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Address:

Scientific Veterinary Institute “Novi Sad”,
21000 Novi Sad, Rumenački put 20
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EVALUATION OF ANTIBACTERIAL ACTIVITY OF *TRICHILIA DREGEANA* SOND (MELIACEAE) CRUDE EXTRACTS AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Tesfa Mossie^{1*}, Temesgen Kasa², Beksisa Urge², Hilena Yifred¹

¹ Ethiopian Institute of Agricultural Research, Jimma
Research Center, Jimma, Ethiopia

² Ethiopian Institute of Agricultural Research, Holeta
Research Center, Holeta, Ethiopia

Abstract

Medicinal plants have been playing a crucial role in drug discovery and development against infectious diseases. The antibacterial activity of methanol crude extract of traditionally used *Trichilia dregeana* Sond was evaluated against common bacterial pathogens. Agar well diffusion and broth dilution assays were used to evaluate the antimicrobial activity and MIC of crude methanol plant extracts. The methanol crude extract of *Trichilia dregeana* Sond showed promising antibacterial activity against all tested bacterial isolates. A significant difference ($p < 0.05$) was observed in susceptibility among *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* species isolates. A higher mean susceptibility or inhibition zone diameter to crude extracts was observed in *Escherichia coli* (19.6 ± 5.9 mm) and *Pseudomonas aeruginosa* (18.4 ± 5.9 mm) compared to *Staphylococcus aureus* isolates. Significant differences were also observed in the susceptibility of tested bacterial isolates at different concentrations of crude extracts. A higher inhibition zone diameter was recorded in *Pseudomonas aeruginosa* (27.2 ± 1.5 mm), followed by *Escherichia coli* (25.2 ± 1.5 mm), and *Staphylococcus aureus* (21.1 ± 1.4 mm) at a concentration of 100 mg/mL. A comparable inhibition zone of crude extracts was observed with that of the positive control at 100 mg/mL concentration against *Staphylococcus aureus* and *Escherichia coli* isolates. The findings indicated that bacterial growth

^{1*} Corresponding Author: tesfa.mossie@eiar.gov.et / mtesfa6@gmail.com

inhibition increased as the concentration of the crude extracts increased. All tested pathogenic bacteria species (gram-positive and gram-negative) were susceptible to plant leaf extract, even at lower levels of concentration. This showed the plant extracts' broad-spectrum activity against both gram-positive and gram-negative bacteria. The methanol crude extract of plants is an effective antibacterial agent for treating bacterial infections since the extract showed significant antibacterial potency comparable with that of the standard antibiotic disk. The findings are promising and encourage further investigation of the crude extracts' phytochemical, toxicological, and pharmacological aspects to support their potential rational use in antimicrobial therapy.

Key words: Antibacterial activity, crude extracts, isolates, MIC, *Trichilia dregeana* Sond

PROCENA ANTIBAKTERIJSKE AKTIVNOSTI SIROVIH EKSTRAKATA *TRICHILIA DREGEANA* SOND (MELIACEAE) PROTIV GRAM-POZITIVNIH I GRAM-NEGATIVNIH BAKTERIJA

Tesfa Mossie¹, Temesgen Kasa², Beksisa Urge², Hilena Yifred¹

¹ Etiopski institut za poljoprivredna istraživanja,
Jimma istraživački centar, Jimma, Etiopija

² Etiopski institut za poljoprivredna istraživanja,
Holeta istraživački centar, Holeta, Etiopija

Kratak sadržaj

Lekovito bilje ima značajnu ulogu u razvijanju lekova za infektivne bolesti. Ispitivano je antibakterijsko dejstvo sirovog ekstrakta dobro poznate *Trichilia dregeana* Sond protiv uobičajenih bakterijskih patogena. Antimikrobna aktivnost i MIC sirovih biljnih ekstrakata evaluirani su pomoću agar difuzionog testa i metoda dilucije u bujonu. Sirovi metanolski ekstrakt *Trichilia dregeana* Sond pokazao je obećavajuću antibakterijsku aktivnost kod svih bakterijskih izolata. Značajna razlika ($p < 0.05$) u pogledu osetljivosti uočena je kod izolata vrsta *Escherichia coli*, *Staphylococcus aureus* i *Pseudomonas aeruginosa*. Viša prosečna osetljivost ili prečnik zone

inhibicije za sirove ekstrakte uočena je kod izolata *Escherichia coli* (19.6 ± 5.9 mm) i *Pseudomonas aeruginosa* (18.4 ± 5.9 mm) u odnosu na izolate *S. aureus*. Značajne razlike u pogledu osetljivosti testiranih bakterijskih izolata uočene su i u odnosu na različite koncentracije sirovih ekstrakata. Veći prečnik zone inhibicije pri koncentraciji od 100 mg/mL zabeležen je kod *Pseudomonas aeruginosa* (27.2 ± 1.5 mm), zatim *Escherichia coli* (25.2 ± 1.5 mm), i *Staphylococcus aureus* (21.1 ± 1.4 mm). Zona inhibicije sirovog ekstrakta uporediva je sa onom uočenom kod pozitivnih kontrola za izolate *Staphylococcus aureus* i *Escherichia coli* pri koncentraciji 100 mg/mL. Ovi nalazi ukazuju na to da se inhibicija bakterijskog rasta povećava sa povećanjem koncentracije sirovog ekstrakta. Sve testirane bakterijske patogene vrste (gram pozitivne i gram negativne) bile su osetljive na ekstrakt lista biljke, čak i na nižim koncentracijama. Ovo ukazuje na širok spektar aktivnosti biljnih ekstrakata kako protiv gram pozitivnih, tako i protiv gram negativnih bakterija. Sirovi metanolski ekstrakt biljaka je efikasan antibakterijski agens za tretman bakterijskih infekcija, s obzirom da je pokazao značajan antibakterijski potencijal koji je uporediv sa onim kod standardnih antibiotskih diskova. Ovi nalazi su obećavajući i ohrabruju dalja ispitivanja fitohemijskih, toksikoloških i farmakoloških aspekata sirovih ekstrakata u cilju njihove potencijalne racionalne primene u okviru antimikrobne terapije.

Ključne reči: antibakterijska aktivnost, sirovi ekstrakti, izolati, MIC, *Trichilia dregeana* Sond

INTRODUCTION

Infectious diseases constitute a significant threat to livestock and public health worldwide due to the emergence of widespread drug resistance, particularly in developing countries (Salam et al., 2023). Many parts of the world continue to have endemic livestock diseases that have devastating effects on animal health and impact national and international trade (Tomley and Shirley, 2009). The economic losses from infectious diseases are mainly significant in Africa, including Ethiopia (Morens et al., 2008). Among infectious diseases of livestock, bacterial-caused diseases account for the most considerable economic losses in the livestock sector in many countries worldwide (Doss et al., 2012; Rathor et al., 2021).

Antibiotics are one of the most significant therapeutic discoveries for infectious diseases, even though synthetic medicines have only been used to treat one-third of infectious infections. This is due to the development of microorganisms resistant to existing chemotherapeutic drugs, as well as widespread indiscriminate and overuse of antibiotics. The widespread use of antibiotics in the treatment and control of infectious diseases has the potential to have public health effects due to drug residues in the food chain. Antibiotic resistance has skyrocketed in recent years, posing treatment challenges (Antara and Batra, 2012). Bacteria can develop a variety of resistance mechanisms to antimicrobial drugs and resistance to both old and new antibiotics is increasing in both human and veterinary medicine. Multidrug-resistant bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP), and vancomycin-resistant enterococci (VRE). As a result, there is an urgent need to develop new and novel antimicrobial compounds with novel chemical structures and modes of action to combat multi-drug-resistant bacterial infections using traditionally used medicinal plants (Talib and Mahasneh, 2010; Bradley, 2002; Gootz, 2010).

Medicinal plants have been used for the treatment of infectious diseases worldwide. More than 80% of people living in Sub-Saharan Africa frequently rely on folk medical practices for their primary health needs, including humans and livestock. This is because of limited access to veterinary services, the emergence of resistant strains, and the high cost and limited affordability of commercial drugs (Beyene et al., 2015). Firm reliance on medicinal plants, mainly in rural areas, is practiced in Ethiopia. Plants have been crucial in drug discovery and development against infectious diseases (Madureira et al., 2012). About 60% of antimicrobial agents are derived from natural products, particularly plant products (Newman and Cragg, 2007). The huge structural diversity and great biodiversity make natural products an outstanding subject for drug discovery and development (Rollinger et al., 2006). Plant extracts produce primary and secondary bioactive molecules that act as a chemical defense against infection. The bioactive molecules are stored in roots, leaves, stems, fruits, and seeds (Seema et al., 2013; Ugwu et al., 2015). Plants' most important bioactive compounds are flavonoids, phenols, phenolic glycosides, saponins, cyanogenic glycosides, and glucosinolates (Tungmunthum et al., 2018). These molecules may have different mechanisms of action than conventional drugs, which have clinical significance in health care improvement by alleviating drug-resistant issues (Eloff, 1998). Traditional medicinal plant extracts have been shown in studies to have antimicrobial activities against infectious diseases (Nayak, 2020). Many African countries, including Ethiopia and other parts of the world, encourage the screening of plants used in tradi-

tional medicine to authenticate their antimicrobial activities. Therefore, the safety and effectiveness of medicinal plants should be investigated through in vitro and in vivo assay methods. The present study investigated the claimed antibacterial properties of medicinal plants traditionally used in Ethiopia and provided scientific validation of their use. The findings were aimed at evaluating the antibacterial activities of methanol crude extracts of *Trichilia dregeana* Sond plants against some gram-positive and gram-negative bacteria pathogens through agar well diffusion and broth dilution methods. The plant assayed in this study is commonly used as a medicinal plant in different parts of Ethiopia.

MATERIAL AND METHODS

Materials and Chemicals

The chemicals used were 99.9% methanol, plant extracts, distilled water, dimethyl sulfoxide (DMSO), reference drugs, Muller-Hilton agar, Muller-Hilton broth media, and nutrient-agar media. The materials used in the study were aluminum foil, mortar and pestle, digital weighing balance, diamond pencil, syringe, What-man No. 1 filter paper, micropipettes, test tubes, Bunsen burner, incubator, lyophilizer, rotavapor, sterile lancet, spatula, flasks, and orbital mixer.

Study Area

Plant materials were collected from selected study areas in the Southwestern Jimma zone of Oromia regional state, Ethiopia. Jimma town, the capital of Jimma Zone, is located in Oromia Regional Administration, 346 km southwest of Addis Ababa. The average annual rainfall is 1216.7 mm, with the main wet season from June to September. The maximum and minimum temperatures of Jimma were 27 °C and 10 °C, respectively. The longitude and latitude of Jimma are 7° 36' N and 36° 50' E, respectively, at an altitude of 1753 m above sea level. The study areas practice mixed farming systems for crop livestock. The area has high humidity and is rich in fauna and flora.

Study Design

The study design was an experimental trial of the in-vitro antibacterial activities of *Trichilia dregeana* Sond plant against infectious diseases. All experimental tests were carried out alongside positive and negative controls.

Preparation and Extraction of Medicinal Plants

Leaves of *Trichilia dregeana* Sond plant were collected from their natural habitats based on their frequent use by informants for treating and controlling infectious diseases. The medicinal plant leaves were washed in tap water, shaded dry at room temperature, and chopped into smaller pieces. The dried medicinal plant was pulverized and ground into powder using a blender machine. The material was weighed before extraction procedures. The powders were labeled and stored in an airtight container until extracted. Four hundred grams (400 g) of dried medicinal plant were macerated in a one-liter solution (800 mL methanol with 200 mL distilled water) for 72 hours at ambient temperature. The 99.9% methanol and distilled water were used as solvents. The macerated medicinal plants were squeezed twice per day for three days. After 72 hours, the macerated medicinal plants were squeezed using double-layer gauze. Then, it was strained using Whatman No. 1 filter paper to get an extract solution free of solids. The plants were again re-macerated in a one-liter solution for 72 hours and squeezed two times per day. The solution was concentrated using a vacuum rotary evaporator to remove the solvent (methanol). Then, the extract was put in a sterile glass bottle and kept in a hot, dry oven at 37°C to evaporate distilled water and the remaining solvent. After removing both methanol and distilled water, the crude extracts were weighed using a sensitive balance and stored at 4°C until a sensitivity test was performed.

Source of Bacterial Strain

Standard bacterial strains, including gram-positive and gram-negative bacteria, were used for the study. The *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* reference bacterial strains were obtained from the Akililu Lema Institute of Pathobiology in Addis Ababa. Prior to testing, the isolates were grown on nutrient agar slants at 4 °C and sub-cultured in nutrient broth for 24 hours. The isolates were used as test pathogens for an antibacterial assay.

Preparation of Crude Extracts Concentration

Five grams (5 g) of crude plant extracts were used to prepare concentration. The crude extracts (5 g) were dissolved or reconstituted in 5 mL of 10% DMSO to prepare a stock solution. Therefore, a crude plant extract stock solution was 1000 mg/mL. From stock solutions, six (6) distinct concentrations of crude extracts (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, and 3.125 mg/mL) were generated for an agar-well diffusion test.

Antibacterial Assay

Agar well diffusion method: The antibacterial activities of the crude extract of *Trichilia dregeana* Sond were evaluated using the agar-well diffusion method (CLSI, 2018). Nutrient and Mueller Hinton agar were used as the culture medium for this assay. Bacterial suspension was prepared from 24-hour sub-cultured colonies using 5 mL sterile saline solution. Agitate the broth culture with a vortex mixer and incubate at 37°C for 8 hours until it achieves 0.5 McFarland turbidity standards. The molten Mueller Hinton agar (MHA) medium was poured into sterile Petri dishes and incubated overnight to check for contamination. The agar plates were homogenously inoculated with the test bacterium using sterile cotton swabs. The plates were allowed to solidify. Then, six wells (6 mm in diameter and about 2 cm apart) were punched with sterile cork borers on the surface of MHA plates. The aliquots of about 100 µL of each concentration of plant extracts were allocated into the respective wells using a sterile micropipette syringe and allowed to diffuse at room temperature for 2 hours. Trimethoprim/Sulfamethoxazole (25 µg) and 10% DMSO were used as positive and negative controls, respectively. The plates were incubated at 37 °C for 24 hours (Yunana et al., 2018). The plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells following 24-hour incubation. The duplicate assay was performed in case of inhibition zone appearance. The diameters of inhibition zones were measured using a clipper. The results of antibacterial activity were interpreted as sensitive (< 14 mm), intermediate (14 - 17 mm), and susceptible (> 17 mm) (Koonan and Subba Rao, 2012; Rahmoun et al., 2014). The results were interpreted as sensitive, intermediate, or resistant, depending on the range recommended by NCCLS (Yunana et al., 2018). The antibacterial activity index of crude extracts (AI) for extract was also calculated using the following formula (Rathnayake et al., 2020).

$$AI = \frac{\text{Inhibition zone diameter of the sample}}{\text{Inhibition zone of the standard}}$$

Minimum Inhibitory concentration: The minimum inhibitory concentration of crude extracts against tested isolates was determined by the tube broth dilution method (CLSI, 2018). MIC is the lowest concentration of crude extracts that results in a complete inhibition of visible growth of bacteria. Stock concentrations (1000 mg/ml) of the methanol extract of the plant were first prepared by reconstituting 5g of weighed dried crude extract into 5 mL of ster-

ile 10% DMSO in a sterile glass beaker. The first concentration was prepared by mixing 9 mL of distilled water with 1 mL of stock solution to get 100 mg/mL. Then, 5 ml was picked from the first concentration (100 mg/mL) and twofold serially diluted in test tubes, each containing 5 mL of distilled water, to get different concentrations ranging from 100 mg/mL to 0.195 mg/mL using sterile test tubes (Quinn et al., 1999). The test bacteria suspension was prepared in sterile normal saline, and its turbidity was adjusted to standard 0.5 McFarland. About 100 μ L of bacterial suspension was added to each test tube containing different plant extract concentrations. Then, the test tubes were incubated at 37°C for 24 hours in aerobic conditions. The lowest concentration of plant extract that inhibited bacterial growth was determined as the MIC. The turbidity that indicates bacterial growth is frequently imperceptible to the naked eye. To determine the bacterial growth in each test tube, one loopful of the solution was streaked on a nutrient agar plate and incubated at 37°C for 24 hours. If no bacterial growth on the nutrient agar plate was observed, then a specific concentration had inhibited the bacterial growth. A tube containing bacterial suspension without plant extracts was used as a positive control, whereas a tube containing plant extracts without broth culture was the negative control.

Data Analysis

The raw data was recorded and stored in the Microsoft Excel database for data management analysis. R software was used for data analysis. Results were expressed as mean $\pm$ SD. All significant levels are set at $p < 0.05$.

RESULTS

The findings of antibacterial activity and MIC values of methanol crude extracts of *Trichilia dregeana* Sond leaves are summarized in Tables 1 and 2, respectively. The methanol crude extract of the plant showed promising antibacterial activity against all tested bacterial isolates at different concentrations. A significant difference ($p < 0.004$) was also observed in terms of susceptibility among *E. coli*, *S. aureus*, and *P. aeruginosa* species isolates (Table 2). The overall antibacterial activity index of plant crude extracts against *P. aeruginosa*, *S. aureus*, and *E. coli* was 80%, 75%, and 60%, respectively (Table 2). Significant differences were observed in the susceptibility of tested bacterial isolates at different concentrations of crude extracts (Table 1). The antibacterial activity of crude plant extract at 100 mg/mL concentration was not significantly different from that of a standard antibiotic disk (Trimethoprim/Sulfamethoxazole) against *S. aureus* and *E. coli* tested isolates. Meanwhile, the antibacterial activ-

ity of crude extracts at a concentration of 100 mg/mL showed significant differences with the positive control against *P. aeruginosa* tested isolate.

The MIC of crude extracts against tested isolates was determined using the broth dilution method. The crude extracts of plants showed different levels of MIC against all tested bacterial isolates (Table 2). The lowest MIC of extracts of *Trichilia dregeana* Sond was 0.39 mg/mL against *E. coli* and *P. aeruginosa* isolates in the present findings. In contrast, the MIC of plant crude extracts against *S. aureus* was 0.78 mg/mL. The growth of *E. coli*, *P. aeruginosa*, and *S. aureus* isolates was observed at concentrations of 0.195 mg/mL and 0.39 mg/m, respectively. The negative and positive control results revealed negative antibacterial results and the presence of bacterial growth, respectively. The absence of bacterial growth can be established by examining the turbidity of the suspension; however, to confirm the presence of bacterial growth, a loopful of the suspension was inoculated on a nutrient agar plate and incubated overnight.

Table 1. Antibacterial activities of crude extracts in agar well diffusion method

Concentrations	Mean inhibition zone $\pm$ SD (mm)		
	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
100 mg/mL	21.1 $\pm$ 1.4 ^a	25.2 $\pm$ 1.5 ^a	27.2 $\pm$ 1.5 ^a
50 mg/mL	17.6 $\pm$ 0.7 ^b	21.5 $\pm$ 1.9 ^{bc}	23.3 $\pm$ 1.6 ^b
25 mg/mL	17.0 $\pm$ 1.1 ^b	18.6 $\pm$ 3.5 ^{cd}	17.2 $\pm$ 0.1 ^c
12.5 mg/mL	13.7 $\pm$ 0.9 ^c	15.1 $\pm$ 0.1 ^{de}	13.9 $\pm$ 0.6 ^d
6.25 mg/mL	11.7 $\pm$ 0.7 ^{cd}	14.6 $\pm$ 0.6 ^{de}	12.8 $\pm$ 0.3 ^d
3.125 mg/mL	9.7 $\pm$ 0.7 ^d	13.1 $\pm$ 0.4 ^e	11.4 $\pm$ 1.8 ^d
Trimethoprim/sulphamethoxazole	20.7 $\pm$ 0.5 ^a	29.5 $\pm$ 1.2 ^a	22.8 $\pm$ 0.3 ^b
F test	***	***	***

*** = significant at 0.001 level, means within a column with different superscripts are significantly different,

Table 2. Mean zone of inhibition and MIC of tested isolates

Isolates	Mean $\pm$ SD (mm)	MICs in mg/mL	Activity index %
<i>Escheria coli</i>	19.6 $\pm$ 5.9 ^a	0.39	60
<i>Staphylococcus aureus</i>	15.9 $\pm$ 4.2 ^b	0.78	75
<i>Pseudomonas aeruginosa</i>	18.4 $\pm$ 5.9 ^a	0.39	80
P value	0.004	-	

MIC= minimum inhibitory concentration, ZOI, zone of inhibition, means within a column with different superscripts are significantly different,

DISCUSSION

The medicinal plant was selected for an experimental study based on its frequent use locally for treating infectious diseases like mastitis. The antibacterial activity of methanol extracts from medicinal plants was evaluated by agar-well diffusion and broth dilution methods against different bacterial isolates. The bacterial species were gram-negative and gram-positive isolates that frequently encountered infectious diseases. *S. aureus* and *P. aeruginosa* are the most common pathogens causing severe infections, while *E. coli* is an opportunistic pathogen. Inhibition zones of plant crude extracts ≥ 10 mm were considered active (Manilal et al., 2023). Significantly higher susceptibility or inhibition zone diameter to crude extracts was observed in *E. coli* (19.6 ± 5.9 mm) and *P. aeruginosa* (18.4 ± 5.9 mm) compared to *S. aureus* isolates. This finding indicated that crude extracts of the plant are more active against gram-negative bacteria (*E. coli* and *P. aeruginosa*) than gram-positive bacterial isolates (*S. aureus*) based on the large inhibition zones produced by crude extracts. The higher antibacterial activity of methanol extracts is due to the higher solubility of the active compounds in this solvent (Geetha et al., 1994). Methanol is a polar solvent with a higher power to extract active antibacterial compounds, mainly phenolic and polyphenolic, from the plant (Do et al., 2014). All tested pathogenic bacterial species (gram-positive and gram-negative) were susceptible to plant leaf extract, even at lower levels of concentration. This showed the broad-spectrum activity of the plant extracts against both gram-positive and gram-negative bacteria. This will significantly help combat the antibiotic-resistant bacteria that have become so common in recent years. The findings also confirm the potential of plants in Ethiopian ethnoveterinary medicine for producing bioactive compounds, which are helpful for rationalizing the use of medicinal plants in primary health care. The overall antibacterial activity index of plant crude extracts against *P. aeruginosa*, *S. aureus*, and *E. coli* was 80%, 75%, and 60%, respectively. The antibacterial activity index is the relative antibacterial activity of solvent crude extracts of plants against bacterial isolates. The inhibition zone of crude extracts was comparable with positive control at 100 mg/mL concentration against *S. aureus* and *E. coli* isolates. The crude extract showed the highest inhibition zone values compared to the positive control, Trimethoprim/Sulfamethoxazole, at 100 mg/ml concentration against *P. aeruginosa* isolates. A higher inhibition zone diameter was recorded in *P. aeruginosa* (27.2 ± 1.5 mm), followed by *E. coli* (25.2 ± 1.5 mm), and *S. aureus* (21.1 ± 1.4 mm) at a concentration of 100 mg/mL. The reference drug used in the experiment showed the highest antibacterial activity against the respective

tested bacterial isolates. The findings indicated that bacterial growth inhibition increased as the concentration of the crude extracts increased. A similar finding was reported by Nigussie et al. (2021) and Bene et al. (2016), as bacterial growth inhibition increased as the concentration of extracts increased.

The MIC of crude extracts against tested isolates was determined using the broth dilution method. The antibacterial activity of plant extracts is grouped depending on the susceptibility tests that produce MIC values in the range of 500-1500 µg/mL (Popović et al., 2015). Antibacterial activity is deemed substantial when MICs are less than 500 µg/ml and moderate when MICs are between 500 and 1500 µg/mL (Kuefe, 2010; Aligiannis et al., 2001). *Trichilia dregeana* Sond was shown to have high antibacterial activity against *E. coli* and *P. aeruginosa* isolates based on this scale. In addition, this plant displays moderate antibacterial activity against *S. aureus* isolates. The present study's observed minimum inhibitory concentration (MIC) values for plant crude extracts fall within the range or are less than those of other plant extracts reported from various regions (Aligiannis et al., 2001; Sama Fonkeng et al., 2015; Yusuf et al., 2022; Manilal et al., 2023). The low MIC values of the isolates indicate the efficacy of the plant extracts (Doughari, 2006). On the other hand, the higher MIC values of the isolates indicate that either the plant extracts are less effective against tested isolates, or the isolates have the potential to develop antibiotic resistance.

CONCLUSION

The current study found that methanol extracts of the *Trichilia dregeana* Sond plant's leaves displayed high antibacterial activity against tested bacterial isolates. The extracts showed potent antibacterial activity against gram-negative and gram-positive bacteria at 0.39 mg/mL and 0.78 mg/mL MIC, respectively. This indicated that the plant could be a good source of antibacterial activity to combat drug-resistant bacterial infections. This provides validity to its ethnobotanical relevance as a broad-spectrum agent in treating infectious diseases. All tested bacterial isolates were significantly susceptible to methanol crude extracts at different concentration levels. The broad-spectrum antibacterial activities of crude extracts of *Trichilia dregeana* Sond were demonstrated. These findings are promising and encourage further investigation of the phytochemical, toxicological, and pharmacological aspects of these crude extracts to support their potential rational use in antimicrobial therapy, particularly for gram-positive and gram-negative bacteria. The study recommends further fractionation and identification of different bioactive compounds from the *Trichilia dregeana* Sond plant that contribute to its antibacterial activity.

Limitation of the Study

Due to chemical reagent limitations and infrastructure issues, phytochemical screening and compound separation were not carried out.

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Author's Contribution

T.M. Concept and design of the study, drafting of the manuscript, data analysis, interpretation of the results, and prepared the final draft of the manuscript. T.K., B.U. and H.Y. carried out laboratory work, recording data, revising the manuscript and final approval of the paper following critical editing. All authors read and approved the final manuscript for publication.

Competing interest

The authors declare that there is no conflict of interest.

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PREVALENCE OF GASTROINTESTINAL HELMINTHS IN FREE-RANGE POULTRY (*GALLUS GALLUS DOMESTICUS*) IN THE NORTHEAST OF TUNISIA

Khaled Kaboudi^{1*}, Nesrine Rhif¹, Rafika Ben Romdhane²,
Nadine Abdallah¹, Mokhtar Dhibi³

¹ University of Manouba, National Veterinary Medicine School,
Department of Poultry Farming and Diseases, Laboratory of
research LR14AGR03, IRESA, Sidi Thabet, Tunisia

² Regional District of Agricultural development of Tunis,
Department of Animal Production, Tunis, Tunisia

³ University of Manouba, National Veterinary Medicine School,
Laboratory of Parasitology, Sidi Thabet, Tunisia

Abstract

This study was carried out to identify and estimate the prevalence of intestinal helminths in free-range poultry (*Gallus gallus domesticus*), from September 2016 to December 2022. A total of 494 birds of both sexes, aged from 6 weeks to 36 weeks, were examined and necropsied. Mucosa and contents of different gastrointestinal tract segments were examined for the helminth parasites. Results showed that 38.86% (192 birds), as overall prevalence, were infected by at least one parasite species. The prevalence of gastrointestinal helminth infections was higher in young animals (under 18 weeks) (32.79%) than adults (6.07%) ($p < 0.05$) and during rainy and cold seasons (autumn: 59.18%; spring: 41.72%; summer: 30.7%; winter: 28.84%) ($p < 0.05$). Nematodes were more prevalent (30.97%) than cestodes (21.66%). The prevalence was significantly higher in young chickens compared to adult animals ($p < 0.05$). Among the helminths species, *Raillietina* genus was the most observed (22.67%) (*Raillietina echinobothrida*: 21.05%; *Raillietina* spp: 1.62%) followed by *Ascaridia galli* (21.25%), *Heterakis gallinarum* (12.35%) and *Capillaria* spp. (4.86%). To our knowledge, this is the first report of gastrointestinal helminths prevalence in free-range chickens

^{1*} Corresponding Author: Khaled Kaboudi, e-mail: khaled.kaboudi@enmv.uma.tn

in the Northeast of Tunisia. Further additional studies are needed to develop better preventive measures in free-range poultry flocks.

Key words: backyard poultry, nematode, cestode, prevalence, Tunisia

PREVALENCA GASTROINTESTINALNIH HELMINTA KOD SLOBODNO DRŽANE ŽIVINE (*GALLUS GALLUS DOMESTICUS*) U SEVEROISTOČNOM TUNISU

Khaled Kaboudi¹, Nesrine Rhif¹, Rafika Ben Romdhane²,
Nadine Abdallah¹, Mokhtar Dhibi³

¹ Univerzitet u Manubi, Nacionalna škola veterinarske medicine, Departman za uzgoj
i bolesti živine, Istraživačka laboratorija LR14AGR03, IRESA, Sidi Thabet, Tunis

² Regionalni okrug za razvoj poljoprivrede Tunisa, Odeljenje
za stočarsku proizvodnju, Tunis, Tunis

³ Univerzitet u Manubi, Nacionalna škola veterinarske medicine,
Laboratorija za parazitologiju, Sidi Thabet, Tunis

Kratak sadržaj

Istraživanje je sprovedeno u cilju identifikovanja i procene prevalencije intestinalnih helminta kod slobodno držane živine (*Gallus gallus domesticus*) u periodu od septembra 2016. do decembra 2022. Obdukovano je i ispitano ukupno 494 uginulih jedinki oba pola, starosti od 6 do 36 nedelja. Sluzokoža i sadržaj iz različitih gastrointestinalnih segmenata su ispitani na prisustvo helmintskih parazita. Rezultati su pokazali da je 38.86% (192 jedinki), u smislu ukupne prevalencije, bilo inficirano bar jednom vrstom parazita. Prevalencija gastrointestinalnih helmintskih infekcija bila je veća kod mladih jedinki (starosti ispod 18 nedelja) (32.79%) nego kod starijih (6.07%) ($p < 0.05$) kao i tokom kišnih i hladnih perioda (jesen: 59.18%; proleće: 41.72%; leto: 30.7%; zima: 28.84%) ($p < 0.05$). Viši stepen prevalencije utvrđen je za nematode (30.97%) u poređenju sa cestodama (21.66%). Od svih vrsta helminta, najčešće su uočeni oni iz roda *Raillietina* (22.67%) (*Raillietina echinobothrida*: 21.05%; *Raillietina* spp: 1.62%), a zatim *Ascaridia galli* (21.25%), *Heterakis gallinarum* (12.35%) i *Capillaria* spp (4.86%). Prema informacijama koje posedujemo, ovo je prvo istraživanje prevalencije gastrointestinalnih helminta kod slobodno držane živine u severoistočnom

Tunisu. Neophodne su dalje studije u cilju razvoja uspješnijih preventivnih mera u jatima u slobodnom uzgoju.

Ključne reči: slobodno držana živina, nematode, cestode, prevalencija, Tunis

INTRODUCTION

The traditional poultry sector holds significant socio-economic importance in rural areas of Tunisia. While it is a major source of income for many families or even villages, free-range poultry only contributed an average of 7% to the national production economy between 2006 and 2010. About 2.773 million layer hens and 1.349 million meat chickens constitute the backyard poultry flocks, according to the last official national estimates in 2010 (GIPAC, 2010). However, the free-range poultry sector contributes to the consumption and supplies local markets for eggs and meat. In this way, free-range chickens participate at least 12% and 9.66% in the national production of poultry meat and eggs, respectively.

