the pigeon and cattle egret and even the hedgehog and squirrels obtained in the current study, except for the values obtained in the cerebrums of the male pigeon and cattle egret and the cerebellum of the female cattle egret, which were higher. In a similar study in goats from Nigeria, Pb levels across five brain regions were observed to range from 0.19 to 0.22 ppm, the lowest levels being in the hippocampus and thalamus and the highest levels being in the cerebellum [50]. A study in 3 different species of birds (Great Kiskadee, Neotropic Cormorant, and Great Grebe) from the Argentina had varied values in the whole brain: 5.16 ± 2.44, 0.44 ± 0.05, and 1.27 ± 0.43 ppm (wet weight) and 11.15 ± 5.04, 1.76 ± 0.17, and 5.34 ± 1.84 ppm (dry weight), respectively [40]. A similar study in wild-captured rodents (African giant rat) from Nigeria, across three ecological zones, showed non-detectable levels of Pb in the serum and brain [22]. These varied results buttress the fact that some animals may have a greater tendency to accumulate some particular metals relative to other animals which may be due to variations in their habitats. Levels of Pb in the brain above 5 µg/g are believed to be indicative of poisoning in birds [52], while levels above 16 µg/g are believed to show an advanced level of exposure [53]. Lead affects every organ in the body, with the nervous system being the primary target. Long-term exposure can result in cognitive deficits, weakness in the extremities, anaemia and kidney damage [44]. Lead values obtained from this study were in some cases far above these values, probably indicating an advanced state of exposure from and in the environment since the location is heavily industrialized metropolitan areas (which could be from the soil, water, or even plants eaten), and posing a significant health hazard to humans to eat these animals as a source of protein and even other predatory animals.

Magnesium

This is an essential nutrient required for various biochemical and physiological functions [3]. Values obtained for magnesium in serum and other regions of the brain remained considerably higher than those obtained for other metals. Literature search revealed that magnesium is usually tested to ascertain or monitor the health status of animals, not commonly for toxicology studies. Magnesium serum concentration in wild northern goshawks (*Accipiter gentilis*) in Pennsylvania was 1.9 ± 0.17 mg/L [54], while a study to establish reference values for Mg in plasma in African grey parrots and Hispaniolan parrots gave results as 2.10 – 3.40 and 1.80 – 3.10 mg/dL, respectively [55]. These values in both these studies were far lower than that obtained from this current study. This result raises a concern on the effect of Mg toxicity, not only in the serum, plasma, or brain, but also in other organs that are generally not tested or seen to be of any concern.

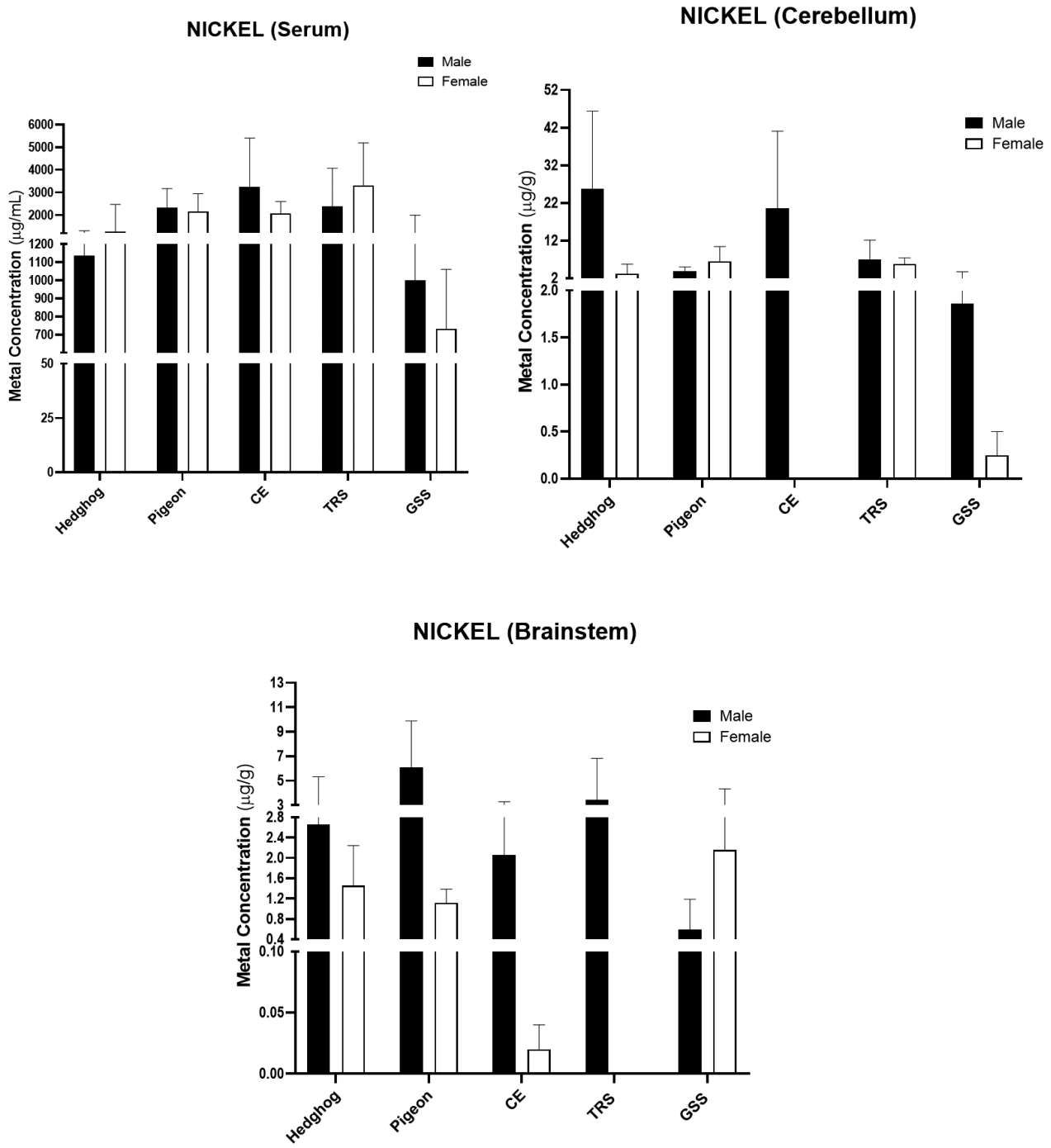

Fig. 8. Bar charts showing concentration of nickel in the sera, cerebellum, and brainstem of male and female hedgehog, pigeon, cattle egret (CE), Thomas's rope squirrel (TRS), and Gambian sun squirrel (GSS).

Manganese

Manganese is involved in the metabolism of proteins, carbohydrates and lipids, while also being a co-factor in many antioxidant enzymes like glutamine synthase. Neurotoxicity of manganese occurs when there is imbalance between antioxidant systems and reactive species. In addition, it can modify epigenetic mechanisms like DNA methylation, altering gene expression and resulting in neurotoxicity [56]. Concentration levels reported in the brains of mallards ranged from 0.28 to 0.40 µg/g [56], while that from different brain regions of the *Liomys irroratus* rodent, obtained from mining areas in Mexico, ranged from 400.1 to 858 µg/kg [34]. These two reports differ greatly from observations in this study, where detectable levels were only observed in the serum of the male *F. anerythrus* and female cattle egret, and no levels in the brain.

Nickel

This is one of the most commonly found heavy metals in wastewater, posing risks to the environment and humans [47]. Bioaccumulation of Ni in brain regions has been reported to result in changes in dopaminergic function and activities like exploratory and memory activities [57]. With values expressed as µg/kg, reports of Ni concentration in rodent brains from a mining area varied from 165.1 ± 89.8 to 572 ± 122.3, with the highest concentration in the nucleus accumbens and the lowest in the striatum [26]. Results from our current study were varied, with the cerebrum having non-detectable levels across all animals, the cerebellum of the female cattle egret and the brainstem of the hedgehog, Gambian sun squirrel, male pigeon, and male cattle egret (see Table 2). Reports from concentration in the mallard brain (where there were detectable levels) – 0.012 ± 0.022 (adults) and 0.020 ± 0.022 (immature birds) [38] showed values lower than those obtained in the current study.

The major means by which living organisms are exposed to heavy metals is by food intake. Grazing animals are exposed by contaminated soil, and the absorbed metal binds to blood cells or blood plasma components [50]. The study of heavy metal accumulation in wildlife is important for ecological and conservation reasons, as exposure to these substances have been known to cause alterations in behaviour, breeding, and survival, resulting in long-term effects that may have deleterious effects on the population of affected animals [26, 31, 33, 42, 58].

Where values were available, metal concentration observed from other results seemed to be generally higher in this study than other reported studies, except in cobalt, where there were no detectable values. This result is alarming and should be a call to increase the awareness of heavy metal pollution and the hazard not only to the animals tested and those that prey on them but also to humans living in these environments and who possibly feed on these.

The reasons for the non-detectable levels in some brain regions in spite of high levels in some other regions in the same animals may possibly be an indicator for the differences in predilection sites for various metals in each animal and differences in environmental factors. But it should be noted that non-detectable levels of some metals in tissue samples have been reported by other authors as well. The irregular pattern and level of bioaccumulation is similar to reports obtained in ducks in Greece [46].

CONCLUSION

The information obtained from this study has provided valuable insights and crucial baseline data into the level of metals in wildlife, which can be useful for future ecological and environmental studies involving the need for conservation of wildlife and flora. The high level of metal concentrations observed generally in this study is a cause for concern, as prolonged exposure to some of these metals (lead, magnesium) has been reported to cause neurodegenerative conditions that may affect behavioural patterns and ultimately alter breeding and survival and subsequently the population and the ecosystem. [30, 32, 33, 34, 55]. Brain regions have been reported to give varied responses when exposed to insults; however, the reason for the sometimes-wide differences in metal concentration across regions in the same species and differences between the sexes still needs investigation. This study has also highlighted the need for surveillance of metal concentration in the environment using animals and wildlife as sentinels since rapid urbanization is increasing the contact of humans with wildlife, meaning that humans are increasingly cohabiting with wildlife, and metal levels in these animals can be essential indicators of metal toxicity in human population.

Ethical Approval and Consent to Participate

This study was performed in line with the principles of the Declaration of Helsinki. Ethical approval was granted by the Animal Care Committee, National Veterinary Research Institute, Vom, Plateau State (Nigeria), ethical code NVRI/AEC/03/116/22.

Clinical Trial Number

Not applicable

Consent to Publication

All authors approved the final version and consented to the publication.

Data Availability Statement

Data are available on reasonable request from corresponding author.

Conflict of Interest

The authors declare there are no conflicts of interest.

Funding

This study was supported by funds from the Humboldt Research Hub for Zoonotic Arboviral Diseases, awarded to JOO.

Competing Interests

All the authors hereby declare that there are no financial or non-financial interests directly or indirectly related to this work.

Generative AI Statement

Authors declare that no generative AI and AI-assisted technologies were used in writing the manuscript.

Authors' Contributions

Conceptualisation – JOO; experimental design – JOO, OOI, FEO; sample collection and analysis – TTG, AAO, ICO, OOI, FEO, OIF, IKP, KIA; manuscript draft – AAO, TTG, ICO and OOI; manuscript editing – JOO and OOI. All authors approved of the final version of the manuscript.

Acknowledgements

The authors gratefully acknowledge the support of Humboldt Research Hub for Zoonotic Arboviral Diseases for financing part of this study.

