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Cytotoxicity Profile and Autophagic Activity of Ranibizumab and Aflibercept on Healthy Human Retina Pigment Epithelium Cells: An In Vitro Experimental Study

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ABSTRACT

Vascular endothelial growth factor (VEGF) plays a central role in retinal neovascular diseases, and anti-VEGF agents such as ranibizumab and aflibercept are widely used to control these conditions. This study aimed to compare the cytotoxic and autophagic effects of ranibizumab and aflibercept on human retinal pigment epithelium (ARPE-19) cells in vitro. ARPE-19 cells were treated with incremental doses of each drug (1.25× to 40× the clinical concentration). Cell viability was assessed using the MTT assay, and mRNA expressions of autophagy-related genes Beclin1 and ATG4 were quantified by qRT-PCR. Ranibizumab significantly reduced cell viability at 20× and 40× doses compared with the control, whereas aflibercept did not show notable cytotoxicity even at the highest tested concentrations. Both drugs influenced autophagy pathways in different ways: ranibizumab caused less inhibition of Beclin1 expression than aflibercept ($p < 0.05$), while both agents induced a non-significant increase in ATG4 expression. These results suggest that aflibercept may have a lower cytotoxic potential at supratherapeutic concentrations and that both drugs can modulate autophagic activity in retinal pigment epithelium cells. Understanding these cellular effects may help optimize the safety and long-term application of anti-VEGF therapy in retinal diseases.

INTRODUCTION

Ocular neovascularization represents a pathological process characterized by the abnormal proliferation of new blood vessels originating from pre-existing vasculature, typically as a compensatory response to ischemia or hypoxia. Retinal ischemia triggers the activation of hypoxia-inducible factor 1-alpha (HIF-1α), which promotes the expression of vascular endothelial growth factor VEGF-A, major regulator of angiogenesis and vascular permeability. The imbalance between pro-angiogenic and anti-angiogenic signaling disrupts the retinal pigment epithelium and the blood-retinal barrier, resulting in vascular leakage, fibrovascular proliferation, and vision loss in conditions such as diabetic retinopathy, retinal vein occlusion, and neovascular age-related macular degeneration (Campochiaro, 2015). Isolation of vascular endothelial growth factor (VEGF) and increased knowledge about its bioactivities have allowed better understanding of ocular neovascularization pathophysiology. In addition to regulating vascular

permeability and promoting the growth of vascular endothelial cells, in vitro studies have also shown that VEGF stimulates the expression of anti-apoptotic proteins Bcl-2 and A1 in these cells (Ferrara et al., 2003).

The advent of anti-VEGF agents in neovascular eye diseases has been a revolutionary development. It has become an inevitable treatment component of multiple ocular pathologies with VEGF-induced pathological vascular growth as diabetic retinopathy, retinal vein occlusion, iris neovascularization, retinopathy of prematurity, neovascular age-related macular degeneration and corneal neovascularization (Adamis and Shima, 2005).

Among the Food and Drug Administration approved anti-VEGF agents, Ranibizumab (Lucentis®, Novartis Pharma AG, Basel, Switzerland) and Aflibercept (Eylea®, Bayer Pharma AG, Berlin, Germany) are the most commonly used ones in ophthalmic clinical practice. Ranibizumab, a recombinant humanized monoclonal antibody, neutralizes all forms of VEGF-A; while Aflibercept, a recombinant fusion protein, neutralizes all forms of VEGF-A as well as VEGF-B and Placental Growth Factor. The differences in structural features and receptor inhibition may provide distinct efficacy and safety profiles in retinal cells (Gillies et al., 2019). Furthermore, recent studies have concluded that autophagy is strongly associated with VEGF and neovascularization formation. This information has led to the emergence of autophagy as a new therapeutic target (Ye et al., 2016; Miaomiao et al., 2016; Du et al., 2017).

In this *in vitro* experimental study, we evaluated the safety profiles of Ranibizumab and Aflibercept in human retina pigment epithelium cell line (RPE) (ARPE-19) at increasing concentrations by employing 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cell viability assay. Additionally, we investigated the relationship between these anti-VEGF agents and the autophagy-related proteins (Beclin1 and ATG4) at sublethal concentrations.

MATERIALS AND METHODS

Cell Culture

ARPE-19 cell line, was purchased from American Type Cell Culture (ARPE-19, [ATCC], catalog No. CRL-2302, Manassas, Virginia, USA). The manufacturer's instructions were followed during the culturing processes (Dunn et al., 1996). ARPE-19 cells used in the experiments were from the 3rd, 4th, and 5th passages. ARPE-19 cells were grown in T25 flasks using 10% Fetal Bovine Serum (FBS, Biochrom GmbH, Germany); 1% penicillin/streptomycin; and a 1:1 mixture (vol/vol) of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (Biochrom GmbH, Germany) at 37°C, 5%CO2, and 95% relative humidity. The cells were subcultured to the confluency of 70-80%. Cell suspensions (6×103 cells/ml) were seeded onto 96-well tissue culture plates. ARPE-19 cells were incubated in 96-well plates for 48 hours prior to the start of the experiment to ensure optimal cell adherence and growth.

Anti-VEGF Treatments: The commercially available off-the-shelf preparations Ranibizumab (0.5 mg/0.05 ml) and Aflibercept (2 mg/0.05 ml) were employed. The clinically accepted intravitreal doses were therefore defined as 0.5 mg/0.05 mL (equivalent to 125 µg/mL) for Ranibizumab and 2 mg/0.05 mL (equivalent to 500 µg/mL) for Aflibercept, as reported by Malik et al. (2014). ARPE-19 cells were exposed to anti-VEGF agents at concentrations of 1.25, 2.5×, 5×, 10×, 20×, and 40× clinically accepted doses for 24 hours. High-dose exposure (up to 40× the clinical concentration) was applied to simulate potential cumulative or localized effects that may occur following repeated intravitreal injections or reduced vitreous clearance. Such high-concentration in vitro models are used to evaluate the cellular tolerance and safety margins of pharmacological agents beyond therapeutic levels. The clinical concentration was estimated by presuming that the quantity of each anti-VEGF agent used clinically in intravitreal injections was uniformly distributed throughout 4 mL of human vitreous volume (Ranibizumab at a concentration of 125 µg/ml and Aflibercept at a concentration of 500 µg/ml, adjusted for potential dilution by the vitreous humor at the epiretinal site) (Malik et al., 2014). The test concentrations for Ranibizumab (clinical dose: 125 µg/ml) were: 1.25× = 156.25 µg/ml, 2.5× = 312.5 µg/ml, 5× = 625 µg/ml, 10× = 1250 µg/ml, 20× = 2500 µg/ml, and 40× = 5000 µg/ml. For Aflibercept (clinical dose: 500 µg/ml), the test concentrations were: 1.25× = 625 µg/ml, 2.5× = 1250 µg/ml, 5× = 2500 µg/ml, 10× = 5000 µg/ml, 20× = 10000 µg/ml, and 40× = 20000 µg/ml. The IC_{50} values for Ranibizumab and Aflibercept were estimated from dose–response curves generated using GraphPad Prism. To evaluate sublethal cellular responses such as autophagy, half of the IC_{50} concentration ($IC_{50}/2$) was selected, as this dose allows assessment of cellular stress without inducing extensive cytotoxicity. In this study, "$IC_{50}/2$" refers to half of the calculated IC_{50} concentration ("half IC_{50}"), corresponding approximately to a dose that maintains around 70–80% cell viability. This concentration was used to assess sublethal stress and autophagic responses without causing excessive cell death. The control group consisted of two subgroups: A positive control group (Maximal Viability, Max V) was considered 100% viable, and no drug was administered. A negative control group (Minimal Viability, Min V) was treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) Triton-X and considered 0% viable.

Cell Viability Assay

The metabolic activity along with the viability of ARPE-19 cells was assessed with MTT assay. This colorimetric assay quantifies the cytotoxicity by measuring absorbance using a multi-well spectrophotometer following a series of reduction reactions. Cell viability (%) was calculated relative to the positive control group (Maximal Viability), and lower absorbance values indicated reduced metabolic activity and higher cytotoxicity. The experiments were conducted in triplicates. Cells incubated for 24 hours at 37°C and 5-6.5% CO2 with different doses of anti-VEGF agents were added 15 µl of the MTT reagent for each well. After 4 hours of incubation of microplates, the Solubilization solution was added. The plate was allowed to stand overnight in the incubator. Finally, the absorbance of the solution with the formazan product was measured. Cytotoxicity was evaluated based on the ratio of dead cells to positive control.

RNA Extraction, Amplification of cDNA, and Quantitative Real-Time PCR (qRT-PCR) Analysis

The ribonucleic acid (RNA) was extracted from ARPE-19 cells using Hybrid-R (GeneAll, Portugal) RNA extraction kit according to the manufacturer's protocol. NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) was used for total RNA

measurements. The concentration and purity of nucleic acid samples were measured using the NanoDrop ND-1000, a fast and convenient device. The absorbance ratio at 260 nm to 280 nm indicates sample purity, with a high ratio indicating a pure nucleic acid sample and a low ratio indicating the presence of contaminants such as proteins. We included samples with an absorbance 260/280 ratio between 2.0 and 2.3 in the study to ensure the purity of the nucleic acid samples. This range is generally considered to indicate high-quality nucleic acid samples that are suitable for downstream applications. If a sample did not meet these parameters, we repeated RNA extraction using spare samples to ensure accuracy and reliability.

Total RNA was used to analyze Beclin1 and ATG4 mRNA expression by real-time PCR (qPCR). From the isolated RNA samples, cDNA was obtained with the NG dART RT kit (Cat No: E0801-02; EURx). Reagents were added and cDNA was synthesized for each sample. Reactions were performed in a PCR device (Applied Biosystems) at 50°C for 40 minutes and 85°C for 5 minutes. BrightGreen 2X qPCR MasterMix-No Dye kit (Cat No: MasterMix-S; Applied Biological Materials Inc. ABM) was used for real-time PCR (qPCR) analysis according to the manufacturer's instructions. Reactions with reagents were performed using a real-time PCR analyzer (Rotor-Gene Q, Qiagen, Germany) at 95°C for 10 minutes (1 cycle), 95°C for 15 seconds, and 60°C for 60 seconds (40 cycles). Each sample was run in duplicate 2 times and mean values were recorded. The expression of target genes according to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated using the 2-ΔΔCT method.

Statistical Analysis

SPSS (version 28.0, Statistical Package for Social Sciences Inc., Chicago, IL, USA) and GraphPad Prism V.7.0 version (GraphPad Software, San Diego, California, USA) were used for the statistical analysis of the data. Each experiment was performed in triplicate. The Mann-Whitney U tests were used to compare the control group with the two anti-VEGF agent groups. In-group comparisons, including the clinical and higher concentrations of the anti-VEGF agents, were also performed using Mann-Whitney U tests. The one-way analysis of variance (ANOVA) was used to determine whether there was a difference between more than two independent groups in qPCR results. The level of significance was set at 0.05. Data were reported as the mean ± standard deviation.

RESULTS

Effects of Ranibizumab and Aflibercept on Cytotoxicity Assay

Based on MTT absorbance values, Ranibizumab and Aflibercept had no significant cytotoxicity on the ARPE cells at the doses applied in this study (Figure 1) ($p>0.05$). These findings indicate that both anti-VEGF agents are generally well tolerated by RPE cells at or below clinical dose ranges, suggesting minimal acute toxicity under short-term exposure conditions.

IC50 values of active pharmaceutical preparations of Ranibizumab and Aflibercept used in our study were much higher than the concentrations used in clinical practice, therefore estimated IC50 values obtained from the graph of dose dilutions calculated with the GraphPad Prism program were utilized in our study. Since the concentrations were within the dilution range used for cytotoxicity analysis, doses corresponding to approximately IC50/2 values were used in the calculations made according to in question IC50 values. The dose corresponding to IC50/2 for Ranibizumab was 428.12 µg/ml, while 1260.88 µg/ml for Aflibercept. Karagöz et al., after examining nanoparticle-loaded anti-VEGF and performing an MTT cytotoxicity test, stated that the existing literature contained no toxicity study reporting an IC50 value. (Karagoz et al., 2021).

Ranibizumab demonstrated a statistically significant decrease in cell viability compared to the positive control group at 20× and 40× clinical doses. Whereas no statistically significant difference was found among positive control and Aflibercept-treated groups in cell viability at 1.25, 2.5×, 5×,10×, 20×, and 40× clinical doses after 24 hours of exposure. There was no statistically significant difference in cytotoxicity when Ranibizumab and Aflibercept were compared separately for each dose range. This suggests that Ranibizumab exerts mild cytotoxic effects only at supratherapeutic levels, while Aflibercept maintains cellular viability even at higher concentrations, consistent with a more favorable safety profile.

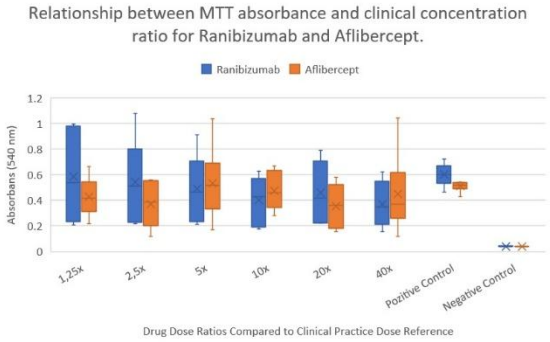

Figure 1. Concentration-dependent change in ARPE-19 cell viability after Ranibizumab and Aflibercept treatments using MTT assay. Three independent experiments were performed in triplicate and all data are expressed as means±SD. The control group consisted of two subgroups: A positive control group was considered 100% viable, and no drug was administered. A negative control group was treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) Triton-X and considered 0% viable.

Effects of Ranibizumab and Aflibercept on Beclin1 and ATG4 Expressions

Statistical analysis of Beclin1 mRNA expression levels in ARPE-19 cells showed a significant difference between the control and Aflibercept groups (*$P\leq0.05$). Compared to the control group, the Ranibizumab-treated group showed an 8% decrease in Beclin1 mRNA expression levels, while the Aflibercept-treated group showed a 54% decrease. These data imply that Aflibercept may more strongly suppress Beclin1-related autophagic activity compared with Ranibizumab, indicating distinct effects on autophagy regulation. Statistical analysis of ATG4 mRNA expression levels showed no significant difference among the groups ($P\geq0.05$). However, both the Ranibizumab-treated group (95%) and the Aflibercept-treated group (81%) had higher levels of ATG4 mRNA expression compared to the control group. This elevation, although not statistically significant, may represent a compensatory cellular response to anti-VEGF exposure, reflecting mild activation of autophagic pathways.

DISCUSSION AND CONCLUSION

Assessing cell viability is crucial for understanding the safety profile of Ranibizumab and Aflibercept, especially at higher concentrations that may induce cytotoxic effects not observed at clinical doses. This study extends previous research by evaluating concentrations up to 40× clinical doses and exploring autophagic pathways through key markers like Beclin1 and ATG4, providing new insights into the impact of these agents on both cell viability and autophagy, which has been minimally addressed in the literature.

In this *in vitro* study, the effects of commercially available anti-VEGF agents on human retina pigment epithelium cells in different concentrations were compared.

Spitzer et al. reported that Ranibizumab did not have any cytotoxicity of at clinical doses on ARPE-19 cell line, parallel to our results (Spitzer et al., 2007). Malik et al. investigated the safety profiles of anti-VEGFs including Ranibizumab and Aflibercept on the ARPE-19 cell line. They exposed the cells to anti-VEGF agents for 24 hours at 0.5×, 1×, 2×, and 10× clinical concentrations. While they found Ranibizumab to be safe on cell viability in all concentrations, they observed decreased cell viability in the Aflibercept group at 10× concentration. They monitored no statistically significant differences in cell numbers at 1/2×, 1×, and 2× therapeutical doses of Ranibizumab and Aflibercept, compared to the untreated cells (Malik et al., 2014). However, there was a dissimilarity in results at 10× doses which may be due to the difference in the cell viability assay method. While an automated trypan blue dye exclusion assay was used in their study, an MTT assay was employed in ours. Ammar et al. investigated the effects of Aflibercept on various ocular cells including ARPE-19. For comparison, they used Ranibizumab as an active control. Their MTT assay revealed that Aflibercept had no in vitro detrimental effect on ARPE-19 cell viability even at up to twice of the recommended dosage (Ammar et al., 2013). Schnichels et al. applied the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) – 2 - (4 - sulfophenyl) - 2H - tetrazolium] assay (a 'one step' MTT assay) and suggested that cell viability of ARPE-19 cells treated with varying concentrations of Ranibizumab and Aflibercept did not show any relevant decrease at different time intervals (1st, 24th, 48th, and 72nd hours) (Schnichels et al., 2013). Puddu et al. have achieved comparable results after the MTS assay that Ranibizumab and Aflibercept displayed no significant difference in viability (Puddu et al., 2016). Saenz-de-Viteri et al. brought a different approach to the in vitro ARPE-19 studies and examined the cumulative effects of anti-VEGF agents including Ranibizumab and Aflibercept. They applied repeated clinical doses of anti-VEGFs. After grouping the cell lines, the medication was performed every other day (days of 0, 2, and 4 days) in Group 1 while daily (days of 0, 1, 2, 3, and 4 days) in Group 2. Both groups tested with MTT assay on the 5th day. There were no statistically significant measurable cytotoxic effects after single or repeated doses of anti-VEGFs under both normal and oxidative stress conditions (Saenz-de-Viteri et al., 2016). Recently, in contrast to previous studies, Emir et al. concluded that even the clinical doses of Ranibizumab and Aflibercept caused reduced cell viability after 24 hours in the MTT analysis of ARPE-19 cells (Emir et al., 2022).

Differences or similarities between our results and previous studies may be attributed to several experimental and biological factors. First, variations in the choice of assay (e.g., MTT, MTS, or trypan blue exclusion) and exposure duration can significantly influence cytotoxicity outcomes, as metabolic assays tend to be more sensitive to early mitochondrial dysfunction. Second, intrinsic physicochemical differences between Ranibizumab and Aflibercept—including molecular weight, VEGF-binding domains, and receptor affinity—may result in distinct cellular responses and autophagic modulation. Third, variations in culture conditions such as cell seeding density, serum percentage, passage number, and medium composition can affect cellular metabolism and VEGF receptor expression, thereby altering the observed responses to anti-VEGF exposure. In addition, potential assay interference caused by protein-rich media or differences in incubation time may contribute to discrepancies in measured viability between studies such as Malik et al. (2014), Schnichels et al. (2013), and Emir et al. (2022). Finally, differences in statistical power and dose scaling beyond clinical relevance can further explain why some studies report minimal toxicity, whereas others find decreased viability or altered autophagy profiles following anti-VEGF exposure. Collectively, these factors provide plausible reasons for the discrepancies observed among reports evaluating anti-VEGF cytotoxicity on retinal pigment epithelial cells

In the present study, no statistically significant difference in cell viability between Ranibizumab and Aflibercept was present, compared to the positive control group at 1.25×, 2.5×, 5× and 10× clinical doses after 24 hours of exposure (Table 1).

On the other hand, Ranibizumab demonstrated decreased cell viability compared to the positive control group at 20× and 40× clinical doses, while Aflibercept did not (Table 2).

In addition, when Ranibizumab was compared to Aflibercept in terms of cell viability for each incremental dose range, no statistically significant difference was found between them (Table 3).

Although several *in vivo* and *in vitro* studies in various dose ranges have been carried out on safety profiles of anti-VEGFs, the present study is the first work that assesses the Ranibizumab and Aflibercept at rates up to 40 × of the clinical doses. The estimated IC50/2 for Ranibizumab in our study was calculated as 428.12 µg/mL, indicating the concentration needed to reduce cell viability by 50% under our experimental conditions. However, significant reductions in cell viability were observed at 20× and 40× clinical doses of Ranibizumab (2500 µg/mL and 5000 µg/mL, respectively), which are much higher than the IC50. This can be explained by the fact that IC50 represents a midpoint in the dose-response curve, and at concentrations far exceeding this threshold, the cytotoxic effects of Ranibizumab accumulate, resulting in more substantial reductions in cell viability. Thus, the cytotoxicity observed at 20× and 40× doses align with the predicted dose-response relationship beyond the IC50 point. Additionally, the IC50 was determined under specific experimental conditions (such as controlled pH, temperature, and assay settings), and it's important to recognize that IC50 values can shift depending on the biological system and environmental factors.