However, in many cases, the birds' productivity can be improved to use the bird's full genetic potential. The traditionally reared poultry farming system exposes chickens to serious health problems because of malnutrition, poor management, lack of biosecurity conditions, failure of vaccination programs, poor housing, and lack of specific veterinary care services. Furthermore, poor genetic potential due to lack of selection and predators pose potential threats to productivity. The backyard chicken feed on a wide range of food substances, varying from grains, fruits, and insects, which may harbor infective stages of parasites, thereby predisposing them to parasitic infection. In this context, parasitic infection is an important health problem in free-range poultry, which handicaps production performances (Mungube et al., 2008).

A central problem of the backyard system is the fact that birds are exposed to a constant risk of infection, mainly cestodes and nematodes, due to direct contact with the feces, the food, the water, and paratenic and intermediate hosts (Permin et al., 2002; Nyoni and Masika, 2012). In addition, a number of epidemiological factors, including the host, sex, age, breed, and environment, may influence the occurrence and the intensity of parasitic infestations (Nadeem et al., 2007).

Helminth infections are usually associated with diarrhea, nutriment spoilage, reduction in growth rate, emaciation, and decreased egg production.

An increased worm burden can even cause the death of animals. In addition, parasites are biological vectors transmitting a number of pathogens. In this way, *Heterakis gallinarum* is considered a potential vector of *Histomonas meleagridis*, which causes blackhead disease in many galliform birds (Soulsby, 1982; Ruff, 1999; Salam, 2015).

Investigating gastrointestinal helminth infections in free-range chickens is crucial to understand the epidemiological status in backyard flocks and define control measures. However, very few scientific reports on the prevalence of helminth infections in backyard chickens in Tunisia have been published (Ben Slimane, 2016). Therefore, this study aimed to investigate the occurrence of gastrointestinal helminths in free-range chickens raised under the traditional system and to identify the most common parasite species.

MATERIAL AND METHODS

Study area and sampling

This study was carried out on 494 dead backyard chickens from September 2012 to June 2018. Animals originated from farms reared under traditional conditions and located in regions around the town of Sidi Thabet in the Ariana Governorate, Tunisia. Sidi Thabet is located northeast of Tunis city center, around 36°51'45"N 10°11'44"E with a hot-summer Mediterranean climate, where winters are mild with moderate rainfall, and summers are hot and dry. Temperatures in July and August can exceed 40 °C (104 °F). Winters are mild, with temperatures rarely exceeding 20 °C (68 °F).

Sample examination

All dead backyard chickens (free-range poultry) were from farms reared under traditional conditions, were of both sexes and aged 6 to 36 weeks. They were classified into young (≤ 18 weeks) and adult animals (> 18 weeks). All the birds were necropsied in the Avian Clinic of the National Veterinary Medicine School of Tunisia and a postmortem examination was performed. After postmortem examination, the gastrointestinal tract was removed, and different portions were opened and rinsed with a physiological saline solution. Mucosa and contents of all segments were placed in Petri dishes containing water in order to be examined under a binocular magnifying glass with a black background surface (Stemi 305-Zeiss) and optic microscope for the research of adult helminths and their eggs. Parasites were identified according to their morphological characteristics using the key criteria mentioned by Soulsby (1982).

Statistical analysis

Comparative analysis of prevalence in chickens was performed using the Chi-deux test (Word Excel, Microsoft office, version 2016). A p -value < 0.05 was considered statistically significant.

RESULTS

All helminth parasites were collected from the intestines and caeca. However, no helminths were observed in the esophagus, crops, proventriculus, and gizzard. The overall prevalence of helminth infections was 38.86% of the chickens examined (494 birds).

Helminth parasites were identified in broilers, pullets, layers, and roosters (Table 1). However, pullets (50.63%) and broilers (31.16%) were most frequently affected, followed by layers (25.71%) and roosters (14.28%) ($\chi^2 = 28.46$; $p < 0.05$).

Table 1. Prevalence of gastro-intestinal helminths in free-range poultry (n = 494) according to the season and the type of production

		No. examined	No. positive	Prevalence (%)	χ^2 (p -value)	Overall prevalence
Season	Autumn	98	58	59.18%	26.94 ($p < 0.001$)	38.86%
	Winter	156	45	28.84%		
	Spring	139	58	41.72%		
	Summer	101	31	30.7%		
Type of production	Broilers	138	43	31.16%	28.46 ($p < 0.001$)	
	Pullets	237	120	50.63%		
	Layers	105	27	25.71%		
	Roosters	14	2	14.28%		

The study, according to the season, showed that helminth infections were diagnosed throughout the year, with highest prevalence noted during autumn (September, October, November) (59.18%), spring (march, April, May) (41.72%), and secondly during summer (June, July, August) (30.7%) and winter (December, January, February) (28.84%) (Table 1) ($\chi^2 = 26.94$; $p < 0.05$).

The prevalence related to the sex of birds showed helminth infections in 25.50% of females and 13.36% of males. The sex had no significant influence on the distribution of infected birds ($\chi^2 = 2.389$; $p > 0.05$) (Table 2). Accord-

ingly, a higher prevalence of helminth infections was observed in young birds (under 18 weeks) (32.80%) than in adults (> 18 weeks) (6.07%). The influence of age was statistically significant ($\chi^2 = 12.304$; $p < 0.05$).

Table 2. Prevalence of gastro-intestinal helminths in free-range poultry (n = 494)

		No. ex- amined	Nematodes		χ^2 (<i>p</i> -value)	Cestodes		χ^2 (<i>p</i> -value)
			No. posi- tive	Prevalence (%)		No. posi- tive	Preva- lence (%)	
Age	Young	375	133	35.46%	14.712	92	24.54%	7.575
	Adults	119	20	16.80%	(<i>p</i> =0.00013)*	15	12.60%	(<i>p</i> =0.006)*
Sex	Male	150	52	34.67%	1.376	31	20.67%	0.125
	Female	344	101	29.36%	(<i>p</i> =0.240)	76	22.09%	(<i>p</i> =0.723)

* significant difference

Among the total of animals necropsied, 41.67% (80 birds) and 20.33% (40 birds) had nematode and cestode infections, respectively. A significant finding was that 37.5% (72 birds) had mixed infections of nematodes and cestodes, with young animals and females being the most infected in each category with significant prevalence ($p < 0.05$).

The prevalence of nematode infections was 30.97% (153 animals). The infection was higher in young (35.46%) and in male birds (34.67%) than in adults (16.80%) and females (29.36%) ($p < 0.05$) (Table 2). Findings of cestode infections indicate an overall prevalence of 21.66% (107 animals). A significant prevalence was observed in young birds (24.54%) than in adults (12.60%) ($p < 0.05$). However, a higher prevalence of cestode infections was noted in females (22.09%) than in males (20.67%).

Different species of intestinal helminths were identified in this study (Table 3). Parasitological investigations showed that *Ascarida galli* (105 birds) (21.25%) (Figure 1A and Figure 2) was the most prevalent parasite followed by *Raillietina echinobothrida* (104 birds) (21.05%) (Figure 1B), *Heterakis gallinarum* (61 birds) (12.35%) (Figure 3 and Figure 4), and *Capillaria* spp (24 birds) (4.86%) (Figure 5).

Table 3. Prevalence of individual parasites in female, male, young (≤ 18 weeks) and adult (> 18 weeks) free-range poultry (n = 192)

Parasite group	Parasite species	Overall prevalence	No. positive (%)		χ^2 (p-value)	No. positive (%)		χ^2 (p-value)
			Female	Male		Young	Adult	
Nematodes	<i>Ascaridia galli</i> (n=105)	21.25%	68 (35.41%)	37 (19.27%)	0.077 (p=0.782)	89 (46.35%)	16 (8.34%)	0.026 (p=0.871)
	<i>Heterakis gallinarum</i> (n=61)	12.35%	45 (23.43%)	16 (8.33%)	2.630 (p=0.105)	55 (28.64%)	6 (3.12%)	2.273 (p=0.132)
	<i>Capillaria</i> spp. (n=24)	4.86%	15 (7.81%)	9 (4.68%)	9.955 (p=0.001)*	17 (8.85%)	7 (3.64%)	3.816 (p=0.05)*
Cestodes	<i>Raillietina echinobothrida</i> (n=104)	21.05%	75 (39.06%)	29 (15.10%)	5.017 (p=0.025)*	92 (47.92%)	12 (6.25%)	1.878 (p=0.171)
	<i>Raillietina</i> sp (n=8)	1.62%	6 (3.12%)	2 (1.04%)	0.325 (p=0.568)	5 (2.60%)	3 (1.56%)	3.030 (p=0.082)

* = significant difference



Figure 1. Infestation of free-range chicken by *Ascarida galli* (A) and *Raillietina* sp (B)



Figure 2. Anterior extremity of *Ascarida galli*



Figure 3. Infestation by *Heterakis gallinarum* in a free-range chicken

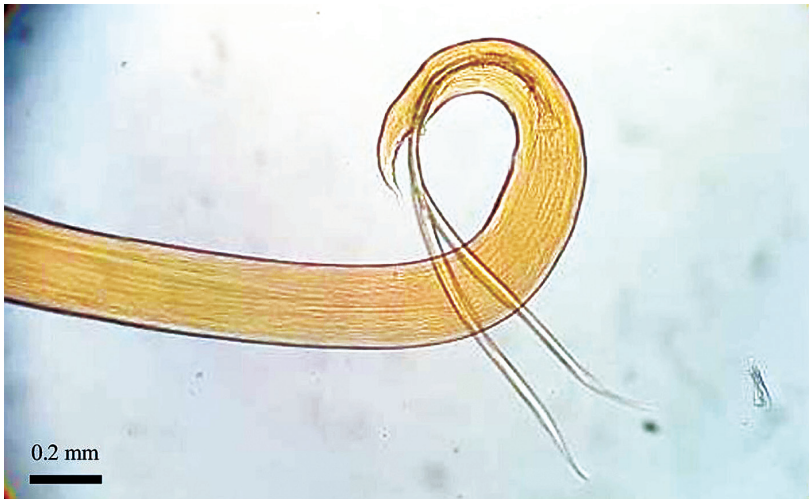


Figure 4. Posterior extremity of a male of *Heterakis gallinarum*



Figure 5. Eggs of *Capillaria* spp

There was a significant prevalence of *Capillaria* spp infections in females (7.81%) ($\chi^2 = 9.955$; $p = 0,001$) and young birds (8.85% ($\chi^2 = 3.816$; $p = 0.05$). Similarly, the presence of *Raillietina echinobothrida* showed a significant association with females ($\chi^2 = 5.017$; $p = 0.025$).

DISCUSSION

This study revealed a relatively low overall prevalence of helminth infections (38.86%) (Total of examined birds: 494), compared to the results of Ben Slimane (2016) (100%), who was interested in thirty animals collected from three regions of Tunisia (Siliana, Boumerdes, and Ghomrasen). This difference could be due to the easier accessibility of the farmers located near the National Veterinary Medicine School, and, consequently, the possibility of veterinary assistance compared to other regions. Our findings were also lower than the reports of many authors in different regions studied worldwide (Table 4).

Table 4. Comparative study of overall prevalence of gastrointestinal helminthes infestation in free-range poultry

Authors	Region of study	Total sample size	Overall prevalence (n = 192)	Nematodes (n = 80)	Cestodes (n = 40)	Mixed infestation (n = 72)
Our study	Northeast of Tunisia	494	38.86%	16.19%	8.09%	15.57%
Naphade and Chaudhari (2013)	Marathwada Region, India	345	84.05%	40%	26.13%	17.97%
Yousfi et al. (2013)	Northwestern, Algeria	124	100%	93.86%	95.61%	90.27%
Mekibib et al. (2014)	Southern, Ethiopia	122	77.80%	67.5%	29.20%	-
Naphade (2014)	Maharashtra state India	317	75.40%	35.96%	22.71%	16.71%
Ben Slimane (2016)	Tunisia: Siliana (north-west); Boumerdas (coastal east); Ghomrasen (south)	30	100%	100%	33.33%	86.66%
Javaregowda et al. (2016)	Shimoga, India	250	73.2%	28.96%	51.36 %	19.67%
Berhe et al. (2019)	Tigray Region, Ethiopia	548	90.98%	84.4%	89%	87.68%
Wuthijaree et al. (2019)	Northern Thailand	211	73.9%	72.5%	27.7%	-

The disparity in these results might be due to a variety of factors, including geographical locations, sample size, study methods, climatic conditions, management practices in poultry flocks, and helminth control measures employed.

Gastrointestinal helminth infections were more frequently observed in young birds under 18 weeks of age (32.80%) compared to adults (> 18 weeks) (6.07%) ($\chi^2 = 12.304$; $p < 0.05$). Females (25.50%) were more frequently infected than males (13.36%) ($\chi^2 = 2.389$; $p > 0.05$). The current findings correspond with the reports of Sarba et al. (2019) regarding the age of animals in different zones of Ethiopia. However, these authors mentioned a higher prevalence in males than females.

The higher prevalence of parasitic infection during rainy and humid seasons is in line with the observations of Mukaratirwa and Hove (2009) in Zimbabwe. In fact, climatic conditions can influence the abundance and the diversity of parasite species.

Among 192 infected animals, 41.67% were found positive for nematodes, 20.33% were found positive for cestodes, and the remaining 37.50% had mixed infections of both cestodes and nematodes. Similarly, Tolossa et al. (2009), Katoch et al. (2012), Baboolal et al. (2012), Mekibib et al. (2014), Ben Slimane (2016), and Wuthijaree et al. (2019) observed a higher prevalence of nematodes than cestodes in backyard chickens in Ethiopia, Jammu (India), Trinidad region, Algeria, Tunisia, and Northern Thailand respectively. Indeed, in agreement with Ruff's report (1999), nematodes constitute the most important group of poultry helminths regarding both infection degree and species number and the resulting tissue damage. On the contrary, Puttalakshamma et al. (2008), Hussien et al. (2012), Idika et al. (2016), Javaregowda et al. (2016), and Berhe et al. (2019) reported a higher prevalence of cestodes followed by nematodes, from different regions.

In the present study, no trematodes were identified. The current findings were in line with the reports of Ben Slimane (2016) and Javaregowda et al. (2016). It might be due to the absence or lower occurrence of the intermediate hosts responsible for the transmission of these parasites in the studied regions (Magwisha et al. 2002).

The relatively high prevalence of gastrointestinal helminths and mixed infections in the present study could be related to environmental factors and poor hygienic conditions in the backyard system, which are very favorable for developing parasitic infections. Animals seek food in the soil, which is frequently contaminated with infective stages of parasites and living organisms (insects, mollusks...) serving as intermediate hosts (Ruff 1999; Yousfi et al., 2013).

The higher prevalence of *Ascaridia galli* (21.25%) followed by *Heterakis gallinarum* (12.35%) has also been reported by several authors (Tolossa et al., 2009; Idika et al., 2016; Javaregowda et al., 2016; Sarba et al. 2019) (Table 5). On the contrary, Yousfi et al. (2013), Mekibib et al. (2014), Abdel Aziz (2016), Ben Slimane (2016), Berhe et al. (2019), and Wuthijaree et al. (2019) reported a higher prevalence of *H. gallinarum* than *A. galli* in local chickens in Algeria, Southern Ethiopia, Egypt, Tunisia, Tigray Region, Ethiopia, and Northern Thailand, respectively.

Table 5. Comparative overall prevalence of intestinal helminthes in free-range poultry

Authors	Region of the study	Total sample size	<i>A. galli</i>	<i>H. gal-linarum</i>	<i>Capil-laria sp</i>	<i>Raillietina echinobo-thrida</i>	<i>Railli-etina sp</i>
Our study	Northeast of Tunisia	494	21.25%	12.35%	4.86%	21.05%	1.62%
Nnadi and George (2010)	South-eastern, Nigeria	261	17.2%	12.6%	5.7%	-	5.7%
Hussen et al. (2012)	Eastern Shewa Zone, Ethiopia	124	32%	37.9%	3.2%**	63.7%	-
Abdullah and Mohammed (2013)	Sulaimani, Iraq	65	31%	81%	1.72%	-	55.17%
Yousfi et al. (2013)	Northwestern, Algeria	124	39.47%	78.07%	35.96%***	85.09%	-
Mekibib et al. (2014)	Southern, Ethiopia	122	45.90%	51.60%	13.1%	17.20%	-
Salam (2015)	Kashmir, India	478	35.35%	4.39%	8.78%**	-	-
Ben Slimane (2016)	Tunisia: Siliana (north-west); Boumerdas (coastal east); Ghomrasen (south)	30	53.33%	100%*	-	-	33.33%
Javaregowda et al. (2016)	Shimoga, India	250	62.3 %	22.6 %	-	9.6 %	-
Berhe et al. (2019)	Tigray Region, Ethiopia	548	68.84%	72.46%	51.45%	39.86%	-
Wuthijaree et al. (2019)	Northern Thailand	211	60.2%	70.9%	44.1%	-	-

Heterakis sp.*; *Capillaria anatis*; ****Capillaria caudinflata*

A. galli is one of poultry's most common and important helminth infections. It causes diarrhea, growth delay, a decrease in body weight and egg drop in laying chickens (Ashenafi and Eshetu 2004). Blood loss due to intestinal mucosa damage, secondary infections, and intestinal obstruction can be observed in severe infections.

Although *H. gallinarum* is generally non-pathogenic, it plays a crucial role as a potential vector of *Histomonas meleagridis*, a highly pathogenic etiologic agent of “Blackhead disease” (histomoniasis) in many avian species (turkeys, chickens, pheasants). Massive infection by *H. gallinarum* in free-range backyard chickens can cause severe debility, morbidity, and mortality in extreme cases (Ashenafi and Eshetu, 2004).

The prevalence of *Capillaria* spp in the current investigation was 4.86%. Our results disagreed with Ben Slimane (2016) and Rama Rao (2018), who did not detect this helminth in studied regions in Tunisia and India, respectively. However, *Capillaria* spp. was identified in several other regions of the world (Abdel Aziz, 2016; Hussien et al., 2012). Recently, a high prevalence of *Capillaria* spp infections was published by Berhe et al. (2019) (51.45%) and Wuthijaree et al. (2019) (44.1%) in backyard poultry flocks in Ethiopia and Northern Thailand, respectively.

The life cycle of *Capillaria* spp can be indirect (*Capillaria caudinflata*, *Capillaria annulata*) or direct (*Capillaria obsignata*) according to the species. Species with a direct life cycle are more frequently detected under intensive farming conditions where constant temperature and humidity are ideal for larval development. However, species with indirect life cycles are particularly abundant in traditional farms, particularly under humid climates (Permin and Hansen, 1998).

The overall prevalence of cestodes in our study (21.66%) was higher than that reported by Hassan et al. (2020) in live-birds market in Nigeria. However, Naphade and Chaudhari (2013) (26.13%), Yousfi et al. (2013) (95.61%), Mekibib et al. (2014) (29.2%), Naphade (2014) (22.71%), Idika et al. (2016) (70.4%), Javaregowda et al. (2016) (51.36 %) and Sarba et al. (2019) (77.8%) showed a higher prevalence. This could be due to the geographic localization, the difference in the season and the climatic conditions, the availability of intermediate hosts, individual host resistance, and ecological parameters.

Among cestodes, *Raillietina echinobothrida* (21.05%) was the most observed, followed by *Raillietina* spp (1.62%), non-identified because of the bad quality of some samples. *Raillietina echinobothrida* infections might be accompanied by growth delay, decreased egg production in layers, diarrhea, intestinal damage, vitamin B deficiency, and mortality in young birds (Ashenafi and Eshetu, 2004).

The current findings correspond with the report of Idika et al. (2016) (64.5%) in local chickens in Nsukka region of southeastern Nigeria. However, this was not in accordance with the observations of Javaregowda et al. (2016) in Shigoma region (India), who reported higher prevalence of *Raillietina tetragona* infections in backyard chickens (77.6%). Similarly, Butt et al. (2014) reported high prevalence of *Cotugnia digonopora* in Pakistan.

Globally, the prevalence of helminth infections did not differ between female and male birds, indicating that helminth species have no natural affinity for either sex of host chicken. The higher prevalence of *Capillaria* spp. and *Raillietina echinobothrida* in female birds ($p < 0.05$) reported in our study can be explained by reduced immune responses and the supply of untreated new female birds to improve egg production. Physiological, morphological, and behavioral differences between females and males could influence the susceptibility to parasite infections. Many helminth surveys have reported no significant differences according to the sex of birds (Hassouni and Belghyti, 2006; Berhe et al., 2019), whereas others have found significant differences in the favor of one or the other sex (Wuthijaree et al., 2019).

Age is also considered a risk factor associated with the occurrence of helminth infections. The high prevalence of different helminth infections reported globally in young animals compared to adults could be attributed to the immature immune system in young birds and the poor acclimatization of young animals to the immediate environment (Ola-Fadunsin et al., 2019). In addition, Tunisian farmers do not use anthelmintic drugs systematically in free-range chickens.

CONCLUSION

This study showed that free-range chickens reared under backyard systems were infected by different helminth species. The relatively high prevalence of gastrointestinal helminth infections might be associated with a considerable decrease in productivity. The most common helminths identified in the current study were *Ascaridia galli* and *Raillietina echinobothrida*. Several factors can influence the prevalence and severity of infections. Age, sex, and season were considered in the current study. In addition, poor management conditions, mixed animal and avian species, and the negligence of anthelmintic application can preserve the infection in the flock. Therefore, the application of strategic control must thus take priority attention in conjunction with good hygienic practices. Finally, studies are needed to elucidate the economic and hygiene impacts of multiple parasitic infections on poultry reared under a backyard system.

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Author's Contribution

K.K. design of the experimental protocol, parasitological investigations, interpretation of the results, writing the manuscript, N.R. sampling, necropsy protocol, R.B.R. sampling, writing the manuscript, N.A. reception and treatment of samples, M.D. parasitological analysis. All authors read and approved the final version of the manuscript.

Competing interest

The authors declare that they have no competing interests.

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GASTROINTESTINAL PARASITES AND ASSOCIATED GUT PATHOLOGY IN CHICKENS SOLD AT LIVE-BIRD MARKETS IN LAGOS STATE, SOUTHWESTERN NIGERIA

Oluwayomi Adeyemi^{1*}, Emmanuel Idowu¹, Bamidele Akinsanya¹,
Isa Jatau², Temitope Oladipo³

¹ University of Lagos, Department of Zoology, Akoka, Yaba, Lagos, Nigeria

² Ahmadu Bello University, Department of Veterinary
Parasitology and Entomology, Zaria, Nigeria

³ Federal University of Abeokuta, Department of
Veterinary Pathology, Abeokuta, Nigeria

Abstract

Gastrointestinal (GI) parasites compromise intestinal health in poultry, causing impaired nutrient uptake, poor growth rate and reduced productivity. The effectiveness of control programs relies on an accurate understanding of parasite occurrence and distribution. This survey aimed to determine the diversity and pathological effects of GI parasites of chickens in Lagos State, Nigeria. Three hundred and sixty-four (364) chickens were randomly sampled from selected live-bird markets. Standard parasitological methods were employed to process and examine gastrointestinal content and mucosal scrapings for parasite identification. Live helminths were preserved in 70% ethanol, cleared in lactic acid and identified at the species level. Sections of the gut were also assessed for microscopic lesions following standard histopathological procedures. Findings revealed that 251 (69%) of the 364 sampled chickens were infected with GI parasites. Eleven nematodes, 5 cestodes and coccidia of the genus *Eimeria* were detected. *Ascaridia galli* (140, 38.5%), *Eimeria* spp. (98, 26.9%) and *Heterakis gallinarum* (82, 22.5%) were the most common parasites found. Helminths were generally more prevalent in local and male chickens ($P < 0.05$) whereas exotic breeds had higher infection rates for *Eimeria* spp. ($P > 0.05$). Histopathological analyses revealed mild to severe inflammation, oedema, mucosal erosion, and hyperplasia in infected chickens. This study showed that GI parasites

^{1*} Corresponding Author: yadeyemi@unilag.edu.ng

are prevalent in chickens and are associated with intestinal damage that can undermine poultry growth and productivity in the study area. Efforts to control must be strengthened to secure chicken production, maintain food security, and prevent economic losses.

Key words: Gastrointestinal parasites, Helminths, Coccidia, Chickens, Nigeria

GASTROINTESTINALNI PARAZITI I PATOLOŠKE PROMENE NA CREVIMA KOD KOKOŠAKA KOJE SE PRODAJU NA PIJACAMA ŽIVIH PTICA U SAVEZNOJ DRŽAVI LAGOS, JUGOZAPADNA NIGERIJA

Oluwayomi Adeyemi¹, Emmanuel Idowu¹, Bamidele Akinsanya¹,
Isa Jatau², Temitope Oladipo³

¹ Univerzitet Lagos, Katedra za zoologiju, Akoka, Yaba, Lagos, Nigerija

² Univerzitet Ahmadu Bello, Katedra veterinarske
parazitologije i entomologije, Zaria, Nigerija

³ Federalni Univerzitet Abeokuta, Katedra za
veterinarsku patologiju, Abeokuta, Nigerija

Kratak sadržaj

Gastrointestinalni (GI) paraziti predstavljaju ozbiljan rizik po zdravlje creva kod živine, izazivajući smanjenu apsorpciju hranljivih materija, usporeni rast i smanjenu produktivnost. Efikasnost programa kontrole zavisi od razumevanja pojave i distribucije parazita. Cilj ovog istraživanja bio je da se utvrde raznolikost i patološki efekti GI parazita kod kokošaka u državi Lagos, Nigerija. Ukupno 364 kokošaka je nasumično uzorkovano na odabranim pijacama živih ptica. Za obradu i ispitivanje gastrointestinalnog sadržaja i uzimanja uzoraka sluzokože korišćene su standardne parazitološke metode kako bi se identifikovali paraziti. Živi helminti su sačuvani u 70% etanolu, očišćeni u mlečnoj kiselini i identifikovani do nivoa vrste. Isečci creva su podvrgnuti mikroskopskom ispitivanju radi utvrđivanja lezija, uz primenu standardnih histopatoloških procedura. Nalazi su pokazali da je 251 (69%) od 364 uzorkovanih jedinki bilo zaraženo gastrointestinalnim parazitima. Identifikovano je 11 vrsta nematoda, 5 vrs-

ta cestoda i kokcidija iz roda *Eimeria*. Najčešće pronađeni paraziti bili su *Ascaridia galli* (140, 38,5%), *Eimeria spp.* (98, 26,9%) i *Heterakis gallinarium* (82, 22,5%). Učestalost nalaženja helminta bila je češća kod lokalnih ptica i to muškog pola ($P < 0,05$), dok su egzotične vrste ptica češće bile inficirane sa *Eimeria spp.* ($P > 0,05$). Histopatološke analize su utvrdile blagu do tešku inflamaciju, edem, eroziju sluzokože i hiperplaziju kod inficiranih jedinki. Ova istraživanja su pokazala da gastrointestinalni paraziti preovlađuju kod kokošaka i da su povezani sa oštećenjem creva, što može negativno utiče na rast i produktivnost živine. Potrebno je uvođenje bolje kontrole parazita kako bi se obezbedila neometana proizvodnja pilića, obezbedila bezbedna hrana i sprečili ekonomski gubici.

Ključne reči: gastrointestinalni paraziti, helminti, kokcidije, kokoške, Nigerija

INTRODUCTION

Poultry is an important source of quality animal protein, income, and employment. It plays a vital role in global socio-economic development. The Nigerian poultry industry contributes approximately 8% to the gross domestic product (GDP) and supplies affordable meat and eggs, enhancing the health and livelihoods of people in both rural and urban areas (Netherlands Africa Business Council [NABC], 2019). The domestic chicken, *Gallus gallus domesticus*, is the most economically significant poultry species raised in Nigeria (Adene and Oguntade, 2006; Ajayi, 2010). It accounts for more than 50% of the estimated poultry population (140-180 million) in the country (Federal Ministry of Agriculture and Rural Development, [FMARD], 2006).

Parasitic infections are a key factor impacting poultry health, welfare, and performance in tropical regions, hindering the poultry industry's ability to keep up with the rising demand for animal protein (NABC, 2019). The most prominent are gastrointestinal (GI) parasites, which damage the gut integrity of bird hosts, impairing nutrient absorption, feed conversion efficiency, growth rate, and overall productivity (Yegani and Korver, 2008). These parasites infect their hosts through the faeco-oral route, either directly (e.g., coccidia and most nematodes) or indirectly via intermediate hosts (e.g. cestodes and trematodes) (Permin and Hansen, 1998). The survival and transmission dynamics of these parasites vary depending on the poultry management practices. Parasites with one-host cycles are more prevalent under intensive farming conditions com-

pared to the heteroxenous forms which prevail in free-range systems where intermediate hosts are readily available (McDougald, 2008a).

While many intestinal parasites are non-pathogenic in chickens, some cause diseases with significant economic consequences (McDougald, 2008a). For example, coccidiosis, caused by intestinal coccidia of the genus *Eimeria*, costs the global poultry industry approximately UK£ 10.4 billion annually (Blake et al., 2020). This estimated loss has been associated with reduced poultry productivity, morbidity and mortality, making disease control highly essential.

Gastrointestinal parasite control primarily relies on good biosecurity and the prophylactic use of antiprotozoal and anthelmintic drugs (Pagani et al., 2008). In order to achieve effective and sustainable control, epidemiological data are required. Previous studies have reported the presence of GI parasites in Nigerian chickens, with prevalence rates varying from 20% to 100% in both exotic and local breeds raised under different farming systems across all geo-climatic regions (Ohaeri and Okwum et al., 2013; Jegede et al., 2015; Afolabi et al., 2016). These studies have shown that *Eimeria* spp. are the most common protozoan parasites while *Ascaridia galli* and *Raillietina* spp. are notable nematodes and cestodes respectively. Despite this plethora of surveys, there is limited and incomprehensive data for GI parasite occurrence among chickens in Lagos State, Nigeria (Adeyemi et al., 2019; Idowu et al., 2019). Such data are essential for developing effective control measures that can support the continued growth of the poultry sector. Therefore, this study aimed to determine the diversity and prevalence of GI parasites and evaluate their pathological impacts in chickens sold at selected live-bird markets in Lagos State, South-west Nigeria.

MATERIAL AND METHODS

Ethics approval and consent to participate

Ethical approval was granted by the Health Research Ethics Committee of the College of Medicine, University of Lagos, reference number CMUL/ACUREC/01/23/1141. Consent to take part in the study was also obtained from all study participants.

Study area

This study was conducted in Lagos State, one of the six southwestern states in Nigeria. It is situated along the narrow plains of the Blight of Benin, approxi-

mately between latitudes 6°35'N and 6.58°N, and longitudes 3°45'E and 3.75°E, and covers a land area of 3577sq.km which is 0.4% of Nigeria's total land mass. It is a wetland dominated by freshwater and mangrove swamps. About 25% of the land area is occupied by lagoons, creeks, and rivers. Lagos State has a humid tropical climate with distinct wet and dry seasons. The wet period spans from April through October with a break in August while the shorter, dry season begins in November and ends in March. The region experiences an average annual rainfall of 1783mm, with a mean relative humidity of 83% and ambient temperatures ranging from 28°C to 33°C (Lagos State Government [LASG], 2023). Poultry production is a lucrative business in Lagos State, which hosts a variety of small to large-scale poultry farms. The state's poultry industry has an estimated population of 2.3 million meat-producing birds, an annual egg production of 133 million, and approximately 30 live bird markets (LBMs) (Olorunwa, 2018; Aminu and Hermanns, 2021).