REFERENCES

1. **Ghorani-Azam, A., Riahi-Zanjani, B., Balali-Mood, M., 2016:** Effects of air pollution on human health and practical measures for prevention in Iran. *J. Res. Med. Sci*., 21, 1, 65. DOI: 10.4103/1735-1995.189646
2. **Luo, L., Wang, B., Jiang, J., Fitzgerald, M., Huang, Q., Yu, Z., et al., 2021:** Heavy metal contaminations in herbal medicines: determination, comprehensive risk assessments, and solutions. *Front. Pharmacol*., 11. DOI: 10.3389/fphar.2020.595335
3. **Tchounwou, P. B, Yedjou, C. G., Patlolla, A. K., Sutton, D. J., 2012:** Heavy metal toxicity and the environment, *EXS,* 101, 133–164. DOI: 10.1007/978-3-7643-8340-4_6
4. **Fukada, T., Kambe, T., 2011:** Molecular and genetic features of zinc transporters in physiology and pathogenesis. *Metallomics*, 3, 7, 662–674. DOI: 10.1039/c1mt00011j
5. **Femi-Akinlosotu, O. M., Olopade, F. E., Obiako, J., Olopade, J.O., Shokunbi, M. T., 2023:** Vanadium improves memory and spatial learning and protects the pyramidal cells of the hippocampus in juvenile hydrocephalic mice. *Front. Neurol.,* 14. DOI: 10.3389/fneur.2023.1116727
6. **Mehri, A., 2020:** Trace elements in human nutrition (II) - an update. *Int. J. Prev. Med.,* 11, 1, 2. DOI: 10.4103/ijpvm.IJPVM_48_19
7. **Ramos, P., Santos, A., Pinto, N. R., Mendes, R., Magalhães, T., Almeida, A., 2014:** Anatomical region differences and age-related changes in copper, zinc, and manganese levels in the human brain. *Biol. Trace. Elem. Res.,* 161, 190–201. DOI: 10.1007/s12011-014-0093-6
8. **Kardos, J., Héja, L., Simon, Á., Jablonkai, I., Kovács, R., Jemnitz, K., 2018:** Copper signalling: causes and consequences. *Cell Commun. Signal.*, 16, 71. DOI: 10.1186/s12964-018-0277-3
9. **Georgieff, M. K., 2008:** The role of iron in neurodevelopment: fetal iron deficiency and the developing hippocampus. *Biochem. Soc. Trans.*, 36, 6, 1267–1271. DOI: 10.1042/BST0361267
10. **Ijomone, O. M., Ifenatuoha, C. W., Aluko, O. M., Ijomone, O. K., Aschner, M., 2020:** The aging brain: impact of heavy metal neurotoxicity. *Crit. Rev. Toxicol.*, 50, 9, 801–814. DOI: 10.1080/10408444.2020.1838441
11. **Jadhav, S. H., Sarkar, S. N., Patil, R. D., Tripathi, H. C., 2007:** Effects of subchronic exposure via drinking water to a mixture of eight water-contaminating metals: A biochemical and histopathological study in male rats. *Arch. Environ. Contam. Toxicol.*, 53, 667–677. DOI: 10.1007/s00244-007-0031-0
12. **Usende, I. L., Alimba, C. G., Emikpe, B. O., Bakare, A. A., Olopade, J. O., 2018:** Intraperitoneal sodium metavanadate exposure induced severe clinicopathological alterations, hepato-renal toxicity and cytogenotoxicity in African giant rats (*Cricetomys gambianus*, Waterhouse, 1840). *Environ. Sci. Pollut. Res.,* 25, 26383–26393. DOI: 10.1007/s11356-018-2588-8
13. **Deng, Y., Wang, M., Tian, T., Lin, S., Xu, P., Zhou, L., et al., 2019:** The Effect of hexavalent chromium on the in-

cidence and mortality of human cancers: A meta-analysis based on published epidemiological cohort studies. *Front. Oncol.,* 9, 24. DOI: 10.3389/fonc.2019.00024

14. **Clancy, H. A., Sun, H., Passantino, L., Kluz, T., Muñoz, A., Zavadil, J., et al., 2012:** Gene expression changes in human lung cells exposed to arsenic, chromium, nickel or vanadium indicate the first steps in cancer. *Metallomics,* 2012, 4, 8, 784–793. DOI: 10.1039/c2mt20074k
15. **Koedrith, P., Kim, H., Weon, J. I., Seo, Y. R., 2013:** Toxicogenomic approaches for understanding molecular mechanisms of heavy metal mutagenicity and carcinogenicity. *Int. J. Hyg. Environ. Health.*, 216, 5, 587–598. DOI: 10.1016/j.ijheh.2013.02.010
16. **Milam, C., Buba, M., Dogara, R. K., Yila, E. Y., 2017:** Assessment of heavy metals (As, Cd, Cr, Cu, Ni, Pb and Zn) in blood samples of sheep and rabbits Jimeta-Yola, Adamawa State, Nigeria. *IJAPBC,* 6, 3, 2277–4688.
17. **Jubril, A. J., Kabiru, M., Olopade, J. O., Taiwo, V. O., 2017:** Biological monitoring of heavy metals in goats exposed to environmental contamination in Bagega, Zamfara State, Nigeria. *Adv. Environ. Biol.*, 11, 6, 11–18.
18. **Jubril, A. J., Obasa, A. A., Mohammed, S. A., Olopade, J. O., Taiwo, V. O., 2019:** Neuropathological lesions in the brains of goats In North-Western Nigeria: possible impact of artisanal mining. *Environ. Sci. Pollut. Res. Int.,* 26, 36, 36589–36597. DOI: 10.1007/s11356-019-06611-y
19. **Hammond, P. B., Aronson, A. L., 1964:** Lead poisoning in cattle and horses in the vicinity of a smelter. *Ann. N. Y. Acad. Sci.,* 111, 2, 595-611. DOI: 10.1111/j.1749-6632.1964.tb53128.x
20. **Tsuchiya, K., 1992:** The discovery of the causal agent of Minamata disease. *Am. J. Ind. Med.,* 21, 2, 275-280.
21. **Roelofs, P., 2021:** Urban renewal in Ibadan, Nigeria: World class but essentially Yoruba, *African Affairs,* 120, 480, 391–415. DOI: 10.1093/afraf/adab021
22. **Olopade, J. O., Mustapha, O. A., Fatola, O. I., Ighorodje, E., Folarin, O. R., Olopade, F. E., et al., 2023:** Neuropathological profile of the African Giant Rat brain (*Cricetomys gambianus*) after natural exposure to heavy metal environmental pollution in the Nigerian Niger Delta. *Environ. Sci. Pollut. Res.*, 30, 120496–120514. https://doi.org/10.1007/s11356-023-30619-0
23. **Igado, O. O., Andrioli, A., Azeez, I. A., Girolamo, F., Errede, M., Aina, O. O., et al., 2020:** The ameliorative effects of a phenolic derivative of Moringa oleifera leave against vanadium-induced neurotoxicity in mice. *IBRO Reports*, 9, 164–182. DOI: 10.1016/j.ibror.2020.07.004
24. **Perkin-Elmer, 1996:** Analytical Methods for Atomic Absorption Spectroscopy. Perkin-Elmer Corporation, USA
25. **Khazaeli, S., Nezamabadi, N., Rabani, M., Panahi, H. A., 2013:** A new functionalized resin and its application in flame atomic absorption spectrophotometric determination of trace amounts of heavy metal ions after solid phase extraction in water samples. *Microchem. J.*, 106, 147–153. DOI: 10.1016/j.microc.2012.06.002
26. **Hernández-Plata, I., Rodríguez, V. M., Tovar-Sánchez, E., Carrizalez, L., Villalobos, P., Mendoza-Trejo, M. S., et al., 2020:** Metal brain bioaccumulation and neurobehavioral effects on the wild rodent (*Liomys irroratus*) inhabiting mine tailing areas. *Environ. Sci. Pollut. Res.,* 27, 29, 36330–36349. DOI: 10.1007/s11356-020-09451-3
27. **Morais, S., Costa, F. G., Pereira, M. L., 2012:** Heavy metals and human health, In *Environmental health – emerging issues and practice.* InTech. DOI: 10.5772/29869
28. **Gall, J. E., Boyd, R. S., Rajakaruna, N., 2015:** Transfer of heavy metals through terrestrial food webs: a review. *Environ. Monit. Assess.*, 187, 201. DOI: 10.1007/s10661-015-4436-3
29. **Basu, N., Scheuhammer, A. M., Sonne, C., Letcher, R. J., Born, E. W., Dietz, R. 2009:** Is dietary mercury of neurotoxicological concern to wild polar bears (*Ursus maritimus*)? *Environ. Toxicol. Chem.*, 28, 1, 133–140. DOI: 10.1897/08-251.1
30. **Krey, A., Kwan, M., Chan, H. M., 2014:** In vivo and in vitro changes in neurochemical parameters related to mercury concentrations from specific brain regions of polar bears (*Ursus maritimus*). *Environ. Toxicol. Chem.*, 33, 11, 2463–2471. DOI: 10.1002/etc.2685
31. **Krey, A., Ostertag, S. K., Chan, H. M., 2015:** Assessment of neurotoxic effects of mercury in beluga whales (*Delphinapterus leucas*), ringed seals (*Pusa hispida*), and polar bears (*Ursus maritimus*) from the Canadian Arctic. *Sci. Total. Env.*, 509–510, 237–247. DOI: 10.1016/j.scitotenv.2014.05.134
32. **Ostertag, S. K., Shaw, A. C., Basu, N., Chan, H. M., 2014:** Molecular and neurochemical biomarkers in Arctic beluga whales (*Delphinapterus leucas*) were correlated to brain mercury and selenium concentrations. *Env. Sci. Technol.*, 48, 19, 11551–11559. DOI: 10.1021/es501369b
33. **Meador, J. P., Ernest, D., Hohn, A. A., Tilbury, K., Gorzelany, J., Worthy, G., et al., 1999:** Comparison of elements in bottlenose dolphins stranded on the beaches of Texas and Florida in the Gulf of Mexico over a one-year period. *Arch. Environ. Contam. Toxicol.*, 36, 87–98. DOI: 10.1007/s002449900446

34. **Dornbos, P., Strom, S., Basu, N., 2013:** Mercury exposure and neurochemical biomarkers in multiple brain regions of Wisconsin River Otters (*Lontra canadensis*). *Ecotoxicology*, 22, 469–475. DOI: 10.1007/s10646-013-1040-6
35. **Erry, B. V., Macnair, M. R., Meharg, A. A., Shore, R. F., 2005:** The distribution of arsenic in the body tissues of wood mice and bank voles. *Arch. Environ. Contam. Toxicol.*, 49, 569–576. DOI: 10.1007/s00244-004-0229-3
36. **Thomson, C., Hahn, C., Johnson, C., 2012:** *Veterinary Neuroanatomy: A Clinical Approach*. Edinburgh: Saunders/ Elsevier, ISBN 9780702034824.
37. **Kim, J. J., Kim, Y. S., Kumar, V. 2019:** Heavy metal toxicity: An update of chelating therapeutic strategies. *J. Trace Elem. Med. Biol.*, 54, 226-231. DOI: 10.1016/j.jtemb.2019.05.003
38. **Kalisińska, E., Salicki, W., Mysłek, P., Kavetska, K. M., Jackowski, A., 2004:** Using the mallard to biomonitor heavy metal contamination of wetlands in north-western Poland. *Sci. Total. Environ.*, 320, 2-3, 145–161. DOI: 10.1016/j.scitotenv.2003.08.014
39. **Kalisinska, E., Lanocha-Arendarczyk, N., Kosik-Bogacka, D., Budis, H., Podlasinska, J., Popiolek, M., et al., 2016.** Brains of native and alien mesocarnivores in biomonitoring of toxic metals in Europe. *PLoS One*, 11, 8, 1–27. DOI: 10.1371/journal.pone.0159935
40. **Cid, F. D., Gatica-Sosa, C., Anton, R. I., Caviedes-Vidal, E., 2009:** Contamination of heavy metals in birds from Embalse La Florida (San Luis, Argentina). *J. Environ. Monit.*, 11, 2044–2051. DOI: 10.1039/b906227k
41. **Lindsay, D., Kerr, W., 2011:** Cobalt close-up. *Nat. Chem.*, 3, 494. DOI: 10.1038/nchem.1053
42. **Jumba, I. O., Kisia, S. M., Kock, R., 2007:** Animal health problems attributed to environmental contamination in Lake Nakuru National Park, Kenya: A case study on heavy metal poisoning in the Waterbuck Kobus ellipsiprymnus defassa (Ruppel, 1835). *Arch. Environ. Contam. Toxicol.*, 52, 270–281. DOI: 10.1007/s00244-005-0241-2
43. **Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., Beeregowda, K. N., 2014:** Toxicity, mechanism and health effects of some heavy metals. *Interdiscip. Toxicol.*, 7, 2, 60–72. DOI: 10.2478/intox-2014-0009
44. **Agency for Toxic Substances and Disease Registry, USA.** https://www.atsdr.cdc.gov/toxicological-profiles/glossary/index.html#heading-c. Accessed May 25, 2025.
45. **Maľová, J., Ciberej, J., Maľa, P., Zigo, F., Semjon, B., 2019:** Heavy metal levels in the tissues of wild living animals from two distinct industrially exploited areas in Slovakia. *Slovak J. Anim. Sci.*, 52, 3, 100–110.
46. **Aloupi, M., Karagianni, A., Kazantzidis, S., Akriotis, T., 2017:** Heavy metals in liver and brain of waterfowl from the Evros Delta, *Greece. Arch. Environ. Contam. Toxicol.*, 72, 215–234. DOI: 10.1007/s00244-016-0349-6
47. **Lambert, M., Leven, B. A., Green, R. M., 2000:** New methods of cleaning up heavy metal in soils and water. *Environmental Science and Technology Briefs for Citizens*.
48. **Kumar, V., Kalita, J., Misra, U. K., Bora, H. K., 2015:** A study of dose response and organ susceptibility of copper toxicity in a rat model. *J. Trace Elem. Med. Biol.*, 29, 269–274. DOI: 10.1016/j.jtemb.2014.06.004
49. **Aaseth, J., Crisponi, G., Andersen, O., 2016:** *Chelation therapy in the treatment of metal intoxication*, 1st edition, Academic Press.
50. **Igado, O. O., Olopade, J. O., Onwuka, S. K., Onwuka, A. C, Daramola, O. A, Ajufo, U. E., 2008:** Evidence of environmental pollution in caprine brains obtained from a relatively unindustrialized area in Nigeria. *African J. Biomed. Res.*, 11, 3, 305–309. DOI: 10.4314/ajbr.v11i3.50744
51. **Valko, M., Morris, H., Cronin, M. T. D., 2005:** Metals, toxicity and oxidative stress. *Curr. Med. Chem.,* 12, 10, 1161–1208. DOI: 10.2174/0929867053764635
52. **Kalisińska, E., 2000:** Lead and other heavy metals in the brain of geese hunted in the vicinity of Slonsk, Poland. *Biol. Bull. Poznań*, 37, 2, 273–286.
53. **Havera, S. P., Wood, S. G., Georgi, M. M., 1992:** Blood and tissue parameters in wild mallards redosed with lead shot. *Bull. Environ. Contam. Toxicol.,* 49, 238–245.
54. **Stout, J. D., Brinker, D. F., Driscoll, C. P., Davison, S., Murphy, L. A., 2010:** Serum biochemistry values, plasma mineral levels, and whole blood heavy metal measurements in wild Northern goshawks (*Accipiter gentilis*). *J. Zoo. Wildl. Med.*, 41, 4, 649–655. DOI: 10.1638/2009-0258.1
55. **de Carvalho, F. M., Gaunt, S. D., Kearney, M. T., Rich, G. A., Tully, T. N. Jr., 2009:** Reference intervals of plasma calcium, phosphorus, and magnesium for African grey parrots (*Psittacus erithacus*) and Hispaniolan parrots (*Amazona ventralis*). *J. Zoo. Wildl. Med.,* 40, 4, 675–679. DOI: 10.1638/2009-0012.1
56. **Tarale, P., Chakrabarti, T., Sivanesan, S., Naoghare, P., Bafana, A., Krishnamurthi, K., 2016:** Potential role of epigenetic mechanism in manganese induced neurotoxicity. *Biomed. Res. Int.*, 2016, 2548792. DOI: 10.1155/2016/2548792