Another aim of the current study was to reveal whether a relationship exists between anti-VEGF agents and autophagy pathways in the ARPE-19 cell line.

Table 1. Statistical comparison of Aflibercept and positive control for each concentration.

	Group	n	Mean Rank	Total Rank	U	p
40× (20000 µg/ml)	Aflibercept	6	4.67	28.00	7.000	0.078
	Positive Control	6	8.17	50.00		
20× (10000 µg/ml)	Aflibercept	6	4.83	29.00	8.000	0.109
	Positive Control	6	8.17	49.00		
10× (5000 µg/ml)	Aflibercept	6	4.83	32.00	8.000	0.179
	Positive Control	6	8.71	49.00		
5× (2500 µg/ml)	Aflibercept	6	6.50	39.00	18.000	1.000
	Positive Control	6	6.50	39.00		
2.5× (1250 µg/ml)	Aflibercept	6	6.67	40.00	17.000	0.873
	Positive Control	6	6.33	38.00		
	Aflibercept	6	5.67	34.00	13.000	0.423
	Positive Control	6	7.33	44.00		

*p<.05

n: Sample size.; u: The difference between the two rank totals.; p: Probability value.; ×: multiples of clinical concentration, µg/ml: micrograms per milliliter.

A positive control group (Maximal Viability, Max V) was considered 100% viable, and no drug was administered. A negative control group (Minimal Viability, Min V) was treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) Triton-X and considered 0% viable.

Table 2. Statistical comparison of Ranibizumab and positive control for each concentration.

	Group	n	Mean Rank	Total Rank	U	p
40× (5000 µg/ml)	Ranibizumab	6	4.17	25.00	4.000	0.025*
	Positive Control	6	8.83	53.00		
20× (2500 µg/ml)	Ranibizumab	6	4.33	26.00	5.000	0.037*
	Positive Control	6	8.67	52.00		
10× (1250 µg/ml)	Ranibizumab	6	5.83	35.00	14.000	0.522
	Positive Control	6	7.17	43.00		
5× (625 µg/ml)	Ranibizumab	6	5.34	32.00	13.000	0.237
	Positive Control	6	8.67	52.00		
2.5× (312.5 µg/ml)	Ranibizumab	6	5.50	33.00	12.000	0.337
	Positive Control	6	7.50	45.00		
1.25× (156.25 µg/ml)	Ranibizumab	6	6.17	37.00	16.000	0.749
	Positive Control	6	6.83	41.00		

*p<.05

n: Sample size.; u: The difference between the two rank totals.; p: Probability value.; ×: multiples of clinical concentration, µg/ml: micrograms per milliliter.

A positive control group (Maximal Viability, Max V) was considered 100% viable, and no drug was administered. A negative control group (Minimal Viability, Min V) was treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) Triton-X and considered 0% viable.

Table 3. Statistical comparison of Aflibercept and Ranibizumab for each concentration.

	Active Substance	n	Mean Rank	Total Rank	U	p
40×	Aflibercept	6	6.67	40.00	17.000	0.873
	Ranibizumab	6	6.33	38.00		
20×	Aflibercept	6	5.50	33.00	12.000	0.337
	Ranibizumab	6	7.50	45.00		
10×	Aflibercept	6	5.83	35.00	14.000	0.552
	Ranibizumab	6	7.17	43.00		
5×	Aflibercept	6	6.50	39.00	18.000	1.000
	Ranibizumab	6	6.50	39.00		
2.5×	Aflibercept	6	7.00	42.00	15.000	0.631
	Ranibizumab	6	6.00	36.00		
1.25×	Aflibercept	6	6.17	37.00	16.000	0.749
	Ranibizumab	6	6.83	41.00		

n: Sample size.; u: The difference between the two rank totals.; p: Probability value.; ×: multiples of clinical concentration.

Angiogenesis is a complex process that involves stages such as endothelial cell proliferation, matrix degradation, migration, tube formation, and vessel maturation. Pathological angiogenesis is linked to several diseases, including cancer, macular degeneration, and diabetic retinopathy. In each of these conditions, inhibition of angiogenesis has been shown to inhibit disease progression (Folkman, 2006; Reddy et al., 2003). VEGF is a transcriptionally regulated survival factor for endothelial cells. Blocking VEGF receptor-mediated signaling via the phosphatidylinositol 3-kinase (PI3K) / protein kinase B (Akt) pathway leads to cell cycle arrest and programmed cell death (Folkman, 2007). Autophagy and apoptosis usually occur in the same cell, often in a sequence where autophagy precedes apoptosis. This is because stress often stimulates an autophagic response, especially when the stress level is not fatal. In most cases, autophagy constitutes a strategy for adapting and coping with stress (Kroemer et al., 2010). However, if the cell initiates apoptosis, autophagy can be inactivated partially due to the caspase-mediated cleavage of essential autophagy proteins. Beyond this general scenario, essential proteins involved in autophagic processes may promote cellular death either by catabolizing vital cell components or facilitating the activation of apoptotic or necrotic programs, respectively (Galluzzi et al., 2009).

In the present study, the decrease in Beclin-1 gene expression levels after anti-VEGF exposure is consistent with this basic information in the literature. There is still no consensus in the literature (whether induction or inhibition) on the relationship between anti-VEGF and autophagy. Lytvynchuk et al. showed in a mouse fibroblast cell line that anti-VEGFs dose-dependently inhibit survival, mitotic activity, and proliferation while increasing cellular heterogeneity, apoptosis, and autophagy. Authors reported that Ranibizumab showed lower antiproliferative and apoptotic activity than Aflibercept and other anti-VEGFs. This result for Ranibizumab, not having a significant effect on proliferation and apoptotic activity despite increasing doses, was explained by the different mechanisms of action (more induction) of Ranibizumab on autophagy (Lytvynchuk et al., 2015). In our results, the effect of Ranibizumab on the autophagy gene Beclin1 (lower inhibitory effect) was significantly dissimilar to Aflibercept (Figure 2).

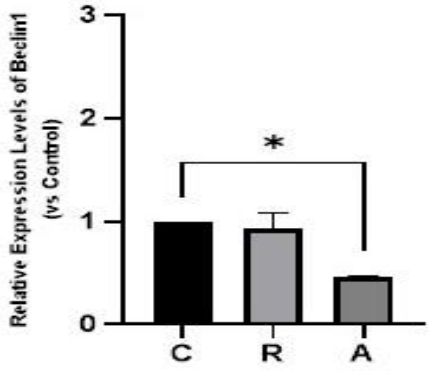

Figure 2. Beclin1 mRNA expression in ARPE-19 cells (n=3). Control (C), Ranibizumab at 50% viability inhibition concentration half (IC50/2) (R), and Aflibercept at 50% viability inhibition concentration half (IC50/2) (A). *P≤0.05(n=3).

Liang et al. investigated the relationship between the anti-VEGF agent Bevacizumab and autophagy in a glioma tumor cell line, concluding that the agent induced autophagy. They attributed this to the fact that Bevacizumab (and other antiangiogenic agents) may cause nutrient deprivation and oxygen stress in the tumor microenvironment by inhibiting the tumor vasculature and induction of the autophagy process (Liang et al., 2015). Liu et al. claimed that autophagy kills tumor cells during tumorigenesis and inhibits tumor growth, but once tumor formation occurs, autophagy protects the survival of tumor cells against various environmental conditions. It has even been hypothesized that autophagy plays a crucial role in suppressing tumorigenesis that as many human cancers, such as breast and prostate cancers, often involve the loss of two core autophagy genes, Beclin1 and/or ATG4. The hypoxia induced autophagy, caused by anti-angiogenesis therapies, is now recognized as an essential contributor to resistance to the drugs (Liu et al., 2016). While autophagy is well-studied in cancer cells, it is less understood in retinal cells. However, insights from tumor biology help us see how VEGF inhibition might influence similar autophagic processes in the retina, especially in diseases with neovascularization. Research on tumor cells shows that blocking VEGF can trigger autophagy, offering clues about how these pathways might work in RPE cells. Indeed, Wang et al.'s study on rhesus monkey choroid/retinal endothelial cell line showed that Ranibizumab and Conbercept triggered autophagy in cells under hypoxia conditions, but Aflibercept has a different effect on autophagy and inhibits the expression of Beclin1. Wang et al. proposed that this autophagy activation could, in turn, promote angiogenesis and thereby weaken the role of anti-angiogenic drugs, so induced autophagy may be the reason for the poor therapeutic effect of anti-angiogenic drugs. They suggested this induced autophagy may be a primary reason for the observed poor therapeutic outcomes associated with certain anti-VEGF treatments. Ultimately, their findings support the conclusions of various clinical trials indicating the superiority of Aflibercept over other currently available anti-VEGF agents. (Wang et al., 2021). Although our study utilized a different cell line than that investigated by Wang et al., we similarly observed Aflibercept's distinct effect, noting its inhibition of Beclin1 expression.

In this study, an increase (statistically not significant) in ATG4 expression was determined in ARPE-19 cell line treated with Ranibizumab and Aflibercept compared to the control group (Figure 3).

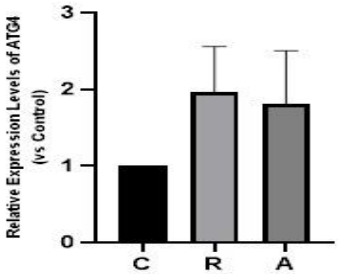

Figure 3. ATG4 mRNA expression in ARPE-19 cells. Control (C), Ranibizumab at 50% viability inhibition concentration half (IC50/2) (R), and Aflibercept at 50% viability inhibition concentration half (IC50/2) (A). *P≤0.05(n=3).

It has been previously reported that anti-VEGF agents induce autophagy (Liang et al., 2015; Liu et al., 2016; Lytvynchuk et al., 2015). In more recent studies, the relationship between anti-VEGF therapies and autophagy has remained the center of attention of researchers. For example, Segatto et al. applied Ranibizumab and Aflibercept to New Zealand white rabbit eyes and evaluated their retinas *in vitro* after enucleation. They found that VEGF inhibition led to changes in neurotrophin signaling and strongly stimulated apoptosis and autophagy. They also noted that the effects caused by Aflibercept at earlier time-points were more pronounced compared to Ranibizumab (Segatto et al., 2019). Schottler et al. found that the autophagosome area increased more using Ranibizumab compared to Aflibercept in a study which they applied Ranibizumab and Aflibercept long-term (12 weeks), and repeated doses to porcine RPE cell line (Schottler et al., 2018). In our study, the increase caused by Ranibizumab in ATG4 gene expression was higher than Aflibercept and in parallel with this study.

Although ATG4, similar to Beclin1, is an essential autophagy protein, its mechanism of action is quite different. Beclin1 initiates the formation of an isolation membrane (also called phagophore) in the first stage of autophagy, the nuclear autophagy pathway (nuclear complex formation), mainly at the contact sites between mitochondria and endoplasmic reticulum (Marino et al., 2014). Unlike other known BH3-only proteins, Beclin1 does not function as a proapoptotic molecule (He and Levine, 2010). Besides this exception, it is noteworthy that most Beclin1-interacting proteins modulate autophagy and apoptosis in the same direction; that is to say, they inhibit or promote both processes (Marino et al., 2014). Unlike Beclin1, ATG4 has a crucial role in autophagosome formation, a more advanced stage of autophagy. Since both the processing and delipidation reactions of ATG8 by ATG4 are essential for autophagosome formation, inhibition of ATG4 leads to the inhibition of autophagy at the autophagosome formation stage (Maruyama and Noda, 2018). In our study, although both are associated with autophagy; the different directions of progress in Beclin1 and ATG4 compared to the control group as decrease and increase respectively, may be explained by this difference in their mechanisms of functioning. The findings from our study regarding Beclin1 and ATG4 expression in ARPE-19 cell line should be interpreted cautiously. While autophagy was influenced by the treatments, the absence of statistically significant changes for ATG4 and the modest decrease in Beclin1 suggest that these results are preliminary. This highlights the need for more targeted autophagy studies in the future, particularly in primary retinal cells or in vivo models of retinal disease.

The current study has several limitations. First, our primary focus was examining the effects of Ranibizumab and Aflibercept on the ARPE-19 cell line. As a result, the generalizability of the findings to other cell types or patient populations may be limited. Second, while the ARPE-19 cell line is commonly used in *in vitro* studies, it may not fully represent the complexity and heterogeneity of the human retina. Therefore, caution is necessary when extrapolating these findings to the *in vivo* settings. Third, we only focused on the short-term (24-hour) effects of anti-VEGF agents. Therefore, we may speculate about their long-term or potential cumulative effects, which requires further investigation.

In conclusion, our study highlights that Ranibizumab reduced cell viability in ARPE-19 cells only at 20× and 40× clinical doses, whereas no significant cytotoxicity was observed at clinical doses. Aflibercept demonstrated less cytotoxicity, maintaining cell viability even at higher doses. Additionally, distinct autophagic responses were observed, with Ranibizumab inhibiting Beclin1 less than Aflibercept. Both drugs increase ATG4 expression suggesting a potential autophagic response. However, this result is preliminary, and further studies are needed to confirm the role of autophagy in retinal cells, using additional markers and assays.

From a clinical perspective, these findings suggest that both Ranibizumab and Aflibercept are safe at standard intravitreal doses, supporting their continued clinical use. The observed cytotoxicity at supratherapeutic concentrations underscores the importance of strict adherence to recommended dosing and monitoring cumulative exposure in patients receiving long-term anti-VEGF therapy. Moreover, the differential autophagic responses between agents may provide a rationale for individualized treatment selection or potential combination strategies targeting both VEGF and autophagy pathways in refractory retinal diseases.

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Ethical Declaration

This study does not involve human or animal participants. Ethical approval and informed consent were not required for this study.

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept: E.E., E.Y., Design: E.E., E.Y., L.D.K. Data Collection or Processing: E.E., B.YD., S.S., M.Y., Analysis or Interpretation: B.G., L.D.K., S.P., R.U., M.Y., Literature Search: E.E., L.D.K., B.G., Writing: E.E.

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Preclinical Interpretation of Oral Urolithin-A Against Respiratory Disease Among Calves: Unlocking The "Gut-Lung Axis"

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ABSTRACT

Pomegranate, as a plant, has long been known for its rich content of ellagitannins. Ellagitannins are completely hydrolyzed to ellagic acid in the intestine, which then gives rise to urolithins. This natural compound may support pulmonary system health as a feed additive, not a pharmacological agent. The aim of the present study was to describe the efficacy of urolithin-a against 17 Holstein calves with respiratory disease. Written owner consent and ethical guidelines report were considered available. No etiological investigation was conducted (outside the purpose of this study, as it was a preclinical, not clinical study). With the support of 3 veterinary experts, respiratory disease among calves was diagnosed based on the University of Wisconsin Calf Health Scoring Schedule (WIN score). Urolithin-a compound was administered perorally to diseased calves at 3 mg/kg for 10 consecutive days. No adverse effects were observed during feed additive administration. WIN scores were investigated before (day 0) and after treatment (day 10). Mean (8.24 vs. 1.82) and median (8.00 vs. 2.00) WIN scores were decreased in association with urolithin-a treatment before and after treatment, respectively ($p<0.0001$). In this preclinical setting, it would not be unfair to suggest that urolithin-a should help in the regression of respiratory disease among the enrolled calves. This nutraceutical administered via the gastrointestinal route was able to reverse respiratory signs indicating the secrets of the 'gut-lung axis' in this field study. The results obtained should be able to change treatment protocols.

INTRODUCTION

Plants such as pomegranate (Cerda et al., 2004) and other relevant ones have high ellagitannin content. Hydrolyzation of ellagitannins into ellagic acid exists within the intestinal tract in which then urolithins are converted by metabolization. Productive concentration depends on the phenotypes of metabolism and diminishes with aging (Cortés-Martín et al., 2020). Moreover furthermore, ellagitannins/ellagic acid metabolic activity have been modified through gut microbiota composition. As an example, *Bifidobacterium pseudocatenulatum* INIA P815 (Gaya et al., 2018) and *Enterococcus faecium* FUA027 (Zhang et al., 2022) exhibited urolithin A, while *Ellagibacter isourolithifaciens* DSM104140T (Selma et al., 2017; Beltrán et al., 2018) and *Gordonibacter urolithinfaciens* DSM 27213 T presented isourolithin A/urolithin C forms of ellagic acid (Watanabe et al., 2020). Urolithin production through ellagitannins has been declared among several different animals. In cattle and sheep, the foremost detectable urolithin compounds were isourolithin A, and urolithin B, whereas urolithin A was generally exhibited thorugh intestinal route (Espin et al., 2013). Given some suggested data (González-Barrio et al., 2012, Alic Ural et al., 2025) is available for urolithin a among cattle, its efficacy has not been validated through gut-lung axis among calves. In the present study the hypothesis was, to determine the efficacy of urolithin A

given in oral route interacts with respiratory disease symptoms. Therefore, in the present study the purpose was to elucidate the efficacy of urolithin-against respiratory disease among calves, further evidence of proof for gut-lung axis.

MATERIALS AND METHODS

Animals and Procedures

Holstein calves (n =17) at the age of 21 to 58 days, of both sexes, were clinically evaluated for respiratory disease (relevant clinical signs) by 3 veterinary surgeons at the same time, individually. In the present study no healthy control group was enrolled due to limited access and permission by the owners. In an attempt to diagnose respiratory disease among calves previously determined, relatively summarized and novel, WIN score (Jaureguiberry et al., 2023) (Table 1) was based on 4-level scoring scales involving nasal (1)/ocular (2) discharge, coughing (3), ear position (4) and rectal temperature (5) (Table 1). Involved calves were classified positive for respiratory disease even if the aggregate score was ≥ 5. Exclusion criteria were enrolled as: 1) evidence of co-morbidity diseases, 2) recent vaccination or drug prescription, 3) and if there exists no evdeince of respiratory signs.

Table 1. Even if a calf with respiratory disease exhibited the aggregate score was ≥ 5, diagnosis was fully supported, based on WIN score (Jaureguiberry et al., 2023).

WIN score for diagnosing respiratory disease (9)	0	1	2	3
nasal discharge (n=)	normal serous discharge	Few amount of unilateral cloudy discharge	bilateral cloudy/ overload mucus discharge	copious bilateral mucopurulent discharge
ocular discharge	0 = normal	1 = few ocular discharge	2 = moderate bilateral ocular discharge	3 = heavy ocular discharge
coughing	0 = none	1 = induced single cough	2 = induced repeated coughs/ or occasional spontaneous coughs	3=repeated spontaneous coughs
ear position	0 = normal	1 = ear flick or head shake	2 = slight unilateral droop	3= head tilt or bilateral droop
rectal temperature	0 = 37.8 to 38.2°C	1 = 38.3 to 38.8°C	2 = 38.9 to 39.4°C	3 = > 39.4°C)

[a,b] Values within a row with different superscripts differ significantly at P<0.0001.

This study was performed with written owner consent and confirmed by Aydın Adnan Menderes University, Local Ethics Committee for Animal Researches (HADYEK) with report no: 64583101/2024/119.

Urolithin-a Preferred at This Study

DQQI Urolithin A Liquid Drops 3000 mg was used at a dosage of 3 mg/kg p.o. Each calf was well accepted and there were no side effects. The drop was safely used perorally with the -help of veterinary surgeons with HADYEK Certificates.During trial not oher nutraceutical or any drug were used. There were no side effects noticed through usage of oral route. All calves accepted well.

Statistical Analyses

Statistical analyses were conducted using SPSS 29.0 (IBM, USA). Descriptive statistics, including means, medians, and standard errors, were calculated for pre-treatment and post-treatment groups. The Wilcoxon test was used to evaluate differences between pre and post treatment groups. The p value less than 0,05 was considered significant in all analyses.