Study design and population

This study was conducted between March 2019 and June 2022, with a break that lasted five (5) months from March to July 2020 due to the imposed COVID-19 lockdown. A cross-sectional survey was conducted on domestic chickens sold and slaughtered in the daily live bird markets (LBMs) in the study area. Three LBMs located in Lagos Mainland (Oyingbo LBM), Ikorodu (Ejina LBM), and Shomolu (Onipanu LBM) Local Government Areas (LGAs) of Lagos State were selected on purpose due to their large size and high turnover rates. The poultry sold in these markets is sourced from various areas within and around Lagos State.

Data and sample collection

A total of 364 chickens designated for slaughter in the LBMs were randomly selected and included in this study. A minimum sample size was determined using Thurfield's formula: $n = z^2 \times pexp (pexp - 1)/d^2$, where n = required sample size; z = confidence interval at 95%; $pexp$ = expected prevalence of 72% based on a previous study (Rufai and Jato, 2017); and d^2 = desired absolute precision of 5%. The breed (exotic or local) and sex (male or female) of each selected chicken were identified at the point of slaughter. Clinical signs of GI infections observed for selected chickens were also noted. Immediately upon slaughtering of the birds, their guts were collected in order to recover the crop, oesophagus, proventriculus, small intestine, large intestine and caeca.

Any lesions observed on the mucosal and serosal surfaces of the tissues were documented. Random tissue sections were taken and kept in 10% formalin for histopathological analysis. Live worms retrieved from the gastrointestinal lumen were collected using forceps and kept in 70% ethanol. Intestinal contents, along with mucosal scrapings, were collected and preserved with a few drops of physiological saline in sterile sample bottles. All samples were properly labeled and transported in cool boxes to the Parasitology laboratory of the Department of Zoology, University of Lagos.

Parasitological analyses

The preserved worms were sorted and counted under a stereo microscope. The worms were then cleared in lactic acid and identified to genus and/or species level under low- and high- power objectives of a compound microscope according to keys provided by Benbrook and Sloss (1961) and Soulsby (1982). Wet preparations of the gastrointestinal contents and mucosal scrapings were made and examined under both low- and high-power objectives of a compound microscope to identify parasites. The samples were also concentrated by sugar-salt floatation method to recover parasite cysts, oocysts, and/or eggs that may be present (Permin and Hansen, 1998). Briefly, the samples were homogenized with additional saline solution and filtered through sterile tea sieves. Fourteen milliliters of the filtrate from each sample were transferred into 15ml Falcon tubes and centrifuged at 1500 rpm for 10 minutes. Exactly 5 ml of saturated sugar-salt solution was then added to the resulting residue in the Falcon tubes. The tubes were then filled with sugar-salt solution until a convex meniscus was formed. Clean, sterile coverslips were placed on top of the tubes, which were then left to stand for 10 minutes. The coverslips were subsequently transferred to clean microscope glass slides and examined under a compound light microscope. Parasite cysts, oocysts, and ova were identified using diagnostic keys from Benbrook and Sloss (1961) and Soulsby (1982).

Histopathological analysis

Formalin-fixed tissues from samples positive for at least one type of GI parasite were processed for routine histopathological examination and stained with haematoxylin and eosin (H & E) as described by Gridley (1960). Fixed tissues that tested negative for parasitic infections were kept as negative controls. The stained tissue sections were then examined under a compound light microscope using 10X and 40X objectives.

Data analyses

Data was entered into Microsoft Excel spreadsheet. Summary statistics of categorical and continuous variables were done on Statistical Package for Social Sciences (SPSS) (IBM SPSS Statistics 20). Pearson’s chi-square test was also performed on SPSS in order to determine the relationship between host variables and presence/absence of parasitic infections. All statistical tests were considered significant at $p < 0.05$ with a confidence interval of 5%.

RESULTS

The study found that 251 (69%) of the 364 chickens sampled at the selected LBMs had parasites in their gut, with 11 species of nematodes, 5 species of cestodes, and *Eimeria* spp. identified. Among the nematodes found, *Ascaridia galli* (n = 140, 38.5%), *Heterakis gallinarium* (n = 82, 22.5%) and *Capillaria obsignata* (n = 64, 17.6%) were the most common, while *Gongylonema inguivicola*, *Tetrameres americana* and *Hartertia gallinarium* had the lowest prevalence rates of 0.3%, infecting only 1 chicken each. The most prevalent cestodes included *Raillietina echinobothrida* and *R. tetragona*, infecting 47 (12.9%) and 21 (5.8%) chickens respectively. Oocysts of *Eimeria*, the only protozoan genus found were detected in 98 (26.9%) chickens (Table 1).

Table 1: Prevalence and distribution of gastro-intestinal tract parasites by breed and sex of chickens slaughtered at selected LBMs in Lagos State

Parasite	All chicken	Breed		<i>p</i> - value	Sex		<i>p</i> - value
	N = 364 n (%)	<i>Exotic</i> (N= 189) n (%)	<i>Local</i> (N= 175) n (%)		<i>Female</i> (N= 223) n (%)	<i>Male</i> (N= 141) n (%)	
Protozoa							
<i>Eimeria</i> spp.	98 (26.9)	61 (32.3)	37 (21.1)	0.017	68 (30.5)	30 (21.3)	0.053
Cestodes							
<i>Rallietina echinobothridia</i>	47 (12.9)	10 (5.3)	37 (21.1)	0.000	18 (8.1)	29 (20.6)	0.001
<i>Rallietina tetragona</i>	21 (5.8)	0 (0.0)	21 (12.0)	0.000	5 (2.2)	16 (11.3)	0.000
<i>Amoebotaenia sphenoides</i>	8 (2.2)	1 (0.5)	7 (4.0)	0.024	2 (0.9)	6 (4.3)	0.033

Parasite	All chicken	Breed		<i>p</i> -value	Sex		<i>p</i> -value
	N = 364 n (%)	Exotic (N= 189) n (%)	Local (N= 175) n (%)		Female (N= 223) n (%)	Male (N= 141) n (%)	
<i>Davainea proglottina</i>	7 (1.7)	4 (2.1)	3 (1.7)	0.780	4 (1.8)	3 (2.1)	0.831
<i>Choanotaenia infundibulum</i>	5 (1.4)	3 (1.6)	2 (1.1)	0.716	3 (1.3)	2 (1.4)	0.953
Nematodes							
<i>Ascaridia galli</i>	140 (38.5)	23 (12.2)	117 (66.9)	0.000	50 (22.4)	90 (63.8)	0.000
<i>Heterakis gallinarum</i>	82 (22.5)	6 (3.2)	76 (43.4)	0.000	22 (9.9)	60 (42.6)	0.000
<i>Capillaria obsignata</i>	64 (17.6)	3 (1.6)	61 (34.9)	0.000	18 (8.1)	46 (32.6)	0.000
<i>Trichostrongylus tenuis</i>	52 (14.3)	0 (0.0)	52 (29.7)	0.000	7 (3.1)	45 (31.9)	0.000
<i>Capillaria contorta</i>	25 (6.9)	0 (0.0)	25 (14.3)	0.000	3 (1.3)	22 (15.6)	0.000
<i>Capillaria anatis</i>	21 (5.8)	0 (0.0)	21 (12.0)	0.000	3 (1.3)	18 (12.7)	0.000
<i>Ascaridia columbae</i>	3 (0.8)	0 (0.0)	3 (1.7)	0.071	0 (0.0)	3 (2.1)	0.029
<i>Syngamus trachea</i>	3 (0.8)	1 (0.5)	2 (1.1)	0.518	1 (0.4)	2 (1.4)	0.319
<i>Gongylonema inguivicola</i>	1 (0.3)	0 (0.0)	1 (0.6)	0.298	0 (0.0)	1 (0.7)	0.208
<i>Tetrameres Americana</i>	1 (0.3)	0 (0.0)	1 (0.6)	0.298	1 (0.4)	0 (0.0)	0.426
<i>Hartertia gallinarium</i>	1 (0.3)	0 (0.0)	1 (0.6)	0.298	0 (0.0)	1 (0.7)	0.208

Based on the breeds of the sampled chickens, local chickens exhibited significantly higher prevalence rates of infection for most cestode and nematode parasites ($p < 0.05$). Conversely, *Eimeria* parasites were more prevalent in exotic chickens than in local chickens ($p < 0.05$), although no significant difference was observed at the species level ($p > 0.05$) (Table 1). Based on the sex of the chickens, the prevalence of most nematodes and *R. echinobothrida* was significantly higher in male chickens compared to females ($p < 0.05$). However, there was no significant difference in the infection rates of *Eimeria* between the sexes ($p > 0.05$) (Table 1).

Co-infections involving multiple parasite species (n = 169, 46.4%) were more common than single-species infections (n = 76, 20.9%) (Table 2: Single

and mixed parasitic infections of the gastrointestinal tract of chickens slaughtered at selected live-bird markets in Lagos State). The prevalence of co-infection was higher in males (n = 109, 77.3%) than in females (n = 60, 26.9%), and in local (n = 144, 82.3%) than in exotic chickens (n = 25, 13.2%). Most birds were positive for one to three parasite species; only a few had up to five (n = 7, 1.9%) or six (n = 1, 0.3%). Infection with more than one species was common in local (n = 144, 82.3%) and in male (n = 109, 77.3%) chickens (Table 2: Single and mixed parasitic infections of the gastrointestinal tract of chickens slaughtered at selected live-bird markets in Lagos State).

Table 2: Single and mixed parasitic infections of the gastrointestinal tract of chickens slaughtered at selected live-bird markets in Lagos State

Infection status	Overall = 364 n (%)	Breed		Sex	
		Exotic = 189 n (%)	Local = 175 n (%)	Male = 141 n (%)	Female = 223 n (%)
Single	76 (20.9)	53 (28.0)	23 (13.1)	22 (15.6)	54 (24.2)
Mixed	169 (46.4)	25 (13.2)	144 (82.3)	109 (77.3)	60 (26.9)
Two	66 (18.1)	18 (9.5)	48 (27.4)	28 (19.9)	38 (17.0)
Three	69 (19.0)	5 (2.6)	64 (36.6)	51 (36.2)	18 (8.1)
Four	26 (7.1)	2 (1.1)	24 (13.7)	23 (16.3)	3 (1.3)
Five	7 (1.9)	0 (0.0)	7 (4.0)	6 (4.3)	1 (0.4)
Six	1 (0.3)	0 (0.0)	1 (0.6)	1 (0.7)	0 (0.0)

The distribution of parasite ova and oocysts along the GI of the sampled chickens is shown in Figures 1 - 3. *Ascaridia galli* eggs were frequently detected in the small and large intestines while *H. gallinarium* was restricted to the caeca. Ova of *Capillaria* species were found throughout the gut, except in the gizzard. *Syngamus trachae* eggs were only found in the oesophagus and proventriculus (Figure 1). Most cestode ova were identified in the small intestines, although those of *C. infundibulum* and *D. proglotinna* were also detected in the caeca (Figure 2).

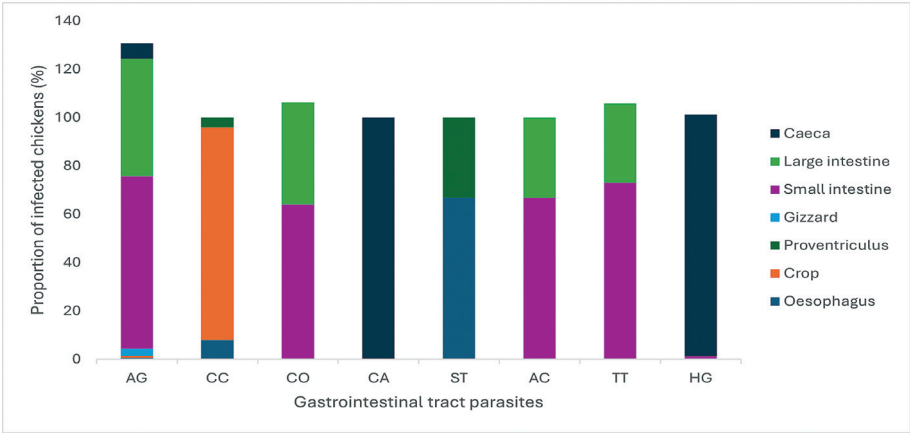


Figure 1: Distribution of nematode ova along the gut of infected chickens slaughtered at selected live-bird markets in Lagos State

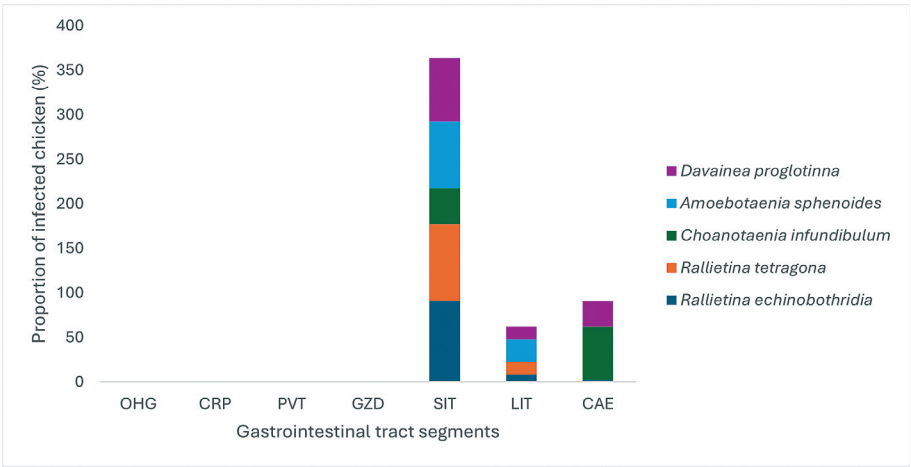


Figure 2: Single and mixed parasitic infections of the gastrointestinal tract of chickens slaughtered at selected live-bird markets in Lagos State

Oocysts of *Eimeria* were mostly detected in the caeca, followed by the large and small intestines; none were found in other parts of the gut (Figure 3).

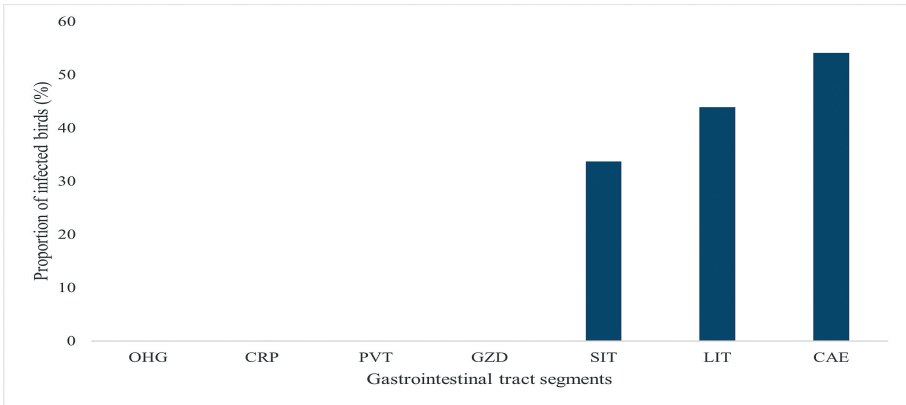


Figure 3: *Eimeria* oocysts distribution in the GI of infected chickens at selected LBMs in Lagos State

Although the sampled birds exhibited no clinical signs of GI infection, approximately 90% of parasitized chickens had gross lesions in at least one part of the gut. These included evidence of necrotic ulcerations on the intestinal mucosa (Figure 4A), severe diffused hemorrhagic enteritis (Figure 4B), severe diffused muroid enteritis (Figure 4C), severe congestion of the intestinal mucosa (Figure 4D), severe diffused necrotic enteritis (Figure 4E) and presence of numerous tapeworms in the intestinal mucosa (Figure 4F).

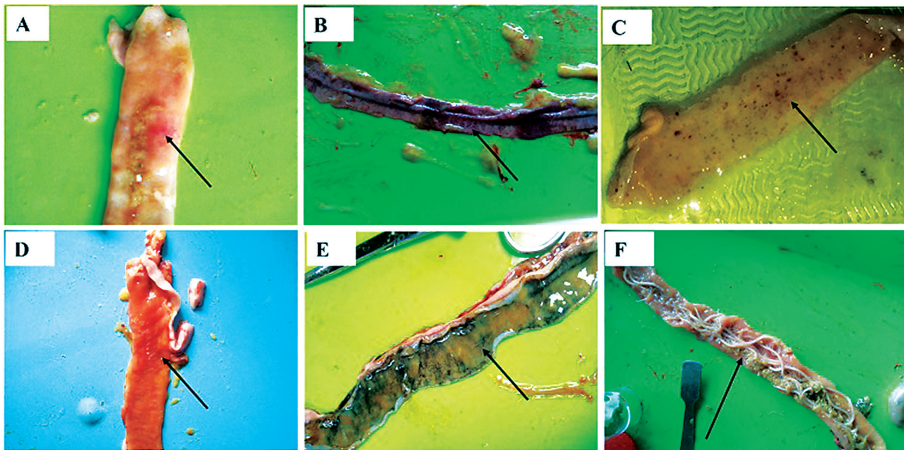


Figure 4: A) focal hemorrhage; B) extensive hemorrhage; C) petechiae; D) whitish coalesced spots on the mucosal surface of intestinal tissue; E) yellow to green mucoid diarrhea in lumen of hemorrhagic small intestine of chickens infected with GI parasites; F) intestinal obstruction with numerous *Ascaridia galli* adults in the lumen of small intestine.

Histopathological analyses revealed the presence of worms in the lumen of the gut, mild to severe infiltration of inflammatory cells, oedema, mucosal erosion, villous atrophy and hyperplasia of the cryptal cells (Figure 5).

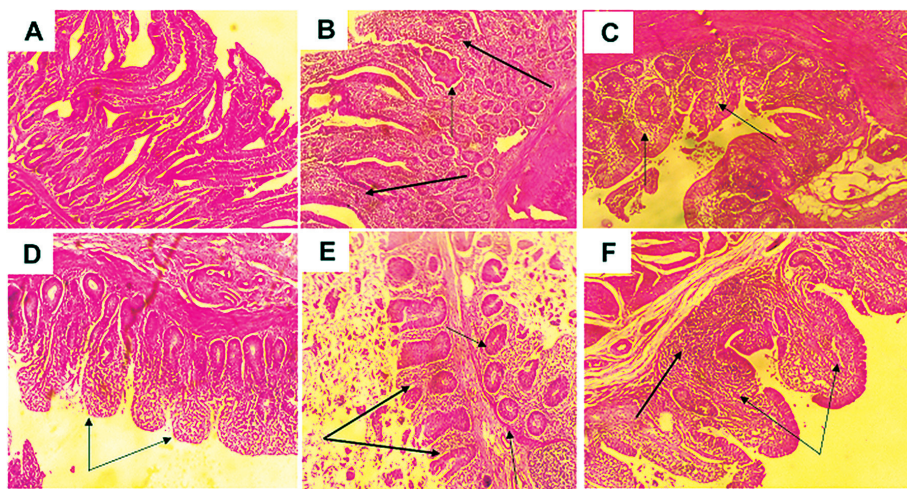


Figure 5: Histopathological lesions observed in the gastrointestinal tract of chickens slaughtered at selected LBMs in Lagos State

DISCUSSION

Poultry meat and eggs are among the most widely consumed and affordable sources of high-quality animal protein for millions of people in low- to middle-income countries (Akintunde and Adeoti, 2014). Gastrointestinal parasites, however, limit poultry health and productivity, undermining the potential of the poultry industry in meeting the growing demand for nutritious food in the developing world (Berhe et al., 2019). Efforts to determine and monitor the occurrence of these infectious agents are essential for planning and implementing effective control programs and policies which in turn ensure food and economic security.

This study revealed a high prevalence of GI parasites in chickens from Lagos State. Previous studies in Nigeria (Ohaeri and Okwum, 2013; Junaidu et al., 2014; Idika et al., 2016; Rufai and Jato, 2017; Elele et al., 2021) have reported similar or even higher prevalence rates, suggesting that gastrointestinal (GI) parasites may contribute to poor poultry productivity, leading to unprofitable poultry production in the country. The high prevalence observed may be a result of the warm, humid climate of the examined area, which supports

the survival of parasites and their intermediate hosts. Inadequate biosecurity practices, hygiene standards and disease control measures may also contribute to parasite survival and dissemination. Furthermore, it is likely that available antiparasitic drugs used for the control of prevalent parasites in the study area have become ineffective.

Nematodes were the most common helminths parasitizing chickens in the study. They have been recognized as the most important helminths of poultry in terms of species diversity, abundance, distribution and pathogenicity (McDougald, 2008a; Ola-Fadunsi et al., 2019). Nematodes are highly resilient organisms that adapt to and thrive in a wide range of ecological settings, including extreme environments (Sapir, 2021). They were found across different parts of the gut of chickens in this study unlike the cestodes which were restricted to the small intestine. These findings are in line with the reports from other parts of Nigeria (Ohaeri and Okwum, 2013; Elele et al., 2021) but differ from those of other authors (Idika et al., 2016; Imam et al., 2017), reflecting the differences that exist between studies in terms of study population, sampling period, sample size, survey design and methods.

Ascaridia galli was the most dominant parasite species found in this study. Similar findings have been reported in other studies conducted in Nigeria (Idika et al., 2016; Rufai and Jato, 2017; Elele et al., 2021; Ozougwu et al., 2021) and might be attributed to the fact that *A. galli* possess a simple and direct life cycle with environmentally resistant eggs. They also feature a wide host range including wild or domestic turkeys, doves, ducks, geese, guinea fowls as well as paratenic hosts such as grasshoppers and earthworms (McDougald, 2008a). The high prevalence of *A. galli* also suggests that chickens might be important reservoir hosts, increasing the risk of exposure to other natural poultry hosts in the study area. Although infection with *A. galli* is usually asymptomatic, high worm burdens, especially in cases of poor management can cause stunted growth, weight loss, intestinal obstruction and death of affected birds which invariably amount to economic losses (Permin and Hansen, 1998).

High prevalence of *Heterakis gallinarium* in this study is noteworthy. The cecal nematode plays an important role in the transmission of *Histomonas meleagridis*, a trichomonad flagellate which can cause a severe disease histomoniasis in gallinaceous birds, and accounts for up to 100% morbidity and mortality in turkeys (Hu et al., 2004; McDougald and Fitz-Coy, 2008). Both *A. galli* and *H. gallinarium* had the highest egg output among the nematodes detected in this study, supporting facts about the high fecundity and robust reproductive capacity of these helminths.

Raillietina species, *R. echinobothrida* and *R. tetragona* were the most common cestodes infecting chickens in this study. This is consistent with the find-

ings of previous studies (Uhuo et al., 2013; Opara et al., 2014; Jegede et al., 2015) and supports the view that these are the most prevalent and widespread tapeworms in poultry (Belete et al., 2016). The use of small ants and beetles as intermediate hosts likely explains their ubiquity (Permin and Hansen, 1998). At high infection intensity, *Raillietina* worms may affect chicken health and productivity. Experimental studies with *R. echinobothrida* have demonstrated nodular granulomas and catarrhal enteritis in the jejunum of the small intestine with infections characterized by reduced growth, emaciation, and weakness (Simon, 2005).

Eimeria species were identified as the sole protozoan parasites infecting chickens in this study. This finding reinforces the recognition of *Eimeria* as one of the most prevalent and widespread poultry parasites, particularly in intensive poultry farming systems where birds are kept in confinement (McDougald, 2008b). They occur everywhere chickens are raised owing to their short and direct life cycle and their highly resistant environmental oocysts which several disinfectants are inactive against (Ola-Fadunsi et al., 2019). Although a study conducted in Akure, Ondo State, reported other gut protozoa, including *Histomonas meleagridis* and *Giardia lamblia* at prevalence rates of 0.6% and 0.3% respectively, *Eimeria* (7.7%) parasites still had the highest prevalence rate of infection (Afolabi et al., 2016).

Most of the helminth parasites were significantly more common in local than exotic chickens. This finding supports the fact that parasitism is prevalent in traditional, free-range systems where local breeds are managed with minimal housing, feeding, and disease control (Permin and Hansen, 1998; McDougald, 2008a). This is supported by Shifaw et al. (2021) who showed that the recent shift from poultry cage systems to extensive free-range or backyard systems is increasing the incidence of helminth infections in developed countries. Indigenous poultry raised in village production systems are free to roam the environment in search of food and water, which exposes them to the infective stages and intermediate hosts of various parasites. This foraging habit also explains why mixed helminth infections were more prevalent in local chickens in this study. Regular deworming, provision of dietary supplements and vitamins will help improve traditional chicken production, increase profitability, and enhance prospects for commercialization. Furthermore, controlling these pathogens in free-range poultry systems will help reduce the risk of transmission to commercial indoor systems.

Eimeria parasites were significantly more prevalent in exotic chickens compared to local breeds. Exotic or improved breeds of chickens are commonly raised in commercial intensive poultry systems characterized by indoor-rearing and high stocking density which favor the accumulation and

rapid spread of monoxenous parasites such as *Eimeria* spp. The risk of exposure to oocysts is increased in these indoor settings, particularly in deep-litter systems where birds have access to feed and water contaminated with faeces. High moisture content resulting from water spillage and warm temperatures in enclosed poultry houses are conditions that also favor the survival, sporulation and abundance of *Eimeria* oocysts (Rao et al., 2015).

Gross lesions characteristic of gastrointestinal tract parasites, including nodules, focal to extensive hemorrhages, petechiae, ballooning, mucus presence, and caecal cores, were observed on the gut mucosa of the infected chickens. These findings provide evidence that parasites are causing varying degrees of damage to the gut integrity of their hosts. Tay et al. (2017) reported similar lesions in the intestinal mucosa of local chickens infected with helminths in Ghana. Previous studies also reported similar findings in backyard and commercial chickens suffering from coccidiosis (Yohannes et al., 2014; Molla and Ali, 2015; Islam et al., 2020).

Histopathological analyses revealed mild to severe inflammation, oedema, loss of crypts, and hyperplasia in the current study. These pathomorphological changes of the intestinal tract have also been reported elsewhere (Shahraki et al., 2018). The observed intestinal pathologies imply that the studied birds are suffering from malabsorption and nutrient deprivation which can lead to reduction in feed conversion efficiency, poor weight gain and lowered productivity.

CONCLUSION

This study determined the prevalence of gastrointestinal parasites in chickens in Lagos State, Nigeria, highlighting their role in causing intestinal tissue damage that can adversely affect poultry performance and productivity. Veterinary health practitioners and poultry extension agents should promote the implementation of strict biosecurity protocols and effective disease control measures among poultry owners and farmers.

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Author's Contribution

OA, EI, BA and IJ conceived and designed this study. OA conducted laboratory analyses, collected and analyzed data, and wrote the original draft of the manuscript. TO interpreted and annotated histopathological data. All authors revised the manuscript critically and together with OA prepared the final draft of the manuscript. All authors read and approved the final manuscript

Competing interest

The authors declare that they have no competing interests.

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DETECTION OF CO-INFECTION IN DISEASED GUPPY (*POECILIA RETICULATA*): *TETRAHYMENA* SP., *MYXOBOLUS* SP. AND OPPORTUNISTIC BACTERIA

Cigdem Urku^{1*}, Mustafa Kemal Meydan²

¹ University of Istanbul, Faculty of Aquatic Sciences,
Department of Fish Disease, Istanbul, Turkey

² ADEC Aquarium and RAS Turnkey Systems, Milas, Turkey

Abstract

The guppy (*Poecilia reticulata*) is one of the world's most popular freshwater aquarium fish. The present study aims to determine the cause of mortality in guppies obtained from the ornamental fish farm in Istanbul province and to detect tissue damage. Externally diseased guppies showed sloughing scales and large ulcerative skin lesions on the body surface. Bacteriological inoculation from internal organs was made onto Tryptic Soy Agar (TSA). After incubation, isolated bacteria were identified as *Aeromonas hydrophila* and *Vibrio parahaemolyticus* according to their physiological, morphological, and API 20E profile. As a result of the parasitological examination, *Tetrahymena* sp. and *Myxobolus* sp. were found on the skin and intestinal lumen of the diseased guppy, respectively. Histopathologically, cytoplasmic vacuolation in the liver, degeneration, and necrosis of the tubular epithelium and granuloma-like structure in the kidney, vacuolization of epithelial cells in the intestinal lumen, hyperplasia of the gill and *Tetrahymena* sp. between necrotic muscles were observed. Interestingly, *Myxobolus* sp. spores were only detected in the intestinal lumen. Co-infection of guppies was determined to cause severe disease with high mortality and tissue damage. The synergistic effects of pathogens may cause more damage to diseased guppies. This report is the first detailed report containing new histopathological findings of tetrahymeniosis and the first detection of *Myxobolus* sp. in the gut of diseased guppy in Turkey.

Key words: *Aeromonas hydrophila*, guppy, histopathology, *Myxobolus* sp., *Tetrahymena* sp., *Vibrio parahaemolyticus*

^{1*} Corresponding Author: curku@istanbul.edu.tr

DETEKCIJA KOINFEKCIJE KOD OBOLELIH GUPIJA (*POECILIA RETICULATA*): *TETRAHYMENA* SP., *MYXOBOLUS* SP. I OPORTUNISTIČKE BAKTERIJE

Cigdem Urku¹, Mustafa Kemal Meydan²

¹Univerzitet Istanbul, Fakultet nauke o vodama,
Katedra za bolesti riba, İstanbul, Turska

²ADEC Akvarijum i RAS sistemi ključ u ruke, Milas, Turska

Kratak sadržaj

Gupi (*Poecilia reticulata*) je jedna od najpopularnijih slatkovodnih akvarijumskih riba širom sveta. Cilj ovog istraživanja je da se utvrdi uzrok smrtnosti gupija sa ornamentalne riblje farme u provinciji Istanbul i detektuje nastalo oštećenje tkiva. Eksterno oboleli gupiji manifestovali su opadanje krljušti i velike ulcerativne kožne lezije na površini tela. Bakteriološka inkulacija iz internih organa urađena je na tripton soja agaru (TSA). Nakon inkubacije izolovane bakterije su identifikovane kao *Aeromonas hydrophila* i *Vibrio parahaemolyticus* na osnovu fiziološkog, morfološkog i API 20E profila. Parazitološko ispitivanje dokazalo je prisustvo *Tetrahymena* sp. i *Myxobolus* sp. na koži odnosno intestinalnom lumenu obolelih gupija. Histopatološki su uočeni citoplazmatska vakuolacija u jetri, degeneracija i nekroza tubularnog epitela i strukture slične granulomima u bubrezima, vakuolizacija epitelних ćelija u intestinalnom lumenu, hiperplazija škrga i *Tetrahymena* sp. između nekrotičnih mišića. Interesantno je da su spore *Myxobolus* sp. detektovane samo u intestinalnom lumenu. Ustanovljeno je da koinfekcija gupija izaziva težak oblik bolesti sa visokom smrtnošću i oštećenjem tkiva. Sinergijski efekat patogena može izazvati još veća oštećenja kod obolelih gupija. Ovaj rad predstavlja prvi detaljan izveštaj koji sadrži nove histopatološke nalaze tetrahimenioze i prvu detekciju *Myxobolus* sp. u crevima obolelih gupija u Turskoj.

Ključne reči: *Aeromonas hydrophila*, gupi, histopatologija, *Myxobolus* sp., *Tetrahymena* sp., *Vibrio parahaemolyticus*.

INTRODUCTION

Ornamental fish production is a profitable industry in the world. The global ornamental fish trade is approximately US\$ 15–30 billion per year (Evers et al., 2019) with 10 % average annual growth (Whittington and Chong, 2007; Dey, 2016). In recent years, it was estimated that 1,471 marine ornamental fish and more than 4,000 species of ornamental freshwater fish were traded globally each year (Sharon et al., 2014). There are 30 predominantly traded freshwater species, of which guppy (*Poecilia reticulata*) and neon tetra (*Paracheirodon innesi*) account for 25% of the market by volume and more than 14% of the total trade value (Dey, 2016; Evers et al., 2019). Guppy, which is native to Trinidad Island and the northern part of South America, lives in natural waters at 24-30 °C and has tolerance to a wide range of salt concentrations. The most popular and widely traded guppy has about 300 subspecies (Sharon et al., 2014).