57. **Tomza-Marciniak, A., Pilarczyk, B., Bąkowska, M., Ligocki, M., Gaik, M., 2012:** Lead, cadmium and other metals in serum of pet dogs from an urban area of NW Poland. *Biol. Trace Elem. Res.*, 149, 345-51. DOI: 10.1007/s12011-012-9433-6

58. **Nam, D. H., Rutkiewicz, J., Basu, N., 2012:** Multiple metals exposure and neurotoxic risk in bald eagles (*Haliaeetus leucocephalus*) from two Great Lakes states. *Environ. Toxicol. Chem.*, 31, 3, 623–631. DOI: 10.1002/etc.1712

sciendo

DOI: 10.2478/fv-2025-0019

FOLIA VETERINARIA, 69, 2: 72—80, 2025

ORIGINAL ARTICLE

AQUEOUS *ALLIUM SATIVUM* EXTRACT SUPPLEMENTATION DELAYS *TRYPANOSOMA BRUCEI* INFECTION IN ALBINO RATS

Abayomi Onaolapo Adeoye[1*], Philip Olusoji Ojo[2], Tolulope Ademola Olakojo[1]

[1]Department of Veterinary Pharmacology and Toxicology, University of Ibadan, Nigeria; [2]Department of Veterinary Parasitology and Entomology, University of Ibadan, Nigeria

OPEN ACCESS

***Correspondence:** yommyadeoye@yahoo.com

Citation: Adeoye, A. O., Ojo, P. O., Olakojo, T. A., 2025: Aqueous *Allium sativum* extract supplementation delays *Trypanosoma brucei* infection in albino rats. Folia Veterinaria, 69, 2, 72–80.

Received: February 2, 2025

Accepted: May 26, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Trypanosomosis is one of the most important diseases of livestock and humans in sub-Saharan Africa. This study evaluated the effect of garlic (*Allium sativum*) aqueous extract supplementation on the course of trypanosomosis in albino rats, considering the phytochemical constituents of the extract, blood parameters, prepatent period, level of parasitaemia, and survival rate of the animals. Phytochemical evaluation of Garlic aqueous extract confirmed the availability of flavonoids, tannins and terpenoids while alkaloids and saponins were absent. All infected rats became parasitaemic 2 days PI (post infection) and climaxed 5 days PI. The post-treatment haematological parameters such as packed cell volume (PCV), haemoglobin (HB), and red blood cell count (RBC) in groups treated with Berenil® and Berenil® plus extract were significantly improved. Groups pretreated with aqueous extract before infection demonstrated delayed parasitaemia for 24 hours and increased survival for 2 days in those with 30 days of pretreatment. However, the extract supplement alone at the selected dosages had no inhibitory effect on *T. brucei* infection. This study concluded that aqueous extract of *Allium sativum* contains flavonoids, tannins, and terpenoids, which acted synergistically with diminazene aceturate against *T. brucei* infection.

Key words: albino rats; garlic; parasitaemia; phytochemistry; *Trypanosoma brucei*

INTRODUCTION

Trypanosomosis is an important insect-borne protozoan disease of animals and humans in sub-saharan Africa, especially in Nigeria. It is described as a disease transmitted biologically by tsetse fly and mechanically by other biting flies [1, 2]. Human African Trypanosomosis (HAT), or sleeping sickness, is caused by *Trypanosoma brucei gam-*

biense or *Trypanosoma brucei rhodesiense*, while American human Trypanosomosis or Chagas disease, is caused by *Trypanosoma cruzi* [3]. Animal African Trypanosomosis (AAT) is caused by different species of trypanosomes, including *Trypanosoma evansi, T. brucei, T. congolense*, and *T. simiae* and it hinders the growth and development of livestock production in endemic parts of Africa [4, 5].

Some of the clinical manifestations of the disease include intermittent fever, anemia, weight loss, infertility, lowered milk yield, abortion, nervous signs, and mortality in affected animals [1]. WHO in 2015 [3] reported that Chagas disease causes about 50,000 deaths annually, while Lejon et al., in 2003 [6], stated an estimated 300,000 to 500,000 Africans carry the disease while 60 million people are at risk of infection. Animals that recover from trypanosome infections are usually unproductive [4]. However, in some cases, infections may be subclinical or chronic showing no clinical signs, although stress factors such as overwork, parturition, malnutrition, and unfavourable environmental conditions may predispose animals to come down with the disease [7].

Efforts to prevent and control clinical trypanosomosis have partially failed due to drug resistance, which occours in an attempt to control the parasites in mammalian hosts, and the inability to find appropriate control measures for the various insect vectors of this disease. The compounds used clinically to control trypanosome infections were introduced in the country more than 50 years ago, but considerable toxicity and resistance have limited their use. This makes searching for efficacious alternative ethno-medicinal plants critical [8, 9].

Garlic, one of nature's wonder plants with its healing ability, exhibits anti-parasitic activity against major human intestinal parasites such as *Entamoeba histolytica, Ascaris lumbricoides* and *Giardia lamblia* [10]; lowers blood pressure [11]; lowers blood cholesterol [12]; and contains anti-tumor properties. It can also boost the immune system to fight off potential diseases and maintain health [13, 14]. Therefore, this study intends to investigate the effect of garlic (*Allium sativum*) aqueous extract supplementation on the course of trypanosomosis in albino rats.

MATERIALS AND METHODS

Plant collection and authentication

The fresh *Allium sativum* bulbs were purchased at Bodija Central Market, Ibadan, Nigeria, and authenticated at the Department of Botany, University of Ibadan.

Preparation of extract

The plant's bulbs were air-dried at room temperature, pulverized, and 1000g of it soaked in 10 liters of distilled water for 24 hours, filtered through Whatman filter paper no1, and concentrated at 50^{o}C with a rotary evaporator. Thereafter, the garlic extract was preserved in the refrigerator until use. 1 gm of the dried extract concentrate was dissolved in 50 ml of distilled water to give a concentration of 20 mg/ml. To ensure that the active principles are preserved, fresh aqueous extracts were prepared every other day. Diminazene aceturate (Berenil$^{(R)}$) manufactured by Kepro Veterinary Company, Holland) was used for the positive control.

Test organism

Trypanosoma brucei brucei was obtained from the Nigeria Institute for Trypanosomiasis Research, Nigeria. The parasites were maintained in the laboratory by continuous passage in rats.

Experimental animals

50 adult male albino rats of about 6 weeks in age, weighing 100 – 150 g, were kept in the experimental animal housing unit of the Department of Veterinary Pharmacology and Toxicology, University of Ibadan. They were acclimatized for 14 days and randomly assigned to groups before the commencement of the experiment and fed with commercial Vital® feed and water *ad libitum.*

Phytochemical analysis of plant extract

Phytochemical screening of aqueous extract of *Allium sativum* was conducted as described by Trease and Evans [15].

Experimental design

The fifty (50) male albino rats of about 6 weeks in age were divided into 10 groups of 5 animals each. Group A: Negative control (no infection and no treatment), B: Infected without treatment, C: Infected and treated with a

single dose of Berenil (7 mg/kg) at day 5 post-infection, D: Infected and treated with Berenil (3.5 mg/kg) + aqueous extract (30 mg/kg) once at day 5 post-infection, E: Infected and treated with extract (30 mg/kg) for 5 days from day 5 post-infection, F: Infected and treated with extract (60 mg/kg) for 5 days from day 5 post-infection, G: 14 days pretreatment with extract (30 mg/kg) + Infection on day 15, H: 14 days pretreatment with extract (60 mg/kg) + Infection on day 15, I: 30 days pretreatment with extract (30mg/kg) + Infection on day 31, J: 30 days pretreatment with extract (60 mg/kg) + Infection on day 31.

Determination of prepatent period and parasitaemia

The level of trypanosome in the blood of the infected rats was monitored daily from the second day post-infection. Blood from the coccygeal vein was used to determine parasitaemia in wet mounts. The trypanosome counts were determined by examination of the wet mount microscopically at x400 magnification using the "rapid matching" method of Herbert & Lumsden [16], which involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with buffered phosphate saline (PBS, pH 7.2).

Blood collection and analysis

The rats were anaesthetized with diethyl ether. Blood samples were collected from the orbital vein of each rat using sodium heparinized micro haematocrit tubes into separate lithium heparinized sample bottles to evaluate haematological and biochemical parameters. The experiment was terminated at day 21 post-infection and blood samples were collected from all surviving animals.

Statistical analysis

All data obtained from the study were presented as means ± standard error of the mean (SEM) and analysed using ANOVA, followed by the post-hoc Tukey's test to evaluate significant differences within the groups. SPSS (Statistical Package for the Social Sciences) was used for the analysis, and the probability level at 0.05 ($P \leq 0.05$) was considered significant.

Table 1. Phytochemical constituents of aqueous extract of *Allium sativum* bulb

Component	Test	Observation	Scoring
Alkaloids	Dragendorff's	Brownish-red colour	-
Tannins	Ferric chloride	Deep red colour	+
Flavonoids	Pew's	Red colour	+
Saponins	Frothing	Persistence foam	-
Terpenoids	Salkowski's	Reddish brown	+

+ indicate presence
- indicate absence

Table 2a. Mean haematological parameters of infected and treated animals with garlic extract and Berenil

PARAMETERS	GROUP A	GROUP B	GROUP C	GROUP D
PCV	41.5 ± 0.3	37.7 ± 2.2 [a]	39.0 ± 1.0	46.5 ± 1.5
HB	13.7 ± 0.2	12.6 ± 0.7 [a]	13.0 ± 0.5	15.6 ± 0.7
RBC	6.8 ± 0.2	6.2 ± 0.5 [a]	6.4 ± 0.2	7.7 ± 0.4
WBC	5437.5 ± 1494.1	3933.3 ± 233.3[a]	3475.0 ± 225.0	5325.0 ± 325.0
PLATELET	114750.0 ±14783.9	90666.7 ±5607.5	84000 ± 28000	118500.0 ± 15500.0
LYMPHOCYTE	56.8 ± 1.6	64.3 ± 1.9	63.5 ± 4.5	70.5 ± 3.5
NEUTROPHIL	39.0 ± 1.8	32.7 ± 1.5[a]	32.0 ± 7.0	25.5 ± 2.5
MONOCYTE	2.0 ± 0.4	1.7 ± 0.3	2.0 ± 1.0	2.5 ± 0.5
EOSINOPHIL	2.3 ± 0.3	1.3 ± 0.9	2.5 ± 1.5	1.5 ± 0.5

Treatment was administered once at day 5 post-infection. All values are expressed as mean ± standard error of mean. Statistical significance is reported at $\alpha^{0.05}$ and indicated with [a] in the table. **GROUP A**: No infection and no treatment; **GROUP B:** Infected with no Berenil and extract treatment; **GROUP C**: Infected and treated with Berenil (7 mg/kg) once on day 5 post-infection; **GROUP D**: Infected and treated with Berenil (3.5 mg/kg) + extract (30 mg/kg) once on day 5 post-infection (PCV: Packed cell volume, HB: Haemoglobin, RBC: Red blood cell count, WBC: White blood cell count)

RESULTS

Phytochemical analysis of *Allium sativum* extract

Phytochemical analysis of the aqueous extract of *Allium sativum* detected flavonoids, tannins, and terpenoids while alkaloids and saponins were not detected (Table 1).