RESULTS

As was also declared at materials and methods section, whether a calf with respiratory disease presented an aggregate score was ≥5, diagnosis was made through WIN score (Jaureguiberry et al., 2023). For this sense; in a total of 17 calves WIN Scores were ranged between 5 to 12 prior to treatment, whereas following urolithin-a intervention given by oral route, scores altered between 0 to 4 (at most). Interestingly 4 calves exhibited scores 0, and other relevant 4 ones as 1 after urolithin-a prescription. Regarding WIN Scores mean values were 8.24 vs. 1.82, following urolithin-a treatment, respectively (Table 2).

DISCUSSION AND CONCLUSION

Urolithin-A was able to suppress lung inflammation, oxidative stress, and apoptosis in acute lung injury in mice (Jiao et al., 2024). Moreover in a prior study has already been elucidated as a treatment target against acute lung injury in mice (Lou et al., 2023). In a prior and interesting research urolithin-A was searched for its efficacy against pulmonary hypertension to those of mice were exposed to hypoxia. In that study urolithin-A alleviated progression of pulmonary hypertension through inhibition of PASMC pyroptosis, indicating the latter natural compound's treatment value (He et al., 2024). Furthermore urolithin-A was able to induce preventive autophagy for suppressing inflammatory respond, oxidation and relevant endoplasmic reticulum stress to those of 1-week-old C57BL/6 mice with pediatric pneumonia (Cao et al., 2022). In the present study urolithin-a significantly decreased (p <0.0001) WIN Scores (Table 2).

In general urolithin-a is the vast majority biologically active and investigated one in contrast to other relevant urolithin species, prominent with several efficacy [i.e. anti-

oxidant, anti-inflammatory, anti-cancer and anti-aging (Lee et al., 2021; Luan et al., 2021; Vini et al., 2023; Huang et al., 2023). Prior investigations denoted that urolithin-a was capable of suppressing NLRP3 inflammasome activation (Tao et al., 2021; Zhang et al., 2021), which potentially alters inflammation. Nevertheless, the therapeutic potential of urolithin a in respiratory disease among calves has not been established. This study reported herein, could thus be the first one investigating the efficacy of urolithin-a as a feed additive prevention and probably curing respiratory disease in a preclinical setting.

Oak leaves involve certain levels of hydrolyzable tannins [composed of gallotannins and ellagitannins] (Salminen et al., 2004). It has been suggested that elevated levels of tannins exhibited in forage inhibited bacteria among intestinal location and diminished ruminant performance, throughly via deducing intake and nutrient digestibility (Smith et al., 2005). It has been postulated that, tannins are capable of exhibiting both adverse and beneficial efficacy among ruminants, which is linked to type/volume nourished (Makkar et al., 1988; Shabtay et al., 2008). Both ellagitannins and ellagic acid paid great attention lately, as because of their antioxidant efficacy (Fukuda et al., 2003; Gil et al., 2000) Elagitannin-rich diets improved plasma lipids, diminished oxidative stress and induced apoptosis all were linked to declined risk for several chronic diseases (Aviram et al., 2000; Larossa et al., 2007; Heber, 2008). All aforementioned efficacy have throughly been dedicated to urolithins, well metabolites of ellagitannins exhibited via microbiota (Cerda et al., 2004, 2005; Gonzalez-Sarrías et al., 2010).

In a prior study it was nominated that ellagitannins were subjected to metabolization in rumen to urolithins. Several urolithins were investigated among ruminal fluid, plasma, urine and feces. Oak leaf ellagitannins declined as they were entirely transformed into urolithins [the vast majority isourolithin A/urolithin B], through rumen/gut microbiota. Finally foremost urolithins exhibited among cattle were detected as isourolithin A and urolithin B (González-Barrio et al., 2012). In the present study we did not have the possibility of detecting metabolites and gut microbiota alterations amon calves enrolled. Apart from this disadvantage we had some limitations. Detailed ultrasonographic examinations (Alic Ural and Ural, 2023), serum zonulin levels (Alic Ural and Ural, 2023), fractional exhaled nitric oxide tests were lacking, which would have helped both supporting diagnostic and prognostic portions, even if performed.

In the present study commercially available urolithin-a (DQQI Urolithin A Liquid Drops 3000 mg) was given p.o. to calves with respiratory disease without any side effects. Feed additive was well accepted. Mean (8.24 vs. 1.82) and median (8.00 vs. 2.00) WIN Scores were declined ($p<0.0001$) in respond to urolithin-a treatment prior to and thereafter, respectively. This novel and interested nutraceutical as given perorally (through gastrointestinal tract) was able to withdraw respiratory signs among calves, indicating proof of 'gut-lung axis' in this preclinical setting, field study. Available data herein, might have helped supportive treatment-regimes.

Acknowledgement

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Ethical Declaration

This study was performed with written owner consent and confirmed by Aydın Adnan Menderes University, Local Ethics Committee for Animal Researches (HADYEK) with report no: 64583101/2024/119.

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

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Assessment and Characterization of Challenges of Poultry Backyard Production in Baidoa District, Somalia

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ABSTRACT

A cross-sectional study was carried between Feb 2024 to July 2024 and employed to assess and characterize the challenges of backyard poultry production in Baidoa district, Bay region Somalia. The sampling technique of the study was multi-stage sampling technique. The researchers first selected four villages in Baidoa district purposively. Then the researcher visits each village and meets household heads, elders, and local representatives to gain permission of data collection and list of households for each village before the data collection. Then the researcher selected 25 households randomly for each village which counts 100 households. A single visit survey was employed by researchers. Primary data was collected using semi-structured questionnaire to take relevant data from the backyard poultry raisers through English in both open-ended and close-ended questions and translated to local language Somali when interviewed the households. Data collected from households were entered into an excel spread sheet then transferred to Statistical Package for the Social Science (SPSS) version 22.0. The study found that the backyard raisers were 77% of female, aged between 36 to 45 years, were 81% of them illiterate. 72% of respondents had a poultry flock between 5 to 40 chickens an average of 12.5 chickens per household. The reasons for keeping poultry in backyard farms 53% of households raised poultry for family consumption. The finding that 41% of backyard poultry producers experienced the loss of 1-10 chickens in the past year, and 24% lost 11-20 chickens. Regarding the seasonality of mortality, the current study's finding that 61% of producers reported peak mortality during the winter months is consistent with previous research. The study found that diseases were the primary cause of mortality (71%), the most common diseases affecting backyard poultry farms in the Baidoa district were Newcastle disease, coccidiosis, respiratory disease, and Fowl pox. In conclusion, the findings of the study depicted that the main challenges faced by backyard poultry raisers in Baidoa district were diseases, inadequate housing, feed availability, predators, lack access to the veterinary services and limited education of most of backyard producers on proper poultry management. Infectious diseases were the primary causes of high mortality in chickens reared by households, Newcastle disease and coccidiosis were the most reported diseases.

INTRODUCTION

Livestock production, particularly chickens, plays important socio-economic roles in developing countries Abebe, A. (2015). Poultry production has a major role in the economies of developing nations, including contributing to poverty alleviation through income generation and household food security Fitsum, M., and Aliy, M. (2014).

Village poultry represents an economical source of animal protein and a means of generating family income. It also promotes self-reliance among women, as they typically manage the sales of poultry and eggs, which provide them with a direct source of income for household expenses and food Assefa, H. (2019). Backyard chickens are particularly advantageous as they require minimal space, feed, and initial investment, making them well-suited to the conditions of extensive farming practices in Somalia.

In Somalia, backyard chickens are the most widespread type of poultry, with traditional breeds like feather chickens, short chickens, naked neck chickens, and American chickens being commonly reared Abdi-Soojeede and Funwie, (2022). These scavenging backyard chickens require limited space, feed, and capital investment compared to other domestic animals kept in Somalia. Backyard chickens play an important role in the livelihood and income of many Somalis, contributing to household food security and providing a readily available source of protein.

Modern poultry production started in Somalia some years ago, primarily initiated by colleges, research stations, and other institutions. Poultry experts began introducing and farming exotic breeds of chickens, along with disseminating improved management, feeding, housing, and healthcare practices to farmers. The introduction of these exotic breeds and modern production methods represents an important development in Somalia's poultry sector. The goal has been to transition some Somali poultry producers away from the traditional extensive systems towards more intensive, commercially oriented production Barre, A. et al. (2023).

However, traditional backyard poultry farming, characterized by small, unimproved indigenous flocks, remains the predominant model in many parts of the country. This extensive system continues to prevail, as the adoption and spread of modern poultry farming techniques have been limited so far Hagi, MO., (2023).

Despite the challenges in replacing traditional practices, the growth of a more efficient, commercial-scale poultry industry could play a significant role as Somalia seeks to enhance food security, increase incomes, and modernize its agricultural systems. Evaluating the progress, obstacles, and potential of these modern poultry production initiatives is crucial for understanding the evolving landscape of the poultry sector in Somalia Barre, A., et al. (2023).

Although backyard poultry farms are exposed to various challenges, there is limited assessment and documentation to characterize the major challenges in Baidoa backyard poultry farms. The overall production system in this region requires further study to better understand the constraints and opportunities for improving productivity and sustainability. By addressing the challenges faced by backyard poultry producers in the Baidoa district, interventions could potentially enhance the contribution of this important sector to household food security and income generation.

Therefore, this study was carried out to characterize the challenges of backyard poultry production in the Baidoa district of Somalia and to identify strategies for improving the productivity and resilience of this vital component of the local agricultural system, enhancing biosecurity strategies, and adopting disease prevention measures.

MATERIALS AND METHODS

Study area

Baidoa is the capital, largest city, and economic center of the Bay region in Southwestern Somalia. The region has borders with the Gedo, Bakool, and Shabelle regions. It is located approximately 250 km southwest of the national capital Mogadishu, in the heart of the inter-riverine region between the Shebelle and Jubba rivers.

The city is situated on a flat, arid plain at an elevation of around 510 meters above sea level. The climate is hot and semi-arid, with two rainy seasons- the gu (spring) rains from April to June and the deyr (Autumn) rains from October to November. The regional economy is primarily agricultural, relying on livestock production, farming, and the harvest of gum arabic.

Sampling

The sampling technique of the study was multi-stage sampling technique. The researchers first selected four villages in Baidoa district purposively. Then the researcher visits each village and meets household heads, elders, and local representatives to gain permission of data collection and list of households for each village before the data collection. Then the researcher selected 25 households randomly for each village which counts 100 households.

Research Population

The target populations for this study were 134 of households raising poultry from Baidoa district, Bay region, Somalia; the target population were households whose engage poultry farming in Baidoa district.

Sample Size

The sample size was determined by using Slovene's formula for sample-size determination:

This study was performed with written owner consent and confirmed by Aydın Adnan Menderes University, Local Ethics Committee for Animal Researches (HADYEK) with report no: 64583101/2024/119.

Where: $n = N / (1 + (N*e^2))$

N = Total Population
n = Sample size
e = is the confidence level at 5%
Substituting into the formula,
n = 134/ (1+ (134*0.0025))
= 134/ 1.335 Therefore n= 100 household heads.

Research Instrument

A semi-structured questionnaire was administered to obtain information from household heads. It contains closed and open-ended questions for collecting backyard poultry raisers their own information. The questionnaire was developed for this study and uploaded as a supplementary file.

Data Gathering Procedures

A single visit survey was employed by researchers. Primary data was collected using semi-structured questionnaire to take relevant data from the backyard

poultry raisers through English in both open-ended and close-ended questions and translated to local language Somali when interviewed the households. Data collection was done by a face-to-face personal interview method, and interviewer visits each household from four villages in Baidoa districts to administer the questionnaire personally. The interview questionnaire was focusing mainly on flock size reared, feed availability, market access, reasons for rearing, major challenges facing, and diseases that cause mortality in chicken of backyard poultry raisers.

Research Design

A cross-sectional study design was employed to assess and characterize the challenges of backyard poultry production in Baidoa district, Bay region Somalia. The study was also quantitative in design which determines the challenges of backyard poultry farms numerically.

Data Analysis

Household data were initially entered into an Excel spreadsheet (2010) and subsequently transferred to the Statistical Package for the Social Sciences (SPSS) version 22.0 for analysis. Descriptive statistics, including frequencies and percentages, were used to identify challenges in backyard poultry production, with the results presented in tables and charts.

RESULTS

The study sample initially included 100 households raising poultry. However, subsequent analysis was based on 76 participants, as 24 reported not having poultry. Among these 76, 74 households stated their poultry had died, while two reported no poultry deaths as shown table 1.

Table 1. How many chickens died from your flock in the past year

No. chicken died	Frequency	Percent	Valid Percent	Cumulative Percent
Non	2	2.0	2.6	2.6
1-10	41	41.0	53.9	56.6
11-20	24	24.0	31.6	88.2
21-30	6	6.0	7.9	96.1
31-40	2	2.0	2.6	98.7
more than 40	1	1.0	1.3	100.0
Total	76	76.0	100.0	

According to the table, the majority of backyard chicken raisers (41%) had 1 to 10 chickens die from their flock in the past year. 24% of them had 11 to 20 chickens die, 6% had 21 to 30 chickens die, 2% had 31 to 40 chickens die, 1% had more than 40 chickens die, but 2% of backyard chicken raisers reported no deaths in their flock in the past year.

The study found that the main challenges faced by backyard poultry farmers were a combination of lack of housing, predators, and diseases, and treatment availability as presented in Table 2. The most common diseases affecting backyard poultry farms in the Baidoa district were Newcastle disease, coccidiosis, respiratory disease, and Fowl pox as demonstrated in Table 3, and the season with the highest mortality peak for the chickens was winter as shown in Table 4.

Table 2. What are the main challenges that you faced in poultry farming

Main challenges that you faced in poultry farming	Frequency	Percent	Valid Percent	Cumulative Percent
Lack of housing, predators, disease	40	40.0	54.1	54.1
Feed, diseases	7	7.0	9.5	63.5
Diseases feed challenge, capacity	4	4.0	5.4	68.9
Feed availability, disease, predator, housing	7	7.0	9.5	78.4
Diseases, Treatment availability	16	16.0	21.6	100.0
Total	74	74.0	100.0	

According to the above table, the main challenges faced by the backyard chicken raisers were a combination of lack of housing, predators, and diseases, reported by 40% of respondents. The remaining respondents cited other challenges, including diseases and treatment availability (16%), feed availability, diseases, predators, and housing (7%), feed and diseases (7%), and diseases, feed challenges, and capacity (4%).

Table 3. Most common diseases that affect your flock

Most common diseases that affect your flock	No of Mentions	Rank of Disease
New castle disease	36	1
Chickenpox	2	7
Nutritional diseases	2	7
Salmonellosis	2	7
Fowl pox	19	4
Coccidiosis	35	2
Respiratory problem	21	3
Predators	4	6
Ectoparasites	7	5

According to the above table, the most common diseases that affected the backyard poultry raisers' flocks were Newcastle disease, which was ranked as the top issue, followed by Coccidiosis as the second most prevalent disease. Other diseases mentioned, in order of ranking, were respiratory problems, fowl pox, ectoparasites, predators, chickenpox, nutritional diseases, and salmonellosis.

Table 4. what season is the morality higher

Season morality higher	Frequency	Percent	Valid Percent	Cumulative Percent
Winter and summer	5	5.0	6.8	6.8
Winter	61	61.0	82.4	89.2
Summer	5	5.0	6.8	95.9
Winter, spring	2	2.0	2.7	98.6
Spring	1	1.0	1.4	100.0
Total	74	74.0	100.0	

According to the above table, the majority of backyard chicken raisers (61%) reported that the season with the highest mortality peak for their chickens was winter. The remaining respondents indicated other seasons with high mortality, including winter and summer (5%), summer (5%), winter and spring (2%), and spring (1%).

DISCUSSION AND CONCLUSION

This study provided information about the assessment of challenges and characterization of poultry backyard production in the Baidoa district of Baay region, Somalia. The study found that the majority of backyard poultry raisers were women (77 %), married (91%) as shown in Figure 1, and aged between 36 and 45 years old (45%) as presented in Figure 2. Most were housewives 69% (Figure 3) and owned and raised backyard chickens (76%) as presented in Figure 4.

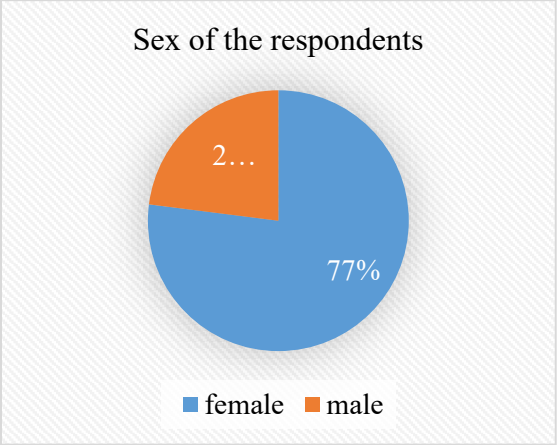

Figure 1. Sex of the respondent

The above chart shows that the majority of the backyard poultry raisers, 77%, were female, while only 23% were male. (Fig. 1).

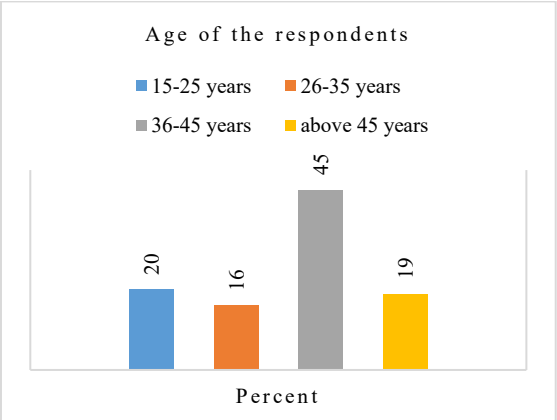

Figure 2. Age of the respondents

The above chart shows that most of the backyard chicken raisers were aged between 36 to 45 years. The other age groups represented were 15 to 25 years (20%), above 45 years (19%), and 26 to 35 years (16%) (Fig. 2).

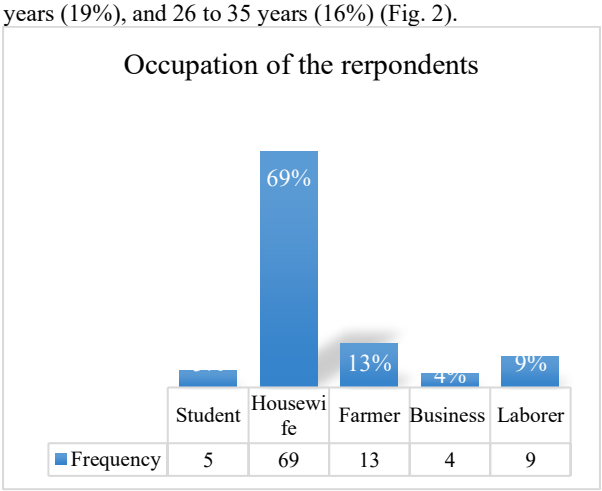

Figure 3. Occupation of respondents

The above chart indicates that the majority of the backyard chicken raisers (69%) were housewives. The remaining respondents were engaged in the following occupations: Farmers (13%), laborers (9%), students (5%), and business owners (4%).

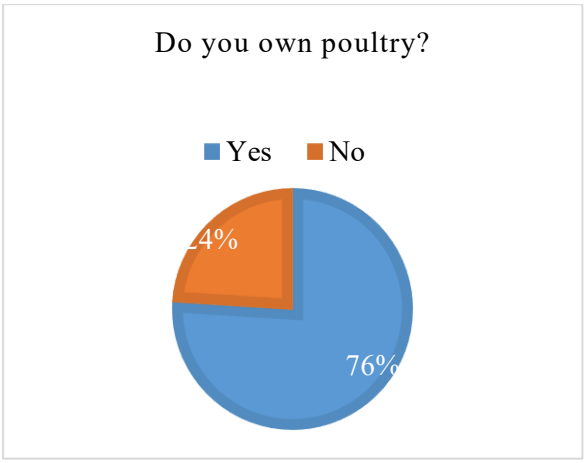

Figure 4. Do you own Poultry?

According to the above chart, 76% of the respondents owned and reared backyard chickens, while 24% did not own any poultry. Since those who did not own poultry were excluded from the rest of the questions, the total number of respondents for the remaining questions was 76.