Tetrahymena is a protozoan parasite, which is the causative agent of tetrahymeniosis or 'guppy killer disease' in tropical aquarium fish, which causes severe economic losses in commercial guppy farms worldwide (Sharon et al., 2014). The species can settle in the scale pockets of the fish and feed on the tissues in that region, as well as penetrate the tissue and settle in the internal organs (Imai et al., 2000). Although it is defined as an invasive pathogen, it can easily cause secondary infection (Sobecka et al., 2012). The first report of tetrahymeniosis in Turkey/Black Sea Region was reported in the guppy (Kayis et al., 2019), and histopathological analyses were not used to detect the tissue damage. Later studies also detected this parasite in *Cichlasoma nigrofasciatum* and *Labidochromis caeruleus* (Kayış et al., 2013).

Myxozoans consist of a species-rich lineage of endoparasitic cnidarians with complex life cycles and have invertebrate and vertebrate hosts in marine and freshwater environments (Okamura et al., 2018). Although *Myxobolus* sp. has been reported in a few aquarium fish, such as Oscar and goldfish fish (Nematollahi et al., 2016), there are limited reports on the detection of this parasite in the guppy (Segovia Salinas et al., 1991; Mohammed et al., 2020).

Most bacterial diseases of ornamental fish result from a combination of factors ranging from environmental conditions, stress, and host susceptibility to pathogen virulence and cause economic losses at every step of the production (Wildgoose, 2001). Known as opportunistic bacteria, *Aeromonas sobria*, *A. hydrophila*, *Shewanella putrefaciens*, *Plesiomonas shigelloides*, *Vibrio cholerae*, *V. parahaemolyticus*, *Pseudomonas* spp., are frequently isolated from diseased ornamental fish (Carnevia et al., 2013; Sicuro et al., 2020; Urku, 2020).

The present study aims to determine the cause of high mortality in dis-

eased guppies obtained from the ornamental fish farm in İstanbul province and to detect tissue damage. This report is the first detailed report containing histopathological findings of tetrahymeniosis and the first detection of *Myxobolus* sp. in the foregut of the diseased guppy in Turkey.

MATERIAL AND METHODS

Fish Samples

Ten diseased guppies were obtained from an aquarium fish-rearing farm in Istanbul/Turkey. There was a 30 % mortality in diseased guppies. The aquarium water temperature and pH values in the are 25-26 °C, and 7, respectively.

Bacteriological Examination

Samples were taken from the liver and kidney of diseased guppies. They were inoculated onto Tryptic Soy Agar (TSA). Petri plates were incubated during 24-48 h at 24-25 °C. The isolates (n=15) recovered from guppy were identified by using the conventional bacteriological method (Gram staining, cell morphology, motility, oxidase and catalase activity, oxidation fermentation test, growth on Thiosulfate Citrate Bile Sucrose (TCBS) medium, vibriostatic test -O129/10,150) and API 20E (Buller, 2004; Austin and Austin, 2016).

Parasitological Examination

The presence of external parasites was investigated by examining fresh samples from the gills and skin of the diseased guppy under a light microscope. After the autopsy, samples taken from the intestine and stomach were also examined for internal parasites (Buchmann, 2007).

Histopathological Examination

After the ventral incision, whole fish (n=5) were immediately fixed in 10% phosphate-buffered formaldehyde, dehydrated with a graded series of ethanol, cleared in chloroform, and embedded in paraffin wax according to standard histological procedures. Paraffin blocks were cut into serial sagittal sections (4-5 µm) with a microtome (Leica RM2125, Germany) and stained with Mayer's hematoxylin and eosin (H&E) and also ZN (Ziehl-Neelsen). Slides were examined under an Olympus BX51 light microscope equipped with an Olym-

pus DP72 digital camera. The width (μm) and length (μm) of detected parasites were measured using ImageJ software version 1.36 (Roberts et al. 2012).

RESULTS

Clinical Findings

Ten diseased guppies exhibited externally large hemorrhagic skin lesions extending to the muscles on the body surface, eroding fins, and loss of the scale (Figure 1).

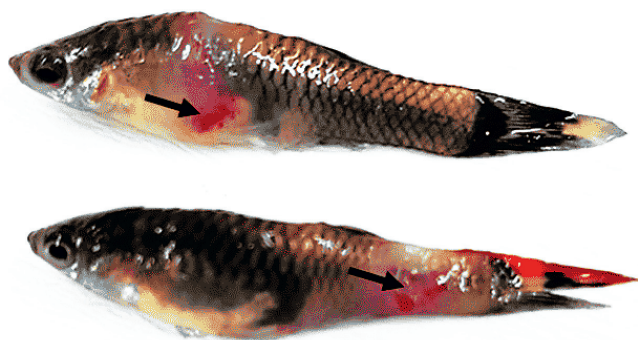


Figure 1. Diseased guppy: Extending to the muscles pronounced hemorrhagic whitish lesions (arrowed) and loss of the scales

Bacteriological Findings

After incubation of liver and kidney swabs, two different colonies were observed on TSA. All isolated bacteria ($n=15$) were motile, Gram-negative basil, oxidase and catalase positive, fermentative, and resistant to O129/10. Five isolates were susceptible to O129/150 and formed green colonies on TCBS (Thiosulfate Citrate Bile Sucrose) medium. According to conventional bacteriological test results, some isolates were identified as *Aeromonas* sp. (10); others were identified as *Vibrio* sp. ($n=5$). In addition, they were defined as

Aeromonas hydrophila (Ah) (n=10) and *Vibrio parahaemolyticus* (Vp) (n=6) with their 304712757 and 410710457 API web profile numbers, respectively.

Parasitological Examination Findings

As a result of the external parasitological examination, *Tetrahymena* sp. was found intensively on the skin of diseased guppy. The parasite (55-35 μ m) with macro (12,35 μ m) and micronuclei (4,362 μ m) had a pear-like body structure covered with cilia (Figure 2a). The presence of any parasite was not detected in the internal parasite examination.

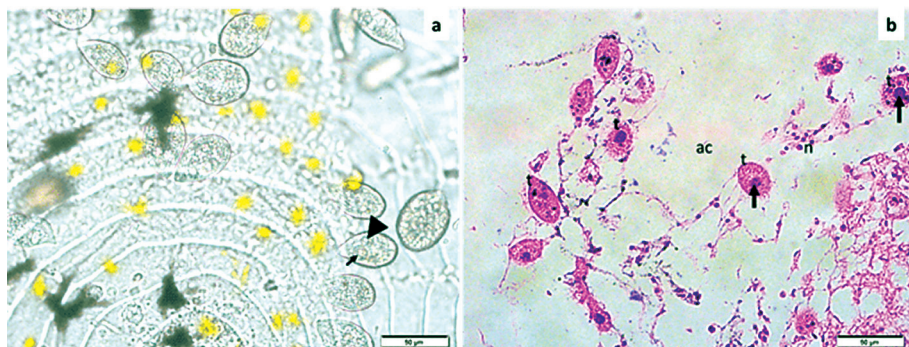


Figure 2. (a) *Tetrahymena* sp. (t) detected on the skin (b) abdominal cavity (ac) (H&E) of the diseased guppy (arrowhead: micronucleus, arrowed: macronucleus, n: leucocytes)

Histopathological Examination Findings

Necrosis of the tubular epithelium and granuloma-like structure and periglomerular and tubular edema in the kidney (Figure 3a, b), cytoplasmic vacuolation and eosinophilic cell infiltration around the necrotic hepatocytes cells (Figure 3c), hemosiderin deposits in the spleen (Figure 3d) (Figure 3c), hyperemia, eosinophilic cell infiltration, and hyperplasia of the gill filaments (Figure 4a, b) were observed. *Tetrahymena* sp. between muscle cells (Figure 5a), pseudobranch (Figure 5b), spinal cord, abdominal cavity (Figure 2b), even between enterocytes (Figure 6a) and around the kidney (Figure 3b), and esophagus (Figure 6d) were noted. Although the presence of any parasite was not detected in the internal parasite examination from the lumen of the intestine and stomach, interestingly mature *Myxobolus* sp spores (35-29 μ m) were observed in the foregut lumen of the diseased guppy (Figure 6b). Vacuoliza-

tion was noted in intestinal epithelial cells (Figure 6c). In addition, acid-fast positive bacterial cells were not observed in ZN-stained preparations.

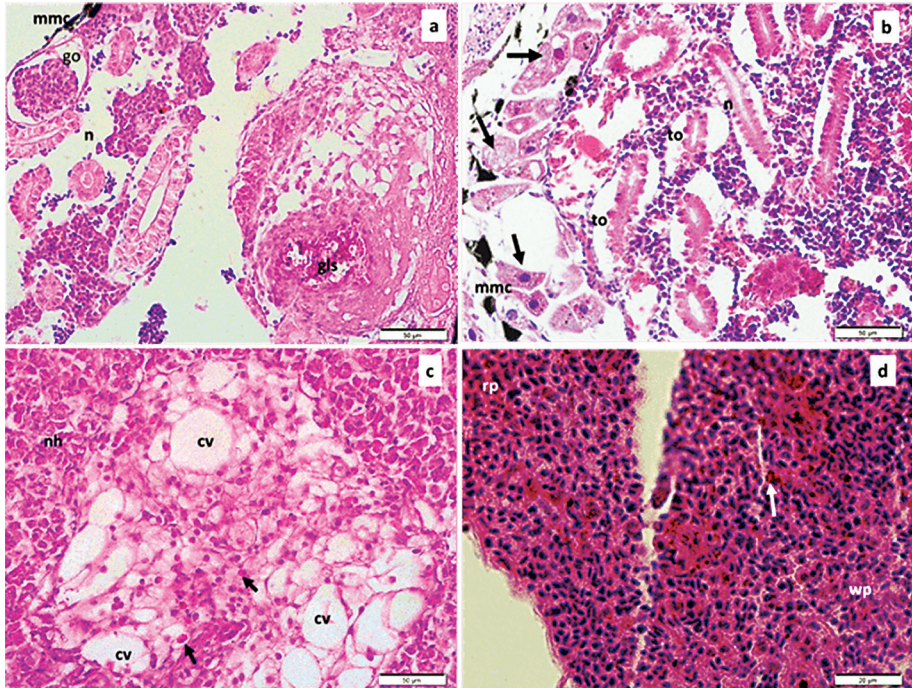


Figure 3. Granuloma-like structure (gls) and discharge of haemopoietic tissue (n), periglomerular (go), tubular (to) edema and melanomacrophage centers (mmc) (a, b); notice *Tetrahymena* sp. (arrowed) around the kidney tissue (b); cytoplasmic vacuolation (cv), and eosinophilic cells (arrowed) among the necrotic hepatocytes (nh) (c); hemosiderin deposits in the spleen (d) (H&E)

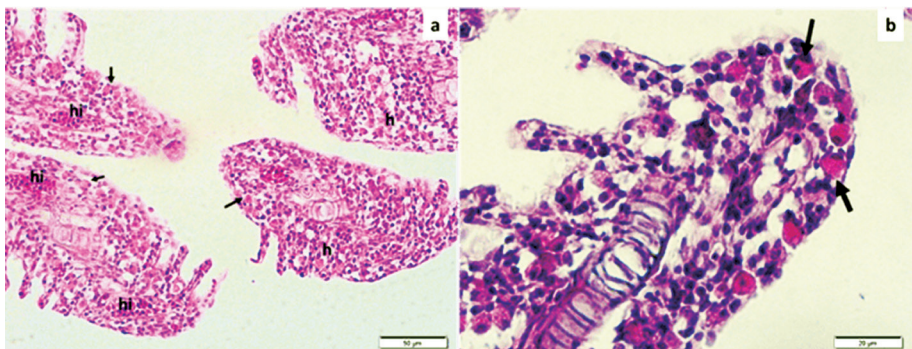


Figure 4. Hyperplasia (h), hyperemia (hi), and eosinophilic cell infiltration (arrowed) in the gill tissue (a, b) (H&E)

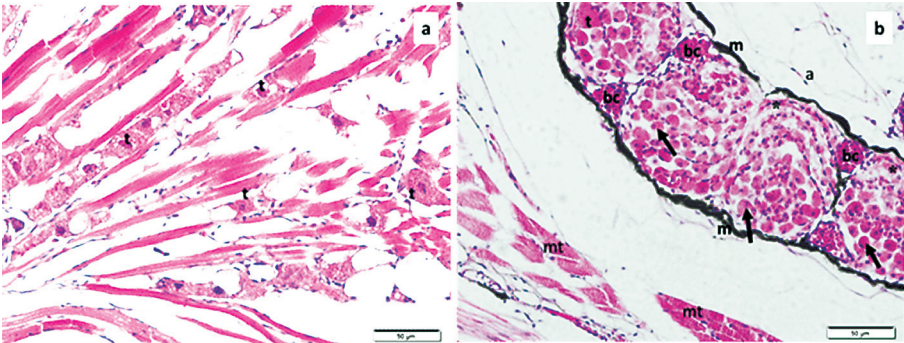


Figure 5. Parasite (t) showed a characteristic pyriform shape penetrating the connective tissue of the muscle cells; note that many parasites are between the muscle fibres with no evident inflammatory response (a); hypertrophy in pseudobranch cells (arrowed) rich in capillary blood vessels (bc); notice *Tetrahymena* sp. (t) between the cells (b) (H&E)

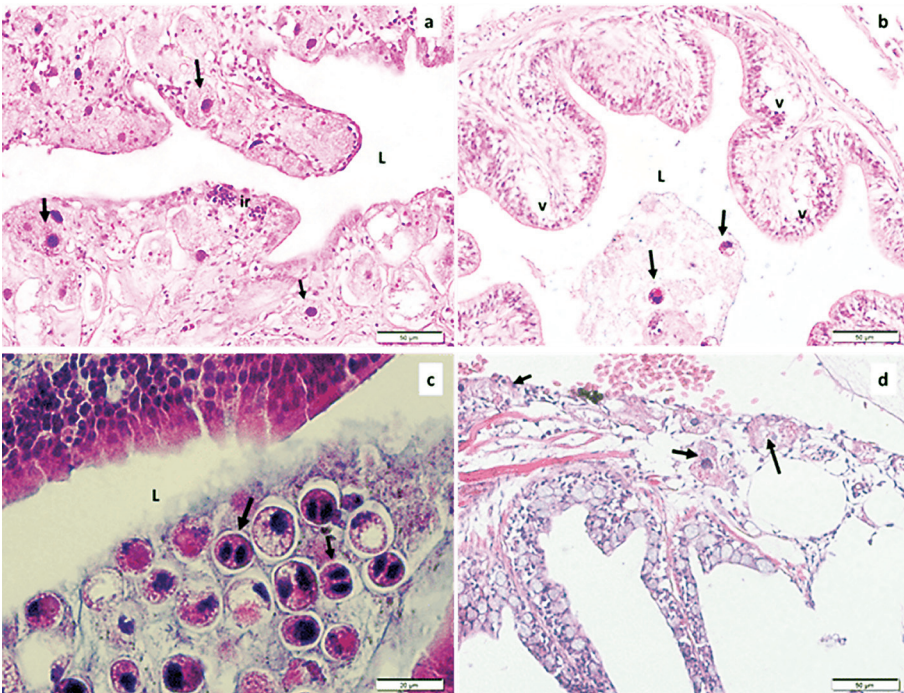


Figure 6. *Tetrahymena* sp. (arrowed) in the midgut, note that many parasites are among the enterocyte with inflammatory reaction (ir) (a) and esophagus (d); vacuolization (v) of epithelial cells of the intestinal lumen of foregut (b); Presence of spores of *Myxobolus* sp. (arrowed) in the foregut (c). (L: lumen) (H&E)

DISCUSSION

This study revealed a co-infection caused by invasive parasites such as *Tetrahymania* sp. and *Myxobolus* sp., and the bacteria *A. hydrophila* and *V. parahaemolyticus*, which were detected as disease agents in the diseased guppy.

Tetrahymaniosis results in parasite invasion of skin, muscle, and even internal organs in many aquarium fish species. Although the main clinical finding of tetrahymaniosis is lethargy or showing characteristic white skin lesions, this parasite causes systemic disease. The disease affects many fish species, and compared to other aquarium fish, guppies are the most susceptible to this disease (Sigh and Buchmann 2002; Leibowitz and Zilberg, 2009; Sharon et al., 2014). In this study, in addition to white skin lesions, which are the main symptoms of the disease, large hemorrhagic skin lesions extending to the muscles on the body surface, eroding fin, and loss of the scale were detected in the fish. This clinical structure, especially hemorrhagic skin lesions, may be caused by the isolated bacteria such as *A. hydrophila* and *V. parahaemolyticus* accompanying the co-infection in the present study.

Myxobolus infection may be coelozoic, most often in the gallbladder and urinary bladder, or histozoic, targeting the skin, muscles, gills, or digestive tract (Lom and Dykova, 2006). Many *myxobolus* species have been reported to cause significant pathological problems such as reduction of epithelial area of the gill, myocarditis of the heart, deformation, displacement, retraction, and compression of capillaries of the gill lamellae and skin nodules (Mathews et al., 2020). In ornamental fish species, it has been reported that *Myxobolus* spp has been detected in the gills of Oscar and goldfish (Nematollahi et al., 2016). Additionally, *Myxobolus nuevoleonensis* has been detected in the bones at the base of the tail, dorsal and anal fins in the molly (*Poecilia mexicana*) and guppy (*P. reticulata*) (Segovia Salinas et al., 1991). As reported by Rasouli et al. (2022) in goldfish, *Myxobolus* sp. was only detected in the intestinal lumen of diseased guppy in this study. Histologically, the parasite sporoplasm, could not be detected in all organs of diseased guppy.

Motile *Aeromonas* Septicemia (MAS), caused by *A. hydrophila* and characterized by hemorrhagic septicemia, is generally characterized by the presence of body surface lesions and hemorrhages, eroded fins, sloughing scales (Anjur et al., 2021). Although clinical findings vary among fish species in vibriosis caused by *V. parahaemolyticus*; skin and muscle are the tissues most affected by the pathogen bacteria. It has been reported to cause black skin lesions in goldfish, deep dermal ulceration, scale loss in tilapia, and ulcerative skin lesions in guppy (Kiani et al., 2016; Adnan Al-Darwesh et al., 2016; Zaher et al.,

2021). In addition, these two bacteria (*A. hydrophila* and *V. parahaemolyticus*) are zoonotic. Therefore, it is essential for aquarists' health.

Tetrahymena invades the host through the skin, reaching the blood vessels and spreading systemically. The organs most affected by the parasite are the gills, heart, kidney, liver, pancreatic tissue, intestinal wall, gonads, and peritoneal cavity (Leibowitz and Zilberg, 2009). It has been reported to cause an intense inflammatory reaction in the tissues where the parasite is localized in the koi and goldfish (Sharon et al., 2014). This study determined that the parasite was not localized in the heart tissue and did not cause a very strong inflammatory reaction in the tissues it penetrated. In addition, it has been observed that the parasite is most densely localized on the skin and around the internal organs such as the kidney and gut.

Although it was reported that *Tetrahymena* sp. was not detected in the intestinal epithelium and lumen (Sharon et al., 2014), parasites localized intensively between the enterocytes drew attention in the present study. Although Imai et al. (2000) did not report any pathological changes in the intestinal mucosa and submucosa, vacuolization was detected in the intestinal epithelial cells of diseased guppy in this study.

The kidney is the most important hemopoietic tissue of fish. Although the presence of the parasite around the kidney tissue was reported, the histopathological changes caused by the *Tetrahymena* sp. in kidney tissue were not described in previous reports (Leibowitz and Zilberg, 2009; Sharon et al., 2014). It destroys tissue cells during parasite migration. In this study, the granuloma-like structure was detected for the first time. Despite the reports that granuloma-like structures in guppy fish are common histological finding in *Mycobacterium* infections (Areechon et al., 2001), the presence of acid-fast positive bacteria was not detected in ZN stained preparations of tissue samples taken from diseased guppy examined in this study.

In general, *Tetrahymena* sp. is believed to accumulate around the blood vessels in the liver of mollies and guppies, having a unique attraction mechanism to these areas. The parasite causes detachment of hepatocytes (Sharon et al., 2014). Granulomas and an increased number of melanomacrophage centers are in the liver of infected fish (Herbert and Graham, 2008; Urku et al., 2018). Although parasites were not detected around the blood vessels in the liver of the fish examined in the present study, cytoplasmic vacuolation and eosinophilic cells among the necrotic hepatocytes were detected. The appearance of a vacuole in the liver is often a parameter of histological liver damage. However, the granuloma reported by Herbert and Graham (2008) was not detected in the liver tissue.

In aquarium fish such as guppy, molly, or angelfish, inflammatory responses are not evident, and the presence of parasite-associated leukocytes was not detected in these fish species (Sharon et al., 2014). This study did not detect the inflammatory reaction in the dermis layer, where the parasite is most intensive; however, leukocytes in the abdominal cavity and inflammatory reaction in the mucosa epithelium were detected.

As a result, the granuloma-like structure pathologically detected in the kidney tissue is not due to mycobacteriosis or myxobolosis; it has been determined that *Myxobolus* spp is found only in the intestinal lumen and not in other tissues and organs. Additionally, acid-fast bacteria were not detected in ZN-stained tissue samples. Therefore, it can be thought that this pathological change is caused by *Tetrahymena* sp.

CONCLUSION

The present report demonstrated that *Tetrahymena* sp. forms a port entrance for opportunistic bacteria such as *Aeromonas* and *Vibrio* accompanied co-infection. In addition, it was histologically determined that the co-infection was associated with *Myxobolus* sp. originating from the live feed. Therefore, disinfection is highly recommended when live feed is used in ornamental fish farms. The synergistic effects of pathogens (invasive parasites and opportunistic bacteria) may histopathologically cause more tissue damage to diseased guppies.

Author's contribution:

CU made contributions to the conception and design of the study, involved in data collection and drafting of the manuscript. MKM provided resources and laboratory work. All authors read and approved the final manuscript.

Competing interests:

Authors declare that they have no competing interests for a work presented.

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SEROTYPING AND ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* ISOLATED FROM DIARRHEIC CALVES IN THE PROVINCE OF TIZI-OUZOU, ALGERIA

Madjid Sadi^{1*}, Madjid Akkou^{1,2,*}, Lamia Bentayeb³, Yacine Titouche⁴

¹ University of Blida, Institute of Veterinary Science, Laboratory of
Biotechnology Related to Animal Reproduction, Blida, Algeria

² University of Médéa, Laboratory of Biology and
Experimental Pharmacology, Algeria

³ University of Mouloud Mammeri, Faculty of Biological
and Agricultural Sciences, Tizi-Ouzou, Algeria

⁴ University of Mouloud Mammeri, Laboratory of Analytical
Biochemistry and Biotechnology, Tizi-Ouzou, Algeria

Abstract

Calf diarrhea is still a problematic disease in the dairy industry world-wide. Several enteropathogens are associated with diarrhea in calves including *Escherichia coli*. This latter is one of the most common causes of diarrhea and septicemia in calves. The primary goal of antimicrobial therapy in calves with diarrhea is to prevent bacteremia and decrease the number of coliform bacteria in the small intestine. The excessive use of antibiotics reduced their effectiveness, which eventually led to the risk of emergence of antibiotic resistance. This study aimed to determine the carriage rate, antimicrobial resistance patterns and serotypes of *E. coli* isolates involved in calf diarrhea in farms from Tizi-Ouzou. A total of 154 fecal samples were collected from diarrheic calves belonging to 22 farms between 2019 and 2021. All the samples were inoculated into MacConkey agar plates (MC) and MacConkey agar supplemented with 2 µg/L of cefotaxime (MC+CTX) for cefotaxime-resistant (CTX^R) *E. coli*. *Escherichia coli* isolates were identified using API20E commercial kits. Antimicrobial susceptibility was determined by a disk diffusion test. The serogrouping of the isolates was realized

^{1*} Corresponding Author: akkoumadj@gmail.com/akkou_madjid@univ_blida.dz

*: The authors contributed equally to this work

with a rapid slide agglutination test. Overall, 128 isolates of *E. coli* (one isolate per sample) were obtained in MC, including three strains in MC+CTX. Furthermore, 47.6 % of *E. coli* strains recovered from MC agar were resistant to at least one antimicrobial. The three *E. coli* isolates recovered from MC+CTX media showed an *ESBL*-producing profile. The serological identification revealed that 67 (52.3%) of the isolated strains were typable with O antisera. The most common serogroup was O55 (13.3%), followed by O128 (10.1%) and O111 (7.8%), O119 (7%), O126 (6.2%), O26 (5.5%), and O114 (2.3%). Antibiotic susceptibility testing revealed that 37.5% (48/128) and 22.7% (29/128) of the isolates were resistant to tetracycline and streptomycin respectively. Furthermore, multidrug resistance was observed in 17.96% (23/128) of the *E. coli* isolates. Our study provides valuable information about *E. coli*-associated calf diarrhea in farms of Tizi-Ouzou. Regular antibiotic resistance monitoring is required to limit the spread of multi-drug-resistant *E. coli* in the barn environment.

Key words: Calves, diarrhea, antimicrobial resistance, serotype, *Escherichia coli*

SEROTIPIZACIJA I ANTIMIKROBNA REZISTENCIJA *ESCHERICHIA COLI* IZOLOVANIH OD TELADI SA DIJAREJOM U PROVINCIJI TIZI-OUZOU, ALŽIR

Madjid Sadi¹, Madjid Akkou^{1,2}, Lamia Bentayeb³, Yacine Titouche⁴

¹ Univerzitet Blida, Institut veterinarskih nauka, Laboratorija za biotehnologiju i animalnu reprodukciju, Blida, Alžir

² Univerzitet Médéa, Laboratorija za biologiju i eksperimentalnu farmakologiju, Alžir

³ Univerzitet Mouloud Mammeri, Fakultet bioloških i poljoprivrednih nauka, Tizi-Ouzou, Alžir

⁴ Univerzitet Mouloud Mammeri, Laboratorija za analitičku biohemiju i biotehnologiju, Tizi-Ouzou, Alžir

Kratak sadržaj

Dijareja teladi još uvek predstavlja problematično oboljenje u industriji mleka i mlečnih proizvoda širom sveta. Više enteropatogena se povezuju sa dijarejom teladi uključujući i vrstu *Escherichia coli*. Ovaj organizam je

jedan od najčešćih uzročnika dijareje i septikemije kod teladi. Primarni cilj antimikrobne terapije kod teladi sa dijarejom je sprečavanje bakteremija i smanjenje broja koliformnih bakterija u tankom crevu. Prekomerna upotreba antibiotika smanjila je njihovu efikasnost, što je na kraju dovelo do rizika od razvoja i širenja antimikrobne rezistencije. Cilj ovog rada je da odredi stopu prenosa, modele antimikrobne rezistencije i serotipove izolata *E. coli* povezane sa dijarejom teladi na farmama u oblasti Tizi-Ouzou. U periodu od 2019. do 2021.godine prikupljeno je ukupno 154 uzorka fecesa teladi sa dijarejom sa 22 farme. Svi uzorci su inokulisani na ploče sa MacConkey (MC) agarom i MacConkey agarom sa dodatkom 2 µg/L cefotaksima (MC+CTX) za cefaksim-rezistentne (CTX^R) *E. coli*. Izolati *Escherichia coli* identifikovani su pomoću komercijalnih kitova API20E. Antimikrobna osetljivost određena je pomoću disk difuzione metode. Serološko grupisanje izolata vršeno je pomoću brzog testa aglutinacije na mikroskopskim pločicama. Ukupno je utvrđeno 128 izolata *E. coli* (po jedan izolat po uzorku) sa MC uključujući tri soja na MC+CTX. Osim toga, 47.6% sojeva *E. coli* dobijenih na MC agaru bilo je rezistentno na barem jedan antimikrobni preparat. Tri izolata *E. coli* dobijena sa MC+CTX podloga manifestovali su *ESBL*-produkujući profil. Serološka identifikacija pokazala je da je tipizacija pomoću O antiseruma bila moguća kod ukupno 67 (52.3%) izolovanih sojeva. Najčešća serogrupa bila je O55 (13.3%), a zatim O128 (10.1%), O111 (7.8%), O119 (7%), O126 (6.2%), O26 (5.5%), i O114 (2.3%). Testovi osetljivosti na antibiotike pokazali su rezistenciju na tetraciklin i streptomycin kod 37.5% (48/128) odnosno 22.7% (29/128) izolata. Višestruka (multi) rezistencija je ustanovljena kod 17.96% (23/128) izolata *E. coli*. Naše istraživanje pružilo je korisne informacije o dijareji teladi izazvanih organizmima *E. coli* na farmama u oblasti Tizi-Ouzou. Neophodno je sprovesti redovan monitoring antimikrobne rezistencija kako bi se ograničilo širenje multirezistentnih *E. coli* u štalama teladi.

Ključne reči: telad, dijareja, antimikrobna rezistencija, serotip, *Escherichia coli*

INTRODUCTION

Cattle farming in Algeria is characterized by substantial heterogeneity in production methods and practices. Gastrointestinal disorders are one of the most prevalent diseases of pre-weaned dairy calves, which cause substantial economic losses due to high morbidity, mortality, growth retardation, and

treatment costs (Bernal-Córdoba et al., 2022).

Calf diarrhea is attributed to both infectious and non-infectious factors. Multiple enteric pathogens such as viruses (e.g., bovine *rotavirus*, bovine *coronavirus*, bovine viral diarrhea virus); bacteria (e.g., *Escherichia coli* K99, *Salmonella* spp., *Clostridium perfringens*) and protozoa (e.g., *Cryptosporidium parvum*, *Giardia duodenalis*, *Eimeria* spp.) are involved in the development of the disease. Co-infection is frequently observed in diarrheic calves. A single primary pathogen can be the cause in some cases (Brunauer et al., 2021).

Affected calves experience dehydration, electrolyte imbalances, metabolic acidosis, and decreased appetite, which, if left untreated, can lead to death (Foster and Smith, 2009; Gomez et al., 2022). Long-term effects of calf diarrhea in dairy heifers include reduced weight gain and development, increased time to first calving, and reduced milk production in the first lactation (Cafarena et al., 2021). Moreover, diarrhea raises serious concerns about newborn calf welfare and remains the primary reason for antimicrobial treatment in beef calf ranches (Waldner et al., 2013). The primary goal of antimicrobial therapy in diarrheic calves is to prevent bacteremia and decrease the number of coliform bacteria in the small intestine. Overusing and misusing of antibiotics is a common practice in the dairy industry and on livestock farms. Careless use of antimicrobial agents and inadequate hygiene and biosecurity contribute to the emergence and spread of antibiotic resistance worldwide (Kaczorek et al., 2017). Furthermore, the intensification of cattle breeding and the intensive use of antibiotics make cattle important reservoirs of resistant bacteria that can be disseminated at the human-animal-environment interface (Rousham et al., 2018).

Escherichia coli is a leading cause of diarrhea and septicemia in calves; as a significant reservoir of human pathogenic *E. coli*, calves can transmit this pathogen to humans (Algammal et al., 2020). There are six major diarrheagenic *E. coli* pathotypes (Jafari et al., 2012). Enterotoxigenic *E. coli* (ETEC) is the confirmed primary causative agent of neonatal calf diarrhea. Enteropathogenic *E. coli* (EPEC), and Shiga toxin-producing *E. coli* (STEC including enterohemorrhagic *E. coli* /EHEC) are also often isolated from diarrheic and healthy calves; still, their role in calf disease remains controversial. EPEC, STEC, and EHEC are important human pathogens, and cattle constitute a major reservoir. Enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC including *Shigella*) are less frequently reported in cattle (Jafari et al., 2012).