Effects of infection and treatment on blood parameters

Following infection with *Trypanosoma brucei,* the PCV (Packed cell volume), HB (Haemoglobin), and RBC (Red blood cell) count were reduced in infected-untreated animals compared to the uninfected control. By day 15 post-treatment, the red cell indices (PCV, HB and RBC) were significantly ($P < 0.05$) improved in groups C and D treated with 7 mg/kg Berenil and 3.5 mg/kg Berenil + 30 mg/kg extract, respectively (Table 2a).

Haematological parameters for groups G, H, I, and J (pretreated and infected) and groups E and F (infected and treated with only the extract) are not recorded because all experimental animals died before the experiment was terminated on day 21 PI.

Following infection with *Trypanosoma brucei*, the plasma total protein, albumin, and globulin were reduced, whereas liver enzymes AST (Aspartate transferase), ALT (Alanine aminotransferase), and ALP (Alkaline phosphatase) were increased in infected groups compared to the uninfected control group. However, on day 21 post-infection, the plasma concentrations of liver enzymes (ALT, AST, and ALP) were reduced in groups C (Infection + treatment with 7 mg/kg Berenil once on day 5 post-infection) and D (Infection + treatment with 3.5 mg/kg Berenil + 30 mg/kg extract once at day 5 post-infection (Table 2b).

Effect of garlic extract on *T. brucei* infected rats

The parasitaemia in the positive control group (infected untreated) was >5.4 on day 2 PI to >8.7 on day 9 PI when all animals in this group died, whereas the negative control group (uninfected), whose animals survived, had no parasitaemia throughout the experiment (Table 3, Figure 1).

Pretreatment with *Allium sativum* extract for 14 (60 mg/kg) and 30 days (30 mg/kg and 60 mg/kg) before infection delayed the prepatent period for 24 hours. The treatment of infected rats with garlic extracts at 30 mg/kg and 60 mg/kg had no inhibitory effect on *Trypanosoma brucei* parasitaemia. There was no significant difference between the parasitaemia levels in the group treated with garlic extract and the positive control group (infection only) with no extract treatment.

The parasitaemia level reduced significantly in groups C (infected + 7 mg/kg Berenil once on day 5 PI), and D (infected + 3.5 mg/kg Berenil + 30 mg/kg extract once on day 5 PI), from >7.8 to <5.4 within 24 hours and then 0 within 48 hrs post-treatment. Both groups had no evidence of relapse throughout the experiment at day 21 PI. The *T. bruccei* parasitaemia levels observed in all the experimental groups are presented in Table 4.

Table 2b. Mean plasma biochemical parameters in infected and treated animals with garlic extract and Berenil

PARAMETERS	GROUP A	GROUP B	GROUP C	GROUP D
TOTAL PROTEIN	7.3 ± 0.1	7.1 ± 0.2	7.5 ± 0.9	7.6 ± 0.4
ALBUMIN	3.0 ± 0.2	2.5 ± 0.2	3.2 ± 0.5	3.3 ± 0.3
GLOBULIN	4.3 ± 0.1	4.6 ± 0.1[a]	4.3 ± 0.4	4.4 ± 0.2
AG RATIO	0.7 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	0.8 ± 0.1
AST	41.3 ± 0.5	44.7 ± 1.5	42.0 ± 3.0	42.5 ± 1.5
ALT	30.3 ± 0.8	32.7 ± 0.9	31.0 ± 3.0	32.5 ± 0.5
ALP	108.8 ± 5.5	124.0 ± 2.0[a]	101.0 ± 2.0	115.0 ± 9.0
BUN	16.4 ± 0.3	16.9 ± 0.3	17.1 ± 0.5	16.8 ± 0.4
CREATININE	0.7 ± 0.1	0.7 ± 0.0	1.0 ± 0.3	0.8 ± 0.0

Treatment was administered once at day 5 post-infection. All values are expressed as mean ± standard error of mean. Statistical significance is reported at $\alpha^{0.05}$ and indicated with [a] in the table. **GROUP A**: No infection and no treatment; **GROUP B:** Infected with no Berenil and extract treatment; **GROUP C**: Infected and treated with Berenil (7 mg/kg) once on day 5 post-infection; **GROUP D**: Infected and treated with Berenil (3.5 mg/kg) + extract (30 mg/kg) once on day 5 post-infection. (AG RATIO: Albumin Globulin ratio, AST: Aspartate transferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, BUN: Blood Urea Nitrogen).

Table 3. Survivability of animals infected and treated with garlic extract and/or Berenil

Days	A	B	C	D	E	F	G	H	I	J
1 (PI)	0/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
2	0/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
3	0/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
4	0/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
5(Treatment)	0/5	4/4	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
6	0/5	4/4	2/5	2/5	3/3	3/3	3/3	3/3	4/4	4/4
7	0/5	2/2	0/5	0/5	1/1	2/2	1/1	1/1	3/3	2/2
8	0/5	1/1	0/5	0/5	1/1	2/2	0/0	1/1	2/2	2/2
9	0/5	0/0	0/5	0/5	0/0	0/0	0/0	0/0	2/2	2/2
10	0/5	0/0	0/5	0/5	0/0	0/0	0/0	0/0	1/1	0/0
12	0/5	0/0	0/5	0/5	0/0	0/0	0/0	0/0	0/0	0/0
15	0/5	0/0	0/5	0/5	0/0	0/0	0/0	0/0	0/0	0/0
17	0/5	0/0	0/5	0/5	0/0	0/0	0/0	0/0	0/0	0/0
19	0/5	0/0	0/5	0/5	0/0	0/0	0/0	0/0	0/0	0/0
21	0/5	0/0	0/5	0/5	0/0	0/0	0/0	0/0	0/0	0/0

PI: Post-infection, Numerator = Number of rats positive for *T. brucei,* Denominator = Number of surviving rats in the group; **A**: No infection and no treatment; **B:** Infected with no Berenil and extract treatment; **C**: Infected and treated with Berenil (7 mg/kg) once on day 5 post-infection; **D**: Infected and treated with Berenil (3.5 mg/kg) + extract (30 mg/kg) once on day 5 post-infection; **E:** Infected and treated with extract (30mg/kg) for 5 days; **F:** Infected and treated with extract (60mg/kg) for 5 days; **G:** 14 days pretreatment with extract (30 mg/kg) + Infection; **H**: 14 days pretreatment with extract (60 mg/kg) + Infection; **I**: 30 days pretreatment with extract (30 mg/kg) + Infection; **J:** 30 days pretreatment with extract (60 mg/kg) + Infection

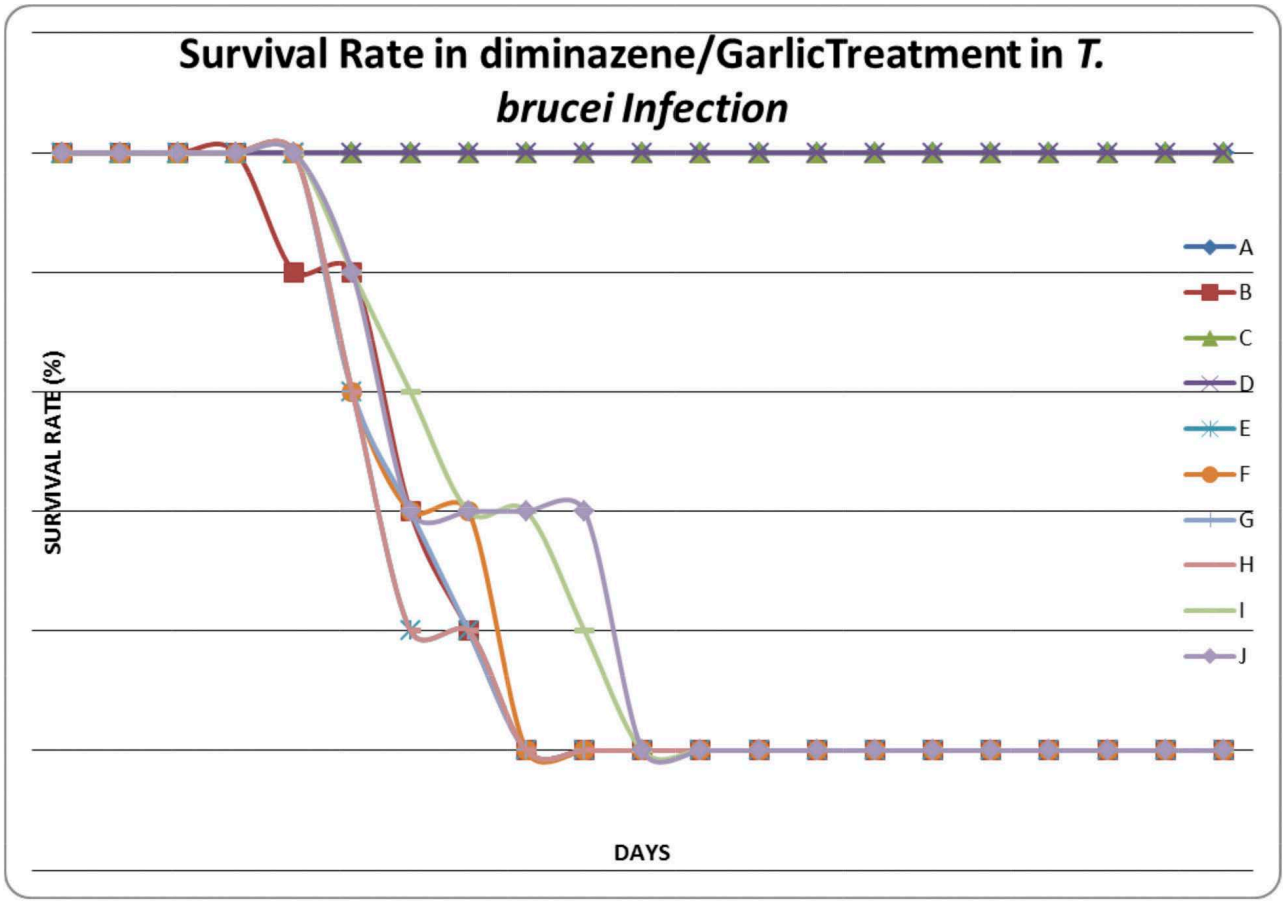

Fig. 1. Survival rate of animals infected and treated with diminazene and/or garlic extract

A: No infection and no treatment; **B:** Infected with no Berenil and extract treatment; **C:** Infected and treated with Berenil (7 mg/kg) once on day 5 post-infection; **D:** Infected and treated with Berenil (3.5 mg/kg) + extract (30 mg/kg) once on day 5 post-infection; **E:** Infected and treated with extract (30mg/kg) for 5 days; **F:** Infected and treated with extract (60 mg/kg) for 5 days; **G:** 14 days pretreatment with extract (30 mg/kg) + Infection; **H:** 14 days pretreatment with extract (60 mg/kg) + Infection; **I:** 30 days pretreatment with extract (30 mg/kg) + Infection; **J:** 30 days pretreatment with extract (60 mg/kg) + Infection

Table 4. Mean Parasitaemia in infected and treated animals with garlic etract and/or Berenil

Days	A	B	C	D	E	F	G	H	I	J
1 (PI)	0	0	0	0	0	0	0	0	0	0
2	0	6.0	6.3	6.0	0	6.0	6.0	0	0	0
3	0	6.6	6.9	6.9	6.3	6.6	6.9	6.3	6.0	6.6
4	0	7.2	7.2	7.2	6.6	6.9	7.5	6.9	6.6	6.9
5(Treatment)	0	7.5	7.8	7.8	7.2	7.5	8.1	7.2	6.9	7.2
6	0	7.8	<5.4	<5.4	7.5	8.1	8.4	7.8	7.2	7.5
7	0	8.4	0	0	8.1	8.4	8.7	8.1	7.5	7.8
8	0	8.7	0	0	8.7	8.7	Dead	8.7	7.8	8.1
9	0	Dead	0	0	Dead	Dead	-	Dead	8.1	8.7
10	0	-	0	0	-	-	-	-	8.4	Dead
11	0	-	0	0	-	-	-	-	Dead	-
15	0	-	0	0	-	-	-	-	-	-
17	0	-	0	0	-	-	-	-	-	-
19	0	-	0	0	-	-	-	-	-	-
21	0	-	0	0	-	-	-	-	-	-

All values are expressed as the mean of base 10 logarithm for the concentration of the parasites per milliliter of blood for reference point. PI: Post-infection; **A**: No infection and no treatment; **B:** Infected with no Berenil and extract treatment; **C**: Infected and treated with Berenil (7 mg/kg) once on day 5 post-infection; **D**: Infected and treated with Berenil (3.5 mg/kg) + extract (30 mg/kg) once on day 5 post-infection; **E:** Infected and treated with extract (30 mg/kg) for 5 days; **F:** Infected and treated with extract (60 mg/kg) for 5 days; **G:** 14 days pretreatment with extract (30 mg/kg) + Infection; **H**: 14 days pretreatment with extract (60 mg/kg) + Infection; **I**: 30 days pretreatment with extract (30 mg/kg) + Infection; **J:** 30 days pretreatment with extract (60 mg/kg) + Infection

DISCUSSION

This study explores a comprehensive phytochemical analysis of the aqueous extract of *Allium sativum* (garlic) and its effects on *T. brucei* infection in white Wistar rats. According to this study, phytochemical constituents of *A. sativum* indicated the presence of flavonoids, tannins, and terpenoids. These findings are consistent with previous studies that have identified similar phytochemicals in garlic, which are known for their various pharmacological properties. Also, these relatively cheap and efficient medicinal components have been reported to have remarkable biological activities, and their therapeutic effects have been substantiated for various ailments [17].