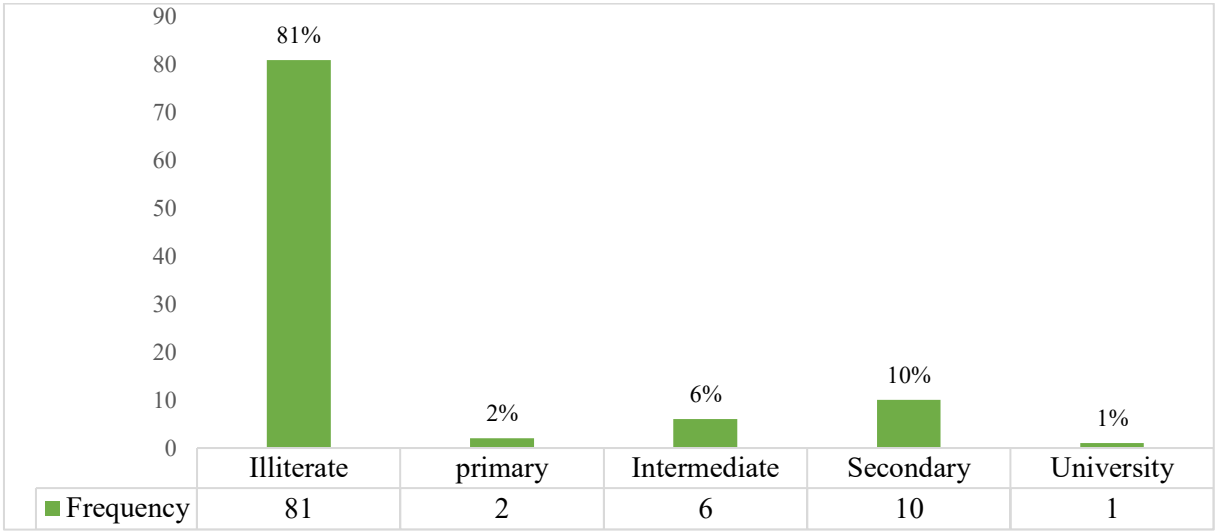

Figure 5. The education level of the respondents

According to the above chart, the majority of the backyard chicken raisers (81%) were illiterate. The remaining respondents had the following levels of education: Secondary (10%), Intermediate (6%), Primary (2%), and University (1%).

The current study revealed that the level of education of most of the family members involved in backyard rearing was illiterate, followed by a secondary education with 81% and 10% respectively as shown in Figure 5. The study has a support for previous study conducted in Bangladesh by Islam, M.S., et al. (2015). who reported that the family members and women typically involved in the rearing activities were mainly illiterate to a secondary educational background.

In this study, almost all poultry owners feed their chickens by giving them grains as indicated in Table 5. This result is close to the previous study conducted in Ethiopia by Abera, D., et al. (2024) who reported chickens was supplemented commonly with grain. Different feed types like maize mixed with sorghum (34.0%), sorghum (34.0%), sorghum, maize, soybean (4.0%) and wheat (2%) were used to supplement their chicken as presented in Table 6.

Table 5. What do you feed your poultry

Feed of chickens	Frequency	Percent	Valid Percent	Cumulative Percent
grain & rice	1	1.0	1.3	1.3
Grain	75	75.0	98.7	100.0
Total	76	76.0	100.0	

According to the table, the majority of backyard chicken raisers (75%) fed their poultry with grain. Only 1% of respondents fed their poultry a mix of grain and rice.

The current study found that 72% of respondents had a poultry flock between 5 to 40 chickens, an average of 12.5 chickens per household as shown in Table 7, the study was supported by a previous study carried out by Moges, F., et al. (2010). in Ethiopia who reported that the average flock size per household was 13 (ranged from 1 to 57). On other hand study conducted in Ethiopia by Abera, D., et al. (2024) showed that the average flock size was 10, ranging from 3 to 43 chickens per household. This difference could be the geographical location, and season carried out the studies, as backyard poultry size could be high in the rainy season due to the availability of scavenging feed in backyard poultry production.

Table 6. What type of grain do you feed your poultry

Type of grain	Frequency	Percent	Valid Percent	Cumulative Percent
maize, and sorghum	34	34.0	44.7	44.7
Sorghum	34	34.0	44.7	89.5
maize and wheat	2	2.0	2.6	92.1
sorghum, and wheat	2	2.0	2.6	94.7
sorghum, maize, soy pean	4	4.0	5.3	100.0
Total	76	76.0	100.0	

Table 7. What is the size of your flock

No. of chickens	Frequency	Percent	Valid Percent	Cumulative Percent
5-20	63	63.0	82.9	82.9
21-40	9	9.0	11.8	94.7
41-60	2	2.0	2.6	97.4
61-80	1	1.0	1.3	98.7
81-100	1	1.0	1.3	100.0
Total	76	76.0	100.0	

According to the table, the majority of the backyard poultry raisers (63%) had a poultry flock size between 5 to 20 chickens. The remaining respondents had the following flock sizes: 21 to 40 chickens (9%), 41 to 60 chickens (2%), and above 61 chickens (2%).

The reasons for keeping poultry in backyard farms were mostly 53% of households raised poultry for family consumption. Only 6% of households kept their poultry for family income as illustrated in Table 8. The study is not in line with other studies carried out in Bangladesh by Shanta, I. S., et al. (2017) who reported that 12% of households raised poultry solely for consumption.

Table 8. Reason for keeping poultry

Reason for keeping chicken	Frequency	Percent	Valid Percent	Cumulative Percent
Sale for income	7	7.0	9.2	9.2
Family consumption	53	53.0	69.7	78.9
Family consumption & sale	16	16.0	21.1	100.0
Total	76	76.0	100.0	

According to the above table, the primary reason for keeping poultry for most backyard chicken raisers (53%) was for family consumption. 16% of them kept poultry for both family consumption and sale, while only 7% kept poultry solely for the purpose of generating sale income.

The finding that 41% of backyard poultry producers experienced the loss of 1-10 chickens in the past year, and 24% lost 11-20 chickens as summarized in Table 1, aligns with a study by Smith, G. A., et al. (2020) that reported high mortality rates in small backyard flocks. However, a contrasting study by Johnson, K. A., et al. (2018) suggested that larger flock sizes were associated with higher mortality, which differs from the current findings.

Regarding the seasonality of mortality, the current study's finding that 61% of producers reported peak mortality during the winter months as presented in Table 4, is consistent with previous research Gómez, Y., et al. (2019) which identified winter as a critical period for increased disease prevalence and mortality in backyard poultry systems, likely due to environmental factors and resource constraints.

The current study's identification of diseases as the primary cause of mortality (71%) as shown in Table 9, is supported by several previous studies. Researchers have consistently found that infectious diseases, such as Newcastle disease, coccidiosis, and avian influenza, are major threats to the health and survival of backyard chickens Patel, K. K., et al. (2021); Lee, S. S., et al. (2016). However, a study by Williams, J. E., et al. (2022) suggests that predation may also be a significant contributor to mortality in some backyard poultry settings.

The current study's finding that the main challenges faced by backyard poultry producers include lack of housing, predators, and diseases (40%) as reflected in Table 2, aligns with previous research. Inadequate housing and protection from predators have been identified as common issues in small-scale poultry operations, which can increase the risk of disease outbreaks and mortality Farrell, P. H., et al. (2018); Sharma, B., et al. (2017). Additionally, the lack of access to treatment and feed availability has been highlighted as a significant challenge for backyard poultry producers in the Baidoa district.

The current study revealed that the most common diseases affecting backyard poultry farms in the Baidoa district were Newcastle disease, coccidiosis, respiratory disease, and Fowl pox as demonstrated in Table 12. This study is in line with previous studies on backyard poultry farming carried out in Ethiopia, Nigeria, Bangladesh, Pakistan, and India which reported similar patterns of diseases in chickens Islam M, et al. (2021), Balami AG, et al. (2014); Abebe E, Gugsa G. (2018).

Table 10. What is the common reason for most of the mortality

Common reason for most of the mortality	Frequency	Percent	Valid Percent	Cumulative Percent
Diseases	71	71.0	95.9	95.9
Diseases, paralysis	1	1.0	1.4	97.3
Diseases, predators	2	2.0	2.7	100.0
Total	74	74.0	100.0	

According to the above table, the most common reason cited by backyard chicken raisers for the mortality of their poultry was diseases, as indicated by 71% of the respondents. A small percentage, 2% and 1% respectively, reported that predators plus diseases and paralysis were the common reasons for poultry deaths.

In conclusion, the findings of the study depicted that the main challenges faced by backyard poultry raisers in Baidoa district were diseases, inadequate housing, feed availability, predators, lack access to the veterinary services and limited education of most of backyard producers on proper poultry management. Infectious diseases were the primary causes of high mortality in chickens reared by households, Newcastle disease and coccidiosis were the most reported diseases. Addressing the identified challenges, such as improving housing, biosecurity, feed availability, disease prevention, and access to veterinary support, and providing education and support to the backyard poultry raisers, could help enhance the sustainability and resilience of backyard poultry production in the Baidoa district.

Recommendations

Based on the conclusions of this study, the following are recommended:

- Authorities should provide education and support to help farmers construct suitable, well-ventilated chicken coops and implement basic biosecurity measures (e.g., fencing, pest control) to reduce the risk of disease outbreaks and predation.
- Authorities should take steps to promote vaccination programs, to protect against common poultry diseases, educate farmers on early disease detection and appropriate treatment options, and improve access to veterinary services and affordable medications.
- Ministry of livestock of Somalia should provide training and resources to help farmers diversify their feed sources and improve the nutritional quality of the feed to contribute to better bird health and productivity.
- Authorities and NGOs should organize training programs on best practices in backyard poultry management, establish demonstration farms or

model poultry units to showcase improved techniques, and facilitate access to information and resources on poultry husbandry, disease prevention, and marketing.
- Local NGOs provide support and guidance to help farmers access local markets, develop value-added products, and diversify their income sources to improve the overall sustainability of their backyard poultry operations.
- Bay region administrators and local veterinarians should facilitate the formation of farmer groups or cooperatives to share knowledge, resources, and collectively address common issues.
- Researchers should carry out further studies to determine the prevalence of poultry diseases prevailing in the study area to help develop a sustainable strategy of disease prevention and control.

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Ethical Declaration
The study was approved by the ethics committee of Somali National University - SNU (in line with the Declaration of Somali Data protection Authority, Mogadishu; (No. 005, 2023). The study follows data protection rules as of the Somali Data Protection act (2023). The researchers informed a written consent to all participants prior of data collection and all study household heads accept consent to participate the study voluntarily.

Conflict of Interest
The authors declare that they have no competing interests.

Authorship contributions
Concept: O.M.S., M.O.S., Design: O.M.S., M.O.S., Data Collection or Processing: O.M.S., M.O.S., M.A.Y.I., Analysis or Interpretation: O.M.S., M.O.S., M.A.Y.I., Literature Search: O.M.S., M.O.S., Writing: O.M.S., M.O.S., M.A.Y.I.,

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Investigation of the Presence of Extended Spectrum Beta Lactamase, Carbapenem and Colistin Resistances in *Salmonella* spp. Isolates by Phenotypic Method and PCR

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ABSTRACT

The study was aimed to investigate extended-spectrum beta lactamase (ESBL) and carbapenem resistance by phenotypically and genotypically in *Salmonella* spp. strains isolated from stool samples of free-range chickens between August 2018 and January 2020. Also it was aimed to investigate colistin resistance gene mcr-1 by PCR. Stool samples collected from flocks' ground from 110 different family-type chicken flocks by veterinarians about at least 25 grams of each flocks into the sterile containers for microbiological examinations in regions and villages of Balıkesir province. *Salmonella* spp. isolation were done according to ISO 6579-2017 method. A total of 34 *Salmonella* spp. isolates were identified based on biochemical characterization and confirmed by PCR targeting the *16S rRNA* gene. ESBL and carbapenem resistance investigated by conventionally according to EUCAST methods. The presence of *blaTEM, blaSHV, blaCTX-M, IMP, OXA-48 like, NDM, KPC* resistance genes was investigated using the polymerase chain reaction (PCR) method. Colistin resistance was investigated by PCR method to determine the presence of mcr-1 gene. One isolate (2.94%) showing a meropenem inhibition zone <25 mm and resistance to piperacillin/tazobactam was classified as carbapenem-resistant. As a result of serotyping of carbapenem resistant isolate was identified as *Salmonella* Infantis. PCR analysis revealed the presence of both the *IMP* and *OXA-48-like* resistance genes in this strain. Phenotypic characterization supported the genotypic findings were resistance to temocillin indicated *OXA-48-like* carbapenemase activity, while the detection of a synergistic effect with dipicolinic acid (DPA) in the combined disk diffusion assay confirmed the functional expression of a metallo-β-lactamase (MBL) associated with the *IMP* gene. To the best of our knowledge, this study represents the first report of the detection of carbapenem resistance in *Salmonella* spp. isolates, isolated from chicken stool in Türkiye. The presence of carbapenem resistance was detected in one *Salmonella* isolate, serotyped as *S.* Infantis.

INTRODUCTION

Carbapenemases are β-lactamase enzymes capable of hydrolyzing a broad spectrum of β-lactam antibiotics, including penicillins, most cephalosporins, carbapenems, and, in some cases, monobactams. Unlike metallo-β-lactamases (MBLs), certain carbapenemases are not inhibited by metal ion chelators. These enzymes represent a significant clinical threat due to their ability to confer resistance to nearly all β-lactam antibiotics and their potential for horizontal gene transfer. Moreover, carbapenemase-producing bacteria often harbor additional resistance mechanisms, resulting in multidrug-resistant phenotypes. Infections caused by such organisms, particularly those belonging to the *Enterobacteriaceae* family, are associated with limited treatment options and high mortality rates (EUCAST, 2017B).

Carbapenem-resistant Enterobacteriaceae (CRE) have been designated as a Priority 1 – Critical pathogen group

in the World Health Organization's 2017 list of antibiotic-resistant bacteria for which the development of new antimicrobial agents is urgently required (WHO, 2017).

Among the various carbapenemases, OXA-48 is currently the most rapidly disseminating type across Europe, with reports of regional outbreaks in several countries. IMP-type carbapenemases, while less prevalent in Europe, are widely distributed in other parts of the world. The clinical significance of carbapenemases lies in their ability to confer resistance to nearly all β-lactam antibiotics, their high potential for horizontal gene transfer, and their frequent co-occurrence with other resistance determinants, leading to multidrug-resistant phenotypes. Infections caused by carbapenemase-producing organisms are associated with limited treatment options and are often linked to high morbidity and mortality rates (EUCAST, 2017b).

Colistin, long regarded as a last-resort antibiotic for the treatment of infections caused by multidrug-resistant Gram-negative bacteria, has increasingly lost its clinical effectiveness due to the emergence of resistance. This resistance arises through chromosomal mutations as well as the acquisition of plasmid-mediated resistance determinants, most notably the mobilized colistin resistance (mcr) genes. The first mcr gene, mcr-1, was initially identified in *Escherichia coli* isolated in China in 2016. Since then, numerous studies have reported a growing diversity of mcr variants, currently ranging from mcr-1 to mcr-10, predominantly among members of the *Enterobacteriaceae* family across different regions of the world. The rapid global dissemination of these plasmid-borne colistin resistance genes represents a serious and escalating threat to public health, significantly compromising the therapeutic value of one of the last available treatment options against critical Gram-negative infections (Mondal et al., 2024).

Salmonella infections are among the zoonotic diseases that cause significant economic losses in poultry farming by leading to reduced productivity and high mortality rates (Babacan and Karadeniz, 2019; Hossain et al., 2021; Yıldırım et al., 2022). These bacteria can also cause infections in humans. *Salmonella* bacteria belong to the family *Enterobacteriaceae* (Babacan and Karadeniz, 2019; Hossain et al., 2021). Chickens are the primary source of transmission for Salmonella infections in humans, and infected animals can transmit the bacteria to humans through the food chain. When these infections are spread through food, they pose a significant public health threat, and Salmonella outbreaks can occur from the consumption of contaminated food. In recent years, there has been a notable increase in the prevalence of zoonotic gastrointestinal diseases worldwide, with *Salmonella* species being more frequently isolated compared to other animals and animal-derived foods. The rise in poultry consumption has also contributed to the increased prevalence of poultry-related zoonotic diseases. *Salmonella* serovars can cause acute or chronic, often subclinical infections in poultry, leading to food poisoning in humans (Akgül et al., 2021; Kırkan et al., 2017; Lozano-Villegas et al., 2024; Quinn et al., 2004; Salar et al., 2015). Monitoring antibiotic resistance in zoonotic and commensal bacteria is highly important for understanding the development and spread of resistance. Therefore, control programs targeting poultry flocks are being implemented in Europe, and in our country, the National Salmonella Control Program was initiated by the Ministry of Agriculture and Forestry in 2018 (Republic of Türkiye, Ministry of Agriculture and Ministry, 2018).

Antibiotics are commonly used in the treatment of Salmonella infections, which contributes to the development of antibiotic resistance. Notably, serovars such as *S.* Enteritidis, *S.* Typhimurium, *S.* Kentucky, and *S.* Infantis can cause infections in humans and are closely monitored for antibiotic resistance. The spread of especially multidrug-resistant *S.* Infantis and *S.* Kentucky strains should be carefully tracked, as *S.* Kentucky shows high resistance to ciprofloxacin. (Akgül et al., 2021; Bénao et al., 2024; Sevük Akkaya and Ak, 2023; Şahan et al., 2016; Waktole et al., 2024).

Animals, particularly poultry, serve as important reservoirs in the transmission of antimicrobial-resistant bacteria to humans, raising critical concerns for food safety and public health. Numerous studies have documented the emergence of carbapenem-resistant *Salmonella* strains isolated from both human clinical specimens and poultry-related sources. (Abdel-Kader et al., 2022).

In both developed and developing countries, the multidrug resistance of *Salmonella* strains isolated from poultry meat poses a serious risk to human health. The transmission of antibiotic-resistant bacteria to humans through food threatens public health and can lead to the transfer of resistance genes to other pathogens. Therefore, the proper use of antibiotics in poultry and the implementation of appropriate preventive health measures are of great importance. The use of drugs that do not lead to antibiotic residues can help prevent the development of multidrug resistance. However, the ease with which antibiotic resistance genes can be transferred between bacteria further complicates the situation (Kırkan et al., 2017; Kutu, 2017; Lozano-Villegas et al., 2024; Şahan et al., 2016; Temelli et al., 2012).

The study was aimed to assess extended- spectrum beta lactamase and carbapenem resistance by phenotypically and genotypically in *Salmonella* strains isolated from stool samples of free-range chickens between August 2018 and January 2020. Also it was aimed to investigate colistin resistance gene *mcr-1* by PCR, according to the multiplex pcr method for carbapenem resistance genes include *mcr-1*.

MATERIALS AND METHODS

Materials and Sampling

Stool samples collected from flocks' ground from 110 different family-type chicken flocks by veterinarians about at least 25 grams of each flocks into the sterile containers for microbiological examinations in different regions and villages of Balıkesir province between August 2018 and January 2020. Samples were delivered to the laboratory under cold chain conditions by veterinarians.

Isolation and Identification of Salmonella spp.

Salmonella spp. isolation were done according to ISO 6579-2017 (ISO, 2017) method from chicken stools. Then, the suspicious colonies growing on XLT_4 agar (Merck, Germany) were identified as *Salmonella* spp. with biochemical tests (Gram negative rod, oxidase negative, catalase positive, H_2S positive, indol negative, gas positive, glikoz fermentative, lactose and sucrose non-fermentative, urease negative, metil red test positive, Voges Preskauer test negative) (ISO, 2017; Quinn et al., 2004) and genus-specific primers targeting the *16S rRNA* gene of *Salmonella enterica* were previously described by Mir et al. (2015) (Table 1), along with amplification conditions (Mir et al., 2015), by PCR method. The serotype of a *Salmonella* spp. isolate, which exhibited carbapenem resistance determined phenotypically and by

PCR (ISO, 2017; Mir et al., 2015; Quinn et al., 2004), was identified through serotyping according to ISO 6579-2017 (ISO, 2017).