To our knowledge, the carriage of pathogenic *E. coli* by calves suffering from diarrhea and the potential zoonotic reservoir represented by cattle are not well documented in Algeria. To address the deficit of epidemiological and

microbiological data concerning serotyping and antimicrobial resistance of *E. coli* isolated from calves with diarrhea, we conducted this study to describe the antimicrobial resistance patterns and serotypes of *E. coli* isolates involved in calf diarrhea.

MATERIAL AND METHODS

Calf diarrhea is a multifactorial syndrome; multiple enteric pathogens are involved in the development of the disease. In this study, we were limited to isolating and identifying *E. coli* in calves with diarrhea without explicitly pointing to case definition.

Study site and sample collection

The study was conducted in the province of Tizi-Ouzou situated in the north of Algeria between 36°28' and 36°55' latitude north and 3°45' and 4°31' east. It covers an area of 2993 km², which represents 0.13% of the national territory. Tizi-Ouzou is bordered from the north by the Mediterranean Sea, from the south by the Department of Bouira, from the east by the Department of Bejaia, and from the west by the Department of Boumerdes.

After obtaining permission from the cattle breeders, 154 fecal swabs were collected from diarrheic calves originating from 22 farms. One fecal sample per animal was obtained rectally using a sterile cotton swab during a veterinarian intervention. All samples were transported in sterile, cold storage jars (+4 °C) to the laboratory for further analysis.

Species identification

After dilution overnight in buffered peptone water at 37 °C, fecal swabs were seeded onto MacConkey agar plates (MC) and MacConkey agar supplemented with 2 µg/L of cefotaxime (MC+ CTX) for cefotaxime resistant (CTX^R) *E. coli* recovery. Plates were then incubated aerobically for 24h to 48h at 37 °C. Following, all suspected colonies (pink and non-mucoid on MacConkey agar, colorless on Sorbitol MacConkey agar) were picked, purified, and primarily identified on the basis of conventional biochemical tests (Indol, Methyl-red, Voges-Proskauer, and Citrate). Final identification of *Escherichia coli* isolates was done using API20E commercial kit (Biomérieux, Marcy l'Etoile, France). After identifying the species, the serotyping was carried out, from a pure and fresh culture of *E. coli* isolated on BHI Agar, by rapid slide agglutination tests using antibody sera which correspond directly to the O antigens. The agglutination reaction occurs when specific antibodies present in serum bind to the

corresponding bacterial antigens and can be observed over a dark surface.

In this study, 13 antigens were used (O111, O55, O26, O86, O119, O127, O125, O126, O128, O114, O124, O142 and O157). The reagents used are based on specific antibodies obtained from the immune sera of rabbits (Bio-Rad[®]).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar (Conda[®]) using the disk diffusion method, according to Clinical Laboratory Standards Institute standard guidelines (CLSI 2020). *E. coli* isolates were tested against the following antimicrobial agents: ampicillin (AMP, 10 µg), amoxicillin + clavulanic acid (AMC: 20 µg + 10 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftazidime (CAZ, 30 µg), cefoxitin (FOX, 30 µg), tetracycline (TET, 30 µg), ciprofloxacin (CIP, 5 µg), trimethoprim-sulfamethoxazole (STX, 1.25 µg + 23.75 µg), gentamicin (GEN, 10 µg), streptomycin (STR, 10 µg) and Chloramphenicol (CHL, 30 µg). The production of ESBL was detected by placing three antibiotic disks in the same line: cefotaxime, ceftazidime, and amoxicillin/clavulanic acid. Isolates were ESBL-positive if the ghost zone between these three antibiotics was visible (CLSI, 2020).

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2016. The chi-square test was used to check whether the prevalence of *E. coli* is dependent on the age of the calves. Results were considered significant when $p < 0.05$.

RESULTS

Occurrence of *E. coli* in calf diarrhea

From 154 cultured samples, 128 (83.1%) strains of *E. coli* were isolated. According to the age groups of the calves, the frequencies of *E. coli* isolation were 43 (33.59%) in the first 2 months old, 67 (52.54%) in aged 2-4 months old, and the remaining 18 (14.06 %) were associated to the calves with 4-6 months of age (Table 1).

Table 1: Distribution of *E. coli* involved in diarrheic calves according to age groups

Age groups	No. of Samples	<i>E. coli</i> positive samples (%)	<i>E. coli</i> negative samples (%)	<i>P</i>
1 day to 2 months	51	43 (84.31%)	8 (15.69%)	0.58
2 - 4 months	83	67 (80.72%)	16 (19,28%)	
4 - 6 months	20	18 (90%)	2 (10%)	
Total	154	128 (83.12%)	26 (16.88%)	

The serological identification of the retrieved isolates revealed that 67 (52.3%) of the isolated strains were typable with O antisera, while 61 isolates (47.6%) were untypable. The most common serogroup was O55 (13.3%), followed by O128 (10.1%) and O111 (7.8%), O119 (7%), O126 (6.2%), O26 (5.5%), and O114 (2.3%). The frequency of *E. coli* serogroups was illustrated in Table 2. There is no statistically significant difference in the prevalence of *E. coli* serogroups ($p > 0.05$).

Table 2: Serogrouping of *E. coli* isolates involved in calf's diarrhea

Serotypes	No. of isolates	Percentage (%)
Non sero-typeable	61	47.65
O55	17	13.30
O128	13	10.10
O111	10	7.80
O119	9	7.0
O126	8	6.20
O26	7	5.50
O114	3	2.30
Total	67	52.34

Occurrence of antimicrobial resistance in *E. coli*

All isolates were tested for susceptibility to a panel of 11 antimicrobials. As shown in Table 3, the results of antimicrobial susceptibility testing in this study showed relatively high resistance rates against most of the antibiotics tested. Resistance to tetracycline was most common (37.5%, n=48), followed by Streptomycin (22.7%, n=29), AMP and SXT (18.7%, n=24), AMC (12.5%, n=16) and GEN (8.6%, n=11), and the lowest resistance level was observed against CHL (6.2%, n=8), CIP (3.1%, n=4), CTX and CAZ (2.3%, n=3). All isolates were susceptible to Cefoxitin (Table 3).

Table 3: Antimicrobial resistance of *E. coli* isolates involved in diarrheic calves

Antibiotics	Abbreviation	Resistant		Susceptible	
		No. of isolates	%	No. of isolates	%
Ampicillin	AMP	24	18.7	104	81.2
Amoxicillin/Cla-vulanic acid	AMC	16	12.5	112	90.6
Cefotaxime	CTX	3	2.3	125	97.7
Ceftazidime	CAZ	3	2.3	125	97.7
Cefoxitin	FOX	0	0	128	100
Ciprofloxacin	CIP	4	3.1	124	96.9
Sulphamethoxazole/Trimethoprim	STX	24	18.7	104	81.2
Tetracycline	TET	48	37.5	80	62.5
Gentamicin	GEN	11	8.6	117	91.4
Streptomycin	STR	29	22.7	103	77.3
Chloramphenicol	CHL	8	6.2	120	93.7

Multiple antimicrobial resistances

Out of 128 isolates tested for resistance against all 11 antibiotics, 67 (52.3%) of the isolates were susceptible to all the antibiotics tested, and 61 (47.7%) were resistant to at least one antibiotic. However, 23 (17.96%) showed multiple drug resistance (resistant to at least three classes of antibiotics). From this latter, 15 isolates (11.6%) were resistant to ≥ 4 antibiotic classes, and 9 (7.03%) were resistant to 5 classes of antibiotics (Table 4, 5).

Table 4: Calve associated *E. coli* isolates resistance according to the number of antibiotics classes

No. antimicrobial families	No. of strains	Percentage (%)
Susceptible	67	52.30
1 class	28	21.87
2 classes	10	7.81
3 classes	8	6.25
4 classes	6	4.68
5 classes	9	7.03

Table 5: Patterns of resistance to antibiotics among *E. coli* recovered from diarrheic calves

Resistance Phenotype	No. of isolates	%	No. of anti-biotic classes with resistance
Susceptible	67	52.34	0
CHL	1	0.78	1
GEN	1	0.78	1
STR	1	0.78	1
TET	22	17.18	1
AMP, AMC	2	1.56	1
AMP, SXT	1	0.78	2
AMP, STR	1	0.78	2
AMP, TET	1	0.78	2
SXT, TET	1	0.78	2
TET, STR	2	1.56	2
GEN, STR	1	0.78	1
CIP, GEN	1	0.78	2
AMP, AMC, GEN	1	0.78	2
AMP, SXT, STR	3	2.34	3
AMP, AMC, TET	1	0.78	2
SXT, TET, STR	4	3.12	3
TET, GEN, STR	1	0.78	2

Resistance Phenotype	No. of isolates	%	No. of anti-biotic classes with resistance
AMP, SXT, TET, STR	1	0.78	4
SXT, TET, GEN, STR	1	0.78	3
AMP, AMC, TET, CHL, STR	1	0.78	4
AMP, CIP, SXT, TET, STR	1	0.78	5
SXT, TET, GEN, STR, CHL	1	0.78	4
AMP, AMC, SXT, TET, STR, CHL	4	3.12	5
AMP, AMC, SXT, TET, GEN, STR	1	0.78	4
AMP, AMC, CTX, CAZ, SXT, TET, STR	2	1.56	4
AMP, AMC, CIP, STX, TET, GEN, STR	2	1.56	5
AMP, AMC, SXT, TET, GEN, STR, CHL	1	0.78	5
AMP, AMC, CTX, CAZ, CIP, SXT, TET, GEN, STR	1	0.78	5

Looking specifically at the ESBL-producing phenotypes, out of 128 *E. coli* isolates tested, three (D78, D104, and D123) were revealed positive. These latter were resistant to at least four antibiotic classes. Two ESBL-producing *E. coli* isolates belonged to serogroup O55 (Table 6).

Table 6: Serogroups and patterns of antibiotic resistance of the ESBL-Producing *E. coli*

Isolates cote	ESBL	Resistance Phenotype	Serogroup
D78	+	AMP, AMC, CTX, CAZ, CIP, STX, TET, GEN, STR	ND
D104	+	AMP, AMC, CTX, CAZ, SXT, TET, STR	O55
D123	+	AMP, AMC, CTX, CAZ, SXT, TET, STR	O55

ND: Not determined

DISCUSSION

Diarrhea within farm animals, especially in neonatal calves, is one of the most challenging clinical syndromes encountered by practicing large animal veterinary practitioners in Algeria (Dadda et al., 2018). Systemically ill calves (depression, anorexia, fever) often suffer from *E. coli* septicemia, and thus, Gram-negative-spectrum antibiotics are advised to treat them. As determined by bacterial examination of fecal samples collected from diarrheic calves, the occurrence of *E. coli* in the present study was 83.1%.

A similar rate was reported in India (85.04%) (Picco et al., 2015). While lower prevalence was reported in Algeria (62%) (Selles et al., 2018) and Egypt (37.4%) (Fouad et al., 2022). The variation in the occurrence of *E. coli* in diarrheic calves could be related to different factors, including the study area, age of calves, farm and sample sizes, management, quality of colostrum, and hygiene measurements (Brunauer et al., 2021). The prevalence of *E. coli* is not statistically different according to age groups ($p = 0.58$). According to a previous study, the frequency of *E. coli* was higher in young calves and subsequently declined as the calves aged (Osman et al., 2013).

Antibiotic resistance is a worldwide problem that has already been identified by several researchers and recognized as a public health problem by the World Health Organization. The unregulated use of antibiotics in bovine farms may enhance the spread of drug-resistant bacteria, particularly ESBL-producing *E. coli*, in the community. In this study, serological tests showed the identification of serogroups O26, O55, O111, O114, O119, O126, and O128 from 52.34% of the *E. coli* isolates. The detected serogroups are frequently associated with pediatric diarrhea (Lanz Uhde et al., 2008; Wani et al., 2013). Similar findings were observed in a previous investigation (Algammal et al., 2020). However, further research revealed different serogroups from those reported in the present study (Lanz Uhde et al., 2008; Vagh and Jani, 2010; Enany et al., 2019). It has been established that food-producing animals may represent an important reservoir of Shiga toxin-producing *E. coli* (STEC); however, highly pathogenic isolates for humans (enterohemorrhagic *E. coli*: EHEC O157:H7) are not detected so frequently in domestic and wild animals. However, the carrier-status is often underestimated, which is due to the sensitivity of detection methods and the shedding and transmission capacity of the EHEC O157:H7 in ruminants and non-ruminants. Furthermore, EHEC O157 can contaminate and colonize vegetables and fresh fruits and also present possible reservoir of this infectious agent (Velhner et al., 2020).

From an antibiotic resistance standpoint, 47.7% of *E. coli* isolates were resistant to at least one antimicrobial agent, while 17.96% were multi-drug re-

sistant (MDR). Similar results were in a study conducted at Tizi-Ouzou province of Algeria on *E. coli* recovered from healthy cow fecal samples (Sadi et al., 2024). Higher levels of multi-drug resistance were reported from different studies around the world (Algammal et al., 2020; Selles et al., 2018; Sobhy et al., 2020; Maciel et al., 2020). Higher rates of multidrug-resistant *E. coli* isolates from diarrheic calves were observed by Selles et al. (2018) in western Algeria 68.75% and Maciel et al. (2020) in Brazil 53.3%.

In the present study, the resistance level for individual antibiotics tested was generally low, with a higher level of resistance against 'older' and commonly used drugs in therapeutics such as tetracycline, followed by streptomycin, sulphamethoxazole/trimethoprim and ampicillin. The resistance rate against newer drug classes, such as cephalosporin, was lower in the present study. In line with our findings, a study from Egypt reported remarkably higher rates of resistance to tetracycline, streptomycin, ampicillin, and trimethoprim-sulfamethoxazole with 79.5%, 67%, 54.5%, and 43%, respectively (Sobhy et al., 2020). Furthermore, resistance to gentamicin, chloramphenicol and ciprofloxacin was recorded in 8.6%, 6.2%, and 3.1% of the tested *E. coli*, respectively. Since 52.34% of the isolated serotypes were involved in human diseases, the observed resistance may be due to the transfer of human bacteria, in which resistance has already been developed to animal hosts. Besides, using human drugs for the treatment of infected animals or, alternatively, the use of wastewater, for irrigation, containing excretions of these antibiotics and their metabolites may enhance the spread of antibiotics resistance (Sobhy et al., 2020).

Analysis of the antimicrobial resistance pattern showed that antimicrobial resistance is a growing phenomenon in Northern Algeria. Thirty phenotypes of antibiotics resistance ranging from one antimicrobial to a combination of seven were observed. This circumstance underscores the need for *E. coli* antibiotic susceptibility testing. From the empirical point of view, we find herein that the most regularly used antibacterial agents in cattle, namely tetracycline, had the most common resistance patterns, which could explain treatment failures.

ESBL-producing *E. coli* has emerged as a major problem worldwide. Primarily, ESBL-producing *E. coli* isolates were only observed in human clinical isolates. However, these bacteria have increased drastically in food-producing animals, making them a natural reservoir and contributing to their spread (Seiffert et al., 2013). Furthermore, bacteria producing enzymes hydrolyzing penicillin and cephalosporins are often resistant to most commonly used antimicrobials (Pfaller and Segreti, 2006; Berthod et al., 2012).

In our study, 2.3% of the isolates were ESBL-producing *E. coli* resistant to at least four classes of antibiotics. Accordingly, previous reports showed lower prevalence of ESBL-producing *E. coli* (0.97%) in a study focusing on *E. coli*

isolated from healthy calves, diarrheic calves and healthy dairy cows from different farms in Khuzestan province, Iran (Mozhdeh et al., 2017), and higher prevalence of ESBL-producing *E. coli* (26 out of 71, 36.6%) in diarrheic calves of Tunisia (Ben Haj Yahia et al., 2023).

Two of the three ESBL-producing *E. coli* isolated in the present study belonged to serogroup O55. Factors such as owning domestic food animals and living near a drainage flow path from a commercial food animal operation may have increased the risk of ESBL-producing *E. coli* carriage among children (Amato et al., 2023). Other factors may have increased the risk of ESBL producing *E. coli* carriage such as household dog ownership and placing animal feces on household land/crops (Mitman et al., 2022; Amato et al., 2023).

CONCLUSION

This study showed important human enteropathogenic serogroups in isolates of *E. coli* from diarrheic calves in Algeria. The intensive use of antibiotics in extensive livestock farming is responsible for the increase in multidrug-resistant *E. coli* isolates. Calves could play a role disseminating enteropathogenic serogroups in humans and antibacterial resistance genes. Furthermore, MDR could spread through the use of livestock feces as manure. Accordingly, there is a need to formulate and enforce policies to regulate the use of antimicrobials in the country; an antimicrobial surveillance program is also necessary. Public health education about the health implications of indiscriminate use of antimicrobials is required.

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Author's Contribution

Conceptualization: MS, MA, LB and YT. Methodology: MS and MA. Formal analysis: YT and LB. Investigation: MS, MA and LB. Writing-original draft preparation, MA, MS and LB. Writing-review and editing: MA and YT. All authors read and approved of the final manuscript.

Competing interest

The authors declare that they have no conflict of interest.

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UNDERSTANDING THE DRIVERS FOR COMMUNITY-BASED PREVENTION OF AFRICAN SWINE FEVER SPREAD: A CASE STUDY OF KASAWO AND KATOSI SUB COUNTIES, MUKONO DISTRICT, UGANDA

Savino Biryomumaisho^{1*}, Geoffrey Otuba¹, John Okongo¹, Frank Mwiine²

¹ Makerere University, School of Veterinary Medicine and Animal Resources, Uganda

² Makerere University, School of Biosecurity, Biotechnical
and Laboratory Sciences, Uganda

Abstract

Pig production in Uganda is a thriving business as the demand for pork and pork products is increasing. Pork consumption per capita in Uganda is estimated to be 3.5 kg and is considered the highest in Africa. Pig production is, however, limited by several factors, including endemic diseases, particularly African swine fever (ASF), which can result in up to 100% mortality. The factors responsible for ASF outbreaks are poorly understood, especially at farm level. A study conducted in Kasawo and Katosi sub-counties between February and March 2023 aimed to examine biosecurity and husbandry practices on pig farms. A questionnaire was distributed to pig farmers and a Focus Group Discussion (FDG) of animal health care workers was held. Blood samples were collected from pigs of all ages and sex in the two sub counties and ASF virus antibodies were tested using competitive ELISA. Out of the 777 collected blood samples, none was seropositive for ASF virus antibodies. Of the 292 households sampled, 70% kept their pigs indoors, while only 0.7% had fenced enclosures, and 4% had functional foot baths. The FGD revealed that animal health workers, who were on daily calls, carried disinfectants to sanitize their personal protective equipment (PPE) every time they left a farm. The lack of centralized slaughter facilities was identified as a key factor contributing to the spread of the ASF virus, as well as the panic selling of pigs during suspected ASF outbreaks. Sharing boars, purchasing and stocking pigs without veterinary health certification

^{1*} Corresponding Author: sbiryomumaisho15@gmail.com

were identified as contributing factors. The results from this study showed that although active ASF infections were not found in pigs, the district experienced ASF outbreaks in the past. The study concluded that educating farmers about the spread and prevention of ASF, ensuring ongoing disinfection of PPE by animal health professionals, and establishing pig slaughter facilities could help eliminate ASF transmission and spread in the area.

Key words: African swine fever virus, biosecurity, seroprevalence

FAKTORI KOJI DOPRINOSU PREVENICIJI ŠIRENJA AFRIČKE KUGE SVINJA U ZAJEDNICAMA: STUDIJA SLUČAJA U OKRUŽIMA KASAVO I KATOSI, MUKONO DISTRIKT U UGANDI

Savino Biryomumaisho¹, Geoffrey Otuba¹, John Okongo¹, Frank Mwiine²

¹ Univerzitet Makerere, Fakultet veterinarske medicine i animalnih resursa, Uganda

² Univerzitet Makerere, Katedra za biosigurnost,
biotehničke i laboratorijske nauke, Uganda

Kratak sadržaj

Proizvodnja svinja u Ugandi predstavlja uspešan privredni sektor, jer potražnja za svinjetinom i svinjskim proizvodima konstantno raste. Procenjuje se da je potrošnja svinjskog mesa po glavi stanovnika u Ugandi 3,5 kg, što ovu zemlju čini liderom u Africi. Međutim, proizvodnja svinja suočava se sa izazovima, među kojima su endemske bolesti, a posebno afrička kuga svinja (AKS), koja može izazvati mortalitet i do 100%. Faktori koji dovode do izbijanja AKS slabo su istraženi, posebno na farmama. Istraživanja sprovedena u oblastima Kasavo i Katosi u periodu između februara i marta 2023. godine imala je za cilj da ispita biosigurnosne mere uzgoja svinja na farmama. Uzgajivačima svinja podeljeni su upitnici, a takođe je održana i Fokus grupa (FDG) sa radnicima iz polja zdravstvene zaštite životinja. Uzorci krvi su prikupljeni od svinja svih uzrasta i pola u dve oblasti, a antitela na virus AKS testirana su ELISA testom. Od 777 prikupljenih uzoraka krvi, nijedan nije bio seropozitivan na antitela na virus AKS. Od 292 uzorkovana domaćinstva, 70% je držalo svoje svinje u zatvorenom prostoru, dok je samo 0,7% imalo ograde, a 4% je imalo funkcionalne dezinfekcione bari-

jere. Fokus grupa (FGD) je ustanovila da su zdravstveni radnici nosili dezinfekciono sredstvo za čišćenje svoje lične zaštitne opreme svaki put kada bi napustili farmu. Nedostatak centralizovanih objekata za klanje prepoznat je kao ključni faktor koji doprinosi širenju virusa AKS, kao i panična prodaja svinja tokom sumnjivih izbijanja bolesti. Utvrđeno je da su deljenje nerastova, kupovina i držanje svinja bez veterinarske zdravstvene potvrde faktori koji doprinose širenju AKS. Rezultati ove studije pokazali su da, iako aktivne infekcije AKS nisu detektovane kod svinja, okrug je u prošlosti imao epidemije ove bolesti. Zaključak istraživanja je da bi edukacija farmera o širenju i prevenciji AKS, stalna dezinfekcije lične zaštitne opreme od strane stručnjaka za zdravlje životinja, kao i upotreba specijalnih objekata za klanje svinja, mogli značajno doprineti smanjenju prenošenja i širenja AKS u ovom području.

Ključne reči: virus afričke kuge svinja, biosigurnost, seroprevalenca

INTRODUCTION

Pig production in Uganda is a thriving business driven by the increasing demand for pork and pork products. Per capita consumption of pork in Uganda at 3.5 kg is the highest in East Africa (Roesel et al., 2019) and is considered the highest in Africa (Atherstone et al., 2021). Most pig farmers are smallholders, keeping 1 to 5 pigs that are tethered around their homesteads (Payne et al., 2022). Pigs are a source of additional income and offer several advantages over other types of livestock: their short production interval and high multiplication rates, little rearing space and ability to convert human food leftovers to calories depending on quantity and nutrient content. The pig industry, however, faces several challenges, including inadequate feed resources, a disorganized marketing system, and endemic diseases, notably African swine fever (ASF), which can result in up to 100% mortality (Kalenzi Atuhaire et al., 2013). Mukono district in central Uganda is among the districts that experienced numerous ASF outbreaks in the past (Ogweng et al., 2020). Epidemiological factors of spread of ASF are not well understood in the context of what is practiced along the pig value chain.

African swine fever (ASF) is an infectious disease that affects domestic and wild pigs of all breeds and ages, caused by ASF virus (ASFV), a member of the *Asfarviridae* family. The clinical symptoms vary from per acute, acute, subacute, to chronic, depending on the virulence of the virus. Acute disease

is characterized by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate (Ngan et al., 2023) caused by ASF virus (ASFV). African swine fever (ASF) is one of the most complex infectious swine diseases. Notification of African Swine Fever (ASF) to the World Organization for Animal Health (WOAH) is mandatory due to its high mortality rate, rapid transmission, and significant sanitary and socioeconomic impact on the international trade of pigs and pork products. ASF has been endemic in over 20 sub-Saharan African countries and in Sardinia since the last century. ASFV has demonstrated its huge capacity for transboundary and transcontinental spread jumping to several hundreds of kilometers away such as Asia (China) and European region (Gallardo et al., 2019). The disease was first detected on the Italian island of Sardinia in 1978. ASF was detected in Georgia in 2007 (Gallardo et al., 2015) and spread to other countries.

African swine fever is endemic in Uganda and several outbreaks of ASF have been reported across the country (Atuhaire et al., 2013). The virus is transmitted through the known sylvatic cycle, domestic and wildlife cycles. African swine fever virus is a unique and complex DNA virus that is unrelated to other viruses, which has contributed to the challenges in developing a vaccine for the disease (Cukor et al., 2020). The virus is the only member of the *Asfarviridae* family and the only known DNA arbovirus. It has remained endemic in Africa since its discovery in Kenya in 1921 (Madden, 2021). The virus is maintained in a sylvatic cycle between *Ornithodoros* soft ticks and warthogs (*Phacochoerus africanus*) which do not develop clinical disease with ASFV infection; these ticks are widespread outside Africa, in parts of Europe and the Americas. Eradicating ASF, which is transmitted through the sylvatic cycle, is extremely difficult because soft ticks can survive for up to five years without feeding, while retaining a significant viral load (Sánchez-Vizcaíno et al., 2009). In addition, the virus can be maintained in soft ticks through sexual transmission, trans-ovarially, and transstadially, allowing the infection to persist without the need for pigs, and enabling it to sustain over long periods.

Another transmission cycle is the domestic cycle, which occurs due to the constant contact between domestic pigs. In this cycle, the infection is primarily spread through oronasal routes (Björnheden, 2011). An infected pig can continue to shed the virus for at least 30 days. Other transmission routes, particularly for spreading the disease to new areas, include swill-feeding with undercooked pork and the movement of contaminated fomites (Penrith and Vosloo, 2009). The virus can survive between 3-6 months in the environment. Contributing factors include stability of the virus in the environment and meat products, low awareness about the disease of pig farmers, laxity on farm bios-

security practices and illegal movements of pigs and their products (Guinat et al., 2016).

Efforts to develop an effective vaccine have not been very successful in the past as vaccines failed to induce effective protective immunity. These have included inactivated DNA, subunit and adenovirus-vectored vaccines (Wu et al., 2021). Genome sequencing of the ASFV shows it is approximately 170-194kb and encodes 105-170 proteins whose functionality remains unclear according to Cackett et al. (2020). Cackett further states that due to its lack of neutralizing antibodies, ASFV is classified on the basis of capsid protein p72 encoded by viral B646L gene, generating 24 different genotypes. Promising live attenuated vaccines have shown protection against homologous challenges, but not against heterologous virus isolates. However, the issue of more virulent strains emerging remains a concern. In the absence of vaccines, observing biosecurity measures becomes the only viable option (Penrith, 2020).

In a serological survey of ASF in different districts in Uganda, Atuhaire et al. (2013) found a prevalence of 26% (47/181 pigs); however, prevalence of ASF in this study was much higher than results in other studies. In a study of slaughtered pigs in Mubende district, central Uganda, 38/997 pigs (3.8%) tested positive to ASF antibodies (Muwonge et al. 2012). Delgado-Baquerizo et al. (2013) collected blood samples from clinically sick pigs from central Uganda districts of Mukono, Wakiso and Nakasongola in a 2007 ASF outbreak, and none of the sera were positive for ASFV antibodies using recommended WOAHA tests *viz.* conventional PCR and immunoblotting assays using an antigen lysate of ASF-infected cells. According to (Björnheden, 2011) in a study carried out in Rakai district in Southern Uganda, a much lower seroprevalence of 2.1% was reported among pigs brought for slaughter. Using archived samples from seven districts of Pallisa, Lira, Abim, Nebbi, Kabarole, Kibaale, and Mukono from 2001-2012, serological examination detected 6/39 positive samples and all of them originated from Abim district; samples from the other six districts (187/193, 96.9%) all tested negative (Kabuuka et al., 2023). However, 8.47% of tissue samples tested positive.

A clear understanding of these factors is essential for designing effective control measures. Mukono district was among those where frequent outbreaks of ASF had been reported in the recent past years (Ogweng et al., 2020). There is a lack of information regarding the epidemiological factors responsible for the outbreaks as well as the level of exposure of pigs to ASFV in Mukono district. Specifically, the biosecurity implementation practices across the entire pig value chain are not well-defined. The extent of the pig population's exposure to ASFV in Kasawo and Katosi sub-counties was not well understood,

nor were effective community-based ASF control strategies clearly defined. Therefore, this study aimed to understand the level of awareness of ASF among farmers, exposure of pigs to ASFV and the interventions aimed to control the spread of ASF. We aim to identify the risk factors behind the recurrent ASF outbreaks in Mukono district and analyze their connection to pig production and marketing systems. We intend to evaluate the extent of the application of biosecurity measures on farms and in villages as well as the role of carrier pigs in ASF epidemiology.

MATERIAL AND METHODS

Study Area

The study was carried out in Mukono District (0.2835°N, 32.7633°E), located in the Central region of Uganda. Mukono District covers an area of 2,986.47 square kilometers and is bordered by Buikwe District to the east, Kayunga District to the north, Wakiso District to the southwest, and Lake Victoria to the south (UBOS, 2017). Mukono district was selected because it has a very high number of pigs (181,846 pigs during the 2008 census), which has increased significantly from that time up until now. This situation contributes to an increased risk of disease outbreaks in the area, including ASF, providing a basis for this study to assess the current conditions and promote early detection. The district is composed of 15 sub-counties, 81 parishes, and 795 villages with a population of 596,804 people and 144,160 households (Mabaya et al., 2021). Katosi and Kasawo sub-counties were selected due to their high pig population, as reported by the District Veterinary Officer (DVO) of Mukono District, and because they have experienced African swine fever outbreaks in the past.

The research protocol was approved by the Department of Veterinary Pharmacy, Clinical and Comparative Medicine Academic Board meeting held on 29th September 2022 at the School of Veterinary Medicine and Animal Resources, Makerere University, Uganda. A consent was sought from each farmer before administering the questionnaire and taking a blood sample from their pigs.

Study on the population

The study was conducted among farmers who reared pigs in their home-steads or on pig farms. Questionnaire interviews were conducted with farmers who agreed to participate in the study and consented to the collection of blood samples from their pigs. Blood samples were collected from pigs of all ages

and both sexes. Any adult member of the selected household who interacted with the pigs participated in the interview, although preference was given to the head or owner of the pigs if they were present during the data collection.

Study design

The study was a cross-sectional study conducted on pig farms across two sub-counties Kasawo and Katosi in Mukono district, from February 2023 to March 2023. This study used a quantitative approach, where questionnaires were administered to farmers involved in the study, and blood samples were collected from the pigs on the farms of the interviewed farmers.

Sampling strategy

A snowball sampling technique was employed during data collection, where a farmer with a pig farm would refer to the research team to the next pig farmer they knew in the village. It was ensured that the whole sub-county would be represented by selecting farmers in each village in the various parishes.

Sample size

The sample size for the study was calculated using the formulae by (Das et al, 2016)

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

Taking a 95% confidence interval, the prevalence of 50% and desired absolute precision of 5%; where n was the required sample size; Z was the multiplier from a standard normal distribution (1.96) at a probability level of 0.05; P was the estimated prevalence at 50%, considering there was no reliable prevalence of ASF in the area, and d was the desired precision for the estimate ($\pm 5\%$). A sample size of 385 pig farmers was required for this study. The sample size was equally divided into the two sub-counties. Pigs of all ages were sampled including piglets, growers, and adults from the pig farms.