Moreover, Huzaifa et al. [18] reported the presence of alkaloids, flavonoids, saponins, tannins and cardiac glycosides in the aqueous extract of garlic bulbs, while Boukeria et al. [19] observed the presence of saponins, alkaloids, and traces of glycosides in *A. sativum* varieties. These findings are consistent with previous studies that have identified similar phytochemicals in garlic, which are known for their various pharmacological properties [20].

Following infection with *T. brucei,* the PCV, HB, and RBC counts were reduced in infected rats compared to the uninfected control, indicating anaemia, a consistent manifestation associated with trypanosomosis [1], which could be due to hemolysis and bone marrow suppression. However, the red cell indices, i.e. PCV, HB and RBC counts were significantly improved in groups C and D treated with Berenil® plus garlic aqueous extract to a level similar to the observation in the uninfected control group. This showed that aqueous extract of garlic was not contraindicated with Berenil®. The animals treated with only the extract before or after the establishment of infection died before the experiment was terminated, suggesting the inability of the extract to inhibit the multiplication of Trypanosome parasites or red cell destruction by *T. brucei* at the selected concentration for this work.

The infection also led to reductions in plasma total protein, albumin, and globulin levels, accompanied by increases in liver enzymes (ALT, AST, and ALP), indicative of hepatic damage and dysfunction. Post-treatment, the groups receiving Berenil alone or in combination with garlic extract showed a significant reduction in these liver enzyme levels by day 21 post-infection, pointing to a res-

toration of liver function. This highlights the therapeutic potential of Berenil, both alone and in combination with garlic extract, in mitigating liver damage induced by trypanosomiasis.

Also, it was observed that albino rats pretreated with aqueous extract for 30 days survived for 2 days more than those pretreated for 14 days and those that were not pretreated. Comparing the effect of pretreatment with the extract alone on the establishment of parasitaemia and survivability of *T. brucei*-infected rats is limited due to a lack of data. Nevertheless, Shubha et al. [21] and El-katcha et al. [22] have reported the immune-stimulating potential of garlic, and this may contribute at least in part to the delay in the establishment of parasitaemia till day 3 in all extract-pretreated rats and the increased survivability (additional 2 days) of *T. brucei*-infected rats pretreated for 30 days.

Treatment of infection with extract alone has no significant effect on survivability, whereas treatment with a combination of 30 mg/kg of extract and 3.5 mg/kg of diminazene aceturate effectively improved the survivability of animals. Pretreatment with *Allium sativum* aqueous extract couldn't effectively prevent the establishment of infection, though it delayed the establishment of parasitaemia for 1 day longer than groups that were not pretreated. Also, treatment with extract alone could not efficiently reduce parasitaemia levels in already established infection, while treatment with 30 mg/kg of extract combined with 3.5 mg/kg of diminazene aceturate was as effective as treatment with only 7 mg/kg of diminazene aceturate in reducing parasitaemia to 0 within 48 hours post-treatment with no evidence of relapse.

This study emphasised the findings of Peni et al. [23], who stated that diminazene aceturate efficacy in managing *Trypanosoma brucei* infection is enhanced when combined with sub-therapeutic doses of *Allium sativum* bulb extract. This synergistic effect of garlic with diminazene aceturate in the treatment of trypanosomosis can be attributed to several mechanisms, some of which include its antioxidant effects, modulation of the host's immune system, and enhanced trypanocidal impact primarily due to its sulfur compound named allicin, which can enhance the Berenil mechanism of action. Arreola et al. [14] previously reported that aqueous garlic extract exerts antioxidant actions by scavenging reactive oxygen species (ROS) and enhancing cellular antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. The immunomodulatory potential of phytochemicals has been largely attributed to their dynamic regulation of molecules such as cytokines and chemokines. This explains the therapeutic effect associated with the different bioactive molecules identified in the garlic plant.

While our study provides valuable insights, we acknowledge some limitations that suggest directions for future research. The sample size was small and might limit the generalizability of findings. In addition, the dosage used had no curative effect when used alone, though it delayed the onset of Trypanosomosis. Future investigation could explore the potential curative effect of the extract at a higher dosage and the possible mechanism of action of garlic as an antitrypanosomal agent. However, these limitations do not diminish the significance of our findings but rather highlight opportunities for more comprehensive future investigations.

CONCLUSION

This study concluded that aqueous extract of *Allium sativum* contains flavonoids, tannins, and terpenoids, which explain the synergy with diminazene aceturate against *T. brucei* infection. Also, 14 days of pretreatment of animals with extract delayed parasitaemia for 24 hours, and 30 days increased survivability for 2 more days, whereas the extract alone at low doses was not curative against *T. brucei* infection. This combination therapy provides a more effective approach to treatment. It may reduce the severity of *Trypanosoma brucei* infection, which may be complicated by already existing drug resistance, toxicity, and high cost of purchase.

Ethical Approval

The experiment was carried out with strict adherence to the international regulations guiding the use of animals for research purposes as outlined in the University of Ibadan Animal Care Use and Research Ethics Committee protocol. Ethical clearance was obtained from the Animal Care Use and Research Ethics Committee (ACUREC), University of Ibadan, with file number UI-ACUREC/17/0034.

Data Availability Statement

The raw data of this study will be made available upon request without any reservation.

Funding

The authors declare that this study has not received any funding.

Declaration of Interests

There is no conflict of interest.

Authors' Contributions

Adeoye conceptualised the design while Ojo and Olakojo were also involved in the literature search, data processing and manuscript writing.

Generative AI statement

Generative AI technologies were not employed in writing this manuscript.

REFERENCES

1. **Ajagbonna, O. P., Mikhail, H. G., Muhammed, B. Y., Onyeyili, P. A, 2003:** Trypanocidal efficacy of *Allium sativum* (Garlic) in rabbits infected with *Trypanosoma brucei*, In A. A. Taiwo, A. M. Raji, D.U. Ogbonna and E. A. Adebowale (eds proceeding of the 28th Annual Conference of the Nigeria Society for Animal Production).
2. **Mandell, Douglas, and Bennett's, 2015:** Principles and Practice of Infectious Diseases. Editors-in-Chief: John E. Bennett, Raphael Dolin and Martin J. Blaser. Eighth Edition.
3. **World Health Organization, 2015:** Chagas disease in Latin America: an epidemiological update based on 2010 estimates. Weekly Epidemiol Rec., 90, 33–44.
4. **FAO, 2006:** A field guide for the diagnosis, treatment and prevention of African animal Trypanosomiasis. FAO of the UN. Rome, pp 1–18, 13–17.
5. **Nok, A. J., Nock, C., 2002:** Transferin coupled azanthraquinone enhances the killing effect on trypanosomes. The role of lysosomal mannosidase. *Parasite*, 9, 4 375–379. DOI: 10.1051/parasite/2002094375
6. **Lejon, D. V., Boelaert, M., Jannin, J., Moore, A., Büscher, P., 2003:** The challenge of *Trypanosoma brucei gambiense* sleeping sickness diagnosis outside Africa, *Lancet Infect. Dis.*, 3, 12, 804–808. DOI: 10.1016/S1473-3099(03)00834-X
7. **Losos, G. T., 1986:** Infectious tropical diseases of domestic animals, Longman Scientific and Technical, in association with the International Development Research Center, Canada, pp 183–212.
8. **Bashir, L., Shittu, O. K., Sani, S., Busari, M. B., Adeniyi, K. A., 2015:** African natural products with potential anti-trypanosomal properties: A review. *Int. J. Biochem. Res. Rev.*, 7, 2, 45–79. DOI: 10.9734/IJBCRR/2015/16039
9. **Kamuanga, M., 2003:** Socio-economic and cultural factors in the research and control of Trypanosomosis, 1-10. Information division FAO, Rome.
10. **Zuhair, D., Mounia, A., Samia, B., 2024:** Garlic (Allium sativum): An in-depth review focusing on its potent antiparasitic activity. *GSC Biol. Pharm. Sci.*, 28, 03, 007–026. DOI: 10.30574/gscbps.2024.28.3.0306
11. **Wang, H. P., Yang, J., Qin, L. Q., Yang, X. J., 2015:** Effect of garlic on blood pressure: A meta-analysis. *J. Clin. Hypertens. (Greenwich)*, 17, 3, 223–231. DOI: 10.1111/jch.12473
12. **Sun, Y. E., Wang, W., Qin, J., 2018:** Anti-hyperlipidaemia of garlic by reducing the level of total cholesterol and low-density lipoprotein: A meta-analysis. *Medicine (Baltimore)*, 97, 18, e0255. DOI: 10.1097/MD.0000000000010255
13. **Abdullah, T. H., Kandil, O., Elkadi, A., Carter, J., 1988:** Garlic revisited: therapeutic for the major diseases of our times? *J. Natl. Med. Assoc.*, 80, 4, 439–445.
14. **Arreola, R., Quintero-Fabián, S., López-Roa, R. I, Flores-Gutiérrez, E. O., Reyes-Grajeda, J. P., Carrera-Quintanar, L., Ortuño-Sahagún, D., 2015:** Immunomodulation and anti-inflammatory effects of garlic compounds. *J. Immunol. Res.*, 2015, 401630. DOI: 10.1155/2015/401630
15. **Evans, W. C., 2009:** Trease and Evans' Pharmacognosy: Sixteenth Edition.
16. **Herbert, W. J., Lumsden, W. H. R., 1976:** *Trypanosoma brucei*: A rapid "matching" method for estimating the host's parasitemia, *Exp. Parasitol.*, 40, 3, 427–431. DOI: 10.1016/0014-4894(76)90110-7
17. **Ullah, A., Munir, S., Badshah, S. L., Khan, N., Ghani, L., Poulson, B. G., Emwas, A. H., Jaremko, M., 2020:** Impor-

tant flavonoids and their role as a therapeutic agent. *Molecules*, 25, 22, 5243. DOI: 10.3390/molecules25225243

18. **Huzaifa, U., Labaran, I., Bello, A. B., Olatunde, A., 2014:** Phytochemical screening of aqueous extract of garlic (*Allium sativum*) bulbs. *Rep. Opinion*, 6, 8, 1–4.
19. **Boukeria, S., Kadi, K., Kalleb, R., Benbott, A., Bendjedou, D., Yahia, A., 2016:** Phytochemical and physicochemical characterization of *Allium sativum* L. and *Allium cepa* L. Essential oils, *J. Mater. Environ. Sci.,* 7, 7, 2362–2368.
20. **Shokrollahi, B., Hesami, S. M., Baneh, H., 2016:** The effect of garlic extract on growth, haematology and cell-mediated immune response of newborn goat kids. *J. Agr. Rural Develop. Trop. Subtrop.*, 117, 2, 225–232.
21. **Shubha, S., Shyam L., 2014:** Medicinal use of garlic (*Allium sativum*) improves fish health and acts as an immunostimulant in aquaculture. 2, 44–47.
22. **El-katcha, M. I., Soltan, M. A., Sharaf, M. M., Hasen, A., 2016:** Growth performance, immune response, blood serum parameters, nutrient digestibility and carcass traits of broiler chicken as affected by dietary supplementation of garlic extract (Allicin). *Alex. J. Vet. Sci.*, 49, 2, 50–64. DOI: 10.5455/ajvs.219261
23. **Peni, I. J., Ribah, M., Ajagbonna, O., Agaie, B., 2012.** Effects of *Allium sativum* bulb extract, diminazene aceturate and their combination on parasitaemia, and biochemical indices in rats experimentally infected with *Trypanosoma Brucei Brucei*. *J. Appl. Pharm. Sci.*, 2, 6, 112-115. DOI: 10.7324/JAPS.2012.2636

sciendo

DOI: 10.2478/fv-2025-0020

FOLIA VETERINARIA, 69, 2: 81—90, 2025

ORIGINAL ARTICLE

IDENTIFICATION OF OCHRATOXIN A-PRODUCING MICROMYCETES

Soňa Pivka, Pavlina Jevinová, Monika Pipová, Ivana Regecová

Department of Food Hygiene, Technology and Safety, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia

OPEN ACCESS

***Correspondence:** sona.pivka@uvlf.sk

Citation: Pivka, S., Jevinová, P., Pipová, M., Regecová, I., 2025: Identification of ochratoxin A-producing micromycetes. Folia Veterinaria, 69, 2, 81–90.