To obtain pure cultures for DNA extraction, all *Salmonella* spp. isolates previously identified through biochemical testing were streaked out into Nutrient Broth (NB, Oxoid, UK) and incubated at 37°C for 18 hours. Following incubation, 1 mL of each culture was centrifuged at 5000 × g for 10 minutes. The supernatant was discarded, and genomic DNA was extracted from the resulting pellet using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA), following the manufacturer's protocol optimized for Gram-negative bacteria. Extracted DNA samples were stored at −20°C until further use in PCR for other molecular analyses.

PCR amplification of the *Salmonella enterica 16S rRNA* gene was performed in a total reaction volume of 25 µL. Each reaction included 5 µL of template DNA and 20 µL of PCR master mix, which consisted of 12.5 µL of DreamTaq PCR Master Mix (2X) (Thermo Scientific, USA), 7.3 µL of DEPC-treated water, 0.1 µL of forward primer (100 pmol/µL), and 0.1 µL of reverse primer (100 pmol/µL). PCR cycling conditions were applied according to the protocol described by Mir et al. (2015), and primer sequences are listed in Table 2.

PCR products (10 µL of amplicon mixed with 2 µL of 10X BlueJuice gel loading buffer; Thermo Scientific, USA) were separated via electrophoresis on 1.5% agarose gels (Prona) prepared in 1X Tris-Borate-EDTA (TBE) buffer. DNA fragments were visualized using a gel documentation system (EBOX CX5 TS EDGE, Vilber). A 100 bp DNA ladder (GeneRuler 100 bp, Thermo Scientific, USA) served as a molecular size marker.

Phenotypic Detection of Extended-Spectrum Beta Lactamase and Carbapenem Resistance

To determine the presence of extended-spectrum beta-lactamase and carbapenem resistance in isolates identified as *Salmonella* spp. through biochemical tests and PCR, conventional methods outlined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Jean et al. (2015) were done (EUCAST, 2017a; EUCAST, 2017b; Jean et al., 2015). According to the EUCAST and Jean et al. (2015) methods, a combined disk diffusion test was performed using cefotaxime 5µg (Liofilchem, Italy), ceftazidime 10µg (Liofilchem, Italy), cefpdoxime 10µg (Liofilchem, Italy), cefotaxime-clavulanic acid 40µg (Liofilchem, Italy) and cefepime 30µg (Liofilchem, Italy), cefepime-clavulanic acid 40µg (Liofilchem, Italy) disks to determine the presence of ESBL. The results of disc diffusion test and inhibition zone diameters were evaluated diameters were evaluated according to the EUCAST standards and Jean et al. (2015) (EUCAST, 2017a; EUCAST, 2017b; Jean et al., 2015).

According to the EUCAST method, a disk diffusion test was performed using meropenem 10µg (Liofilchem, Italy), meropenem + phenylboronic acid (Liofilchem, Italy), meropenem + dipicolinic acid (DPA) (Liofilchem, Italy), meropenem + cloxacillin (Liofilchem, Italy), temocillin 30µg (Liofilchem, Italy), piperacillin-tazobactam 110µg (Oxoid, UK) disks to determine carbapenem resistance. Except for piperacillin-tazobactam, the results were evaluated according to the EUCAST standards and van Dijk et al. (2014). (EUCAST, 2017a; EUCAST, 2017b; van Dijk et al., 2014). Piperacillin- tazobactam results was evaluated according to CLSI guideline (CLSI, 2020).

Genomic DNA Extraction and Detection of Antimicrobial Resistance Genes via PCR

Additionally, PCR was performed using specific primers and amplification conditions described by Bektaş et al. (2018) for ESBL genes to detect the presence of *blaTEM*, *blaSHV*, *blaCTX-M* and Hatrongjit et al. (2018) for carbapenem resistance genes (*IMP*, *OXA-48 like*, *NDM* and *KPC*) and one of the colistin resistance gene *mcr-1* (Bauer et al., 1966; Bektaş et al., 2018; EUCAST, 2017a; EUCAST, 2017b; Hatrongjit et al., 2018) (Table 3 and Table 4). Colistin resistance was investigated using the PCR method including multiplex pcr with carbapenem resistance genes according to to determine the presence of the *mcr-1* gene (Hatrongjit et al., 2018).

Polymerase Chain Reaction (PCR) assays were employed to detect genes associated with extended-spectrum β-lactamases (ESBLs), carbapenemases, and colistin resistance. Target gene regions were amplified using specific primer sets, synthesized commercially, based on sequences previously reported by Bektaş et al. (2018) and Hatrongjit et al., 2018 respectively (Bektaş et al., 2018; Hatrongjit et al., 2018).

For the detection of ESBL genes (*blaTEM*, *blaSHV*, *blaCTX-M*), PCR reactions were carried out in a final volume of 25 µL, which included 5 µL of template DNA and 20 µL of PCR master mix. The PCR mix consisted of 12.5 µL of DreamTaq PCR Master Mix (2X) (Thermo Scientific, USA), 7.3 µL of DEPC-treated water, and 0.1 µL of each forward and reverse primer (100 pmol/µL). Amplification conditions were applied according to the protocol described by Bektaş et al., (2018).

Carbapenemase and colistin resistance genes were screened through multiplex PCR, following the methodology described by Hatrongjit et al., (2018). Each 15 µL reaction included 2 µL of extracted DNA and 13 µL of PCR master mix. This mix contained 8.8 µL of DreamTaq PCR Master Mix (2X), 2.2 µL of DEPC-treated water, and 0.2 µL of each forward and reverse primer (100 pmol/µL). Amplification conditions followed the protocol of Hatrongjit et al., (2018), as detailed in Table 3.

All PCR products, including those amplified for carbapenemase, *mcr-1*, and ESBL genes, were resolved by gel electrophoresis. A total of 10 µL of PCR product was mixed with 2 µL of 10X BlueJuice gel loading buffer (Thermo Scientific, USA) and loaded onto a 1.5% agarose gel (Prona) prepared in 1X Tris-Borate-EDTA (TBE) buffer. Electrophoresis was conducted, and DNA bands were visualized using a gel documentation system (EBOX CX5 TS EDGE, Vilber). A 100 bp DNA ladder (GeneRuler 100 bp, Thermo Scientific, USA) was used as a molecular weight marker.

In the study, reference strains of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC® 13076™), *E. coli* (ATCC® 25922™), IMP: *E. coli* NCTC 13476, NDM: *K. pneumonia* NCTC 13443, KPC: *K. pneumonia* CCUG 56233, mcr-1: *E. coli* NCTC 13846, and CTX-M: *E. coli* CCUG 62975 were used as positive controls in conventional and molecular methods obtained from the Ministry of Agriculture and Forestry Giresun Food Control Laboratory and the Ministry of Health, General Directorate of Public Health, Microbiology Reference Laboratory. PCR master mix (without DNA) was used as the negative control in the PCR method.

Table 1. Genus-specific primers targeting the *16S rRNA* gene of *Salmonella enterica.*

Primers	Sequence	Target Gene	Base Pairs	Reference
16S rRNA-F	TGTTGTGGTTAATAACCGCA	*16S rRNA*	574 bp	Mir et al. (2015)
16S Rrna-R	CACAAATCCATCTCTGGA			

Table 2. *Salmonella 16S rRNA* gene's amplification conditions in PCR

PCR Steps	Cycles Conditions	Cycles numbers	References
Initial Denaturation	94°C, 2 min.	1 cycle	
Denaturation	94°C, 20 sec.		
Annealing	54°C, 20 sec.	30 cycles	Mir et al. (2015)
Extention	72°C, 30 sec.		
Final Extention	72°C, 2 min.	1 cyle	

Table 3. Carbapenem resistance and *mcr-1* genes' amplification condiditons in PCR

PCR Steps	Cycles Conditions	Cycles numbers	References
Initial Denaturation	95°C, 30 sec.	1 cycle	
Denaturation	95°C, 30 sec.		
Annealing	56°C, 30 sec.	30 cycles	Hatrongjit et al. (2018)
Extention	72°C, 45 sec.		
Final Extention	72°C, 5 min.	1 cyle	

RESULTS

A total of 34 *Salmonella* spp. were isolated using the ISO 6579-2017 method (ISO, 2017) (Figure 1) from stool samples and identified with biochemical tests (ISO, 2017; Quinn et. al., 2004) and genus-specific primers targeting the *16S rRNA* gene of *Salmonella enterica* (Mir et al., 2015) in PCR (Figure 2).

No extended-spectrum beta-lactamase activity was detected in 34 *Salmonella* spp. isolates using the disk diffusion test according to EUCAST (EUCAST, 2017a; EUCAST, 2017b), Jean et al. (2015) and PCR results.

According to the method specified in the EUCAST standard (EUCAST, 2017b), one *Salmonella* spp. isolate was identified as carbapenem-resistant, which had a meropenem zone <25mm and resistance to piperacillin/tazobactam according to EUCAST and CLSI guidelines, respectively (CLSI, 2020; EUCAST, 2017b). 33 of 34 *Salmonella* isolate no showed carbapenem resistance according to EUCAST guideline (EUCAST, 2017b).

Phenotypic analysis of resistance mechanisms was conducted in accordance with EUCAST guidelines (EUCAST, 2017b) and the method described by van Dijk et al. (2014). Resistance to temocillin was indicative of the presence of *OXA-48-like* genes. In one carbapenem-resistant *Salmonella* spp. isolate, the presence of a metallo-β-lactamase (MBL) mechanism was confirmed by enhanced synergy in the combined disk diffusion test using dipicolinic acid (DPA), supporting the presence of the *IMP* gene (EUCAST, 2017b; van Dijk et al., 2014).

Serotyping (ISO, 2017) were done to only this carbapenem-resistant one *Salmonella* spp. strain in Microbiology Department (Salmonella reference laboratory) in Ankara University Faculty of Veterinary Medicine, Türkiye. Because this strain is epidemiologically important as well as fo One Health principles. The carbapenem-resistant isolate was serologically typed as *S.* Infantis according to Kauffman-White scheme (ISO, 2017) by Salmonella referance laboratory.

According to the EUCAST procedure, an isolate phenotypically determined to be resistant to carbapenems and serotyped as *Salmonella* Infantis was found to carry the *IMP* and *OXA-48-like* carbapenem resistance genes based on PCR results (Figure 3). Carbapenem resistance genes were not detected by PCR in 33 of 34 *Salmonella* isolates; it was not detected phenotypically according to EUCAST procedure. Phenotypic results were found to be consistent with genotypic findings.

As a result of, carbapenem resistance was identified both phenotypically and genotypically in one isolate, which was serotyped as *Salmonella* Infantis. The colistin resistance gene *mcr-1* was not detected in 34 *Salmonella* isolates by PCR. All results were shown on Table 4.

Figure 1. Isolated *Salmonella* spp. On XLT_4 agar

Table 4. Results of isolation, identfication, carbapenem resistance and ESBL, *mcr-1* gene

Number of Stool	Isolated and Identified *Salmonella* spp.	Carbapenem resistant *Salmonella* spp. by phenotypic method	Carbapenem resistant *Salmonella* spp. by PCR	ESBL positive *Salmonella* spp. by phenotypic method and PCR
110	34 (30.9%)	1 (2.94%)	1 (2.94%) (*IMP* and *OXA-48-like* genes detected)	-

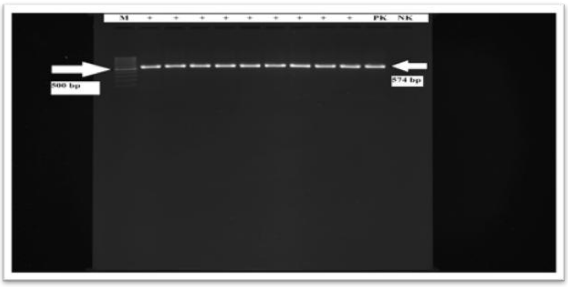

Figure 2. PCR results of *16S rRNA* gene of *Salmonella enterica*

M: Marker (100 bp DNA ladder (GeneRuler 100 bp, Thermo Scientific, USA), +: *16S rRNA* gene of *Salmonella enterica* positive strains, PK: Positive control, NK: Negative control.

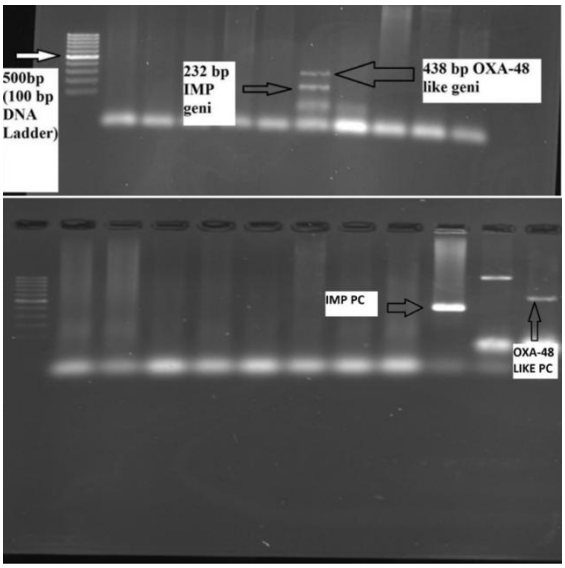

Figure 3. PCR results for carbapenemase resistance genes identified the presence of the *IMP* and *OXA-48-like* genes in *Salmonella* isolates.

M: Marker (100 bp DNA ladder (GeneRuler 100 bp, Thermo Scientific, USA), -: carbapenem resistance genes

DISCUSSION AND CONCLUSION

This study reports, for the first time, the presence of carbapenem resistance in *Salmonella* isolates obtained from chicken stool in Türkiye. Yıldız and Demirbilek (2024) have reported that they have found carbapenem resistant *S.* Enteritidis from dog isolates.

Dishan et al. (2024) reported that, in a study conducted in Türkiye, they isolated 112 *Salmonella enterica* strains from 293 chicken meat samples using the ISO 6579 method, and that all of these isolates were susceptible to meropenem.

Given the significance of Salmonella infections in poultry and their potential for zoonotic transmission through the food chain within the One Health approach, the identification of a carbapenem-resistant *Salmonella* isolate was deemed to be of considerable epidemiological importance.

The development of antimicrobial resistance (AMR) is associated with the inappropriate use of primary treatment drugs and the extensive application of antimicrobial compounds, along with the rising demand for animal-derived food products (Lozano-Villegas, 2024).

The development of antibiotic resistance in *Salmonella* spp. isolated from animal-derived food products predominantly occurs via two main pathways: co-resistance, in which a single genetic determinant confers resistance to multiple antimicrobial agents, and the simultaneous presence of distinct resistance genes targeting different antibiotic classes. Co-resistance facilitates the persistence and dissemination of *Salmonella* strains with resistance to a broad spectrum of antibiotics, thereby contributing to the emergence of multidrug-resistant (MDR) isolates, defined as those exhibiting resistance to three or more classes of antimicrobials. The proliferation of MDR *Salmonella* poses a serious public health risk, particularly due to their resistance to critically important antibiotics such as carbapenems, fluoroquinolones and third-generation cephalosporins (Oh et. al., 2025).

According to the 2024 EFSA (European Food Safety Authority) report, multidrug resistance is observed in *Salmonella* strains isolated from poultry meat in both developed and developing countries. In particular, the emergence of carbapenem-resistant *Salmonella* spp. isolates has been reported in 2021 and 2022 (EFSA, 2024).

Di Taranto et al. (2025) were declared that among the 128 *Salmonella* strains analyzed, 16 isolates (12.5%) were identified as extended-spectrum β-lactamase (ESBL) producers, all of which also displayed MDR profiles. These findings reinforce the role of chicken products as a significant reservoir of *Salmonella* spp. and emphasize that *S.* Infantis was the most frequently detected serotype, accounting for 85.93% of all isolates.

Dehdasti et al. (2024) were declared that they found NDM-1 gene in 6 of 39 *Salmonella* strains.

Kanaan et al. (2022) were showed that among the 20 *S.* Enteritidis isolates resistant to carbapenems, the most frequently detected carbapenemase gene was *blaIMP* (35.0%, n = 7), followed by *blaOXA−48−like* (25.0%, n = 5) and *blaNDM* (10.0%, n = 2). Notably, no isolates harbored the *blaKPC*or *blaVIM* genes.

Similar with Kanaan et al. (2022), in this study, *IMP* and *OXA-48-like* carbapenem resistance genes was detected in *S.* Infantis isolate.

In the report published by the European Food Safety Authority (EFSA) in 2017, *Salmonella Infantis* was reported as the fourth most common serotype in humans, and notably, it was identified as the most prevalent

serotype in poultry over the past five years, accounting for 33.6% of cases (Torun and Müştak, 2019).

In our country, studies conducted under the Salmonella Control Program initiated by the Ministry of Agriculture and Forestry in 2018 have shown that *S.* Infantis the most common *Salmonella* serotype in chicken isolates in Türkiye (Republic of Türkiye, Ministry of Agriculture and Ministry, 2018). Also Yapıcıer and Sareyyüpoğlu (2022) was reported that the most isolated seroype was *S.* Infantis in their study. The detection of carbapenem resistance in the *S.* Infantis serotype in this study is considered significant due to the prevalence of this serotype in our country. From a public health perspective, especially considering that carbapenem antibiotics are used for human treatment, it is believed that carbapenem antibiotics may not be effective in treating foodborne *S.* Infantis infections, and resistance may spread among bacteria.

This study reports, for the first time, the presence of carbapenem resistance in *Salmonella* isolates obtained from chicken stool and the *S.* Infantis serotype in Türkiye. In this study, carbapenem resistance was detected in one *Salmonella* isolate identified as *S.* Infantis. However, it was thought that this resistance may be part of the global increase in carbapenem resistance and multidrug resistance (MDR) observed in *Salmonella* spp..

Phenotypic results were found to be consistent with genotypic findings for carbapenem resistance. An isolate phenotypically determined to be resistant to carbapenems and serotyped as *S.* Infantis was found to carry the *IMP* and *OXA-48-like* carbapenem resistance genes based on PCR results. Carbapenem resistance genes were not detected by PCR in 33 of 34 *Salmonella* isolates; it was not detected phenotypically according to EUCAST procedure. In this study, this result demonstrated that the carbapenem resistance determined phenotypically was also genetically expressed.

The identification of carbapenem resistance genes via PCR in an isolate serotyped as *S. Infantis*, which exhibited phenotypic resistance based on the EUCAST guidelines, was considered epidemiologically important within the scope of the One Health concept, due to the possibility of horizontal gene transfer.

Carbapenemases are regarded as highly significant from an epidemiological perspective, especially when they reduce the effectiveness of carbapenem antibiotics such as imipenem, meropenem, ertapenem, and doripenem. These enzymes are particularly concerning due to their capacity to mediate resistance against nearly all β-lactam antibiotics and their ability to spread efficiently through horizontal gene transfer. Consequently, carbapenemase-producing organisms often exhibit multidrug resistance and are associated with infections that carry substantial mortality risks (EUCAST, 2017b). OXA-48-like carbapenemases are swiftly proliferating across Europe (Hopkins et al., 2019). Considering that *Salmonella* is most commonly transmitted to humans through poultry products as a foodborne pathogen, the detection of a carbapenem-resistant *Salmonella* isolate in this study is of particular significance within the One Health concept, especially given the critical role of carbapenem antibiotics in human medicine. It was considered that conducting both phenotypic and genotypic surveillance including all carbapenem genes for carbapenem resistance in bacteria could be beneficial. In this study, carbapenem resistance was detected in only one of the *Salmonella* spp. isolates and one province region of country; however, as the World Health Organization (WHO, 2017) has reported the global dissemination of carbapenem-resistant *Enterobacteriaceae* strains, further investigations are considered necessary for identified all carbapenem resistance genes and all regions of country. In future studies, plasmids or other mobile genetic elements could be identified for epidemiological typing, allowing the determination of clonal relationships and the potential risk of outbreaks. Moreover, it is considered important to perform both phenotypic and genotypic screenings for carbapenem resistance in isolates obtained from animals. Assessing the genetic similarities between carbapenem-resistant isolates from human and animal sources may provide valuable insights into their potential epidemiological linkage.

Particularly, as carbapenem-resistant isolates may cause infections in humans as foodborne pathogens, it is considered that the continuous monitoring of carbapenem resistance through the One Health approach is essential.

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Ethical Declaration

Stool, feces or litter collected from coop floor and clinical applications for diagnosis and treatment are not subject to ethics committee approval according to Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmelik 8-k, ethics committee permission is not required for this study.