Sample collection and laboratory sample analysis

A blood sample (2-4 mL) was collected from the anterior vena cava in

younger pigs (younger than 6 months) and from the jugular vein in older pigs into plain vacutainers, and it was stored in a cool box with ice packs and transported to the laboratory. Upon arrival at the laboratory, serum was carefully pipetted into cryotubes. In some cases, centrifugation was performed, and the serum was then stored at -20°C until analysis. The serum was analysed for presence of African swine fever IgG antibodies using commercial kits of competitive Enzyme-linked immunosorbent assay (c-ELISA). Optical densities against positive and negative controls were read using a digital ELISA reader. Positive cases cut-offs were calculated by subtracting the mean positive controls from mean negative controls and by dividing the difference with 0.5 according to the manufacturer's instructions. Mean negative cut offs were calculated by subtracting mean positive controls from mean negative controls and dividing the difference by 0.4.

A sample optical density was positive if the cell reading OD was less than the positive cut-off (0.953) and negative if the cell OD was greater than the negative cut-off point (1.128). A sample was considered suspicious if the cell OD was greater than the positive cut-off (0.953) but less than the negative cut-off (1.128).

RESULTS

The most common pig rearing system was intensively practiced by 62% of farmers. Free range (roaming) pig rearing was the least practiced by 1.7% of farmers. In both sub counties, only 21% kept local breeds of pigs. The rest kept crossbreeds with exotic breeds. The majority (57%) fed their pigs using feed troughs. In terms of cleaning, only a few farmers (39%) regularly cleaned the drinkers and feeders on a daily basis. Most households (97%) kept small pig herds sized between 1-10 pigs (Table 1). Regarding breeding, the majority of farmers used boar for mating; the boar was either owned by a farmer or borrowed from a neighbor. Although artificial insemination has been introduced, it has not yet been widely practiced, especially in Kasawo sub-county. As for weaning piglets, the majority of farmers (52%) weaned their piglets at or just over four weeks, while others weaned them at different ages after farrowing.

Table 1: Pig farm management practices

Variable	Characteristic	Kasawo, n=174	Katosi, n=118	Overall, N=292	(95% CI)
Pig breed	Both exotic and local	34 (19.5)	25 (21.2)	59 (20)	(15.85, 25.37)
	Exotic	93 (53.4)	79 (66.9)	172 (59)	(53.01, 64.56)
	Local	47 (27.0)	14 (11.9)	61 (21)	(16.47, 26.10)
Breeding method	Artificial in-semination	12 (6.9)	1 (0.8)	13 (4.5)	(2.493, 7.677)
	Boar from neighbour	85 (48.9)	80 (67.8)	165 (57)	(50.60, 62.24)
	Farmers' own boar	63 (36.2)	31 (26.3)	94 (32)	(26.93, 37.93)
	Village boar	14 (8.0)	6 (5.1)	20 (6.8)	(4.341, 10.54)
Pig herd size	1-10	152 (87.4)	103 (87.3)	255(87)	(82.83, 90.81)
Method of keeping pigs	Above 10	22 (12.6)	15 (12.7)	37(13)	(9.187, 17.17)
	Intensive	104 (59.8)	78 (66.1)	182 (62)	(56.47, 67.86)
	Roaming or free ranging	2 (1.2)	3 (2.5)	5 (1.7)	(0.632, 4.179)
	Semi-intensive	18 (10.3)	15 (12.7)	33 (11)	(8.016, 15.64)
	Tethering	50 (28.7)	22 (18.6)	72 (25)	(19.91, 30.09)
Feed type	Both home-made and commercial feed	66 (37.9)	20 (16.9)	86 (29)	(24.36, 35.10)
	Commercial feeds	7 (4.0)	11 (9.3)	18 (6.2)	(3.800, 9.736)
	Food leftovers from school	1 (0.6)	1 (0.8)	2 (0.7)	(0.119, 2.723)
	Homemade pig feed	77 (44.3)	33 (28.0)	110 (38)	(32.14, 43.53)
	Kitchen leftovers	21 (12.1)	50 (42.4)	71 (24)	(19.59, 29.73)
	Roam around	2 (1.1)	3 (2.5)	5 (1.7)	(0.632, 4.179)
Caretaker	Female children	14 (8.0)	7 (5.9)	21 (7.2)	(4.614, 10.94)
	Hired person	9 (5.2)	6 (5.1)	15 (5.1)	(3.007, 8.508)
	Housewife	87 (50)	80 (67.8)	167 (57)	(51.29, 62.90)
	Husband	30 (17.2)	16 (13.6)	46 (16)	(11.87, 20.56)
	Male children	34 (79.1)	9 (7.6)	43 (15)	(10.97, 19.44)

Variable	Characteristic	Kasawo, n=174	Katosi, n=118	Overall, N=292	(95% CI)
Feed and water	Cemented floor	16 (9.2)	23 (19.5)	39 (13)	(9.778, 17.93)
	Feeding troughs	99 (56.9)	68 (57.6)	167 (57)	(51.29, 62.90)
	Non ce- mented floor	59 (33.9)	27 (22.9)	86 (29)	(24.36, 35.10)
Cleaning frequency of troughs	After every two days	23 (13.2)	38 (32.2)	61 (21)	(16.47, 26.10)
	Daily	64 (36.8)	49 (41.5)	113 (39)	(33.13, 44.57)
	Never	46 (26.4)	13 (11.0)	59 (20)	(15.85, 25.37)
	Once a week	26 (14.9)	13 (11.0)	39 (13)	(9.778, 17.93)
	Twice a week	15 (8.6)	5 (4.2)	20 (6.8)	(4.341, 10.54)
Keeping both adult pigs and piglets	No	131 (75.3)	84 (71.2)	215 (74)	(68.11, 78.51)
	Yes	43 (24.7)	34 (28.8)	77 (26)	(21.49, 31.89)
Sow care after birth	Both home-made and commercial feed	70 (40.2)	14 (11.9)	84 (29)	(23.72, 34.39)
	Commercial feeds	6 (3.4)	16 (9.2)	22 (7.5)	(4.890, 11.34)
	Homemade feeds	87 (50)	43 (36.4)	130 (45)	(38.76, 50.43)
	Kitchen leftovers	10 (5.7)	41 (34.7)	51 (17)	(13.39, 22.42)
	Left to roam around	1 (0.6)	4 (3.4)	3 (1.0)	(0.266, 3.225)
Water source	Borehole	141 (81.0)	6 (5.1)	147 (50)	(44.47, 56.20)
	Rainwater	3 (1.7)	9 (7.6)	12 (4.1)	(2.241, 7.256)
	River	0 (0.0)	11 (9.3)	11 (3.8)	(1.993, 6.832)
	Spring	1 (0.6)	12 (10.2)	13 (4.5)	(2.493, 7.677)
	Tap	1 (0.6)	9 (7.6)	10 (3.4)	(1.750, 6.404)
	Well	28 (16.1)	71 (60.2)	99 (34)	(28.55, 39.69)
Weaning age	>4 weeks	90 (51.7)	61 (51.7)	151 (52)	(46.16, 57.92)
	1 week	5 (2.9)	2 (1.7)	7 (2.4)	(1.062, 5.124)
	2 weeks	21 (12.1)	6 (5.1)	27 (9.3)	(6.334, 13.40)
	3 weeks	26 (14.9)	11 (9.3)	37 (13)	(9.251, 17.28)
	4 weeks	30 (17.2)	38 (32.2)	68 (23)	(18.78, 28.83)

Variable	Characteristic	Kasawo, n=174	Katosi, n=118	Overall, N=292	(95% CI)
Piglet feed	Both home-made and commercial feeds	68 (39.1)	10 (8.5)	78 (27)	(21.80, 32.24)
	Commercial feeds	5 (2.9)	14 (11.9)	19 (6.5)	(4.069, 10.14)
	Home kitchen leftovers	16 (9.2)	44 (37.3)	60 (21)	(16.16, 25.73)
	Homemade feeds	82 (47.1)	40 (33.9)	122 (42)	(36.10, 47.68)
	Leftovers from school/hospital	1 (0.6)	1 (0.8)	2 (0.7)	(0.119, 2.723)
	Roam around	2 (1.1)	9 (7.6)	11 (3.8)	(1.993, 6.832)
House cleaning frequency	Every 2 days	27 (15.5)	43 (36.4)	70 (24)	(19.28, 29.37)
	Daily	70 (40.2)	34 (28.8)	104 (36)	(30.18, 41.44)
	Never	38 (21.8)	21 (17.8)	59 (20)	(15.85, 25.37)
	Occasionally	22 (12.6)	9 (7.6)	31 (11)	(7.436, 14.87)
	Once a week	17 (9.8)	11 (9.3)	28 (9.6)	(6.575, 13.70)

Table 2: Biosecurity practices at pig farms in Kasawo and Katosi sub-counties

Variable	Category	Kasawo, N=174	Katosi, N=118	Overall, N=292	95% CI
Presence of footbath	No	162 (93.1)	117 (99.2)	279 (96.0)	(92.32, 97.51)
	Yes	12 (6.9)	1 (0.8)	13 (4.0)	(2.493, 7.677)
Pig rearing biosecurity	Fenced facility	1 (0.6)	1 (0.8)	2 (0.7)	(0.119, 2.723)
	Free roaming	1 (0.6)	4 (3.4)	5 (1.7)	(0.632, 4.179)
	Housed	117 (67.2)	88 (74.6)	205 (70.0)	(64.55, 75.32)
	Tethering	55 (31.6)	25 (21.2)	80 (27.0)	(51.49, 79.19)
Changing footbath	Everyday	6 (3.4)	0 (0.0)	6 (13.0)	(5.186, 25.94)
	Irregularly	2 (1.1)	0 (0.0)	2 (4.2)	(0.725, 15.43)
	Never	32 (18.4)	0 (0.0)	32 (67.0)	(22.44, 32.96)
	Once a week	6 (3.4)	1 (0.6)	7 (15.0)	(6.545, 28.38)
	Twice a week	1 (100.0)	0 (0.0)	1 (2.1)	(0.109, 12.47)

Variable	Category	Kasawo, N=174	Katosi, N=118	Overall, N=292	95% CI
Visitors access to farm	Multiple	145 (83.3)	112 (94.9)	257 (88.0)	(83.59, 91.40)
	Only through the main entrance	10 (5.7)	2 (1.7)	12 (4.1)	(2.241, 7.256)
	Visitors not allowed	19 (10.9)	4 (3.4)	23 (7.9)	(5.167, 11.74)
Vehicles accessing farm	No	167 (96.0)	116 (98.3)	283 (97.0)	(94.03, 98.49)
	Yes	7 (4.0)	2 (1.7)	9 (3.1)	(1.512, 5.971)
Source of pigs	Born	78 (44.8)	28 (23.7)	106 (36.3)	(30.83, 42.14)
	Both	2 (1.1)	0 (0.0)	2 (0.7)	(0.119, 2.723)
	Brought	94 (54.0)	90 (76.3)	184 (63.0)	(57.17, 68.51)
Veterinary inspection of farm	No	111 (63.8)	90 (76.3)	201 (83.0)	(77.24, 87.13)
	Yes	32 (36.2)	10 (23.7)	42 (17.0)	(12.87, 22.76)
Disposal of dead pigs	Buried	145 (83.3)	97 (82.2)	242 (83.0)	(77.95, 86.92)
	Burned	13 (7.5)	10 (8.5)	23 (7.9)	(5.167, 11.74)
	Cooked and fed to other pigs	1 (0.6)	0 (0.0)	1 (0.3)	(0.018, 2.194)
	Slaughtered and eaten	15 (8.6)	11 (9.3)	26 (8.9)	(6.007, 12.92)
Disposal of faeces	Composite pit	31 (17.8)	27 (22.9)	58 (20.0)	(15.54, 25.00)
	Thrown nearby	143 (82.2)	91 (77.1)	234 (80.0)	(75.00, 84.46)
Feral animals cited at farm	No	127 (73.0)	95 (80.5)	222 (76.0)	(70.63, 80.72)
	Yes	47 (27.0)	23 (19.5)	70 (24.0)	(19.28, 29.37)
Pig slaughter in the village	No	140 (80.5)	108 (91.5)	248 (87.0)	(82.10, 90.32)
	Yes	28 (19.5)	10 (8.3)	38 (13.0)	(9.684, 17.90)
Pork inspection	No	63 (36.2)	17 (14.4)	80 (80.0)	(70.57, 87.08)
	Yes	15 (8.6)	5 (4.2)	20 (20.0)	(12.92, 29.43)
Qualified vet/meat inspector	No	144 (82.8)	113 (95.8)	257 (91.0)	(86.68, 93.80)
	Yes	21 (12.1)	5 (4.2)	26 (9.2)	(6.201, 13.32)

Variable	Category	Kasawo, N=174	Katosi, N=118	Overall, N=292	95% CI
Running water availability	No	147 (84.5)	105 (89.0)	252 (89.0)	(84.67, 92.32)
	Yes	18 (10.3)	13 (11.0)	31 (11.0)	(7.676, 15.33)
Effluent disposal	Buried	69 (39.7)	12 (10.2)	81 (28.0)	(22.76, 33.32)
	Burnt	27 (15.5)	8 (6.8)	35 (12.0)	(8.599, 16.41)
	Bush	78 (44.8)	98 (83.1)	176 (60.0)	(54.39, 65.88)
Centralized slaughter	No	48 (27.6)	20 (16.9)	68 (23.0)	(18.65, 28.64)
	Yes	126 (72.4)	98 (83.1)	224 (77.0)	(71.36, 81.35)

The majority of farms (70%) had their pigs confined in temporary or semi-permanent structures. Most of the farmers did not have a footbath on the farms (96%) and 88% of the farm visitors had access to the pig units. Homesteads which had running water were few n=31 (11%); majority accessed spring and well water. Dead pigs were buried by majority of farmers (83%); most of the farmers disposed of pig feces by throwing it nearby. Pigs that were purchased or exchanged were most commonly not subjected to veterinary inspection; only 17% of the pig acquisitions underwent prior veterinary inspection and certification (Table 2).

Focus Group discussion with animal health workers

Three main topics that were discussed were the following: pig slaughter status in the district, implementation of biosecurity measures during their day-to-day work and pig breeding methods. They highlighted the absence of centralized pig slaughter facilities in the sub-counties and noted that individual veterinarians are unable to travel across the entire sub-county on a daily basis to conduct pork inspections. This called for the need to establish centralized slaughter facilities where all pigs can be slaughtered on designated days, with all pork subjected to inspection in order to promote public health and facilitate the orderly collection of taxes for local governments.

In terms of breeding, some had been trained in pig artificial insemination (AI) but not fully operational. Once AI input supply chain is in place, more pigs will be serviced by AI. They noted that sharing boars for breeding female pigs was common and can be a potential source of spread of diseases particularly ASF in case of an outbreak.

Regarding on-farm biosecurity practices, all animal health workers carried disinfectants on their motorcycles and disinfected their personal protective equipment (PPE) whenever they left a pig farm. They had previously attended training on observing biosecurity measures. Additionally, after completing work at a farm, they disinfected the tires of their motorcycles.

Prevalence of African swine fever virus

A total of 777 blood samples were analyzed in the laboratory for the presence of African swine fever antibodies from both Katosi and Kasawo sub-counties. The prevalence of African swine fever was zero for both Kasawo and Katosi sub-counties.

Table 3: Blood samples of pigs analysed and the prevalence of ASF virus in pigs

Sub-county	Female pigs	Male pigs	Total number of samples	Prevalence (%)
Kasawo	267	193	460	0
Katosi	196	121	317	0
Overall	463	314	777	0

DISCUSSION

ASF seroprevalence for both Katosi and Katosi sub-counties was 0%. This finding is comparable to those reported by other scholars. Some studies have also reported similar findings of 0% in South West and Central Kenya (Okoth et al., 2013; Abworo et al., 2017; Drider et al., 2022) and 0% in Central Uganda (Muhangi et al., 2015). The finding of no seropositive pigs in the two sub counties was partly attributed to highly virulent ASFV strains that cause very high mortality in the affected pigs. At the time of sampling, all affected pigs had died. Additionally, farmers keep pigs for a shorter period of time, and it is possible that survivor pigs after ASF outbreaks are sold off. This significantly reduces the chances of finding serologically positive pigs on farms.

However, other studies have reported higher seroprevalence for ASF. Patrick et al. (2020) obtained 37% in South Kivu Province in DRC; Abwage et al. (2015) and Patrick et al. (2020) reported 13.3% in Taraba state in North East

Nigeria. The differences in the regional findings could probably be explained by the type of husbandry system used as well as the circulating regional ASFV strains (Eblé et al., 2019). Additionally, the higher seropositivity could be attributed to the fact that over time, pigs may recover from the carrier state and continue to test positive serologically. The higher seropositivity could also be due to the pathogenicity of the circulating virus strains. Pigs infected with low pathogenic virus strains may recover and become carriers and therefore remain serologically positive (Eblé et al., 2019). When blood samples, tonsils and lymph nodes from slaughtered pigs in Kampala metropolitan area were collected simultaneously, Okwasiimire, (2022) identified that 0.15%, n=1,208 serum samples only had detectable antibodies yet 59.5% were positive for ASFV DNA. Similarly, other studies in Uganda reported seropositivity of 0.2% in Mubende (Muwonge et al., 2012). The high values were attributed to sampling from pig abattoirs; notably, sampled pigs could have already been in incubation state. Farmers usually sell off their pigs when they suspect a potential disease outbreak in order to avoid losses when a disease outbreak has been confirmed (Muwonge et al., 2012; Atuhaire et al., 2013; Muhangi et al., 2015; Asambe et al., 2019).

In a comparative study at the Western border of Kenya and the Eastern border of Uganda, Abworo et al. (2017) compared different diagnostic techniques for ASF on asymptomatic pigs. It was found that samples taken from tissues including tonsils, lymph nodes, spleen, heart, lungs and liver had better chances of detecting ASFV compared to serological techniques. This further highlights the role of asymptomatic carrier pigs in the spread of ASF. Notably, these samples were obtained after pigs were slaughtered/sacrificed. The last outbreak in Kasawo sub-county had been reported at least six months before samples were taken in the present study. However, the reports were not substantiated with laboratory evidence suggesting possible occurrence of other diseases that present similar or related clinical signs as ASF. Such diseases include swine erysipelas, septicaemic salmonellosis, highly pathogenic porcine reproductive and respiratory syndrome, porcine dermatitis nephropathy among others (Porras et al., 2024).

The majority of farmers kept their pigs confined (99.4%). This could be due to the limited grazing or roaming space and the presence of crop farming activities, as most farmers own small plots of land where they engage in various agricultural enterprises. This practice of confining pigs limits physical interaction among pigs from different farms, which occurs in a free-range system and therefore minimizes the risk of transmission of pig diseases including ASF. Several biosecurity concerns were identified at the farm level, including the sharing of mating boars between pig farms, unauthorized access to pig

units by visitors, the slaughter of sick or dead pigs for sale and consumption, and the open disposal of effluent from pig slaughter. Some of these practices had also been reported by other scholars in earlier studies in other areas of Uganda (Tejler, 2012; Muhangi et al., 2014). The management practices of the majority of farmers in this study was low input in terms of investment and therefore pig farmers may be reluctant to implement the basic biosecurity measures at the farms as they see that as an additional cost of production (Nantima et al., 2015). The slaughter or sale of sick pigs is often done to recover some monetary value and prevent a total loss, disregarding the risk of further spreading the disease. Farmers reported practices of slaughtering sick pigs for sale or consumption, as well as improper disposal of carcasses through open dumping.

The study also identified gaps in terms of hygiene for most of the pigsty and feeders/troughs. Only 39% and 36% of the farmers cleaned the troughs/feeders and pig houses daily respectively; for example, some feeders/troughs and pigsties were found in extremely dirty conditions. This practice may stem from farmers being occupied with other activities, leading to limited attention given to the piggery project, or from a lack of understanding regarding the importance of hygiene. These poor sanitary practices are a risk factor for emergence and spread of other pig diseases at the farm. The farmers also mentioned the most common signs of ASF in pigs including shivering, lack of appetite, weakness, recumbence, high fever, redness of the skin at the body extremities, and death. This suggests the farmers' ability to identify the ASF disease and report it in case of an outbreak in the community. Nonetheless, laboratory confirmation is necessary to rule out other diseases of pigs that present similar or related clinical signs as ASF. However, the obstacle to reporting could be attributed to fear of losses in case of imposition of quarantine since there is no compensation to affected pig farmers (Tejler, 2012).

Animal health providers, veterinary paraprofessionals in particular operating in the areas of both Katosi and Kasawo sub-counties were equipped with disinfectant for decontaminating on their motorcycles and their footwear at all times when entering and leaving farms they visit. This is a commendable biosecurity practice and potentially plays a significant role in limiting the spread of pig diseases between farms. In outbreaks, fomites such as trucks and veterinary equipment, clothes, gumboots among others can carry the virus from farm to farm when proper disinfection is not carried out (Blome et al., 2020). Farmers also reported practices of slaughtering sick pigs for sale or consumption, improper disposal of carcasses by open dumping. These practices have also been reported in other studies (Chenais et al., 2017) and contribute to

spreading the ASF virus during outbreaks.

Lack of authorized and centralized pig slaughter places in local government areas of jurisdiction was identified as one of the factors contributing to introduction and spread of ASFV in the environment in cases of outbreaks or when a pig is slaughtered in a rural setting (Dione et al., 2018). In that case, environmental contamination with blood and body effluent is disposed of in the local environment. It has been established that the ASFV can stay in the environment for a varied length of time. In Armenian conditions, the ASFV survived in the environment for at least six months (Arzumanyan et al., 2021). Scientific research conducted in the 20th century has shown that the ASFV is resistant to heat exposure, desiccation, and decay (McKercher et al., 1978). Other investigations report that the best place for infective ASF virion would be the bone marrow from intact tubular bones e.g. the femur of buried carcasses. Outside the pig carcass, infective virions may not stay beyond one month during summer (Karalyan et al., 2019). However, it has been shown that ASFV can persist in tissues for several months; the virus can survive for more than 100 days in Iberian-cured pork products and sausages (Farez and Morley, 1997). It has been estimated that contaminated pens in tropical regions can remain infectious to domestic pigs for up to three days. In an experiment carried out in order to estimate how long ASF virions remain infective in a contaminated environment by exposing uninfected pigs to ASFV-contaminated pens for varied length of time, pens remained infective for one day. Exposure of 2-7 days did not result in infection confirming that although ASFV environmental contamination results in infection, it happens in a short window (Olesen et al., 2018). In fact, resistance of the virus to inactivation means transmission by fomites such as clothing, equipment and vehicles remain risks (Wilkinson, 1989). That is why it is imperative for animal health workers to disinfect their vehicle tyres as well as their protective clothes between farms.

The results from this study showed that there are no centralised pig slaughter places in the district. Centralised slaughter places would offer several advantages, the first being enhanced public health. All slaughtered pigs would undergo meat inspection, and any pigs found to carry zoonotic diseases, such as porcine cysticercosis, would be condemned. In the present arrangement, less than 10% of slaughtered pigs are inspected by qualified veterinarians or public health practitioners simply because an animal health worker may not move into all villages where slaughter takes place on a daily basis while performing other designated duties. Secondly, the spread of the virus in the environment would be minimised. All effluent would be disposed of safely in a designated place while at the same time any effluent treatment would be done

centrally. Thirdly, revenue collection for the local government would be enhanced leading to better provision of social services to the local population. The following simple model illustrates this point: if 60 pigs are slaughtered daily across the entire district, and a local tax of 5,000 Uganda Shillings (UShs) is charged per pig, it would generate 9,000,000 UShs monthly, which translates to 108,000,000 UShs annually (approximately 30,000 USD). This revenue would be used for the maintenance of slaughter facilities as well as providing other essential services in the community and district at large. If the numbers of slaughtered pigs increased, the revenue would proportionately increase.

CONCLUSION

This study revealed key drivers through which ASF is transmitted in local communities. The practice of ASFV being transmitted via fomites was lowered by animal health workers carrying disinfectants on motorcycles. Since these personnel move from farm to farm, the risk would be significant if this measure was not being implemented. Secondly, increasing farmers awareness of recognizing the disease in pigs and actions to take when suspected cases are observed need to be enhanced. Very few homesteads practiced biosecurity measures at pig pens, a practice that should be widely practiced. Lastly, there is a need to establish centralized pig slaughter facilities as there are many advantages to this practice: establishment of centralized collection and disposal of effluent from the slaughter process. There is also an improvement in public health, as pork is inspected by qualified personnel, with appropriate actions taken, especially if pigs carry zoonotic diseases like porcine cysticercosis. This would allow local authorities to better manage the facility, improve data collection on pig health in the area, implement effective disease control measures, and boost local revenue collection.

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Author's Contribution:

SB and FM conceptualised the study; GO, JO and SB collected field data. GO and SB analysed the data; SB, GO and FM wrote the manuscript.

Competing interest

The authors declare that they have no financial or personal relationships that could have improperly influenced the writing of this article.

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THE PRESENCE OF *SALMONELLA ENTERICA* AND *LISTERIA MONOCYTOGENES* ON POULTRY SLAUGHTER PLANT EQUIPMENT AFTER SANITATION PROCEDURES

Suzana Vidaković Knežević^{1*}, Miloš Pelić¹, Marina Žekić¹,
Slobodan Knežević¹, Jelena Vranešević¹, Dragana Ljubojević Pelić¹, Sara Savić¹

¹Scientific Veterinary Institute „Novi Sad”, Novi Sad, Republic of Serbia

Abstract

The persistence of *Salmonella enterica* and *Listeria monocytogenes* on poultry slaughter plant equipment and food contact surfaces is an important factor for food contamination. Both bacterial foodborne pathogens can form biofilms and survive stress caused by sanitation procedures. The aim of this study was to evaluate the degree of contamination of poultry slaughter plant equipment by *Salmonella enterica* and *Listeria monocytogenes* after cleaning and disinfection. **Samples were taken from** four areas of the poultry slaughter plant (i.e., slaughter line, cutting line, meat processing, and packing area) and evaluated using real-time quantitative PCR. In total 32.5% of samples were positive for *Salmonella enterica*, while 37.5% were positive for *Listeria monocytogenes*. Both *Salmonella enterica* and *Listeria monocytogenes* were mostly detected on the slaughter line, mostly after evisceration. Evisceration, or removal of the birds' internal organs, may present a high-risk step of cross-contamination due to gut breakage and transfer of the contents to the skin, other carcass surfaces, and on equipment surfaces. This study indicates that poultry slaughter plant equipment may harbor foodborne pathogens like *Salmonella enterica* and *Listeria monocytogenes* after applying standard sanitation procedures.

Key words: foodborne pathogens, cross-contamination, real-time quantitative PCR, food contamination

^{1*} Corresponding Author: suzana@niv.ns.ac.rs

PRISUSTVO *SALMONELLA ENTERICA* I *LISTERIA MONOCYTOGENES* NA OPREMI U POGONU ZA KLANJE ŽIVINE NAKON SPROVEDENE PROCEDURE SANITACIJE

Suzana Vidaković Knežević¹, Miloš Pelić¹, Marina Žekić¹,
Slobodan Knežević¹, Jelena Vranešević¹, Dragana Ljubojević Pelić¹, Sara Savić¹

¹Naučni institut za veterinarstvo „Novi Sad”, Novi Sad, Republika Srbija

Kratak sadržaj

Perzistentnost organizama *Salmonella enterica* i *Listeria monocytogenes* na opremi u pogonima za klanje živine kao i na površinama koje su u kontaktu sa hranom predstavljaju značajan faktor u pogledu kontaminacije hrane. Oba pomenuta bakterijska patogena mogu da formiraju biofilmove i da prežive na površinama nakon procesa sanitacije. Cilj ovog istraživanja bio je da se proceni stepen kontaminacije opreme u pogonu za klanje živine bakterijama *Salmonella enterica* i *Listeria monocytogenes* nakon čišćenja i dezinfekcije. Uzorci su sakupljeni na četiri lokacije u pogonu za klanje (linija klanja, linija rasecanja, zona za obradu mesa i zona za pakovanje) i analizirani pomoću real-time kvantitativne PCR metode. Ukupno 32,5% uzoraka bilo je pozitivno na *Salmonella enterica*, a 37,5% na *Listeria monocytogenes*. Obe bakterije, *Salmonella enterica* i *Listeria monocytogenes* su najčešće detektovane na liniji klanja, uglavnom nakon evisceracije. Evisceracija odnosno uklanjanje unutrašnjih organa, može predstavljati korak visokog rizika za unakrsnu kontaminaciju zbog pucanja creva i prenosa crevnog sadržaja na kožu i ostale površine trupa, kao i na površinu opreme. Ova studija je pokazala da se na opremi u pogonima za klanje živine i nakon primene standardnih procedura sanitacije mogu zadržavati patogeni poreklom iz hrane poput *Salmonella enterica* i *Listeria monocytogenes*.

Cljučne reči: patogeni hrane, unakrsna kontaminacija, kvantitativni PCR u realnom vremenu, kontaminacija hrane

INTRODUCTION

Poultry meat is one of the primary animal protein sources in human nutrition. Due to the short production period, poultry meat production is highly

economical (Uzundumlu and Dilli, 2023). The modern poultry meat industry has achieved great progress in the field of food safety and hygiene of the production process by applying good production and hygiene practices, Hazard Analysis Critical Control Points (HACCP), and other food safety systems. Despite all that, it still faces the problem of foodborne pathogenic bacteria control, such as *Salmonella enterica* subspecies *enterica*, and *Listeria monocytogenes*. These foodborne pathogenic bacteria, in addition to being responsible for a large number of hospitalized people worldwide, also affect the shelf life of poultry meat and meat products (Redmond and Griffith, 2006).

According to Zoonoses Directive 2003/99/EC, foodborne outbreaks caused by *Salmonella* and *Listeria monocytogenes* are mandatory for reporting in Europe. *Salmonella enterica* is the etiologic agent of salmonellosis, while *Listeria monocytogenes* causes listeriosis (EFSA and ECDC, 2021). The risk groups for both foodborne diseases are mostly people with compromised immune systems, elderly, and children. Pregnant women are at an increased risk of developing listeriosis (Serenio et al., 2019). *Salmonella enterica* is a Gram-negative bacterium that can survive at pH 4.0 - 9.0, water activity > 0.94, and with temperature growth range 5 - 45 °C (Gast and Porter, 2020), while *Listeria monocytogenes* is a Gram-positive bacterium, which can survive at pH 4.6 - 9.5, water activity > 0.92, and with temperature growth range from a few degrees below 0 °C to 45 °C (Carpentier and Cerf, 2011).

Microbial contamination of poultry meat can occur during the entire poultry production chain with bacteria from the digestive tract, skin, and feathers, especially as a result of wrong techniques during slaughtering. Evisceration is a critical point of carcass contamination due to the presence of many bacteria in the gastrointestinal tract of broilers. Additionally, microbial contamination may occur from water, air, and equipment surfaces (Rouger et al., 2017).

On equipment and machine surfaces, such as stainless steel and polystyrene, at poultry slaughterhouse plants, *Salmonella enterica* and *Listeria monocytogenes* can easily adhere and form microcolonies. Further bacterial reproduction leads to biofilm formation (Vidaković Knežević et al., 2021), an association of microorganisms firmly attached to the surface, encased within an extracellular polymeric substance matrix. Biofilm acts as the constant source of microbial contamination in food processing environments (Abebe, 2020).