Received: May 6, 2025

Accepted: May 26, 2025

Published: June 16, 2025

Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

This study focused on the identification of microscopic filamentous fungi isolated from food commodities (chicken eggs, fermented green coffee beans, and dried marjoram) and the evaluation of their ability to produce ochratoxin A (OTA). A total of 179 isolates (93 from eggshells, 34 from coffee, 52 from marjoram) underwent phenotypic and genotypic identification. Phenotypic traits were assessed macroscopically and microscopically; genotypic identification was performed using conventional PCR and verified by ITS (internal transcribed spacers) region sequencing. Three complementary methods were used to assess OTA production: detection of the *otanps*PN gene, cultivation on coconut cream agar, and thin-layer chromatography. Identified species included *Aspergillus niger*, *A. ochraceus*, *A. westerdijkiae*, *A. carbonarius*, *A. tubingensis*, and *Penicillium verrucosum*. Molecular analysis corrected the misclassification of two *A. carbonarius* and one *A. tubingensis* isolate, reidentified as *A. niger*. OTA production was confirmed by all methods in most isolates. Notably, only one *A. niger* isolate from coffee carried the biosynthetic gene. Two *A. tubingensis* isolates, despite being considered non-ochratoxigenic, also showed OTA production. The findings underscore the importance of combining morphological and molecular tools in fungal identification and highlight the need for multi-method approaches in evaluating the toxigenic risk of foodborne fungi.

Key words: chicken egg, coffee, marjoram, microscopic filamentous fungus, mycotoxin

INTRODUCTION

Microscopic filamentous fungi (MFF) are widely distributed microorganisms capable of colonizing a variety of food substrates at different stages of processing, storage, and distribution. Their presence in food products is undesirable not only due to the deterioration of sensory properties but, more importantly, because of their ability to produce secondary metabolites with potent toxic effects—mycotoxins. One of the most hazardous and frequently

monitored mycotoxins within the food chain is ochratoxin A (OTA), predominantly produced by species belonging to the genera *Aspergillus* and *Penicillium* [1, 2, 3].

Contamination by OTA-producing microscopic filamentous fungi has been demonstrated across a range of food commodities, including cereals, coffee, dried fruits, herbs, spices, or wine. The contamination can occur either primarily (during cultivation) or secondarily (during harvesting, drying, storage, or transportation) [4, 5, 6, 7, 8]. OTA is remarkably stable under high temperatures and standard food processing conditions, implying that even processed foods may pose a significant risk to consumers [9].

Preventive measures and control of OTA contamination are implemented at all stages of food production and processing. An integral part of this effort is the effective and sensitive detection of microscopic fungi and their toxic metabolites. Although traditional phenotypic methods based on morphological characteristics are valuable, they exhibit limitations in distinguishing closely related species [10]. Consequently, increasing emphasis is placed on molecular analyses, which enable precise species-level identification and the detection of specific toxigenic lineages. Complementary methods, such as chemical analyses of mycotoxins, serve to confirm the production capacity of toxic metabolites under defined conditions [11].

From a public health perspective, OTA is classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC), exhibiting nephrotoxic, immunotoxic, hepatotoxic, and teratogenic effects [12]. Long-term exposure, even at low doses, may lead to serious chronic diseases, making OTA one of the most intensively monitored substances in food safety [13]. OTA is rapidly absorbed and distributed but slowly eliminated and excreted, leading to potential accumulation in the body, mainly due to binding to plasma proteins and a low rate of metabolism. Plasma half-lives range from several days to several weeks, which poses a serious risk to humans [12].

Although extensive monitoring has been conducted for OTA in cereals and coffee, fewer studies have focused on fungal contamination in animal-origin products such as eggs. Moreover, the role of less common substrates like dried herbs (e.g., marjoram) remains underexplored. Identifying OTA-producing fungi in such commodities is critical to understanding hidden routes of contamination and improving food safety controls.

This study was designed to fill these gaps by applying a polyphasic approach to identify and characterize OTA-producing fungi from chicken eggshells, green coffee beans, and dried marjoram. By integrating phenotypic, genotypic, and chemical analyses, we aimed to evaluate both species diversity and the toxigenic potential of the isolates.

Our findings contribute to a more nuanced understanding of OTA risk across food matrices and underscore the need for precise diagnostic methods in food microbiology. Therefore, systematic monitoring and research focused on toxigenic fungal species are crucial prerequisites for safeguarding public health.

MATERIALS AND METHODS

Isolation of microscopic filamentous fungi

The strains of microscopic filamentous fungi were isolated from the surface of moldy eggshells (n=90), fermented green coffee samples (n=3), and dried marjoram samples (n=7) according to the instructions of STN EN ISO 21527-1/O1 and STN EN ISO 21527-2/O1 [14, 15].

Phenotypic identification

Phenotypic identification of microscopic filamentous fungi isolates was carried out according to the criteria established by several authors [10, 11, 17, 16]. Isolated colonies presumed to belong to different mold genera or species were inoculated onto the surface of yeast extract sucrose agar (YES), malt extract agar (MEA), Czapek yeast extract agar (CYE), Czapek yeast autolysate agar (CYA), and 25 % glycerol nitrate agar (G25N; Hi-Media, Mumbai, India). Incubation was performed for 7 days at 25 °C (YES, MEA, CYE, and CYA) and 5 °C (CYA and G25N). Macroscopic evaluation focused on the colony growth rate, color, texture, corrugation, and the presence of exudates and pigments. Microscopic evaluation was conducted on agar preparations stained with lactophenol cotton blue using an optical microscope. Micromorphological structures were measured and documented using the B-290TB optical microscope and Optika Vision Lite 2.13 software (OPTIKA® Microscopes, Ponteranica, Italy). Isolates preliminarily classified as *Penicillium* and *Aspergillus* underwent the creatine test as described by Frisvad and Samson [16].

DNA isolation

Total genomic DNA of MMF was isolated using a two-step procedure involving zirconium and glass beads, Proteinase K (Macherey-Nagel GmbH & Co., Dueren, Germany), ultrasonic disruption, and the commercially available E.Z.N.A.® Fungal DNA Mini Kit (OMEGA Bio-tek, Inc., Norcross, GA, USA) according to Regecová et al. [18]. DNA purity and concentration were measured using a BioSpec spectrophotometer (SHIMADZU, Kyoto, Japan).

Genotypic identification by PCR

Fungal isolates were identified using conventional PCR methods. Primers used in this study (Table 1) were selected based on available literature and synthesized commercially by Amplia Ltd. (Bratislava, Slovakia).

Amplification was carried out in a 20 µL reaction volume containing 4.0 µL of HOT Firepol® Blend Master Mix (Solis BioDyne, Tartu, Estonia), 0.5 µL of each primer (10 pmol/µL concentration), and 1–10 ng of template DNA using a TC-512 thermal cycler (Techne, Staffordshire, UK). The PCR cycling conditions included an initial denaturation at 95 °C for 12 minutes, followed by 40 cycles of denaturation at 95 °C for 20 seconds, primer annealing at 46–60 °C for 60 seconds (depending on the primers), and elongation at 72 °C for 2 minutes. Amplification was terminated by cooling to 6 °C.

Detection of PCR products

Amplified PCR products were detected by electrophoresis on a 1% agarose gel prepared in TBE buffer and stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., Fremont, CA, USA). Amplicons were visualized by UV transillumination using a Mini Bis Pro® system (DNR Bio-Imaging Systems Ltd., Neve Yamin, Israel). A 100 bp

Table 1. Primers used and their characteristics

Primer name	Primer sequence (5′-3′)	Annealing temperature (°C)	PCR product size	Gen-Bank-EMBL accession number	Study
Penicillium spp.					
ITS 212d	AAATATAAATTATTTAAAACTTTC	46	336 bp	LC 317718.1	[19]
ITS 546	CTGGATAAAAATTTGGGTTG				
Penicillium verrucosum					
VERF	TCGTAACAAGGTTTCCGTAGG	59	607 bp	DQ681351.1	[20]
VERR	TTTCCTTCCGCCTTATTGAT				
Aspergillus spp.					
AspNest1	TCTTGGTTCCGGCATCGAT	55	240 bp	AF138288	[21]
AspNest2	TGACAAAGCCCCATACGCT				
AspNest3	GAAGAACGCAGCGAAATGC		146 bp		
AspNest4	AACACACAAGCCGTGCTTGA				
Aspergillus ochraceus					
OCRAF	CTTTTTCTTTTAGGGGGCACAG	60	430 bp	FM995527	[22]
OCRAR	CAACCTGGAAAAATAGTTGGTTG				
Aspergillus niger					
ASPU	ACTACCGATTGAATGGCTCG	46	295 bp	TIMM0113	[23]
Ni1R	ACGCTTTCAGACAGTGTTCG				
Aspergillus carbonarius					
CAR1	GCATCTCTGCCCCTCGG	53	389 bp	GQ888685.1	[24]
CAR2	GGTTGGAGTTGTCGGCAG				
Aspergillus westerdijkiae					
WESTF	CTTCCTTAGGGGTGGCACAG	60	430 bp	FN185739	[22]
WESTR	CAACCTGATGAAATAGATTGGTTG				
ITS1–5.8S–ITS2 region					
ITS1	TCCGTAGGTGAACCTGCGG	53	variable	-	[25]
ITS4	TCCTCCGCTTATTGATATGC				

DNA ladder (Sigma-Aldrich, Saint Louis, MO, USA) was used as a molecular weight marker.

Sequencing

ITS1–5.8S–ITS2 PCR products were sequenced by the Sanger method (SeqMe, Dobříš, Czech Republic). The obtained sequences were analyzed for homology with sequences available in the GenBank-EMBL database using the BLAST program (NCBI, software package 3.40, Bethesda, MD, USA).

Detection of ochratoxin A

The ability of isolates to produce ochratoxin A was determined by detecting the gene encoding its production and by demonstrating its presence in agar media. Detection of the specific *otanpsPN* gene using real-time PCR was performed with specific primers designed by Rodríguez et al. (Table 2) [26].

Amplification was conducted in a 20 µL reaction volume containing 10.0 µL of Q SYBR® Green Supermix (BIO-RAD, USA), 1.0 µL of each primer (10 pmol/µL concentration), and 1–10 ng of DNA using a CFX Opus 96 thermal cycler (BIO-RAD, USA). The PCR conditions included an initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 56 °C for 60 seconds, and a melt curve analysis from 65 °C to 95 °C with 5-second increments. The specific melting temperature (Tm) was considered to be 84 ± 0.5 °C. The cultivation screening assay on coconut cream agar (CCA) and thin-layer chromatography (TLC) were performed according to Jevinová et al. (2023) [11].

Statistical analysis

Chi-square (χ^2) tests of independence were used to compare phenotypic and molecular identification results and OTA production capabilities among species, performed in Jamovi software (version 2.6.44.0).

RESULTS

A total of 179 isolates of microscopic filamentous fungi were subjected to phenotypic and genotypic identification, including 93 isolates from the surface of eggshells, 34 from coffee beans, and 52 from marjoram. Based on characteristic macroscopic and microscopic features, 12 isolates were identified as *Penicillium verrucosum*, 5 as *Aspergillus carbonarius*, 43 as *A. niger*, 11 as *A. ochraceus*, 8 as *A. westerdijkiae*, and 4 as *A. tubingensis*.