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept:Concept: O.B., Design: O.B., Data Collection or Processing: O.B., Analysis and Interpretation: O.B., Literature Search: O.B., Writing: O.B.

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Investigation of the Prevalence of Lungworm According to Fecal Examination in Cats in Kırklareli Region/Türkiye

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ABSTRACT

The aim of this study is to investigate the prevalence of lungworm according to fecal examination in cats in Kırklareli region. For this purpose, stool samples were taken from the defecation areas of 100 owned and stray cats in the Kırklareli region. Stool samples taken were delivered to Kırıkkale University, Faculty of Veterinary Medicine, Routine and Epidemiology laboratory in accordance with the cold chain rules. Stool samples were examined for lungworm using the Fülleborn Flotation technique and the Baermann technique. McMaster test was used to determine the number of larvae/eggs per gram of feces, which were positive for the larvae of lungworm. As a result of the study, *Aelurostrongylus abstrusus* larvae, one of the lungworm of cats, were found in 8 (8%) of the cat feces examined by the Baermann method, while all feces were negative in the Fülleborn flotation method. There was no statistically significant difference in terms of *A. abstrusus* positivity according to age, breed and gender. A minimum of 50 and a maximum of 500 larvae were found in per gram of feces using the McMaster technique. In conclusion, this study is the first to determine lungworm in cats in Kırklareli region. It has been demonstrated once again that *A. abstrusus* is the dominant species as the lungworm in cats in Türkiye.

INTRODUCTION

Aelurostrongylus abstrusus, *Troglostrongylus brevior*, *T. subcrenatus*, *Oslerus rostratus* (Syn: *Anafilaroides rostratus*) and *Capillaria aerophila* (Syn: *Eucoleus aerophilus*) have been reported as lungworm in domestic cats (Traversa et al., 2009; Jefferies et al., 2010; Brianti et al., 2012; Brianti et al., 2014; Di Cesare et al., 2014; Traversa and Di Cesare, 2016; Giannelli et al., 2017). The infectious stage depends on the species and it is first or third stage larvae. Some species develop directly while others have an indirect development (Doğanay et al., 2018).

Aelurostrongylus abstrusus is a nematode classified in the family Angiostrongylidae of the superfamily Metastrongyloidea (Conboy and Sykes, 2023). It is considered the most important respiratory system parasite of domestic cats in terms of its world and the clinical signs it causes (Traversa and Di Cesare, 2013; Colella et al., 2019). Adult *A. abstrusus* inhabit nodules in the alveoli, alveolar ducts, and bronchioles of infected hosts (Traversa and Di Cesare, 2016). Females are 9-10 mm length and 100 µm width, and males are 5-6 mm length and 70 µm width (Szatmari, 2016). *Aelurostrongylus abstrusus* is a nematode parasite with an indirect life cycle. While the final hosts of the parasite are cats, the intermediate hosts are snails and slugs. Mice, birds, reptiles, and amphibians serve as paratenic hosts for this parasite. Research has suggested that some arthropods, including cockroaches, can be serve as paratenic hosts for *A. abstrusus* (Falsone et al., 2017).

Although nematodes of the genus *Troglostrongylus* were previously considered parasites of wild cats (Traversa and Di Cesare, 2013; Brianti et al., 2014), infections in domestic cats, mostly in young animals, are increasingly reported. In recent years, *T. brevior* has been considered the second most common lungworm of domestic cats after *A. abstrusus*. The adult parasite colonizes the bronchi and bronchioles of infected hosts

(Traversa and Di Cesare, 2016). Adult parasites show sexual dimorphism. Females are 9.6–16.8 mm length and 0.26–0.40 mm width, while males are 6.6–7.2 mm length and 0.2–0.23 mm width (Crisi et al., 2018). The life cycles of *T. brevior* and *A. abstrusus* are similar. In experimental studies, *Helicella barbesiana, H. ustalis, Limax flavus*, *Monaca syriaca*, *Retinella nitellina*, *Theba pisana* and *Helix aspersa* have been reported to act as intermediate hosts for *T. brevior* (Gerichter, 1949; Giannelli et al., 2014; Crisi et al., 2018). *Cornu aspersum* (*H. aspersa*) has also been reported to transmit the agent in natural infections (Morelli et al., 2020). Animals become infected by ingesting the intermediate host containing L3 or mostly paratenic hosts (rodents, frogs, lizards, snakes and birds) (Gerichter, 1949; Anderson, 2000; Bowman et al., 2002). Recent studies suggest that *T. brevior* may be transmitted from infected mothers to their offspring via the galactogenous route (Brianti et al., 2013).

Capillaria aerophila is a nematode in the Trichuridae family that parasitizes the respiratory tracts of cats, dogs, wild carnivores, and rarely humans (Conboy, 2009; Traversa et al., 2011; Khatat et al., 2016). It is considered a low-pathogenic species in cats. Adult parasites are thin, whitish, and filamentous, and are located under the epithelium in the bronchi, bronchioles, and trachea (Bowman et al., 2002; Conboy, 2009). Female parasites measure 16-41 mm in length, while males range from 10-25 mm (Szatmari, 2016). The parasite has a direct life cycle. The eggs that female parasites release after mating are swallowed after coughing and passed into the feces of the infected animal. They reach the infectious stage within 1-2 months in the environment (Bowman et al., 2002). Although it has been reported that earthworms can serve as facultative intermediate or paratenic hosts, it has not been proven that these organisms play a role in the biology of these parasites (Bowman et al., 2002; Conboy, 2009).

The aim of this study is to investigate the prevalence of lungworm in cats in the Kırklareli region according to fecal examination. There are a limited number of studies in Türkiye on the prevalence of lungworm in cats. In the region where the study was conducted, no research has been done on this topic until today, and there is no data on the existence of the parasites. With this study, the first information on the presence and prevalence of lungworm in owned and stray cats in the area has been presented.

MATERIALS AND METHODS

Collection of fecal samples

Within the scope of the study, fecal samples were collected from pet clinics and defecation areas of owned cats in Kırklareli. Permissions for the collection of fecal samples from cats were obtained from the Kırıkkale University Animal Experiments Local Ethics Committee (letter dated 31.01.2022 and numbered E.74224). The 100 cat fecal samples were delivered to the Kırıkkale University, Faculty of Veterinary Medicine, Parasitology Department, Routine and Epidemiology Laboratory under cold chain. Information such as age, breed, gender, whether they showed clinical signs related to respiratory system were recorded. The cats from which the samples were taken were of 12 different breeds (Table 1), 53 were females and 47 were males. While 44 cats were 1 year old and under, 56 were animals over 1 year old.

Fecal analysis

Fecal samples were examined for the presence of eggs and/or first stage larvae of lungworm by using Fülleborn flotation and Baermann method in the laboratory. In order to determine the parasite density in the feces in the samples detected positive for lungworm, the number of eggs and/or larvae per gram (g) of feces was determined using the McMaster method. The Baermann method was performed in accordance with the procedures outlined by Zajac and Conboy (2012), while the Fülleborn flotation and McMaster technique were conducted following the methodology described by Şenlik (2016). Species identification of the detected larvae was carried out following the criteria described by Traversa and Di Cesare (2016) and Morelli et al. (2021). In addition to, the general condition and examination findings of the cats from which the samples were taken were recorded and the number of eggs and/or larvae were compared with the presence and intensity of clinical findings.

Table 1. Number and ratio of animals from which fecal samples were taken according to breed

Breed	Number (n)	Ratio (%)
Crossbred	64	64
British shorthair	10	10
Persian cat	5	5
Bombay cat	5	5
Chinchilla	4	4
Scottish	3	3
Siamese cat	3	3
Ankara cat	2	2
Van cat	1	1
Ragdoll	1	1
Norwegian Forest Cat	1	1
Russian	1	1
Total	**100**	**100**

Statistical analysis

The study results were analyzed using the Chi-Square test in IBM SPSS Statistics 20 program and the results were evaluated at 0.05% confidence interval. Due to low expected cell counts, Fisher's exact test was used to evaluate the association between age and infection status.

RESULTS

During the study, *A. abstrusus* first stage larvae (L1) were detected in eight (8%) of 100 fecal samples examined by the Baermann method, while eggs and/or larvae of other lungworm were not detected (Fig 1). The same feces were subjected to the Fülleborn flotation method, and no larvae and/or eggs of any lung nematode were detected in this method.

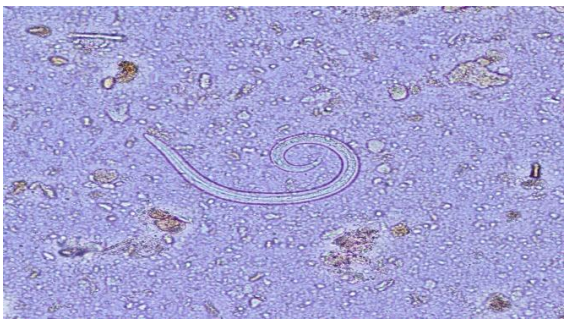

Figure 1. *Aelurostrongylus abstrusus* first stage larvae (L1)

Of the cats in which *A. abstrusus* L1 was detected, one cat was 1 year old or younger (≤1) and seven cats were over 1 year old (>1) (Table 2). However, no significant difference was found between ≤1 year old and >1 year old

cats in terms of *A. abstrusus* L1 positivity (p>0.05). The association between age group and *A. abstrusus* infection status, assessed using the Baermann method, is summarized in Table 3. Infection was detected in 7 of 56 animals older than one year (12.5%), whereas only 1 of 44 animals aged one year or younger (2.3%) tested positive. Fisher's exact test revealed no statistically significant association between age group and infection status (p = 0.075). Although a higher proportion of infections was observed in animals older than one year, this difference did not reach statistical significance.

Of the cats that were positive for *A. abstrusus*, 4 were female and 4 were male. It was determined that 7.5% of the female and 8.5% of the males were infected with *A. abstrusus* (Table 4). There was no statistically significant difference in the presence of *A. abstrusus* between male and female cats (p>0.05).

Table 2. Distribution of *A. abstrusus* presence according to age

			A. abstrusus		Total
			Positive	Negative	
Age	≤ 1	Number	1	43	44
		Age %	2.3	97.7	100.0
		A. abstrusus %	12.5	46.7	44.0
		Total%	1.0	43.0	44.0
	>1	Number	7	49	56
		Age %	12.5	87.5	100.0
		A. abstrusus %	87.5	53.3	56.0
		Total %	7.0	49.0	56.0
Total		Number	8	92	100
		Age %	8.0	92.0	100.0
		A. abtsrusus %	100.0	100.0	100.0
		Total %	8.0	92.0	100.0

Chi-Square: 3.502 p:0.062

Table 3. Association between age group and *A. abstrusus* infection status (Baermann Method)

Age group	**Negative**	***A. abstrusus* positive**	**Total**
> 1 year	49	7	56
≤ 1 year	43	1	44
Total	92	8	100

Table 4. Distribution of *A. abstrusus* presence according to gender

			A. abstrusus		**Total**
			Positive	**Negative**	
Gender	Female	Number	4	49	53
		Gender %	7.5	92.5	100.0
		A. abstrusus %	50.0	53.3	53.0
		Total%	4.0	49.0	53.0
	Male	Number	4	43	47
		Gender %	8.5	91.5	100.0
		A. abstrusus %	50.0	46.7	47.0
		Total %	4.0	43.0	47.0
Total		Number	8	92	100
		Gender %	8.0	92.0	100.0
		A. abstrusus %	100.0	100.0	100.0
		Total %	8.0	92.0	100.0

Chi-Square: 0.31, p:0.573

According to the breeds, six (6%) of the cats found positive for *A. abstrusus* were crossbred, one (1%) was Bombay and one (1%) was Persian. Of the infected cats, 75% were crossbred, 12.5% were Bombay and 12.5% were Persian cats. The infection rate was 9.4% in crossbred cats, 20% in Bombay cats and 20% in Persian cats. However, there was no statistically significant difference between cat breeds in terms of the presence of *A. abstrusus* (p>0.05).

In fecal samples positive for *A. abstrusus*, larval counts per gram, as determined by the McMaster technique, ranged between 50 and 500 larvae (Table 5). Severe respiratory symptoms were observed in three cats in which larval counts of 450 or higher per gram were detected. No clinical signs were observed in the other cats testing positive for *A. abstrusus*.

Table 5 Number of larvae detected per gram of feces in cats infected with *A. abstrusus*

Cat Number	Gender	Age	Breed	Per gram larvae number (n)
1	Male	>1	Crossbred	500
2	Female	>1	Crossbred	500
3	Male	>1	Crossbred	450
4	Male	>1	Crossbred	350
5	Female	>1	Crossbred	350
6	Female	>1	Persian cat	250
7	Female	≤1	Crossbred	100
8	Male	>1	Bombay cat	50

DISCUSSION AND CONCLUSION

While there are many studies in various countries in the world to determine the lungworm in cats, the number of studies conducted in Türkiye to determine the presence and prevalence of these parasites is limited. These studies conducted in Türkiye are generally in the form of case reports (Tuzer et al., 2002; Burgu and Sarımehmetoglu, 2004; Atasever and Yazar, 2009; Gokpinar and Yildiz, 2010; Yildiz et al., 2011; Yildiz and Gokpinar, 2011; Umur et al., 2020), and only two studies were found to determine the prevalence of these parasites (Asılıoglu and Gokpinar, 2021; Yildirim et al., 2023).

In this study, the rate of *A. abstrusus* was determined as 8% in cats in the Kırklareli region according to fecal examination. In previous prevalence studies conducted in Türkiye, the rate of this parasite was determined as 4% in cats in the Kırıkkale and Ankara regions (Asılıoglu and Gokpinar, 2021) and 5% in cats in the Balıkesir region (Yildirim et al., 2023). This rate was found 0.40% in Colombia (Lopez-Osorio et al., 2021), 0.49% in Sweden (Grandi et al., 2017), 0.80% in Switzerland (Giannelli et al., 2017), 0.92% in Belgium (Giannelli et al., 2017), 0.83-17.40% in Portugal (Payo-Puente et al., 2008; Nabais et al., 2014; Waap et al., 2014; Giannelli et al., 2017), 1.70% in the UK (Elsheikha et al., 2019), 1.98% in Brazil (Farago et al., 2022), 1-5% in Spain (Miro et al., 2004; Giannelli et al., 2017), 2.60% in the Netherlands (Robben et al., 2004), 4.34% in France (Giannelli et al., 2017), 2.07% in the USA (Carruth et al., 2019), 5-17.80% in Italy (Traversa et al., 2008; Di Cesare et al., 2015; Giannelli et al., 2015; Giannelli et al., 2017), 6.10% in Romania (Ciopaşiu et al., 2018), 6.60% in Germany (Barutzki and Schaper, 2013), 8% in Greece (Diakou et al., 2015), 8.86-13.60% in Denmark (Olsen et al., 2015; Hansen et al., 2017), 19.8-22.5% in Hungary (Kiszely et al., 2019), 33.3-35.8% in Bulgaria (Stoichev et al., 1982; Giannelli et al., 2017). While the rate of *A. abstrusus* detected in our study was similar to the studies conducted in Greece, Romania, Germany and Denmark, it was lower than in Bulgaria and Hungary, and higher than the studies conducted in Colombia, Switzerland and Belgium. The number of fecal samples examined, the different techniques used in diagnosis, the care and feeding habits of the cats taken as samples, the climate and habitats of the regions where the studies were conducted and the differences in the prevalence of intermediate and/or paratenic hosts are thought to be effective in the emergence of these different results.

When the effect of age on the prevalence of *A. abstrusus* in cats is examined, different results have been obtained in studies conducted worldwide. Although some of these studies reported a statistically significant difference in the presence of *A. abstrusus* in cats according to age groups (Hansen et al., 2017; Carruth et al., 2019), some studies found no significant difference (Asılıoglu and Gokpinar, 2021). Asılıoglu and Gokpinar (2021) reported that they found the rate of *A. abstrusus* to be higher in cats ≤1 year old than in cats >1 year old, but there was no significant difference in the presence of *A. abstrusus* between age groups. Ciopaşiu et al. (2018) reported the rate of *A. abstrusus* to be lower in cats 1-2 years old and older than 2 years old compared to cats between 2 months and 1 year old. Hansen et al. (2017) reported that they encountered this parasite mostly in cats aged 11-51 weeks, and that the positivity rate they detected was statistically significant compared to cats younger than 10 weeks, 1-3 years old, and older than 3 years old. Carruth et al. (2019) found a higher rate of *A. abstrusus* in cats aged 1-12 months compared to cats older than 12 months and found that there was a significant difference between age groups in terms of the presence of this parasite. In a study conducted by Giannelli et al. (2017) in various European countries, the rate of *A. abstrusus* was found to be higher in cats older than 2 years of age compared to cats <6 months, 6-12 months and 1-2 years old. In the present study, 7 of the 8 cats positive for *A. abstrusus* were >1 year old, while one was ≤1 year old. However, no significant difference was found between age groups in terms of the presence of *A. abstrusus*. Contrary to most of the other studies evaluating according to age, in this study, higher levels of *A. abstrusus* were found in adult cats compared to young cats. The reason why this agent was found more in cats older than one year is thought to be due to the fact that the number of samples taken in this study was higher in this age group and that these animals had more encounters with the parasite's intermediate or paratenic hosts at some point in their lives.

In the present study, 7.5% of female cats and 8.5% of male cats whose feces were examined were found to be positive for *A. abstrusus* L1. However, there was no significant difference between males and females in terms of the presence of this parasite ($p>0.05$). Similar to this study, Carruth et al. (2019), and Asılıoglu and Gokpinar (2021) found the rate of *A. abstrusus* to be higher in male cats than in females, but reported that there was no statistically significant difference between the two gender in terms of the presence of this parasite. Hansen et al. (2017) found that the rate of *A. abstrusus* was higher in females than in males, although there was no statistically significant difference between the gender. Elsheikha et al. (2019) determined that there was no significant difference between the gender in their study in England. When the study results were evaluated, the results of the studies conducted to date show that there is no gender predisposition for *A. abstrusus* in cats.

In this study, six of the eight cats in which *A. abstrusus* L1 were detected were crossbreds (75%), one was Bombay (12.5%), and one was Persian (12.5%). Although the infection rate in crossbred cats was higher than in other breeds, no statistically significant difference was found between cat breeds in terms of the presence of *A. abstrusus*. The high number of infected crossbred cats is

due to the fact that most of the sampled cats (64%) were crossbred. Asılıoglu and Gokpinar (2021) evaluated the cats they sampled as crossbreds and purebreds and detected a higher rate of *A. abstrusus* in crossbred cats than in purebreds. However, the researchers reported that there was no significant difference between crossbred and purebred cats in terms of the presence of this parasite.

The McMaster technique was applied to determine the number of larvae per gram in stool samples that tested positive for *A. abstrusus*. Accordingly, the number of larvae per gram of feces was determined as minimum 50 and maximum 500. To date, no study has been found to determine the number of larvae per gram of feces in cats infected with *A. abstrusus*. In the present study, respiratory symptoms were observed in three infected cats with fecal larval counts of ≥450 larvae per gram, suggesting a potential association between pulmonary parasite burden and clinical condition. Nevertheless, further investigations involving a larger number of cats infected with *A. abstrusus* are required to better clarify the relationship between lung parasite load and the severity of clinical signs.

To date, *T. brevior* has not been detected in fecal examinations of domestic cats in Türkiye. Umur et al. (2020) reported that they *T. brevior* was detected in the lung tissue of two domestic cats necropsied. In studies conducted in various countries, the rate of *T. brevior* was found to be 1.2-14% in domestic cats. No larvae of this parasite were found in any of the fecal samples examined in this study. In order to determine the prevalence of this parasite in Türkiye, we believe that more studies should be conducted and more cat feces should be examined.

In this study, *C. aerophila* eggs were not found in the examined cat feces. In previous studies conducted in Türkiye, the *C. aerophila* rate was determined as 3.3% in Ankara (Mimioğlu, 1951) and 4% in Elazığ (Altaş and Taşan, 1999). In studies conducted worldwide, *C. aerophila* was detected at an average rate of 6.6% in seven different countries of Europe (Rehbein et al., 2014), 11% in Italy (Traversa et al., 2009), and 8.3% in Serbia (Iliç, 2009). We believe that the fact that all of the cats from which fecal samples were taken in this study were owned cats and had little contact with the environment, and that the study had different climatic conditions compared to the regions in Türkiye where the agent was detected, were effective in obtaining this result.