Real-time PCR is a valuable and sensitive method for investigating foodborne outbreaks and identifying pathogens. This method provides fast results, contrary to conventional culture methods that require at least five or more days to confirm the identification of *Salmonella enterica* and *Listeria monocytogenes* in positive samples. Besides replacing conventional time-consuming

methods, the advantages of the real-time PCR method are minimized cross-contamination and reduced manpower (Bohaychuk et al., 2007).

This study was conducted at a slaughterhouse plant to evaluate the contamination rate of equipment after sanitation and disinfection with *Salmonella enterica* and *Listeria monocytogenes*, using a real-time PCR method.

MATERIALS AND METHODS

Sample collection

The study was performed at a small-scale poultry slaughter plant in the Srem district, Republic of Serbia. This poultry slaughter plant has implemented a Hazard Analysis and Critical Control Point (HACCP) system and Good Hygiene Practice (GHP). Its main products are fresh meat in the form of whole, half, or quarter carcasses or smaller meat cuts. Further, this poultry slaughter plant processes fresh poultry meat into cured, smoked, canned, and other meat products. The sampling protocol applied in the poultry slaughter plant included sampling the equipment with cotton swabs under SRPS EN ISO 18593:2018 within 2 hours after cleaning and disinfection. Samples were taken from four different areas of the poultry slaughter plant, including the slaughter line (19 samples), the cutting line (11 samples), the processing area (8 samples), and the packing area (2 samples). A total of 40 samples were collected. All samples were transported to the laboratory under cooled conditions and processed the same day.

DNA extraction and real-time PCR

Genomic DNA extraction from swabs was performed using the Genesig Easy extraction kit (Primedesign™ Ltd, Eastleigh, United Kingdom) through a six-step process (Žekić Stošić et al., 2021). The detection and quantification of *Salmonella enterica* and *Listeria monocytogenes* genomes were performed using the commercial genesig real-time PCR kits (*Salmonella enterica* Genesig® Easy kit, and *Listeria monocytogenes*, Primedesign™ Ltd, Eastleigh, United Kingdom). Both kits contain primers representing 100% homology with over 95% of the National Center for Biotechnology Information Database reference sequences. The *Salmonella enterica* kit targets the *invA* gene, while the *Listeria monocytogenes* kit contains specific primers for quantifying all *Listeria monocytogenes* and does not detect other *Listeria* species. Under optimal PCR conditions, detection kits have very high priming efficiencies of > 95% and

can detect less than 100 copies of the target template. In both of these qPCR protocols, there are 50 cycles. Every cycle consisted of enzyme activation at 95 °C for two minutes, followed by denaturation at 95 °C for 10 seconds and data collection at 60 °C for one minute. Genomic DNA from *Salmonella Enteritidis* ATCC 13076 and *Listeria monocytogenes* ATCC 19118 served as positive controls, while sterile water was a negative control.

RESULTS

According to the preslaughter documentation, the flock slaughtered on the sampling day had an official *Salmonella enterica* negative status. In total, 40 samples (Table 1.) were collected from four areas of the poultry slaughter plant after cleaning and disinfection. Thirteen samples were *Salmonella enterica* positive (32.5%), and fifteen were *Listeria monocytogenes* positive (37.5%) by qPCR method. Eleven samples were positive for both *Salmonella enterica* and *Listeria monocytogenes* (27.5%). Along the slaughter line, both *Salmonella enterica* and *Listeria monocytogenes* were first detected in the evisceration line. From the vent cutter drip tray to the giblet sorting table, only the foot loop of the eviscerator and the neck skin cutter were free from *Salmonella enterica*. Similarly, *Listeria monocytogenes* was detected throughout the evisceration line, starting from the vent opener cutter assembly (blade). In total, 12 (63.16%) and 13 (68.42%) of the 19 samples taken from the cleaned and disinfected slaughter line were *Salmonella enterica* and *Listeria monocytogenes* positive, respectively. *Salmonella enterica* and *Listeria monocytogenes* positive samples were taken from the slaughter line (57.89%).

Table 1. Distribution of *Salmonella enterica* and *Listeria monocytogenes* on poultry slaughter plant equipment surfaces.

No.	Areas of poultry slaughter plant	Surfaces	<i>Salmonella enterica</i>	<i>Listeria monocytogenes</i>
1	Slaughter line	Live bird hang-on area	Negative	Negative
2		Waterbath Stunner	Negative	Negative
3		Shackle of the slaughtering conveyor	Negative	Negative
4		Rubber fingers of plucker	Negative	Negative
5		Vent cutter assembly (blade)	Negative	Negative
6		Vent cutter drip tray	Positive	Negative
7		Vent opener cutter assembly (blade)	Positive	Positive
8		Vent opener drip tray	Positive	Positive
9		Evisceration spoon	Positive	Positive
10		Foot loop of eviscerator	Negative	Positive
11		Wing grid of eviscerator	Positive	Positive
12		Breastplate of eviscerator	Positive	Positive
13		Frame (base of the machine) of eviscerator	Positive	Positive
14		Glaze wheel of eviscerator	Positive	Positive
15		Cropping machine cleaning mandrel	Positive	Positive
16		Neck skin cutter	Negative	Positive
17		Throat slitting machine stand	Positive	Positive
18		Giblet harvesting	Positive	Positive
19		Giblet sorting table	Positive	Positive

No.	Areas of poultry slaughter plant	Surfaces	<i>Salmonella enterica</i>	<i>Listeria monocytogenes</i>
20	Cutting line	Shackle	Negative	Negative
21		Whole wing cutter	Negative	Negative
22		Saddle cutter	Negative	Negative
23		Drum and thigh cutter	Negative	Negative
24		Breast cap cutter	Positive	Negative
25		Portioning spindle (cone)	Negative	Positive
26		Chicken breast filleting machine conveyor belt	Negative	Negative
27		Sloped belt conveyor for boneless chicken breasts	Negative	Negative
28		Planed belt conveyor for boneless chicken breasts	Negative	Positive
29		Whole wings conveyor belt	Negative	Negative
30		Drums and thighs conveyor belt	Negative	Negative
31	Processing area	Wolf meat grinder machine No. 1	Negative	Negative
32		Wolf meat grinder machine No. 2	Negative	Negative
33		Baader Meat Separator	Negative	Negative
34		Meat Filling Machine	Negative	Negative
35		Cutter	Negative	Negative
36		Tumbler	Negative	Negative
37		Meat cart	Negative	Negative
38		Floor drain	Negative	Negative
39	Packing area	Meat slicer knife	Negative	Negative
40		Meat slicer conveyor belt	Negative	Negative

One more *Salmonella enterica* positive sample was collected in the cutting line from the knife of the breast cap cutter machine. This sample was negative for *Listeria monocytogenes*. However, *Listeria monocytogenes* positive samples in this area of the poultry slaughter plant were portioning spindle (cone) and the planed belt conveyor for boneless chicken breasts.

Both the processing and packing area tested negative for *Salmonella enterica* and *Listeria monocytogenes*.

DISCUSSION

At the four stages of the poultry production chain investigated (the slaughter line, the cutting line, the processing area, and the packing area), the prevalence of *Salmonella enterica* ranged from 0% to 63.16%, while the prevalence of *Listeria monocytogenes* ranged from 0% to 68.42%.

As we only looked into one specific poultry slaughter plant, the prevalence found at the various production stages cannot necessarily be generalized. Besides, PCR may be too sensitive and detect microorganisms that are present at non-pathogenic levels (Mackay, 2004). However, the result was expected because the area with the highest prevalence is also the dirtiest in the poultry slaughter plant. The observed difference in prevalence could also be explained by the very different number of samples or by the variable efficiency of sanitation practices. Earlier studies show differences in cleaning and disinfection when performed by the slaughterhouse staff and external professional cleaning firms (Zeng et al., 2021). In our study, cleaning and disinfection are performed by the staff working in the specific poultry slaughter plant area. The difference in cleaning and disinfection results could be caused by the lack of food safety awareness and the level of training, motivation, etc. Besides that, the accessibility of surfaces for cleaning and disinfection plays an important role in *Salmonella enterica* and *Listeria monocytogenes* presence. Additionally, the positive rate of swabs at the slaughter and evisceration line is because *Salmonella enterica* and *Listeria monocytogenes* are commensally pathogens of the gut for many animals, including poultry, from where they can contaminate equipment surfaces during the evisceration and enter into the food chain. It has been previously suggested that *Salmonella enterica* **serovar** Enteritidis, *Salmonella enterica* **serovar** Typhimurium, and *Listeria monocytogenes* could persist in a meat processing environment by attaching firmly to stainless steel and plastic surfaces and forming biofilms (Vidaković Knežević et al., 2021; Vidaković Knežević et al., 2023). Forming biofilms is an important survival strategy for bacteria due to unfavorable conditions. Adhered cells tend to be more resistant to cleaning and disinfection than suspended bacteria. Once established, biofilms act as permanent sources of contamination and dispersal in the environment, leading to cross-contamination (Alvarez-Ordóñez et al., 2019).

The total elimination of *Salmonella enterica* and *Listeria monocytogenes*

from food processing environments is almost impossible because of their ubiquitous presence in nature and the many potential avenues for entry into these processing environments (Duze et al., 2021). Additionally, the constant use of the same disinfectants and the sub-lethal concentrations may lead to resistance of these foodborne pathogens to the antibacterial agent applied (Langsrud et al., 2003). Therefore, controlling *Salmonella enterica* and *Listeria monocytogenes* in poultry slaughterhouses is complex.

CONCLUSION

The prevalence of *Salmonella enterica* and *Listeria monocytogenes* in the poultry slaughter plant studied was very high, with up to over 60% of equipment surfaces. The most critical point is the stage of evisceration. The complex machines, containing many small and hard-to-reach parts, make cleaning and disinfection processes challenging. In order consider these results as food safety and health risks, it is necessary to isolate and identify *Salmonella enterica* and *Listeria monocytogenes* because those methods are irreplaceable as confirmatory methods in the microbiology of food. Elementary principles for hygiene must be respected, including effective mechanical cleaning, the recommended concentrations of antibacterial agents, contact time, rinsing, and drying. All workers involved in hygiene must be well-trained.

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Author's Contribution

SVK basic idea, conception, and design, drafting the manuscript; SVK and MP sampling; MŽ and SS examination; SK and JV interpretation of results and manuscript revision; DLJP conception and design. All authors read and approved the final version of the manuscript.

Competing interest

The authors declare that they have no competing interests

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THE IMPACT OF COVID-19 ON HUMAN-PET RELATIONSHIPS IN MALAYSIA AND INDONESIA: A PUBLIC SENTIMENT ANALYSIS

Nazhan Ilias¹, Fathrurahiman Saidin¹, Nur Indah Ahmad²,
Moniza Waheed³, Murni Wan Mohd Nor⁴, Mokrish Ajat^{1,5*}

¹ Universiti Putra Malaysia, Faculty of Veterinary Medicine, Department
of Veterinary Preclinical Sciences, Serdang, Selangor, Malaysia

² Universiti Putra Malaysia, Faculty of Veterinary Medicine, Department of
Veterinary Pathology and Microbiology, Serdang, Selangor, Malaysia

³ Universiti Putra Malaysia, Faculty of Modern Languages and Communication,
Department of Communication, Serdang, Selangor, Malaysia

⁴ Universiti Putra Malaysia, Faculty of Human Ecology, Department of
Government and Civilization Studies, Serdang, Selangor, Malaysia

⁵ Universiti Putra Malaysia, Institute of Bioscience, Natural Medicines and
Products Research Laboratory (NaturMeds), Serdang, Selangor, Malaysia

Abstract

Human stress levels escalated amid the COVID-19 epidemic as a result of restrictions on social interactions and movement. Furthermore, due to the lack of awareness about the disease's characteristics, numerous cases of violations of animal welfare occurred. The study is focused on the macro-level analysis regarding the impact of COVID-19 on human-pet interactions in Malaysia and Indonesia. A total of 1,829 tweets related to human-pet interactions during COVID-19 were retrieved from Twitter between March 17th and September 17th, 2020. Natural Language Toolkit (NLTK) was utilized to analyze the tweets with human moderation. The analysis revealed a large number of neutral and positive sentiments in the initial stage of the study. Later, positive public sentiment (50%, n = 27/54) rose in Malaysia significantly as the Twitter users were demanding justice for the abused animals. Meanwhile, the sentiments in Indonesia were pre-

^{1*} Corresponding author: mokrish@upm.edu.my

dominantly both neutral (42%, $n = 52/123$) and positive (34%, $n = 42/123$), with the sentiment shifting after an incidence of animal cruelty went viral. Following a study in the United Kingdom reporting positive COVID-19 cases in cats, an upward trend in negative public reaction was observed in Malaysia (35%, $n = 7/20$) and Indonesia (48.8%, $n = 40/82$). In conclusion, the public sentiment regarding the impact of COVID-19 on human-pet interactions affects individuals due to the associated health risks.

Key words: COVID-19, Twitter users, pet owners, sentiment analysis, Malaysia, Indonesia, animal welfare, pandemic effects

UTICAJ COVID-19 NA ODNOS IZMEĐU LJUDI I KUĆNIH LJUBIMACA U MALEZIJI I INDONEZIJI: ANALIZA JAVNOG MNJENJA

Nazhan Ilias¹, Fathrurahiman Saidin¹, Nur Indah Ahmad², Moniza Waheed³, Murni Wan Mohd Nor⁴, Mokrish Ajat^{1,5}

¹ Univerzitet Putra Malezija, Fakultet veterinarske medicine, Katedra za predkliničku veterinarsku medicinu, Serdang, Selangor, Malezija

² Univerzitet Putra Malezija, Fakultet veterinarske medicine, Katedra za veterinarsku patologiju i mikrobiologiju, Serdang, Selangor, Malezija

³ Univerzitet Putra Malezija, Fakultet modernih jezika i komunikacije, Katedra za komunikacije, Serdang, Selangor, Malezija

⁴ Univerzitet Putra Malezija, Fakultet humane ekologije, Katedra za vladine i civilizacijske studije, Serdang, Selangor, Malezija

⁵ Univerzitet Putra Malezija, Institut za biološke nauke, Laboratorija za istraživanje prirodnih lekova i proizvoda (NaturMeds), Serdang, Selangor, Malezija

Kratak sadržaj

Stres kod ljudi eskalirao je tokom pandemije COVID-19 kao posledica ograničenja društvenih interakcija i slobode kretanja. Usled nedovoljne informisanosti o karakteristikama bolesti, došlo je do brojnih slučajeva narušavanja dobrobiti životinja. Ovaj rad analizira na makro nivou uticaj COVID-19 na interakcije između ljudi i kućnih ljubimaca u Maleziji i Indoneziji. Ukupno 1.829 tvitova vezanih za interakcije ljudi i kućnih ljubimaca tokom pandemije COVID-19 preuzeto je sa Tvitera u periodu

od 17. marta do 17. septembra 2020. godine. Korišćeni su alati za obradu prirodnog jezika (NLTK) kako bi se izvršila analiza tvitova, uz moderiranje sadržaja vezanog za ljude. Analiza je otkrila veliki broj neutralnih i pozitivnih sentimenata prema životinjama u početnoj fazi studije. Kasnije je pozitivno raspoloženje javnosti (50%, $n = 27/54$) značajno poraslo u Maleziji, jer su korisnici Tvitera tražili pravdu za zlostavljane životinje. U međuvremenu, sentiment prema ljubimcima u Indoneziji bio je pretežno neutralan (42%, $n = 52/123$) i pozitivan (34%, $n = 42/123$), a do promena je došlo je nakon što je okrutnost prema životinjama eskalirala. Nakon studije iz Ujedinjenog Kraljevstva u kojoj su navedeni pozitivni slučajevi COVID-19 kod mačaka, u Maleziji (35%, $n = 7/20$) i Indoneziji (48,8%, $n = 40/82$) uočen je trend porasta negativnih reakcija javnosti. Odnos javnog mnjenja prema uticaju COVID-19 na odnos između ljudi i ljubimaca utiče na pojedince zbog rizika koji nosi po zdravlje ljudi.

Ključne reči: COVID-19, korisnici Twitter-a, vlasnici kućnih ljubimaca, analiza javnog mnjenja, Malezija, Indonezija, dobrobit životinja, uticaji pandemije

INTRODUCTION

The COVID-19 pandemic has caused unprecedented changes to human society, affecting various aspects of our lives, including interactions with animals. The imposed movement restrictions and social distancing measures have led to increased stress and anxiety among individuals, prompting many to seek solace in the companionship of pets (Martin et al., 2021). However, the pandemic has also been accompanied by concerning reports of animal abuse and abandonment (Carroll et al., 2022), raising questions about the impact of public sentiments on human-animal interactions during this challenging period. Early in the pandemic, concerns arose regarding the potential for SARS-CoV-2 transmission from humans and pets. Researchers initiated investigations to determine if pets, particularly dogs and cats, could serve as reservoirs or vectors for the virus (Hobbs and Reid, 2021). Several studies have reported instances of pets testing positive for SARS-CoV-2, primarily after close contact with COVID-19-positive owners (Sit et al., 2020; Garigliany et al., 2020; Newman et al., 2020; Barrs et al., 2020). Cats appear to be more susceptible than dogs, with some ferrets and other domesticated animals also showing susceptibility (Meisner et al., 2022). These cases sparked interest in understanding the

dynamics of human-to-animal transmission. While the primary concern was initially focused on human-to-pet transmission, research has also explored the possibility of reverse zoonotic transmission, where infected pets might transmit the virus back to humans (Meisner et al., 2022).

During crises, timely and accurate information is crucial. Sentiment analysis aids in crisis communication by assessing public reactions, identifying misinformation, and evaluating the effectiveness of emergency response efforts (Aldosery et al., 2024). This is particularly evident in natural disasters, public health emergencies, and other crises (Li et al., 2020). As the use of public sentiment in scientific research grows, literature also addresses ethical considerations and challenges (Hung et al., 2020). Issues related to privacy, bias in sentiment analysis algorithms, and the responsible use of data have emerged as critical issues for researchers and policymakers (Ligthart et al., 2021). Twitter datasets are a valuable data resource for investigating various research problems to enhance knowledge, innovation, and discovery in various sectors (Thakur, 2022). Studying the pattern of tweets may enable researchers to analyze the severity of public emotion towards the pandemic and its effect on humans and pets.

Social media, especially Twitter, has emerged as a powerful platform for sharing opinions, with its open text-sharing format providing valuable insight into public sentiment worldwide (Aldosery et al., 2024). Public sentiments, expressed through various channels such as social media, have become a valuable resource for scientific inquiry. In the context of public health, monitoring public sentiments on social media has proven to be a valuable tool for early disease surveillance and outbreak detection (Cui et al., 2023). Research has demonstrated the feasibility of using sentiment analysis to identify trends, detect emerging health concerns, and track the spread of diseases, including infectious diseases like flu and COVID-19 (Tan et al., 2021). Researchers investigating social issues and public opinion turn to sentiment analysis to evaluate societal attitudes. This demonstrates how sentiments expressed on social media platforms reflect public opinions on diverse topics, including social justice, equality, and human rights (Drus and Khalid, 2019).

Early coordinated responses to the pandemic are often linked to general positivity expressed in public sentiments (Lwin et al., 2022). However, negative sentiments have appeared as the public's frustration grows, with the pandemic affecting their health mental physical health. As the lockdown and restriction continued, certain countries experienced superstition, fatalism or anxiety, while others showed more positive reaction. The majority of the citizens accepted the restrictive measures, trusting the authorities' health measure and

availability of adequate public information (Lim et al., 2021). In this study, a macro-level analysis of public sentiment around COVID-19 is conducted and how these collective attitudes have impacted human-pet relationships in Malaysia and Indonesia is examined. Utilizing Twitter as the primary data source, this research investigates trends in public discourse related to pets during the pandemic, exploring how shared experiences and concerns influence pet ownership, care practices, and emotional reliance on pets. The analysis highlights the changing role of pets in offering companionship and support during times of crisis, influenced by the regional cultural and social contexts of these two countries.

MATERIALS AND METHODS

Authentication

Twitter developer account authentication is a process that allows developers to access Twitter's API on behalf of users. A Twitter developer account was obtained as required for this study. This process grants three main components: the bearer token, the access token, and the access token secret. To authenticate a request to the Twitter API, these tokens were included. The use of these tokens is essential for authentication in Python, enabling the data mining of tweets. (see Appendix A).

Installation of tools

PyCharm Community Edition was installed with the goal of providing the Python coding notebook. Several tools were installed in the notebook terminal, such as *ConfigParser*, *Tweepy*, *Pandas*, and *NLTK*. The installation required codes in the terminal (see Appendix B). *Configparser* was installed in order to allow the authentication tokens to be read in the codes; *Tweepy* was used for tweet search and collection; *Pandas* enabled the tweets mined by *Tweepy* to be read and saved; and the tweets were analysed by *NLTK*.

Data mining using Twitter API

The tweets were mined using *Tweepy* with authentication tokens obtained using Python codes (see Appendix C). To specify the tweets for this study, the keywords for query input are needed. In this study, the search term "cat OR dog OR pet from Malaysia (Wuhan virus OR corona OR COVID-19 OR

COVID OR lockdown OR MCO) since:2020-03-17 until:2020-09-17” was inputted in the queries. The words that were located outside of the bracket indicated the main keyword to be searched for; in this case, “cat,” “dog,” and “pet.” The location of either Malaysia or Indonesia was inputted by “from:” and the country of the subject. The words in parentheses indicated as the word to search matches with the main keyword and time duration of the search were enclosed by the inputted “since” and “until.” For the purpose of this study, the analysis comprised a total of 1,829 country-identifiable tweets from Malaysia (636) and Indonesia (1193).

Analysis of public sentiment

The data was then analyzed using the sentiment analyzer in the *NLTK* (see Appendix D). The tweets were systematically classified on a 1 to 5 scale to assess the public sentiment regarding the impact of COVID-19 on human-pet interactions. This scale was designed to capture a range of emotional responses expressed by the public during the pandemic. A score of 1 was assigned to tweets that conveyed a very negative sentiment, reflecting strong disapproval or concern about the effects of COVID-19 on human-pet relationships. A score of 2 indicated a negative sentiment, where the tone was generally unfavorable, expressing dissatisfaction or anxiety about the situation. Tweets with a score of 3 were classified as neutral, reflecting an absence of strong emotional reaction, indicating that the content did not exhibit a clear positive or negative stance on the issue. A score of 4 represented positive sentiment, with tweets expressing a generally favorable view of how COVID-19 influenced human-pet interactions, perhaps highlighting positive outcomes or coping mechanisms. Finally, tweets receiving a score of 5 were deemed very positive, reflecting enthusiasm, appreciation, or optimism regarding the bond between humans and pets amidst the challenges posed by the pandemic. This classification provided a comprehensive means to evaluate the public’s emotional response to the evolving dynamics between humans and pets during the COVID-19 pandemic. To generate an accurate hybrid public sentiment approach, human moderation was implemented along with the analysis. In human moderation, each one of the tweets was read and re-rated to avoid being mistakenly classified. The examples of tweets that were data-mined from Malaysia (Table 1) and Indonesia (Table 2) were rated 1 to 5 based on the public sentiments.

Table 1. Examples of public sentiments tweets rated in Malaysia

Rating	Public Senti-ments	Examples
1	Very Negative	Original: Bodoh ye kucing ni kena-pa tak kene corona ek Translation: This cat is stupid, why doesn't it have covid?
2	Negative	Original: Boleh sangat nak bela ambil kucing tepi jalan.... Tapi risau takut ada corona hmmm Translation: You can really adopt the street cat... But I'm worried about covid hmmm
3	Neutral	Original: @detikcom Ini bukan hal baru. memang ada corona virus pada kucing. Translation: @detikcom This is nothing new. There is indeed a corona virus in cats.
4	Positive	Original: Ketika negara mu lockdown tapi kucing adalah yang utama Translation: When your country is in lockdown, but cats are the main thing
5	Very Positive	Original: Pujuk hatiii .. hg bnyk kongsi dgn kucing hg ... Tu yg rezeki mai semulaaaa ... Macehhh kucing ² kakak .. maceh sbb teman time mco Tq moni sushi batik n moty .. i gonna miss u play around .. Translation: Persuading my heart ... you have shared a lot with your cats ... They were the one that provided sustenance ... Thank you my cats .. Thanks for accompanying me during MCO Thank you Moni, Sushi, Batik, and Monty .. I'm going to miss you playing around ..

Table 2. Examples of public sentiments tweets rated in Indonesia

Rating	Public Senti-ments	Examples
1	Very Negative	Original: @kumparan Besok minta Ke-mentan mbuka peternakan kucing hitam. Tuh Vietnam sukses menekan corona Translation: kumparan Tomorrow ask the Ministry of Agriculture to open a black cat ‘farm’. Vietnam has succeeded in suppressing covid.
2	Negative	Original: @thisiscakelat Nongki terus, g takut corona kucing muu Translation: @thisiscakelat Keep hanging out, don’t be afraid of covid your cat
3	Neutral	Original: Si Moren, kucing di rumah udah mulai bersin-bersin, flu mungkin. Ini kucing ga bisa jadi carrier COVID-19 kan? Anybody? Translation: Moren, the cat at home has started sneezing, maybe it’s a cold. This cat can’t be a carrier of COVID-19, right? Anyone?
4	Positive	Original: Mantan model di Gowa, Sulawesi Selatan, selamatkan puluhan kucing yang telantar di saat pandemi COVID-19. #IndosiarBerita Baiknya... Translation: A former model in Gowa, South Sulawesi, rescued dozens of abandoned cats during the COVID-19 pandemic. #IndosiarBerita Kind...
5	Very Positive	Original: Puasa kali ini bener-bener beda kondisinya, tapi situasinya tetep sama aja sendiri dirumah ditemani kucing kesayangan 🐱 Alhamdulillah atas segala rezeki yg dikasih Allah SWT #corona #COVID-19 Translation: The conditions of fasting this time are really different, but the situation is still the same, alone at home accompanied by your beloved cat 🐱 Thank God for all the good fortune given by Allah SWT #corona #COVID-19

Relation of public sentiment to events

Events were identified through mentions in tweets and subsequently analyzed for their impact on changes in public sentiment. Briefly, events were identified by scanning the collected tweets for mentions of specific keywords, phrases, or hashtags related to significant occurrences during the study period. These events could include government action, animal welfare, research on COVID-19 regarding pets, or any other significant events that could potentially affect public perception. Tweets over time related to each event were analyzed to identify associations and changes in public sentiment.

RESULTS

The following section provides a detailed analysis of the volume of tweets, public sentiment scores, and the qualitative labels assigned to sentiments by each country, alongside key global and local responses. In general, positive and neutral sentiments were the most prevalent among Malaysian and Indonesian Twitter users, respectively, particularly at the outset of the study. However, a noticeable shift in public sentiment occurred in both countries over the course of the study, reflecting the evolving circumstances. This change in sentiment was influenced by various events during the study period, which were closely tied to the progression of the pandemic. The trend of positive COVID-19 cases in both Malaysia and Indonesia, as depicted in Figure 1, provides a contextual backdrop for these shifts in public sentiment.

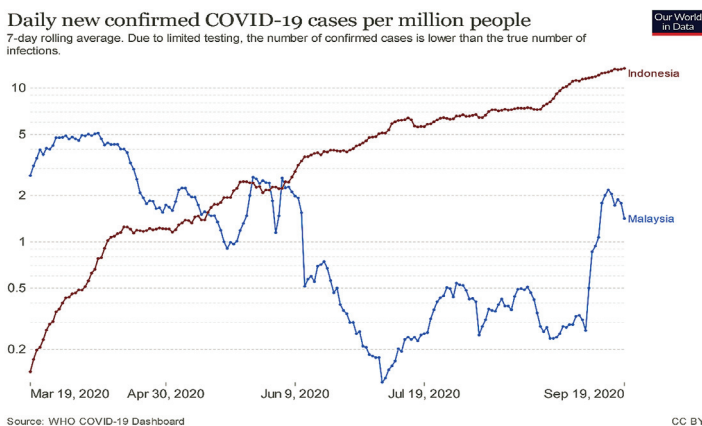


Figure 1. Logged number of positive COVID-19 in Malaysia and Indonesia from 17th March 2020 to 17th September 2020. Adapted from Our World in Data, by University of Oxford, n.d.: <https://ourworldindata.org/coronavirus/country/malaysia?country=MYS~IDN>)

The initial response (March 18, 2020)

At the outset of this study, on March 18th, 2020, coinciding with the initial stages of the COVID-19 pandemic (Figure 2), the public sentiment expressed by Malaysian Twitter users was largely neutral. Namely, 31.1% (n = 14/45) of the tweets reflected neutral sentiment. Meanwhile, both positive and very positive sentiments were recorded at 26.7% (n = 12/45), indicating a moderate level of optimism. In contrast, negative sentiment was relatively less pronounced, with 13.3% (n = 6/45) of tweets expressing dissatisfaction, while only 2.2% (n = 1/45) conveyed very negative sentiments.

Similarly, on March 18th, 2020, as shown in Figure 3, the sentiment among Indonesian Twitter users at the pandemic's onset was marked by a dominant positive response, with 47.8% (n = 11/23) of tweets reflecting positive emotions. Additionally, 26.1% (n = 6/23) of the tweets were classified as either neutral or very positive, further emphasizing a generally favourable perspective.

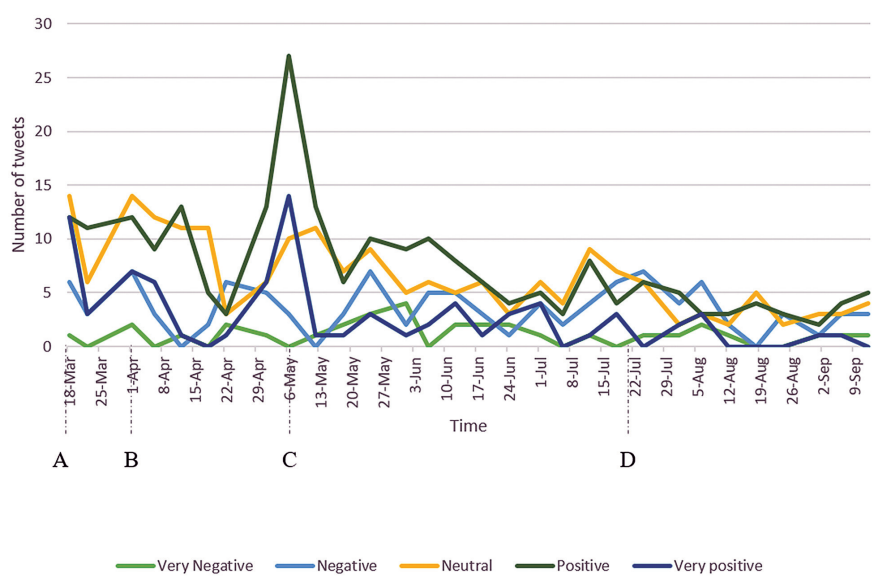


Figure 2. Public sentiments about COVID-19 Influencing the Interaction Between Pets and Human in Malaysia. (A) Movement control order (MCO) commenced, (B) second week of MCO (first extension of MCO), (C) outcry against animal cruelty, and (D) News report of COVID-19 positive in cats in United Kingdom

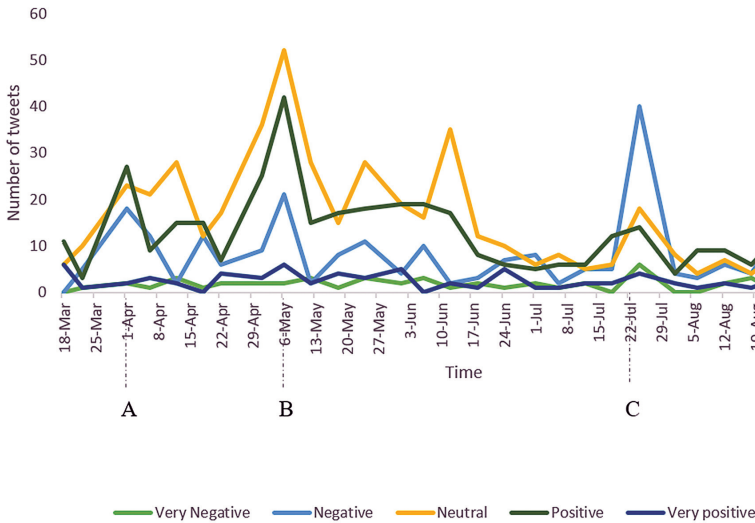


Figure 3. Public sentiments of COVID-19 in Influencing the Interaction Between Pets and Human in Indonesia. (A) Government refusal in movement restriction action, (B) outcry against animal cruelty, and (C) news report of COVID-19 positive in cats in United Kingdom

The Second Week of Movement Control Order (April 1st, 2020)

As the second week of the Movement Control Order (MCO) commenced, the public sentiment expressed by Malaysian Twitter users began to shift, with a very negative sentiment recorded at 4.8% ($n = 2/42$) and negative sentiment at 16.7% ($n = 7/42$). However, neutral and positive sentiments continued to dominate the discourse, accounting for 33.3% ($n = 14/42$) and 28.6% ($n = 12/42$) of the tweets, respectively, as depicted in Figure 2. Notably, the proportion of very positive sentiment declined to 16.7% ($n = 7/42$), which is a decrease when compared to the data collected on March 18th, 2020. In Indonesia, amidst growing public apprehension and the government's decision not to impose further movement restrictions, a noticeable shift in sentiment occurred. As shown in Figure 3, there was an increase in both very negative sentiment (2.8%, $n = 2/72$) and negative sentiment (25%, $n = 18/72$). The percentage of very positive sentiment remained low at 2.8% ($n = 2/72$), while positive (37.5%, $n = 27/72$) and neutral (31.9%, $n = 23/72$) sentiments continued to be predominant.