The isolates were further analyzed using conventional PCR at both the genus and species levels. A total of 78 isolates were assigned to the genus *Penicillium*, of which 8 were specifically identified as *P. verrucosum*. Additionally, 109 isolates were assigned to the genus *Aspergillus*, including 46 identified as *A. niger*, 10 as *A. ochraceus*, 8 as *A. westerdijkiae*, and 3 as *A. carbonarius*. Two isolates preliminarily identified phenotypically as *A. carbonarius* and one isolate identified as *A. tubingensis* were reclassi-

Table 2. Primers for the detection of OTA and their characteristics

Primer name	Primer sequence (5′-3′)	Annealing temperature (°C)	PCR product size	Gen-Bank-EMBL accession number	Study
*otanps*PN					
F-npstr	GCCGCCCTCTGTCATTCCAAG	56	117 bp	AY557343	[26]
R-npstr	GCCATCTCCAAACTCAAGCGTG				

Table 3. Number of isolates identified by the methods used

Species	Commodity	Phenotype	PCR	Sequencing
P. verrucosum	chicken eggs	12	8	8
A. carbonarius	marjoram	5	3	3
A. niger	coffee, marjoram	43	46	46
A. ochraceus	coffee	11	10	10
A. westerdijkiae	coffee	8	8	8
A. tubingensis	coffee, marjoram	4	-	3

fied as *A. niger* by PCR analysis. Three isolates initially classified as *A. tubingensis* based on morphology were not confirmed by PCR.

The fungal strains identified by conventional PCR were subsequently subjected to sequencing for confirmation. Sequencing was also performed on isolates phenotypically identified as *A. tubingensis*. Amplification of the ITS1–5.8S–ITS2 region of ribosomal DNA (rDNA), characterized by low intraspecific polymorphism and high interspecific variability, was carried out using the ITS1 and ITS4 primers. The amplicons were sequenced using the Sanger method. Sequencing validated all species identifications determined by PCR and genetically confirmed three isolates preliminarily classified as *A. tubingensis* by morphology (Table 3).

The ability of fungal isolates to produce ochratoxin A (OTA) was assessed using three methods. OTA production was confirmed by at least one method in 70 isolates (8 eggs, 18 coffee, 44 marjoram). TLC and coconut agar assays correlated strongly with molecular detection of the *otanpsPN* gene (detected in 73 isolates). Among *A. niger* isolates from coffee, the ability to produce OTA was rare; gene encoding OTA production was detected only in one isolate (Table 4). Unexpectedly, OTA production was confirmed in three *A. tubingensis* isolates, suggesting possible variation in toxigenic capacity.

Statistical analysis showed no significant differences between phenotypic and PCR identification ($p = 0.138$), nor between phenotypic identification and sequencing ($p = 0.138$). PCR and sequencing identification results were in complete agreement.

Statistical analysis also showed no significant differences among species in OTA production capability across the different detection methods ($p > 0.05$; coconut cream agar: $p = 0.508$; TLC: $p = 0.508$; real-time qPCR: $p = 0.305$).

DISCUSSION

This study focused on the identification and characterization of microscopic filamentous fungi isolated from the surface of chicken eggshells, fermented green coffee beans, and dried marjoram. Combining phenotypic and genotypic methods, several potentially toxigenic species of the genera *Penicillium* and *Aspergillus* were identified.

Of the 179 isolates, the majority originated from eggshell surfaces (93 isolates), suggesting that eggs can serve as significant carriers of environmental contaminants, including filamentous fungi, consistent with previous findings [27, 28, 29, 30]. Notably, the ochratoxigenic species *P. verrucosum*, typically associated with cereal substrates, was also identified among the eggshell isolates, as previously described by Schmidt-Heydt et al. [31].

In green coffee beans (34 isolates), species of sections *Nigri* (*A. niger*, *A. tubingensis*) and *Circumdati* (*A. westerdijkiae*, *A. ochraceus*) were predominantly detected, aligning with reports of thermotolerant and xerotolerant fungi in coffee [32, 33, 34, 35]. Although coffee is considered a high-risk commodity for OTA contamination, we observed notable variability in OTA production among isolates.

Dried marjoram (52 isolates) exhibited the highest contamination rate with OTA-producing fungi, primarily *A. niger*, *A. carbonarius*, and *A. tubingensis*, supporting findings by Dimić et al. [36] and other studies [37, 38, 39].

Phenotypic identification based on macroscopic and microscopic traits was verified by conventional PCR and sequencing of the internal transcribed spacer (ITS) region. While phenotypic methods allowed preliminary classification, molecular techniques revealed misclassifications, particularly distinguishing *A. niger* from *A. tubingensis*

Table 4. Number of isolates producing ochratoxin A

Species	Commodity	Number of isolates	qPCR	CCA	TLC
P. verrucosum	chicken eggs	8	8	8	8
A. carbonarius	marjoram	3	3	3	3
A. niger	coffee	4	1	0	0
	marjoram	42	41	40	40
A. ochraceus	coffee	10	10	9	9
A. westerdijkiae	coffee	8	8	8	8
A. tubingensis	coffee	1	1	1	1
	marjoram	2	1	1	1

and *A. carbonarius*. These findings highlight the morphological similarity within section *Nigri* and the essential role of molecular methods [10, 40]. Results corroborate previous reports emphasizing the necessity of ITS sequencing and PCR for accurate species-level identification in morphologically similar fungi [41].

Sequencing fully confirmed PCR-based identifications, and variability in ITS amplicon sizes proved the genomic region's suitability for distinguishing related species. This underscores the robustness of molecular approaches in mycological diagnostics.

Ochratoxin A (OTA) production was evaluated using three methods: *otanps*PN gene detection, cultivation on coconut cream agar, and TLC. Among the *Aspergillus* species, *A. ochraceus* is widely recognized as the principal OTA producer [42], followed by *A. carbonarius* and *A. niger*, which are also frequently implicated in OTA contamination of food commodities [43]. Additionally, *A. westerdijkiae* has been identified as a significant OTA-producing species, with toxigenic potential comparable to that of *A. ochraceus* [44].

While *P. verrucosum*, *A. ochraceus*, *A. carbonarius*, and *A. westerdijkiae* showed OTA production by all methods, among coffee-derived *A. niger* isolates, the biosynthetic gene was detected in only one, and no OTA was visible on agar, diverging from reports of consistent OTA production in this species [35, 45, 46]. This discrepancy may be attributed to lower incubation temperatures and shorter duration (25 °C and 5 °C for 7 days), which are known to suppress OTA biosynthesis [47]. According to Medina et al., elevated temperatures (e.g., 37 °C) may stimulate secondary metabolite biosynthesis as a stress response [48]. Similarly, Belli and Akar note that OTA is produced during the stationary growth phase, typically after 14 days of incubation [49].

Interestingly, OTA production was detected in three *A. tubingensis* isolates, a species generally considered non-ochratoxigenic [39, 50, 51, 52]. Similar findings by Gherbawy et al. suggest that under certain conditions, *A. tubingensis* may produce OTA, emphasizing the need for individual strain assessment [53]. This highlights the need for individualized evaluation of toxigenic potential, even in species not classically considered OTA producers.

Statistical analysis showed strong concordance between phenotypic and molecular identification, confirming the reliability of PCR and ITS sequencing while acknowledging the limitations of morphology-based approaches [10].

This study provides novel insights into the presence and toxigenic profiles of microscopic filamentous fungi in commonly consumed food matrices of both animal and plant origin. It demonstrates that even species traditionally considered non-toxigenic may pose potential risks under specific environmental conditions. By integrating morphological and molecular methods with chemical detection of mycotoxins, the study highlights the necessity of a multi-tiered approach to ensure accurate fungal identification and mycotoxin risk assessment. These findings contribute valuable knowledge to the fields of food safety, mycotoxicology, and fungal diagnostics and may support future improvements in regulatory monitoring practices.

CONCLUSIONS

This study addressed the identification of microscopic filamentous fungi isolated from chicken egg surfaces, fermented green coffee beans, and dried marjoram and evaluated their ability to produce ochratoxin A (OTA). By combining phenotypic methods with molecular (PCR, ITS sequencing) and chemical approaches, several species of *Aspergillus* (*A. niger*, *A. tubingensis*, *A. ochraceus*, *A. carbonarius*, *A. westerdijkiae*) and *Penicillium verrucosum* were successfully identified.

Molecular analyses confirmed the reliability of phenotypic identification but also revealed discrepancies among closely related species, emphasizing the need for integrated methods. OTA production testing through coconut cream agar, thin-layer chromatography, and PCR detection of the *otanpsPN* gene showed a high level of concordance, with the highest proportion of positive isolates found in marjoram. Integrated approach revealed species previously underrecognized as OTA sources, including *P. verrucosum* in eggs and A. *tubingensis* in herbs. The occurrence of OTA production in isolates traditionally deemed non-toxigenic highlights the necessity for ongoing verification and strain-specific evaluation.

The study highlights the importance of rigorous monitoring for OTA-producing fungi in both plant- and animal-derived foods. Combining morphological, molecular, and chemical analyses provides a comprehensive strategy for fungal identification and mycotoxin risk assessment, contributing valuable insights for food safety and public health protection.

Future research should focus on studies refining food safety standards and supporting regulatory efforts in mitigating mycotoxin exposure risks globally, such as study of environmental and genetic factors influencing OTA biosynthesis in atypical producers like *A. tubingensis*; longitudinal studies assessing OTA accumulation across production and storage phases; and development of rapid, field-deployable molecular assays for early screening of OTA producers in diverse food environments.

Data Availability Statement

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

Ethical Statement

No ethical approval was needed for this study.

Conflict of Interest

The authors declare no conflict of interest.

Funding

This work was funded by the Scientific Grant Agency of the Ministry of Education, Research, Development and Youth of the Slovak Republic and the Slovak Academy of Sciences, grant number (VEGA 1/0038/25).

Generative AI Statement

The authors declare no generative AI and AI-assisted technologies were used in writing the manuscript.

Authors' Contributions

Soňa Pivka: writing—original draft, writing—review and editing, conceptualization, visualization, investigation, methodology, formal analysis, data curation. Pavlina Jevinová: writing—review and editing, visualization, investigation, methodology, data curation. Monika Pipová: methodology, data curation. Ivana Regecová: methodology, data curation.

REFERENCES

1. **Bryła, M., Ksieniewicz-Woźniak, E., Stępniewska, S., Modrzewska, M., Waskiewicz, A., Szymczyk, K., Szafrańska, A., 2021:** Transformation of ochratoxin A during bread-making processes. *Food Control*, 125, 1, 107950. DOI: 10.1016/j.foodcont.2021.107950
2. **Chen, W., Li, C., Zhang, B., Zhou, Z., Shen, Y., Liao, X., Yang, J., Wang, Y., Li, X., Li, Y., Shen, X. L., 2018:** Advances in biodetoxification of ochratoxin A-A review of the past five decades. *Front. Microbiol.*, 9, 1, 1386. DOI: 10.3389/fmicb.2018.01386
3. **Gu, K., Ryu, D., Lee, H. J., 2021:** Ochratoxin A and its reaction products affected by sugars during heat processing. *Food Chem.*, 348, 129038. DOI: 10.1016/j.foodchem.2021.129038
4. **Alshannaq, A., Yu, J.-H., 2017:** Occurrence, toxicity, and analysis of major mycotoxins in food. *Int. J. Environ. Res. Public Health.*, 14, 6, 632. DOI: 10.3390/ijerph14060632
5. **Arce-López, B., Lizarraga, E., Vettorazzi, A., González-Peñas, E., 2020:** Human biomonitoring of mycotoxins in blood, plasma and serum in recent years: A review. *Toxins*, 12, 3, 147. DOI: 10.3390/toxins12030147
6. **Banahene, J. C. M., Ofosu, I. W., Odai, B. T., Lutterodt, H. E., Agyemang, P. A., Ellis, W. O., 2024:** Ochratoxin A in food commodities: A review of occurrence, toxicity, and management strategies. *Heliyon*, 10, 20, e39313. DOI: 10.1016/j.heliyon.2024.e39313
7. **Banahene, J. C. M., Ofosu, I. W., Odai, B. T., 2023:** Surveillance of ochratoxin A in cocoa beans from cocoa-growing regions of Ghana. *Heliyon*, 9, 7, e18206. DOI: 10.1016/j.heliyon.2023.e18206
8. **Yazdanfar, N., Mahmudiono, T., Fakhri, Y., Mahvi, A., Sadighara, P., Mohammadi, A., Yousefi, M., 2022:** Concentration of ochratoxin A in coffee products and probabilistic health risk assessment. *Arab. J. Chem.*, 15, 104376. DOI: 10.1016/j.arabjc.2022.104376
9. **Kőszegi, T., Poór, M., 2016:** Ochratoxin A: molecular interactions, mechanisms of toxicity and prevention at the molecular level. *Toxins*, 8, 4, 111. DOI: 10.3390/toxins8040111
10. **Houbraken, J., Visagie, C. M., Meijer, M., Frisvad, J. C., Busby, P. E., Pitt, J. I., Seifert, K. A., Louis-Seize, G., Demirel, R., Yilmaz, N., Jacobs, K., Christensen, M., Samson, R. A., 2014:** A taxonomic and phylogenetic revision of *Penicillium* section *Aspergilloides*. *Stud. Mycol.*, 78, 373–451. DOI: 10.1016/j.simyco.2014.09.002