In conclusion, *A. abstrusus* was detected for the first time in cats in Kırklareli region. *T. brevior* and *C. aerophila* were not found in cats in the region. As in previous studies, it has been demonstrated once again that *A. abstrusus* is the dominant species of lungworm in cats in Türkiye. It is thought that *A. abstrusus* should also be taken into consideration in cats with respiratory system symptoms. We believe that more studies should be conducted to determine lungworms in cats both in the region and in Türkiye.

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Ethical Declaration

Permissions for the collection of fecal samples from cats were obtained from the Kırıkkale University Animal Experiments Local Ethics Committee (letter dated 31.01.2022 and numbered E.74224).

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept: B.A., S.G., Design: S.G., B.A., Data Collection or Processing: S.G., B.A., Analysis or Interpretation: B.A., S.G., Literature Search: B.A., S.G., Writing: S.G., B.A.

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International Journal of Veterinary and Animal Research 8(3): 99-101, 2025 **CASE REPORT**

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Post-Mortem Findings of Ascaridia Species Infection in Shikra (*Accipiter badius*)

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ABSTRACT

A Shikra (*Accipiter badius*) weighing 178 grams, and measuring 9 inches (from head to tail) was brought to the Veterinary Clinical Complex at College of Veterinary Science and Animal Husbandry, MHOW with a history of lethargy, inability to fly, and gasping. Based on clinical condition and prevailing environmental factors, dehydration and heat stroke were suspected and oral fluid therapy was administered. The following day, the shikra was found deceased and a post-mortem examination was performed. On opening the thoraco-abdominal cavity, congestion in the liver and intestines was observed. Pulmonary haemorrhages were present, with the right lung more severely affected. On opening the oesophagus and gastrointestinal tract, several roundworms were detected in the oesophagus, crop, gizzard, and proximal part of intestine. Microscopic examination of roundworms revealed 3 lips on the cranial end and transverse annulations throughout the length of the worm, characteristic of the Ascaridia species. In conclusion, the cause of death was probably due to roundworm infection, causing anaemia and pneumonia, resulting in hypoxia.

INTRODUCTION

The Shikra (*Accipiter badius*) is a small, agile raptor native to Central Asia and Southern Persia, and ranges from India and Myanmar to Sri Lanka and Southern China. This species is characterized by several subspecies, each varying in size, plumage, and migratory behavior. The widely distributed subspecies *A. b. dussumieri* is common in India, particularly in regions such as the North-West Frontier Province, Kashmir, Northern Assam, and the Himalayan foothills up to 5,000 feet. In contrast, the smaller and darker *A. b. badius* is found in the southern parts of India, including Travancore and Sri Lanka. Notably, while some subspecies are year-round residents, others, such as the larger and paler *A. b. cenchroides* from Central Asia migrate seasonally to regions like Balochistan, Sindh, and Punjab during the winter months (Fatima et al., 2016).

In terms of conservation status, the Shikra is classified as "Least Concern" on the IUCN Red List (2021), reflecting its relatively stable population. Additionally, it enjoys the highest level of legal protection under Schedule I of the Wildlife Protection Act, 1972, ensuring measures to safeguard its habitats and mitigate potential threats.

The present study aims to determine the cause of death in a Shikra (*Accipiter badius*) through a thorough post-mortem examination and to contribute to the literature on avian parasitology, pathology, and raptor health management.

MATERIALS AND METHODS

An adult Shikra (*Accipiter badius*) was rescued and brought to the Veterinary Clinical Complex at the College of Veterinary Science and Animal Husbandry, MHOW. The bird weighed 178 grams and measured approximately 23 cm from head to tail. Its exact age could not be determined, as it was wild, but it was presumed to be an adult based on its morphological features and the observation of a regressed bursa of Fabricius during necropsy. The bird was identified as male due to the presence of testes. It had been rescued from the roadside and presented with symptoms of lethargy, gasping, and an inability to fly. Based on the clinical signs and the prevailing summer season, dehydration and heatstroke were suspected, and oral fluid therapy was administered. However, the following day, the Shikra was found deceased and submitted for post-mortem examination.

Initially, the bird was inspected for external injuries or discharges. A transverse incision was made at the caudal end of the sternum using scissors, and the skin was retracted to expose the pectoral muscles. The thoraco-abdominal cavity was opened by cutting the ribs with scissors and retracting the sternum. Upon opening the cavity, the visceral organs, lungs, air sacs, heart, and peritoneum were examined, and gross lesions were documented photographically. The trachea and gastrointestinal tract were incised to assess the luminal content, mucosal condition, and presence of parasites.

Representative tissue samples from the lungs, kidneys, liver, trachea, and intestines were collected and preserved in 10% neutral buffered formalin for 48 hours. These samples were then dehydrated using increasing concentrations of alcohol, cleared in xylene, and embedded in wax blocks. Thin sections (4-5 µm) were cut and prepared for slide examination. The sections were stained with hematoxylin and eosin (H&E) to observe microscopic lesions. Additionally, roundworms collected from the gastrointestinal tract were cleared in lactophenol for 48 hours for further examination.

RESULTS

Upon inspection, no external injuries were observed, and there was no discharge from the nostrils, mouth, or cloaca. The muscles appeared normal, and no subcutaneous hemorrhages were detected. Upon opening the thoraco-abdominal cavity, gross lesions were identified in multiple organs, including the lungs, liver, kidneys, intestines, gizzard, proventriculus, and trachea. The liver showed signs of congestion and oedematous enlargement evidenced by roundening of the edges of the liver, accompanied by necrotic foci. Histopathological analysis revealed focal necrosis and infiltration of mononuclear cells (Figure 1).

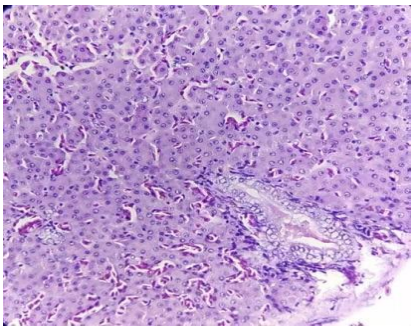

Figure 1. Liver (400x), showing infiltration of mononuclear cells and degenerative changes

The lungs exhibited extensive hemorrhages, with the right lung being more severely affected (Figure 2). Histopathological examination indicated red hepatization characteristised by alveolar lumen filled with erythrocytes, consolidation, edema in peribronchial lesion, and hemorrhage around the bronchi, parabronchi, and air capillaries (Figure 3). Congestion and oedema was observed in the trachea (Figure 4). In the kidneys, histopathology revealed glomerular atrophy, inflammatory mononuclear cell infiltration, and degenerative changes in the renal tubules (fig. 5).

Figure 2. Gross lesions visible after opening the thoraco-abdominal cavity: haemorrhage in liver and lungs

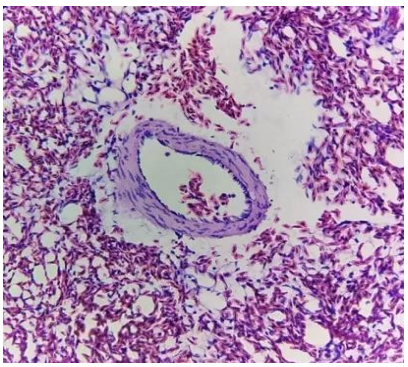

Figure 3. Lungs (400x), showing haemorrhage in bronchi and lungs parenchyma

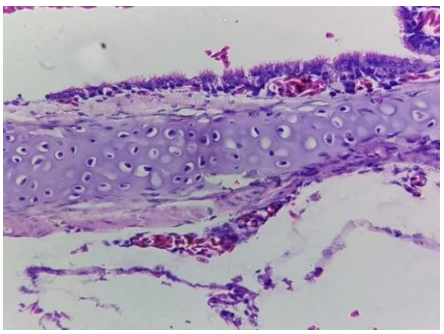

Figure 4. Trachea (400x), showing congestion and oedema

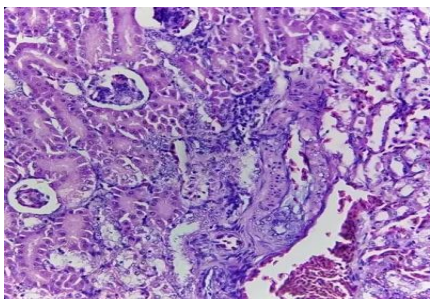

Figure 5. Kidney (400x), showing glomerular atrophy, tubular degeneration, and congestion.

In the gastrointestinal tract, several roundworms were found in the esophagus, crop, gizzard, and proximal intestines. Microscopic examination revealed that these roundworms had three lips on their anterior end and transverse annulations along their length, characteristics typical of the *Ascarididae* family (fig. 7). However, because no molecular tests for species-level diagnosis were performed, identification could not be made beyond morphological characteristics.

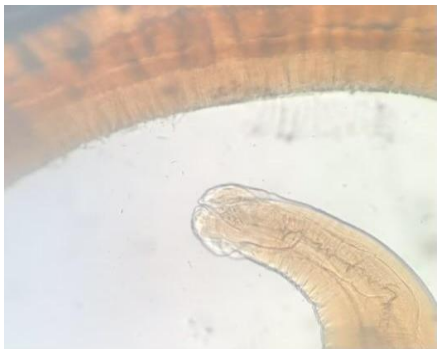

Figure 7. Roundworm (100x), anterior end having 3 lips

In conclusion, the Shikra (*Accipiter badius*) was found to be infected with nematodes, likely *Ascaridia* spp., as indicated by anatomical examination. The presumed cause of death was respiratory distress and hypoxia resulting from severe pneumonia.

DISCUSSION AND CONCLUSION

Despite being a common bird of prey in India and neighboring regions, there is limited research on the diseases and parasites of the Shikra. Previous studies have documented roundworms in other birds of the Accipitriformes group, which are closely related to the Shikra. For instance, Oyarzún-Ruiz et al. (2022) studied the endoparasites and ectoparasites of Harris's hawk (*Parabuteo unicinctus*) in central and southern Chile. Among 29 birds necropsied, 17 (58.6%) were found to be parasitized by helminths. Several species of nematodes were recorded, including *Porrocaecum depressum* (3.5%) from the small intestine, *Physaloptera alata* (6.9%) from the esophagus, *Microtetrameres* sp. (20.7%) from the proventriculus and small intestine, *Cyathostoma (Hovorkonema) americana* (3.5%) from the air sacs and lungs, and *Capillaria tenuissima* (13.8%) primarily from the small intestine.

Additionally, Yao C. Su and Andrew Chang Y. Fei (2004) reported parasitic species in crested goshawks (*Accipiter trivirgatus formosae*) from Taiwan, including *Lutztrema monoteron*, *Ascaridia perspicillus*, *Dispharynx nasuta*, *Spirocerca sanguinolenta*, *Raphidascaris* sp., and *Caryospora* sp. Majda Globokar et al. (2017) and Morgan and Schiller (1950) noted that *Porrocaecum angusticolle* and *Porrocaecum depressum* are found in both Accipitriformes and Falconiformes, with a global distribution. In a case report by Fatima et al. (2016), a Shikra rescued at Madras Veterinary College in Chennai was found to be infested with a tapeworm, suspected to be *Hymenolepis* spp., identified through fecal centrifugal sedimentation.

This case report contributes to the limited body of literature on Shikra parasites by documenting a roundworm infection in a rescued Shikra from Madhya Pradesh. The nematode was morphologically consistent with Ascaridia spp.; however, molecular confirmation was not performed. Further research is warranted to comprehensively characterize the parasite fauna of Shikra and to elucidate their implications for avian health and conservation.

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Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Writing: K.S., Analysis or Interpretation: N.S., Literature Search: K.S.

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Use of Tannins in Livestock Nutrition

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ABSTRACT

This study aimed to evaluate the effects of tannins and tannin-containing plants at different inclusion levels in animal diets by reviewing various feeding studies involving different livestock species. Tannins are phenolic compounds produced by plants, particularly woody species, as a defense mechanism against external factors. They are classified into two major groups: hydrolysable tannins and condensed tannins. Tannins are known for their ability to bind proteins, inhibit microbial enzymes, and exert antibacterial effects by disrupting bacterial membranes. In ruminants, condensed tannins form complexes with proteins within the pH range of 3.5–7.5, thereby reducing microbial degradation in the rumen and improving bypass protein availability. Positive effects such as increased growth hormone levels, inhibition of gastrointestinal parasite larvae, and improved nitrogen utilization have been observed, especially in ruminants. However, high tannin levels (above 60 g/kg DM) may reduce feed palatability and intake due to their astringent taste. Safe inclusion levels have been reported as 8–10% in goats, 3–5% in cattle, and approximately 1% in poultry. In conclusion, tannins can be used strategically in livestock nutrition to improve health and performance parameters when administered at species-appropriate and controlled levels.

INTRODUCTION

Tannins are defined as phenolic compounds that are found in greater amounts in the structure of perennial plants, characterized by the ability to bind proteins and other nutrients, are easy to find in all plant kingdoms and agricultural by-products, and are produced by plants to protect themselves against external factors (Boğa et al., 2021; Menchi et al., 2021). Their molecular weights range from 500 Da to 3000 Da and are found in the leaves, bark, fruit, woody stems and roots of plants. Tannins are soluble in water (20-35°C temperature range) except for some high molecular weight structures (Dehghanian et al., 2022).

Tannins can greatly affect digestion, protein, cellulose and fat metabolism in ruminants, as they suppress general rumen microflora activity. Tannins can improve the bypass protein ratio up to certain levels due to their protein binding properties and can cause increased intestinal absorption of amino acids. In addition, this situation can provide environmental benefits by reducing methane and ammonia emissions from ruminant animals. It has been reported that by reducing saturated fatty acids and increasing polyunsaturated fatty acids and biohydrogenation (BH) intermediates, it can provide increases in the nutritional quality of ruminant animals (Menchi et al., 2021).

Structure and Classification

Tannin is a group of substances that have the ability to bind to proteins in aqueous solutions chemically (Makkar, 2003). The most common method used in the classification of tannins is the analytical method and according to this method, tannins are divided into two large classes as hydrolyzable tannins and condensed tannins. Hydrolyzable tannins include two subgroups as gallo tannins and ellagitannins. The non-hydrolyzable group is classified as oligomeric and polymeric proanthocyanidins 'condensed tannins'. Complex tannins, which contain the characteristic structural elements of both ellagitannins and condensed tannins, have been called 'unclassified tannins' so far (Molino et al., 2023).

The most common form of tannin is condensed tannin, which consists of two or more monomeric (-) epicatechin or (+) catechin units. These types of condensed tannins (CT) are called procyanidins (Dehghanian et al., 2022). While oak leaves and broad bean fruits contain more hydrolyzed tannins, condensed tannins have been reported in sorghum and clover species. Hydrolyzed tannins are broken down into 1 mole of glucose and 7 moles of gallic acid by enzymes (İmik and Şeker, 1999).

Effect of tannin on animals

Boğa et al. (2021), reported that condensed tannins inhibit the larval development of digestive tract parasites and show an antiparasitic effect by combining with proteins in the rumen and preventing microbial degradation. It has been recorded that giving tannin-containing rations to lambs and sheep with parasite infestation improves live weight gain and reduces parasite eggs excreted with feces by 20-50% (Kamalak et al., 2005). It has been reported that growth levels increase in animals to which condensed tannin is added in addition to anthelmintic application in lambs with parasite infestation (Üstün and Aydın, 2007).

It has been reported that tannins show antibacterial effect by inhibiting microbial enzymes such as protease and lipase (Kaya and Yalçın, 1999). Tannic acid (TA) prevents the use of iron by bacteria by forming a chelation with iron and inhibits the growth of microorganisms found in the intestine such as *Bacteriodes fragilis, Clostridium perfiringens, Escherichia coli* and *Enterobacter cloacae*. It has also been reported that tannins are effective against biofilm-forming bacterial infections such as *S. Aereus* (Ünver et al., 2014).

Effect of tannin on nutrition

Tannins can cause color changes and decrease in flavor in feeds due to the enzymatic changes they cause and the astringent taste they give (Singh et al., 2023). Deterioration in the flavor of feed and a decrease in feed consumption due to disgust were observed in animals fed with feeds containing more than 60 grams of tannin per kilogram (Kamalak et al., 2005). It has been observed that the addition of grain to the ration at levels of 8-10% in goats, 3-5% in cattle, and 1% in poultry does not cause negative effects (Gürsoy, 2022). Köse and Kardeş (2021), reported that the astringent and bitter taste of foods containing tannin can be reduced by processes such as boiling (heat treatment) or peeling the shells.

While feed consumption decreased in sheep fed with feed containing 55 grams of tannin per kilogram, this effect was either less or not seen at all in smaller amounts (Kamalak et al., 2005). Tannin in feeds forms a complex with glycoprotein in saliva. In some animals, as they adapt to diets containing high amounts of tannin, the amount of proline-rich proteins in their saliva increases. These proteins form bonds with tannin, preventing it from forming compounds with other proteins in the diet (Kamalak et al., 2005).

Toxicity

It is known that tannins can cause structural problems after absorption in the digestive system by forming a complex structure with other compounds in feeds, can lead to essential amino acid deficiency and can cause toxicity (İmik and Şeker, 1999).

They can prevent digestion and absorption by binding to proteins, carbohydrates and minerals. It has also been reported that tannins can reduce the function of digestive enzymes and nutrient absorption by damaging the digestive system membrane by forming complexes. Tannins slow down digestion by binding to carbohydrates. When tannins combine with proteins, they can reduce digestion and reduce the animal's amino acid supply. This can reduce the energy level of the ration. Tannins can prevent absorption by binding minerals and lead to iron, zinc and copper deficiency. They can reduce protein bioavailability or increase fecal nitrogen, which can cause protein digestibility in humans and animals. They also inhibit amylase, chymotrypsin and lipase activity, prevent iron absorption and reduce protein digestibility (Singh et al., 2023). Gallic acid and pyrogallol, which are hydrolysis products of tannins, can cause hemolysis. Gallic acid and pyrogallol are more toxic than tannins. Tannins precipitate albuminous substances and, through their astringent activity, reduce mucosal secretions and mucosal permeability. They also affect water absorption and salivary flow. If food and water intake decrease, the process can progress to dehydration. When consumed in high concentrations, loss of appetite and constipation develop. Excessive amounts can cause gastroenteritis ulcerosa (Üstün and Aydın, 2007).

EFFECT OF TANNIN USE IN LIVESTOCK ON PRODUCTION CHARACTERISTICS

Since tannins reduce enzyme activity and protein digestibility in poultry and other monogastric animals, these animals are more affected than ruminants (Ünver et al., 2014). Condensed tannins form complexes with proteins and carbohydrates and inhibit microbial enzymes (Kaya and Yalçın, 1999). High consumption of tannins by poultry, negatively affects performance, decreases in live weight, decreases in egg production, retards growth, negatively affects feed consumption, and decreases feed utilization. This is evidenced by the presence of histopathological findings in the liver and kidneys, as well as the negative impact on energy, protein, arginine, leucine, methionine, phenylalanine, and starch digestibility, which can result in mortality in cases of consumption exceeding 5% (Gürsoy, 2022). When used in poultry diets, it has been determined that it binds to methionine, reduces the biological value of protein and reduces the metabolizable energy level of feed (Özen, 1980). It has been determined that 1% Tannic Asid added to poultry diets does not change egg yield, and 2% significantly reduces egg yield. In egg yolks, it creates pale spots and abnormal olive green color disorders (Özen, 1980).