The Outcry Against Animal Cruelty (May 6th, 2020)

On May 6th, 2020, a widely publicized case of animal cruelty marked a pivotal moment in the public sentiment expressed by Malaysian Twitter users, as shown in Figure 2. In the wake of this incident, positive sentiment surged significantly, reaching 50% ($n = 27/50$) of the tweets. Conversely, no tweets reflected a very negative sentiment ($n = 0$), while negative sentiment accounted for only 6% ($n = 3/50$), and neutral sentiment was observed in 20% ($n = 10/50$). Very positive sentiment, which expressed strong approval, was recorded at 28% ($n = 14/50$). Similarly, this event had a profound effect on Indonesian Twitter users, as illustrated in Figure 3, where neutral and positive sentiments were predominant, comprising 42% ($n = 52/123$) and 34% ($n = 42/123$) of the tweets, respectively.

Concerns Over COVID-19 in Cats (July 22, 2020)

On July 22nd, 2020, public sentiment among Malaysian Twitter users underwent another significant shift, this time in response to reports of COVID-19 cases in cats in the United Kingdom. As illustrated in Figure 2, negative sentiment rose notably, reaching 35% ($n = 7/20$) of the tweets. Similarly, there was also a shift in sentiment among Indonesian Twitter users beginning on July 22nd, 2020, in reaction to the same news about COVID-19 in cats in the United Kingdom. As shown in Figure 3, a negative sentiment increased substantially, accounting for 48.8% ($n = 40/82$) of the tweets.

DISCUSSION

This study provides a comprehensive analysis of public sentiment on COVID-19 and examines its impact on human-pet relationships in Malaysia and Indonesia. Data was obtained and classified into 4 courses of events which are the following: 1) the initial response (March 18th, 2020), 2) the second week of MCO (April 1st, 2020), 3) the outcry against animal cruelty (May 5th, 2020), and 4) concerns over COVID-19 in cats (July 22nd, 2020). By leveraging Twitter as a key data source, this research looks into how pandemic-related discourse has shaped perceptions and interactions with pets in these regions. Findings reveal a range of public attitudes towards pets throughout the courses, highlighting how these relationships have been both challenged and deepened in response to the pandemic's stressors.

Overall, public sentiment toward pets has been dynamic, reflecting vary-

ing stress levels in Malaysia and Indonesia, where higher daily COVID-19 case counts appeared to correlate with heightened stress among citizens (Manchia et al., 2022). During the initial response on March 18th, 2020, it was observed that Malaysians who interacted with pets or stray animals displayed predominantly positive attitudes in managing the early stages of the COVID-19 situation. A high proportion of neutral sentiment may indicate early-stage adaptation and uncertainty (Yang et al., 2020). Similarly, further findings on Indonesian Twitter users particularly those engaged with pets or stray animals, exhibited a notably optimistic outlook in managing the early challenges of the COVID-19 pandemic. A significant proportion of positive sentiment may reflect a broader sense of confidence in their ability to navigate the crisis effectively, likely influenced by their close interactions with animals, which could have provided comfort and emotional stability during uncertain times. Throughout this period, many pet owners reported spending more time with their pets, which they recognized as beneficial for reducing stress and anxiety during the crisis (Ogata et al., 2023).

On the second week of MCO (April 1, 2020), increased concerns among Malaysian and Indonesian Twitter users regarding the potential transmission of SARS-CoV-2 between humans and pets, as well as the reverse transmission from pets to humans were observed. This outcome could potentially be the result of a surge of misinformation, conspiracy theories, and unverified claims about COVID-19. Such content, often including misleading narratives, significantly impacted public perceptions, heightening concerns about the virus (Hughes et al., 2022). Clearly, public sentiment regarding the potential transmission of COVID-19 from pets to humans has been mixed. Some individuals expressed concerns about the possibility of pets serving as reservoirs for the virus, while others emphasized the limited evidence of human-to-pet transmission (Agle and Xiao, 2021). Subsequent research has confirmed that SARS-CoV-2, the virus responsible for COVID-19, can be transmitted from humans to animals through close contact. Notably, domestic pets such as cats and dogs have contracted the virus, typically following direct exposure to infected individuals (Meisner et al., 2022). Although the likelihood of pets transmitting COVID-19 to humans is minimal, pets can occasionally experience severe illness due to the virus, although such cases remain extremely rare (Nielsen et al., 2023). This divergence in opinion underscores the need for continued research to clarify the complexities of disease transmission, particularly regarding cross-species interactions and the role of environmental factors. Furthermore, ongoing studies are essential to deepen our understanding of information control during the pandemic, particularly in relation to how

public perceptions have been shaped by the flow of information during this critical period. In response, the media played a crucial role in health communication, striving to keep the public well-informed and to foster a supportive and healthy environment (Mian and Khan, 2020).

Other important highlights that took place during COVID-19 pandemic were the outcry against animal cruelty on 6th May 2020 and the concerns over COVID-19 in cats on 22nd July 2020. This shift in sentiment reflected widespread concern regarding the potential health risks posed by the virus to both humans and their pets. The news sparked a sense of anxiety, as Malaysians and Indonesians feared the possibility of their pets becoming infected, which could potentially exacerbate the challenges they were already facing in managing the pandemic. According to research from Harvard, misinformation about zoonotic disease contributed greatly to these outcomes. Due to unclear information about how the virus could spread between humans and animals, many pet owners made hasty, and often unnecessary, choices concerning their pets' well-being (Harvard Law School, 2021). Additionally, when the virus was initially linked to a wet market, attention quickly turned to wildlife trade practices and market conditions worldwide. These markets, where animals are confined in close, often unsanitary conditions, have long been linked to zoonotic disease risks. Studies suggest that the pandemic amplified public demands for stricter regulations or even the closure of these wildlife markets, as concerns for both animal welfare and public health grew more urgent (Young, 2024). Besides, the urgent need for COVID-19 treatments and vaccines led to a significant increase in animal testing, which in turn raised ethical concerns about the welfare of animals involved in such rapid scientific efforts. This heightened focus has prompted researchers and advocates to call for alternatives to animal models, such as *in vitro* techniques and computational simulations, particularly for future studies where these alternatives may be feasible. These approaches are seen as essential to reduce reliance on animal testing while still achieving scientific progress (Harvard Law School, 2021). During this phase, there was widespread outrage and emotional response to the cruelty incident, signaling a collective demand for justice and reform in the treatment of animals. The significant presence of positive sentiment highlights the strong emotional bond between pet owners and animal advocates, as well as a powerful call for justice in response to acts of animal abuse. The incident acted as a catalyst, sparking a wave of empathy and advocacy for animal welfare, as users voiced their concerns and called for stronger protection of animals. This emotional outpouring reflects broader societal values regarding the ethical treatment of animals and the collective demand for accountability in cases of abuse.

CONCLUSION

The COVID-19 pandemic has undoubtedly impacted human-animal interactions, particularly in Malaysia and Indonesia. Our analysis of public sentiments surrounding the pandemic and its influence on human-pet interactions has revealed several key insights. In conclusion, the COVID-19 pandemic has highlighted the dynamic nature of public sentiment and its influence on human-pet interactions. While positive sentiment generally prevails, external factors such as viral events and perceived health risks can trigger shifts in perception. Addressing animal welfare issues during times of crisis requires a nuanced understanding of public sentiment, responsible media reporting, and clear communication from experts to safeguard the well-being of both humans and animals.

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Author's Contribution

NIA, MW, MWMN and MA made contributions to conception and design of the study and revised the manuscript critically. FS involved in data collection and analysis, and drafting the manuscript. NI performed the statistical analysis, revised the manuscript critically and prepared the final draft of the manuscript. All authors read and approved of the final manuscript.

Competing interest

The authors declare that they have no conflicts of interest.

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Appendix A

```
import tweepy
# Set up your Twitter API credentials
consumer_key = 'YourConsumerKey'
```

```
consumer_secret = 'YourConsumerSecret'  
access_token = 'YourAccessToken'  
access_token_secret = 'YourAccessTokenSecret'  
# Authenticate with Twitter API  
auth = tweepy.OauthHandler (consumer_key, consumer_secret)  
auth.set_access_token (access_token, access_token_secret)
```

Appendix B

```
#Installation of tools  
PS C:\Users\user\PycharmProjects\pythonProject> pip install configparser  
PS C:\Users\user\PycharmProjects\pythonProject> pip install tweepy  
PS C:\Users\user\PycharmProjects\pythonProject> pip install pandas  
PS C:\Users\user\PycharmProjects\pythonProject> pip install nltk
```

Appendix C

```
# Create the API object  
import tweepy  
# Set your Twitter API credentials  
consumer_key = 'your_consumer_key'  
consumer_secret = 'your_consumer_secret'  
access_token = 'your_access_token'  
access_token_secret = 'your_access_token_secret'  
# Authenticate with Twitter API  
auth = tweepy.OAuthHandler(consumer_key, consumer_secret)  
auth.set_access_token(access_token, access_token_secret)  
api = tweepy.API(auth)  
# Define your search query  
query = " cat OR dog OR pet from:Malaysia (wuhan virus OR corona  
OR COVID-19 OR covid OR lockdown OR MCO) since:2020-03-17  
until:2020-09-17"  
# Search for tweets  
tweets = tweepy.Cursor(api.search, q=query, lang="msa").items()  
# Iterate through the results and print tweets  
for tweet in tweets:print(tweet.text)
```

Appendix D

```
# Import library
import pandas as pd
from nltk.sentiment import SentimentIntensityAnalyzer
# Change csv to data frame
df = pd.read_csv('your_file.csv')
# Analyse data frame and create new column in csv
from nltk.sentiment import SentimentIntensityAnalyzer
sia = SentimentIntensityAnalyzer()
df['Sentiment'] = df['Text_Column'].apply(lambda x: sia.polarity_
scores(x)['compound'])
# Saving the files
df.to_csv('sentiment_results.csv', index=False)
```

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PRELIMINARY REPORT ON AFLATOXIN M1 IN PIG URINE AS A MARKER OF AFLATOXIN B1 EXPOSURE IN SERBIA

Miroslava Polovinski Horvatić^{1*}, Saša Krstović¹, Darko Guljaš¹,
Marko Damjanović¹, Mile Mirkov¹, Igor Jajić¹

¹ Faculty of Agriculture, University of Novi Sad, Novi Sad, Republic of Serbia

Abstract

In contemporary agricultural practices, it is increasingly difficult imagine production processes that are unaffected by the challenges posed by climate change. The aflatoxin B1 (AFB1) is a mycotoxin which was not very prominent in food and feed in Europe. However, in recent decades, climate change has emerged as a significant issue, raising concerns about food safety and animal production. One of the main problems regarding AFB1 is an uneven distribution in feed samples, and quite often feed sample analysis does not provide a proper insight into the exposure to this mycotoxin. This study aimed to investigate the occurrence of AFB1 in feed and its biomarker aflatoxin M1 (AFM1) in urine of fattening pigs on one small family farm. Urinary biomarker levels were subsequently used to estimate the probable daily intake and concentration of AFB1 in feed, employing established mathematical models. Four samples of a complete feeding mixture for fattening pigs originating from one farm were analyzed and the average AFB1 concentration of $0.046.3 \pm 0.0048$ mg/kg was determined. Seven urine samples collected from the slaughter fatteners that were fed the above mentioned feed were analyzed for AFM1 levels. All samples contained AFM1, with an average concentration of 6.7 ± 2.7 µg/l. The estimation of the AFB1 daily intake enabled us to assess the level in feed. The mathematical model revealed a level of 0.269 mg/kg that overestimated the AFB1 content obtained by analyzing the feed.

Key words: feed, daily intake, mathematical model, mycotoxins

^{1*} Corresponding Author: miroslavapolovinski@yahoo.com

PRELIMARNI IZVEŠTAJ O ANALIZI AFLATOKSINA M1 U URINU SVINJA U SRBIJI KAO MARKERA IZLOŽENOSTI AFLATOKSINU B1

Miroslava Polovinski Horvatić¹, Saša Krstović¹, Darko Guljaš¹,
Marko Damjanović¹, Mile Mirkov¹, Igor Jajić¹

¹ Univerzitet Novi Sad, Poljoprivredni fakultet, Novi Sad, Republika Srbija

Kratak sadržaj

U savremenoj poljoprivrednoj praksi, skoro je nemoguće zamisliti proizvodnju bez nekog problema prouzrokovanog klimatskim promenama. Aflatoksin B1 (AFB1) je mikotoksin koji nije bio previše izražen u hrani i hranivima u Evropi. Međutim poslednjih nekoliko decenija usled klimatskih promena, postao je veliki problem i izazvao zabrinutost u pogledu bezbednosti hrane i animalne proizvodnje. Jedan od glavnih problema vezanih za AFB1 je neravnomerna distribucija u bilo kom uzorku hraniva, i često se dešava da analiza uzoraka hraniva ne da odgovarajući uvid izloženosti ovom mikotoksinu. Ova studija je imala za cilj da istraži pojavu AFB1 u hranivu i njegovog biomarkera aflatoksina M1 (AFM1) u urinu kod tovljenika na maloj porodičnoj farmi. Nivoi biomarkera u urinu su korišćeni za izračunavanje verovatnog dnevnog unosa i koncentracije AFB1 u hrani za tovljenike korišćenjem prethodno utvrđenih matematičkih modela. Četiri uzorka kompletne smeše za ishranu tovljenika sa jedne farme je analizirano i utvrđena prosečna koncentracija AFB1 je iznosila $0.046.3 \pm 0.0048$ mg/kg. Sedam uzoraka urina uzetih sa linije klanja od tovljenika hranjenih pomenutom hranom je analizirano na AFM1. Svi uzorci su sadržali AFM1, prosečne koncentracije 6.7 ± 2.7 µg/l. Procenjen dnevni unos AFB1 korišćen je za procenu koncentracija u hrani za tovljenike. Matematički model je ukazao na nivoe od 0.269 mg/kg što premašuje nivoe AFB1 dobijene analizom hrane za životinje.

Ključne reči: hrana za životinje, dnevni unos, matematički model, mikotoksini

INTRODUCTION

Aflatoxin B1 (AFB1) is a mycotoxin that has garnered significant attention in this region of Europe over the past two decades. (Cavallarin et al., 2004; Medina et al., 2014; de Rijk et al., 2015). At the beginning of the research of AFB1 in Europe, its occurrence in feed or food was linked to the imported food and feed in Europe from other continents (Blount 1961; EFSA 2005). However, the occurrence of AFB1 in corn produce in some European countries like Italy (EFSA 2005) or some countries in the Balkans (including Serbia) became frequent with occasional incidences of major outbreaks in recent decades (de Rijk et al. 2015; Horvatic et al. 2018). The outbreak consequently influenced dairy production by reducing demand for milk products. The ultimate result was a significant reduction in the number of dairy herds in Serbia. There are a lot of published studies on the occurrence of aflatoxin M1 (AFM1) in milk or milk products in Serbia (Kos et al. 2013; Kos et al. 2014; Jajić et al. 2019).

At the same time, corn was used in the rearing and fattening of monogastric animals such as pigs. There is no concern regarding food safety issues when it comes to the occurrence of AFB1 in animal feed because there are no significant residues in the meat of these animals (Pleadin et al. 2021). Moreover, due to the controls of feed, cases of acute poisoning were rare even in the years the aflatoxins outbreak in corn. However, it is widely recognized that mycotoxins negatively impact production, influencing the immune status of the affected animals and key performance parameters, such as weight gain (Kanora and Maes 2009; Pu et al. 2021).

In intensive animal production, such as pig farming, animals are often at the brink of their biological limits. This mode of production encounters various challenges, with mycotoxin-related issues frequently being suspected. However, due to the uneven distribution of mycotoxins in feed, the results of feed analyses can vary significantly. For instance, during the outbreak of AFB1 in the Balkans in 2013, mycotoxin levels among replicated sample test results ranged from 21 to 204 µg/kg (de Rijk et al. 2015), all exceeding the European Union (EU) legal limit for AFB1 in corn for feed, set at 20 µg/kg (EC 2002). Despite this, the contaminated corn successfully passed import controls and was distributed to farms, raising concerns about how such substantial shipments with elevated AFB1 levels evaded detection by the EU.

AFM1 is produced from AFB1. It is excreted in urine, feces, and milk. In order to determine the mycotoxin AFB1 intake through feed, urine concentration of AFM1 can be used (Thieu and Pettersson 2009; Gambacorta et al., 2019; Tkaczyk and Piotr 2021). A good correlation was observed between the

ingested amount of mycotoxin and excreted biomarkers in the urine of pigs (Gambacorta et al. 2013). The mean percentage of dietary AFM1 excreted as a biomarker in 24 h post-dose urine was 2.5% for AFB1 (Gambacorta et al. 2013; Gambacorta et al. 2019).

The aim of this paper was to examine the concentration of AFM1 in urine and employ the existing mathematical model (Gambacorta et al., 2019) to evaluate exposure to AFB1 on a pig farm in Serbia. The samples were taken from a small-scale producer, that fed pigs with corn produced locally on their land on a farm in Vojvodina province, Serbia. At the same time, the complete feeding mixture produced by a farmer for fattening pigs was analyzed for AFB1.

MATERIAL AND METHODS

Animals

The animals (N=7) originated from a small farm in Sot, Srem, Vojvodina, Serbia. The animals were reared in controlled conditions on the farm. The study included piglets-fatteners obtained from F1 generation sows and terminal sires. All fattening pigs were reared on the same premises and fed the same feed. Fattening of the hogs was divided into three stages: the starting, growing, and finishing stage. After the end of the fattening period, when they were approximately six months old (weighing 105-110 kg), they were taken to the local slaughterhouse where the samples were taken.

Sampling

Urine samples were collected from seven randomly chosen pigs from the slaughter line. The whole bladders full of urine were taken and frozen at -20 °C. After thawing the required volume of urine, the samples were taken for analysis. The samples of feed were taken randomly from the feeding place.

Determination of AFB1 in feed

Precisely 20 g of samples were measured and placed in a 150 mL beaker. AFB1 was extracted with 100 mL of 70% methanol in the water on an Ultra Turrax T18 homogenizer (IKA, Germany) for 3 min at 11,000 rpm. The crude extract was then filtered through a quantitative slow filtration filter paper (Filtros Anoia, Spain). The immunochemical analysis was performed using the Veratox, Aflatoxin (Total), and Quantitative Test Kit (Neogen, Lansing, MI,

USA) with four calibration standard solutions (0, 5, 15 and 50 µg/kg). The analytical procedure was carried out according to the manufacturer's procedure. AFB1 quantification was carried out on an ELISA reader equipped with a 630 nm filter (BioTec Instruments, USA).

Determination of AFM1 in urine

After defrosting, 12 mL of urine was incubated with the β-glucuronidase (obtained from Sigma Aldrich, USA) for 14 hours in order to clear conjugates. Following the incubation with β-glucuronidase, the samples were cleaned up by immunoaffinity columns AflaStar R M1 (Romer Labs, USA). Immunoaffinity columns were dried using a nitrogen stream, and the AFM1 bound to the columns was eluted with 3 mL of methanol. The methanol extract was evaporated in a stream of nitrogen, and all remains were dissolved in 200 µL of n-hexane (Sigma Aldrich, USA). Also, 200 µL of trifluoroacetic acid (Sigma Aldrich, USA) was added for derivatization. This mixture was heated for 10 minutes at 40 °C. After 10 minutes, the mixture was evaporated in a stream of nitrogen, dissolved in 300 µL of acetonitrile/water (75:25), and transferred to a vial for analysis. The analysis was carried out on HPLC with fluorescent detection (1260 Infinity, Agilent Technologies, USA), using the following conditions: column flow was set to 1.25 mL/min. The mobile phase was acetonitrile/water 75:25. Acetonitrile (HPLC grade) was purchased from Sigma Aldrich, USA. FLD excitation wavelength was set at 365 nm, and the emission wavelength was set at 440 nm. The chromatography column used for the analysis was C18 Zorbax Hypersil ODS 150×4,6 mm, 5 µm in particle size (Agilent Technologies, USA). The column was kept at 30 °C. All the analyses were done in triplicate.

Method quality control was confirmed by carrying out recovery (standard addition method), precision, and linearity tests. Recovery was tested at 1, 10, and 50 µg/L resulting in an average recovery rate of 91.3%. Precision was calculated after 6 consecutive injections of spiked pig urine samples for repeatability and another 6 injections on the next day for interlaboratory reproducibility calculation. Relative standard deviations for repeatability and interlaboratory reproducibility were 4.9% (N=6) and 7.7% (N=12), respectively. Linearity was calculated after injecting a series of 7 standard solutions (ranging from 2.5 to 25 µg/L), resulting in a regression coefficient of $R^2=0.9993$. Limits of detection and quantification were 0.3 and 1.0 µg/L, respectively. They were calculated based on the signal-to-noise ratio.

Mathematical model

The mathematical model applied in this study was adapted from a previous paper that used in vivo experiment to determine the relationship between the intake of AFB1 through feed and the excretion of its metabolite AFM1 in urine over a 24-hour period (Gambacorta et al. 2019). That study established the average excretion rate of AFM1 in urine based on different AFB1 concentrations in the feed. It was conducted on pig fatteners weighing 110 kg on average, which is also a typical weight at the end of the fattening phase in the Republic of Serbia. Therefore, in our study, we also used pig fatteners with an average body weight of 110 kg. The formulas used to calculate the probable daily intake of AFB1 were sourced from this earlier research, which also analyzed other mycotoxins in urine (although we only used data for AFB1). The average 24-hour urine excretion values incorporated into the formula are from the studies conducted by Gambacorta et al. (2013; 2019).

Estimation of mycotoxin intake (Gambacorta et al. 2019) was as follows:

$$PDI = (C \times V \times 100) / (W \times E)$$

Where:

PDI - probable daily intake of AFB1 mycotoxin ($\mu\text{g/kg}$ body weight - BW)

C - pig urinary concentration ($\mu\text{g/L}$)

V - mean 24 h pig urine volume (2.5 l)

W - mean pig body weight

E - mean urinary excretion rate of 2.5% for AFB1 excreted as AFM1

Estimation of feed contamination:

$$ML = PDI \times W / V$$

Where:

ML - mycotoxin level in the feed ($\mu\text{g/kg}$)

PDI - probably daily intake of each mycotoxin ($\mu\text{g/kg}$ body weight)

W - mean pig body weight

V- mean 24h pig urine volume (2.5 L)

RESULTS AND DISCUSSION

The results of the analyzed feed and pig's urine are presented in Tables 1 and 2. Prior to the analysis of feed and pig urine samples, the farmer reported issues, including a decline in production parameters. The occurrence of AFB1 in feed was from 0.041 - 0.051 mg/kg. The analyzed feed samples revealed

concentrations two or more times higher than the maximum permissible level of 0.02 mg/kg established by Serbian legislation. (Serbian Regulation, 2014).

Table 1. AFB1 (mg/kg) content in complete feeding mixtures for fattening pigs (N=4).

Samples	AFB1 (mg/kg)
1	0.041 ± 0.009
2	0.051 ± 0.013
3	0.042 ± 0.011
4	0.051 ± 0.010
Average	0.046 ± 0.005

Table 2 shows the results of analyzed samples of pig urine. Based on the average weight on the slaughter line and probably daily intake, the occurrence of AFB1 in feed was calculated. The presumed concentrations of AFB1 in feed were higher than the levels actually determined in the complete feed mixture.

Moreover, the average concentration was five times higher than the one analyzed in feed and almost 13 times higher than allowed for this category of animals.

Table 2. AFM1 content in pig's urine and AFB1 levels in feed calculated using the mathematical model (N=7)

Samples	AFM1 in urine (µg/l)	PDI (µg/kg BW)	ML (mg/kg)
1	6.33 ± 1.7	5.75	0.253
2	12.21 ± 2.9	11.10	0.488
3	8.41 ± 2.2	7.65	0.336
4	7.18 ± 2.3	6.53	0.287
5	5.17 ± 0.8	4.70	0.207
6	4.44 ± 0.8	4.04	0.177
7	3.32 ± 0.6	3.02	0.133
Average	6.72 ± 2.7	6.11	0.269

PDI: calculated probable daily intake of AFB1 (µg/kg body weight)

ML: calculated AFB1 level in the feed (mg/kg)

The detected concentrations of urinary AFM1 were surprisingly high. The concentrations were much higher than in the study of Gambacorta et al. (2019). However, this study was done in Sweden, a country with very good agricultural practices and traditionally this region of Europe is not linked with a high level of aflatoxin in feed. On the other hand, the study done in Vietnam indicated that pigs are commonly exposed to aflatoxin and the highest found concentration in 15 samples of AFM1 in pigs' urine was 7.8 ng/ml (Thieu and Pettersson, 2009) or 13.66 µg/kg (Lee et al., 2017).

The use of mycotoxin biomarkers in pig urine is commonly employed in certain countries as an indicator of mycotoxin exposure. (Vidal et al., 2018; Lee et al., 2017). However, to our knowledge, this approach has not been utilized in Serbia. It is widely recognized that in recent years, farmers in Serbia have struggled to meet the permissible concentration of AFM1 in milk due to the prevalence of AFB1, primarily in corn. (Kos, et al. 2013; Kos et al. 2014; Kos et al. 2018; Jajić et al. 2019). Small family farms produce relatively small amounts of feed which they use as feed locally for their animals. Feed or feed ingredients are not commonly tested. However, there is no actual data regarding the occurrence of aflatoxin in feed for fattening animals such as pigs on such farms. On small farms, when producers are aware of corn contamination, they typically avoid including it in the feed mixture for dairy cattle; however, there are no instances of completely excluding corn from the diet. It is used for animals for which it is thought that are less susceptible to mycotoxins. The farmers do not have enough economic power to completely avoid the contaminated corn.

It is nearly inconceivable that this farm experienced no issues related to the presence of AFB1 in the feed. This may be attributed to either a lack of viable options for the farmer to mitigate the problem or a complete unawareness of the associated risks. AFB1 is recognized for its impact on production in intensively fattened pigs (Smith et al., 2005). By utilizing biomarkers in urine to assess exposure to AFB1, it is possible to monitor the effects of various mycotoxin deactivators in pig feed. (Lauwers et al., 2019).

CONCLUSION

The results of this study indicate high concentrations of AFM1 in pig urine. However, it is important to note that the samples were taken from animals already suspected of consuming contaminated corn. Feed samples indicated AFB1 levels that were double the legal limit, while urine samples suggested that AFB1 concentrations in the feed that exceeded the permitted limit by more than twelve times. This discrepancy raises the question of whether urine

biomarkers overestimate AFB1 exposure or if AFB1 levels in feed are being underestimated due to inadequate sampling methods.

Given that AFM1 in urine has been established as a reliable indicator of AFB1 exposure (Gambacorta et al., 2019), we believe that the AFB1 levels in complete feed mixtures should align more closely with those suggested by the urine samples. Serbia, as one of the world's leading corn exporters, faces significant economic consequences due to AFB1 contamination. This issue disproportionately affects smaller producers who may lack the financial resources to address the problem effectively. While AFB1 contamination in fattening animals does not pose a direct safety risk to consumers, it still negatively impacts animal production. Further extensive research, particularly at slaughter lines, is necessary to better understand the incidence of AFB1 contamination in fattening pigs and to determine the full scope of the problem.

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Author's Contribution:

MPH wrote the manuscript with input from all authors; SK verified the analytical methods; DG and MD performed the analysis; MM sampling and statistics; IJ contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript. All authors discussed the results and approved the final manuscript.

Competing interest

The authors declare that there is no conflict of interest.

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2. Chen J. and McClane B.A. 2015. Characterization of *Clostridium perfringens* TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. *Infection and Immunity*, 83, 2369–2381. doi:10.1128/IAI.03136-14.
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5. Ficken, M. D. and Wages, D. P. 1997. Necrotic enteritis in Diseases of Poultry, Eds. B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif, Iowa State University Press, Ames, Iowa, USA, 10th edition, ISBN xxx-xxx-xxxxx-xx-x.

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6. Plumb J.A. and Hanson L.A. 2011. Sturgeon viruses. In *Health maintenance and principal microbial diseases of cultured fishes*. Eds. J.A. Plumb, L.A. Hanson, 3rd edition, Blackwell Publishing, 219-225.

Articles in proceedings:

7. Giangaspero A., Marangi M., Pati S., Cafiero M.A., Camarda C., Sparagano O.A.E. 2011. Investigating the presence of acaricide residues in laying hens naturally infected by the red mite *Dermanyssus gallinae*. In *Book of Abstracts*, The 12th Asian food conference 2011, BITEC Bangna, Bangkok, Thailand, 27.
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9. Lazić G., Lazić S., Bugarski D., Grubač S., Lupulović D., Samojlović M., Petrović T. 2018. Human enteroviruses in river water and sewage in Vojvodina. In *Book of Abstracts*, International Scientific Conference “Green economy and environment protection”, Belgrade, 23-25. April 2018, edited by Larisa Jovanović, Belgrade, Naučno stručno društvo za zaštitu životne sredine “ECOLOGICA”, 95-96. ISBN 978-86-89061-11-6.

Laws and Regulations:

10. European Union. 2003. Commission Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, Official Journal of the European Union, L 268:29. <https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2003R1831:20100901:EN:PDF>

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11. European Food Safety Authority. 2016. Peer review of the pesticide risk assessment of the active substance benzoic acid. EFSA Journal, 14(12):4657-n/a. <http://dx.doi.org/10.2903/j.efsa.2016.4657>.

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