11. **Jevinová, P., Koréneková, B., Pipová, M., Regecová, I., 2023:** *Instructions for practical exercises in mycology and mycotoxicology of feed and food*. Košice: University of Veterinary Medicine and Pharmacy in Košice, 2023. ISBN 978-80-8077-794-4.178 pp.
12. **Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Malir, J., Toman, J., 2016:** Ochratoxin A: 50 years of research. *Toxins*, 8, 7, 191. DOI: 10.3390/toxins8070191
13. **Więckowska, M., Szelenberger, R., Niemcewicz, M., Harmata, P., Poplawski, T., Bijak, M., 2023:** Ochratoxin A-The Current knowledge concerning hepatotoxicity, mode of action and possible prevention. *Molecules* (*Basel, Switzerland*), 28, 18, 6617. DOI: 10.3390/molecules28186617
14. **STN EN ISO 21527-1/O1, 2008:** Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds. Part 1: Colony count technique in products with water activity greater than 0,95.
15. **STN EN ISO 21527-2/O1, 2008:** Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds. Part 2: Colony count technique in products with water activity less than or equal to 0,95.
16. **Frisvad, J., Samson, R., 2004:** Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identif cation of food and airborne terverticillate *Penicillia* and their mycotoxins. *Studies in Mycology,* 1, 1, 1-173.
17. **Tančinová, D., Mašková, Z., Feišociová, S., Dovičičová, M., Barboráková, Z., 2012:** *Introduction to food mycology: A key to the identification of food-important filamentous microscopic fungi.* Nitra: Slovak University of Agriculture in Nitra, 2012. ISBN 978-80-200-1703-1. 286 pp.
18. **Regecová, I., Pipová, M., Jevinová, P., Demjanová, S., Semjon, B., 2020:** Quality and mycobiota composition of stored eggs. *Ital. J. Food Sci.*, 32, 3, 540–561. DOI: 10.14674/IJFS-1702
19. **Pedersen, L. H., Skouboe, P., Boysen, M., Soule, J., Rossen, L., 1997:** Detection of *Penicillium* species in complex food samples using the polymerase chain reaction. *Int. J. Food Microbiol.*, 35, 2, 169–177. DOI: 10.1016/s0168-1605(96)01216-0
20. **Demjanová, S., Jevinová, P., Pipová, M., Regecová, I., 2021:** Identification of *Penicillium verrucosum*, *Penicillium commune*, and *Penicillium crustosum* isolated from chicken eggs. *Processes*, 9, 1, 53. DOI: 10.3390/pr9010053
21. **Buess, M., Cathomas, G., Halter, J., Junker, L., Grendelmeier, P., Tamm, M., Stolz, D., 2012:** *Aspergillus*-PCR in bronchoalveolar lavage for detection of invasive pulmonary aspergillosis in immunocompromised patients. *BMC Infect. Dis.*, 12, 237. DOI: 10.1186/1471-2334-12-237
22. **Gil-Serna, J., Vázquez, C., Sardiñas, N., González-Jaén, M. T., Patiño, B., 2009:** Discrimination of the main Ochratoxin A-producing species in *Aspergillus* section *Circumdati* by specific PCR assays. *Int. J. Food Microbiol.*, 136, 1, 83–87. DOI: 10.1016/j.ijfoodmicro.2009.09.018
23. **Sugita, C., Makimura, K., Uchida, K., Yamaguchi, H., Nagai, A., 2004:** PCR identification system for the genus *Aspergillus* and three major pathogenic species: *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. *Med. Mycol.*, 42, 5, 433–437. DOI: 10.1080/13693780310001656786
24. **González-Salgado, A., Patiño, B., Vázquez, C., González-Jaén, M. T., 2005:** Discrimination of *Aspergillus niger* and other *Aspergillus* species belonging to section Nigri by PCR assays. *FEMS Microbiol. Lett.*, 245, 2, 353–361. DOI: 10.1016/j.femsle.2005.03.023
25. **White, T. J., 1991:** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*. New York: Academic Press, 1990. ISBN -978-0-12372180-8. 315-322 pp.
26. **Rodríguez, A., Rodríguez, M., Luque, M. I., Justesen, A. F., Córdoba, J. J., 2011:** Quantification of ochratoxin A-producing molds in food products by SYBR Green and TaqMan real-time PCR methods. *Int. J. Food Microbiol.*, 149, 3, 226–235. DOI: 10.1016/j.ijfoodmicro.2011.06.019
27. **Gaweł, A., Siedlecka, M., Karwańska, M., Skrok, M., Przychodniak, I., Knap, P., Chmielina, A., Truszko, V., Kuczkowski, M., Bierowiec, K., Woźniak-Biel, A., Szczepankiewicz, B., Bobrek, K., 2025:** Occurrence of fungi on duck egg shells and drug resistance analysis of Aspergillus spp. isolates. *Appl. Sci.*, 15, 2, 860. DOI: 10.3390/app15020860
28. **Melo, M. V. C., , dos Santos, F. C. P., da Silva, I. N. G., Rodrigues, J. L. C., Freire, J. E. C., 2025:** Fungal profile of eggshells from commercial and free-range hens of a supermarket chain in Fortaleza, CE, Brazil. *Biosci. J.*, 40, e40026. DOI: 10.14393/BJ-v40n0a2024-69507
29. **Perez-Nadales, E., Nogueira, M. F., Baldin, C., Castanheira, S., El Ghalid, M., Grund, E., Lengeler, K., Marchegiani, E., Mehrotra, P. V., Moretti, M., Naik, V., Oses-Ruiz, M., Oskarsson, T., Schäfer, K., Wasserstrom, L., Brakhage, A. A., Gow, N. A., Kahmann, R., Lebrun, M. H., Perez-Martin, J., Wendland, J., 2014:** Fungal model systems and the elucidation of pathogenicity determinants. *Fungal Genet Biol.*, 70, 100, 42–67. DOI: 10.1016/j.fgb.2014.06.011

30. **Tomczyk, Ł., Stępień, Ł., Urbaniak, M., Szablewski, T., Cegielska-Radziejewska, R., Stuper-Szablewska, K., 2018:** Characterisation of the mycobiota on the shell surface of table eggs acquired from different egg-laying hen breeding systems. *Toxins*, 10, 7, 293. DOI: 10.3390/toxins10070293
31. **Schmidt-Heydt, M., Graf, E., Stoll, D., Geisen, R., 2012:** The biosynthesis of ochratoxin A by *Penicillium* as one mechanism for adaptation to NaCl rich foods. *Food Microbiol.*, 29, 2, 233–241. DOI: 10.1016/j.fm.2011.08.003
32. **Hagos, L., Guta, M., Bacha, K., 2024:** Prevalence of mycotoxigenic fungi and ochratoxin A in coffee (*Coffea arabica* L.). *Cogent Food Agric.*, 10. DOI: 10.1080/23311932.2024.2407524
33. **Hlebová, M., Uzsáková, V., Podhorská, L., Vešelényiová, D., Mrkvová, M., Čuboň, J., Hleba, L., 2022:** Mycobiota and co-occrurence of mycotoxins in green and roasted coffee beans. *J. Microbiol. Biotechnol. Food Sci.*, 11, e5779. DOI: 10.55251/jmbfs.5779
34. **Lu, L., Tibpromma, S., Karunarathna, S. C., Jayawardena, R. S., Lumyong, S., Xu, J., Hyde, K. D., 2022:** Comprehensive review of fungi on coffee. *Pathogens (Basel, Switzerland)*, 11, 4, 411. DOI: 10.3390/pathogens11040411
35. **Maman, M., Sangchote, S., Piasai, O., Leesutthiphonchai, W., Sukorini, H., Khewkhom, N., 2021:** Storage fungi and ochratoxin A associated with arabica coffee bean in post-harvest processes in Northern Thailand. *Food Control*, 130, 108351. DOI: 10.1016/j.foodcont.2021.108351
36. **Dimić, G. R., Kocić-Tanackov, S. D., Tepić, A. N., Vujičić, B. L., Šumić, Z. M., 2008:** Mycopopulation of spices. *Acta Period. Technol.*, 39, 1-9.
37. **Garcia, M. V., Parussolo, G., Moro, C. B., Bernardi, A. O., Copetti, M. V., 2018:** Fungi in spices and mycotoxigenic potential of some *Aspergilli* isolated. *Food microbiol.*, 73, 93–98. DOI: 10.1016/j.fm.2018.01.013
38. **Krstić, M., Stupar, M., Đukić-Ćosić, D., Baralić, K., Svetlana, Đ., 2021:** Health risk assessment of toxic metals and toxigenic fungi in commercial herbal tea samples from Belgrade, Serbia. *J. Food Compos. Anal.*, 104, 104159. DOI: 10.1016/j.jfca.2021.104159
39. **Nascimento, A., Lopez, B., Silva, B., Munaretto, M., Souza, R., Nazário, C., Oliveira, V., Paula, C., Maia, M., Júnior, D., 2024:** Microbiological quality of dehydrated aromatic condiments and spices. *Int. J. Curr. Microbiol. Appl. Sci.,* 13, 3, 1–26. DOI: 10.20546/ijcmas.2024.1303.001
40. **Silva, D. M., Batista, L. R., Rezende, E. F., Fungaro, M. H., Sartori, D., Alves, E., 2011:** Identification of fungi of the genus *Aspergillus* section *Nigri* using polyphasic taxonomy. *Braz. J. Microbiol.,* 42, 2, 761–773. DOI: 10.1590/S1517-83822011000200044
41. **Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., et al, 2012:** Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl. Acad. Sci. U.S.A.*, 109, 16, 6241–6246. DOI: 10.1073/pnas.1117018109
42. **Mantle, P., 2002:** Risk assessment and the importance of ochratoxins. *Int. Biodeterior. Biodegrad.,* 50, 1, 143-146. DOI: 10.1016/S0964-8305(02)00079-3
43. **Hayat, A., Rhouati, A., Marty, J., Barthelmebs, L., 2012:** Recent advances in ochratoxin A-producing fungi detection based on PCR methods and ochratoxin A analysis in food matrices. *Food Control,* 26, 1, 401–415. DOI: 10.1016/j.foodcont.2012.01.060
44. **Frisvad, J. C., Frank, J. M., Houbraken, J. A. M. P., Kuijpers, A. F. A., Samson, R. A., 2004:** New ochratoxin A producing species of *Aspergillus* section *Circumdati*. *Stud. Mycol.*, 50, 23-43.
45. **Varga, J., Kevei, E., Rinyu, E., Téren, J., Kozakiewicz, Z., 1996:** Ochratoxin production by *Aspergillus* species. *Appl. Environ. Microbiol.*, 62, 12, 4461–4464. DOI: 10.1128/aem.62.12.4461-4464.1996
46. **Frisvad, J. C., Larsen, T. O., Thrane, U., Meijer, M., Varga, J., Samson, R. A., Nielsen, K. F., 2011:** Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. *PLOS One*, 6, 8, e23496. DOI: 10.1371/journal.pone.0023496
47. **da Silva, S., Pereira, R., Azevedo, N., da Gloria, E., Chalfoun, S., Batista, L., 2020:** Fungi associated to beans infested with coffee berry borer and the risk of ochratoxin A. *Food Control*, 113, 107204. DOI: 10.1016/j.foodcont.2020.107204
48. **Medina, A., Fraga, M. E., Sanchis, V., 2005:** Fungal contamination and mycotoxin production in maize grown in Spain under different environmental conditions. *Mycol. Res.*, 109, 11, 1185-1191.
49. **Belli, N., Akar, T., 2004:** Production of ochratoxin A by *Aspergillus ochraceus* in relation to various environmental conditions. *Food Control*, 15, 1-3, 123-128.
50. **Wang, Y., Wang, L., Liu, F., 2016:** Ochratoxin A producing fungi, biosynthetic pathway and regulatory mechanisms. *Toxins (Basel)*, 8, 3, 83. DOI: 10.3390/toxins8030083
51. **Choque, E., Klopp, C., Valiere, S., Raynal, J., Mathieu, F., 2018:** Whole-genome sequencing of *Aspergillus tubin-*

gensis G131 and overview of its secondary metabolism potential. *BMC Genom.*, 19, 1, 200. DOI: 10.1186/s12864-018-4574-4

52. **Storari, M., Bigler, L., Gessler, C., Broggini, G. A., 2012:** Assessment of the ochratoxin A production ability of *Aspergillus tubingensis*. *Food Addit. Contam. - Chem. Anal. Control Expo. Risk Assess.*, *29*, 9, 1450–1454. DOI: 10.1080/19440049.2012.698656

53. **Gherbawy, Y. A., Hamza, L. H. A., Maghraby, T. A., Shebany, Y. M., Abdelwahab, S. F., El-Dawy, E. G. A. M., 2025:** Characterization of *Aspergillus tubingensis* from grains in upper Egypt and their potential for ochratoxins production. *Food Biotechnol.*, 39, 1, 28–50. DOI: 10.1080/08905436.2025.2458242