It has been reported that the inclusion of 10–40 g/kg of condensed tannins in dry matter (DM) has beneficial effects on ruminant nutrition (Kamalak et al., 2005). Condensed tannins form complexes with proteins within a pH range of 3.5 to 7.5, preventing their degradation by rumen microorganisms and thereby reducing the rate of microbial digestion (Ünver et al., 2014). These tannin-protein complexes bypass the rumen and are broken down in the abomasum and small intestine, allowing the released proteins to be absorbed more efficiently in the small intestine. Additionally, tannins in the ration stimulate saliva production, which facilitates the recycling of urea nitrogen back into the rumen, enhancing microbial protein synthesis and overall productivity (Kamalak et al., 2005). The effectiveness of tannins in ruminant diets depends on factors such as their concentration, chemical structure, the overall composition of the ration, and the animal's adaptation to tannin-rich feeds (Ünver et al., 2014). The inclusion of 20-45 g/kg DM of condensed tannins has been associated with improvements in milk and wool yield, as well as reproductive performance, whereas levels exceeding 55 g/kg DM may negatively affect feed intake, digestibility, growth, and wool production (Öztürk, 2015). Furthermore, condensed tannins can help mitigate ruminal tympani caused by highly soluble proteins in feed. Supplementation with tannin-containing plants at levels of 5 g/kg DM or higher has been shown to significantly

reduce rumen gas production and prevent bloat by precipitating foam (Üstün and Aydın, 2007).

A study reported that approximately 25% of global CH_4 emissions are formed by enteric fermentation of animals (Önel et al., 2021). It was determined that the amount of methane gas in the atmosphere has doubled over the last few centuries, and it was stated that the effect of methane on global warming is 21 times greater than carbon dioxide gas. (Öztürk, 2015). According to the Kyoto Protocol, which Turkey joined on August 26, 2009, it was reported that greenhouse gas emissions in the world should be reduced to the levels of 1990 (Öztürk, 2015). A 13% decrease in methane emissions was achieved by using 2.5% acacia tannin in dry matter in sheep rations (Meral and Biricik, 2013). In a feeding study conducted on lactating Holstein cows, a mixture of tannin obtained from Quebracho and chestnut trees was added to the rations of the experimental groups. The mixture was added to DM at two different rates of 0.45% and 1.8%. The results of the study, as reported by Keser and Kutay (2021), showed a decrease in daily methane emissions per animal of 56 and 48 g in the low and high tannin groups, respectively.

Condensed tannins have been widely studied for their ability to form complexes with proteins within the pH range of 3.5-7.5, preventing microbial degradation in the rumen and allowing more proteins to reach the small intestine for absorption (Ünver et al., 2014). Kamalak et al. (2005), reported that supplementing 10-40 g/kg DM of condensed tannins in ruminant diets increases protein efficiency by enhancing bypass protein and stimulating saliva production, which promotes nitrogen recycling via urea return to the rumen. Boğa et al. (2021), found that supplementing sheep rations with 20-40 g/kg DM condensed tannins resulted in a 62% increase in essential amino acid absorption, a 20% improvement in milk yield and milk protein, and a significant rise in wool yield. Similarly, in cows, tannin supplementation lowered milk urea nitrogen (MUN) and ruminal ammonia nitrogen levels without negatively impacting milk protein content.

Moderate levels of condensed tannins (20-45 g/kg DM) have been shown to enhance milk and wool yield and reproductive parameters. However, inclusion rates exceeding 55 g/kg DM have been observed to have a negative impact on feed intake, digestibility, growth, and wool yield (Öztürk, 2015). Üstün and Aydın (2007), observed that diets containing more than 55 g/kg DM condensed grain reduced feed consumption and digestibility. Moreover, the incorporation of condensed tannins at levels of ≥5 g/kg DM has been demonstrated to curtail rumen gas production and efficaciously forestall bloat by precipitating foam.

Williams et al. (2020) conducted a study on eight Holstein cows using ruminal cannulation to evaluate the effects of tannin and cottonseed oil supplementation. Four diets were tested: control, 800 g/day cottonseed oil, 400 g/day tannin, and a combination of both. Methane production decreased by 14% with cottonseed oil, 11% with tannins, and 20% with the combined supplementation. Similarly, the addition of *Lotus corniculatus* (27 g/kg DM) to dairy cow diets led to a milk yield of 16.5 kg, while the inclusion of polyethylene glycol (PEG) alongside *L. corniculatus* reduced yield to 13.8 kg.

A study was conducted by İmik and Şeker (1999) to investigate the effects of oak leaves, tea factory waste, and sorghum as part of the rations for Akkaraman yearlings. The study found that oak leaves did not significantly affect the live weight or wool quality of the subjects, indicating their suitability as roughage. Sorghum, which was found to contain high levels of tannins, was found to be safe up to a level of 400 g included in the ration. In a subsequent study, Kamalak et al. (2005), reported that the supplementation of condensed tannins at levels of 22-38 g/kg DM resulted in a 10% increase in wool production, while the inclusion of 50 g/kg had deleterious effects.

Güçlü and Yalçın (2004), found that treating cottonseed meal (CSM) with 3-9% tannic acid and 5-10% lignosulfonate reduced crude protein digestion in rams. Aktaş and Akkan (2011), evaluated the effects of acorn tannins (3% and 4%) on in vitro rumen fermentation. They observed no impact on rumen pH but found significant reductions in ammonia levels and protein degradability, suggesting improved protein efficiency.

Şentürk et al. (2015), studied the effects of tannin supplementation (90 g *Quebracho Colorado* per animal) on negative energy balance in dairy cows. Blood and milk samples collected before and after calving revealed significant reductions in BHB levels at parturition and on days 7 and 14 postpartum. This suggests that tannin may help mitigate the effects of negative energy balance during early lactation.

İmik et al. (2003), replaced barley with dehulled *Sorghum vulgare* at 8–32% in lamb diets and observed improved live weight gain, feed intake, and digestibility. Ibrahim and Hassen (2022), evaluated unencapsulated and encapsulated *Acacia mearnsii* tannins in Merino lambs. Both forms reduced enteric methane and regulated rumen fermentation without compromising dry matter intake or growth. Encapsulated tannins had a stronger effect on methane reduction.

Getachew et al. (2008) reported that increasing levels of tannic acid (30-90 g/kg DM) reduced NH_4-N concentrations by up to 67% in sheep diets. PEG addition reversed this effect, highlighting the protein-binding role of tannins. TA also reduced isovalerate production, with no significant changes in blood metabolites or enzymes. Menchi et al. (2021), compared tannin extracts (kebrako and kebrako + chestnut) and found that the mixture increased the CO_2/CH_4 ratio and influenced rumen biohydrogenation.

Buccioni et al. (2011), showed that chestnut and quebracho tannins altered the rumen bacterial fatty acid profile and increased C18:1 trans11 accumulation in vitro, supporting their role in modulating fatty acid metabolism. Orlandi et al. (2020), found that 7.7 g/kg DM Acacia tannin reduced urinary urea excretion in sheep without affecting net flow of urea, ammonia or glucose. Sarnataro and Spanghero (2020), compared chestnut tannins and *Stevia rebaudiana* (SB) extract, finding that chestnut tannins reduced rumen ammonia and protozoa counts, while SB extract significantly decreased protozoa population without affecting ammonia levels.

CONCLUSION

Poultry and other monogastric animals are more affected by the negative effects of tannins than ruminants. Tannins can provide an increase in protein efficiency by allowing valuable protein sources to pass through the rumen without being destroyed and allowing more protein to flow into the small intestine. When plants containing condensed tannin at moderate levels are added to the diet, an increase in essential amino acid absorption in the intestine can be achieved, while when used in high concentrations, it can combine with proteins, reduce digestion and reduce the animal's amino acid supply. Adding tannin to the diet can

increase milk yield by limiting grain intake and can also be used to protect against negative energy balance. Adding certain levels of condensed tannin to ruminant diets positively affects wool yield and reproductive parameters. However, it is recommended not to add more than 55 g/kg DM of condensed tannin to the diet because it negatively affects feed consumption, digestion rate, growth and wool yield.

Consequently, the utilisation of specific quantities of condensed tannin in animal rations may either not exert any influence on animal production parameters or may engender favourable outcomes. However, elevated levels have the potential to yield adverse consequences.

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Conflict of Interest
The authors declare that they have no competing interests.

Authorship contributions
Concept: Ö.B., M.B., Design: Ö.B., M.B., Literature Search: Ö.B., M.B Writing: Ö.B., M.B

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Interplay of Environmental Factors in The COVID-19 Pandemic: Transmission, Dynamics and Implications

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ABSTRACT

This review paper critically examines the multifaceted relationship between environmental determinants and the transmission dynamics of severe respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for coronavirus disease 2019 (COVID-19). Through an extensive analysis of existing literature, it elucidates the intriguing connections between air pollution, particularly particulate matter (PM), and the incidence and severity of COVID-19 cases worldwide. Particles within the aerosol size range, known as aerosolized particles, can remain suspended in the air for extended periods and be transported over distances, thereby contributing to infection clusters in confined spaces, poorly ventilated areas, and close-contact settings. Additionally, this review investigates the influence of weather conditions, including temperature, humidity, and solar radiation, on COVID-19 spread and severity, emphasizing the complex interplay of environmental factors in viral transmission. Furthermore, the presence of SARS-CoV-2 in water sources has raised questions about waterborne transmission. Studies have detected viral RNA in wastewater (WW), rivers, and sewage, highlighting the potential for fecal-oral transmission. The review also evaluates the utility of WW-based epidemiology as a tool for early detection and surveillance of COVID-19 outbreaks, despite existing challenges in standardizing detection methods and correlating viral levels with clinical cases. Soil is also being examined as a potential reservoir and transmission medium for SARS-CoV-2. Meanwhile, studies have identified the virus in soil samples, mainly in areas with heavy contamination from infected individuals or medical waste. As a result, understanding the transmission dynamics of SARS-CoV-2 through air, water, and soil is crucial for developing effective control strategies and preventive measures.

INTRODUCTION

Over the last two decades, the world has experienced three significant pandemics, with coronavirus disease 2019 (COVID-19) triggered by severe respiratory syndrome coronavirus 2 (SARS-CoV-2) emerging most recently, preceded by the SARS outbreak in 2003 and MERS in 2012. All three pandemics resulted from zoonotic transmission, emphasizing the persistent threat of diseases from animal sources (Ramadan Shaib, 2019). SARS-CoV-2 spreads rapidly through direct contact or respiratory droplets from speaking, sneezing, or coughing (Anand et al., 2021). As of March 17, 2024, the world health organization (WHO) recorded 774,954,393 confirmed COVID-19 cases worldwide, along with 7,040,264 reported deaths, with figures continuing to rise (WHO, 2024). In this context, it is crucial to keep in mind that other potential causes of virus dissemination include pollution (Bontempi, 2020), and meteorological and socioeconomic variables such as trade exchanges. Because of its worldwide spread, recurring outbreaks, high fatality rate, and rapid transmission among vulnerable populations, COVID-19 remains a significant public health threat (Shrestha et al., 2022). The pandemic has caused significant human casualties on a global scale and posed an unprecedented threat to the economy, ecosystem, and healthcare industry. Therefore, huge international scientific efforts are being made in a variety of fields to better understand the variables affecting the new

coronavirus's transmission and infectiousness with the hopes of limiting its spread, slowing the rate of diffusion, and creating novel therapeutic interventions or vaccines (Lundstrom et al., 2023).

Understanding the behavior and dynamics of SARS-CoV-2 in the environment is essential for preventing future outbreaks. Healthy individuals are most commonly infected by inhaling virus particles released by infected individuals during everyday activities like speaking, sneezing, and coughing (Chatterjee et al., 2020). Jin et al. (2020) suggests that the primary mode of transmission for SARS-CoV-2 is through respiratory droplets (particles >5µm). In May 2021, the Centers for Disease Control and Prevention (CDC) updated its COVID-19 guidelines to acknowledge that aerosolized particles smaller than droplets can linger in indoor air for minutes to hours, increasing the risk of exposure. It's also important to consider that SARS-CoV-2 may spread via other routes beyond contaminated droplets (Morawska et al., 2009; Piscitelli et al., 2022a).Surfaces touched by infected individuals, as well as water, sewage, trash, or soil, can serve as channels for transmission (Gogoi et al., 2023; Onakpoya et al., 2021). However, the duration for which infectious virus particles can survive in airborne suspension is still debated. The risk of COVID-19 infection decreases as the distance from the source increases and as more time passes since exhalation. Heavier respiratory droplets carrying the virus fall to the ground or surfaces due to gravity, while smaller droplets and aerosols stay suspended in the air and disperse as they mix with larger volumes and flows of air (CDC, 2020, 2021). Environmental factors such as temperature, humidity, and UV radiation can also influence the degradation of viral particles over time (CDC, 2020, 2021). These elements may have a significant role in the developing seasonal pattern of the SARS-CoV-2 epidemic waves.

Meanwhile, several studies have indicated that SARS-CoV-2 can persist in the human gastrointestinal system, suggesting that human excreta may represent a new route of transmission for the virus (Machkovech et al., 2024). Notably, SARS-CoV-1 nucleic acids were detected in patient excreta and urine, remaining viable for 3 to 17 days (Parida et al., 2023). Hung et al. (2004) found up to 10^7 copies of SARS-CoV-2 RNA per milliliter of stool and 2.5 x 10^4 copies per milliliter of urine. Additionally, Xiao et al. (2020) reported that 39 of 73 hospitalized SARS-CoV-2-infected patients had positive stool samples, with 23.29% of them continuing to test positive for the virus even after viral RNA was no longer detectable in the respiratory tract. SARS-CoV-2 was also found in the stool of an asymptomatic child whose respiratory samples were negative for the virus (Tang et al., 2020a). A recent study examined the durability of different types of personal protective equipment (PPE) widely used by medical professionals and the general public during the pandemic (Kasloff et al., 2021). Their research showed that SARS-CoV-2 RNA could remain on various PPE for different lengths of time. For example, the virus was detectable on face shield plastic and N-95 masks for up to 21 days, while Tyvek, which is a synthetic material made from high-density polyethylene (HDPE) fibers maintained the virus for 14 days, nitrile gloves for 7 days, and cotton fabric for about 4 hours at 20°C and a relative humidity of 35% to 40%. These results suggest that the existing water infrastructure connected to hospitals, public spaces, homes, toilets, drains, runoff, and water treatment systems could play a role in the widespread transmission of the virus. The study highlights the importance of proper PPE management in high-risk settings and suggests using cotton-based materials, such as cotton masks, as they show lower viral persistence, potentially helping control the spread of COVID-19. Similar to how norovirus and rotavirus have been documented to spread through aerosolization during wastewater (WW) and sludge treatment (Pasalari et al., 2019), SARS-CoV-2 could also be transmitted through water-soil-food pathways. Therefore, further research is needed to evaluate the public health risks associated with the aerosolization of SARS-CoV-2-contaminated WW and the inhalation of infectious bioaerosols (Kanwar et al., 2023).While water and air have received considerable attention regarding SARS-CoV-2 transmission, soil as a potential secondary transmission route has been relatively understudied. Moreover, storm water runoff from agricultural regions can carry contaminants, including SARS-CoV-2, into surface or groundwater bodies (Kanwar et al., 2023). Continuous sewage discharge can also impact soil ecosystems, potentially serving as a reservoir for the virus and contributing to secondary transmission sources. To fully understand SARS-CoV-2 transmission, it is essential to investigate the interactions of various environmental factors, including air, water, soil, and food. Figure 1 illustrates potential pathways for the SARS-CoV-2 in water and soil environments, highlighting the need for continued research in this area.

This review aims to explore the unprecedented environmental impacts of the COVID-19 pandemic, drawing on a thorough synthesis of global data from multiple fields. The novelty lies in its examination of the intricate interactions between the virus and environmental factors, encompassing air quality, water quality, noise pollution, and greenhouse gas emissions. Furthermore, it aims to elucidate the pathways through which SARS-CoV-2 transmission is influenced by environmental conditions, paving the way for effective mitigation strategies. Through this endeavor, the review underscore the broader implications of the pandemic on environmental sustainability and advocate for transformative practices that prioritize both human health and ecological well-being.

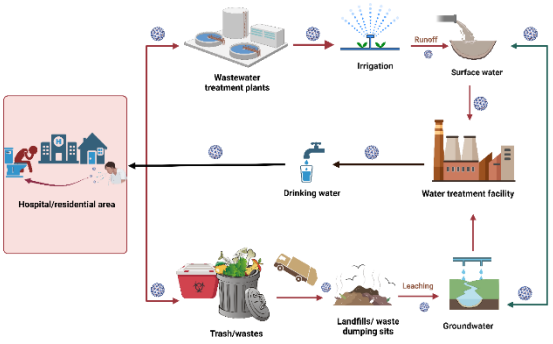

Figure 1. Potential pathways for SARS-CoV-2 transmission in aquatic and soil ecosystems.

ENVIRONMENTAL DETERMINANTS OF SARS-COV-2 TRANSMISSION

Research Group on COVID-19 and air pollution Investigating the relationship with particulate matter: A growing body of evidence suggests an interesting

connection between air pollution levels and the incidence of COVID-19. For instance, regions with elevated levels of particulate matter (PM) have reported higher rates of COVID-19 cases (Domingo and Rovira, 2020). Additionally, studies have detected SARS-CoV-2, the virus responsible for COVID-19, in outdoor air PM in urban areas of Northern Italy and the United States of America (USA) (Setti et al., 2020a). Furthermore, Linillos-Pradillo et al. (2021) examined the presence of SARS-CoV-2 RNA in outdoor air samples of PM-10, PM-2.5, and PM-1, using data collected between May 4 and May 22, 2020, in Madrid. The study employed MCV high-volume samplers with three inlets to gather samples using quartz fiber filters. The RNA extraction and amplification procedures were conducted following methods established by Setti et al. (2020) in Italy. The researchers concluded that the lack of detectable viral genomes could be due to several factors, including reduced social interactions, widespread mask usage, and economic restrictions, all of which likely helped curb the spread of the virus. Additionally, lower daily PM levels and rising temperatures during the spring season may have contributed to the observed findings.

Regarding the assessment of prolonged air pollution exposure and a potential rise in the severity of COVID-19 health effects, including mortality, Wu et al. (2020d) addressed the problems and outlined prospective paths and prospects. The same authors had previously noted that after adjusting for various area-level factors, higher historical exposures to PM-2.5 in the USA were associated with increased COVID-19 death rates at the county level (X. Wu et al., 2020e). However, the published study remains preliminary in assessing the impact of air pollution on the geographic spread of the disease, both locally and globally. Beyond any potential connection to COVID-19 transmission, there are many other compelling reasons to take strong action to reduce air pollution. The WHO 2021 report highlights that exposure to ambient air pollution is responsible for 4.2 million preventable deaths annually worldwide, along with numerous adverse health effects, including respiratory and cardiovascular diseases. In a study across 36 Organisation for Economic Co-operation and Development (OECD) countries, Barnett-Itzhaki and Levi (2021) examined the relationship between long-term, population-weighted exposure to PM-2.5 and NOx and the resulting morbidity and mortality over time following the first confirmed COVID-19 case. PM-2.5 levels were significantly associated with COVID-19 morbidity and mortality at 10, 20, 40, and 60 days, while NOx concentrations and population density correlated with these outcomes at 60 days. Continued exposure to air pollution above WHO guidelines may increase COVID-19 morbidity and mortality.

De Angelis et al. (2021) conducted an ecological study investigating the effects of prolonged exposure to PM and nitrogen dioxide (NO_2) on COVID-19 incidence and overall mortality. Their findings revealed a significant increase in COVID-19 cases as levels of PM-2.5 and PM-10 rose (58% and 34%, respectively). Additionally, a 10μg/m^3 annual increase in PM-2.5 was linked to a 23% increase in all-cause mortality. In contrast, NO_2 levels were negatively correlated with both COVID-19 incidence and all-cause mortality. Similar results were observed by Mele et al. (2021) and Gujral and Sinha (2021), who used separate neural networks to monitor these trends in Paris, Lyon, Marseille, Los Angeles, and Ventura. Moreover, Sangkham et al. (2021) conducted research in the Bangkok Metropolitan area, also highlighting the impact of air quality on viral dissemination. Evidence suggests that both short- and prolonged exposure to air pollution exacerbates respiratory disease symptoms and raises mortality rates, aligning with early investigations of COVID-19 death rates. However, these findings require further verification and support, considering individual-level risk factors (Piscitelli et al., 2022a). Zhu et al. (2021) highlighted the detrimental effects of PM on various aspects of human health, including the respiratory, circulatory, neurological, and immune systems, as well as their potential toxicological mechanisms. In addition, studies of the early COVID-19 outbreak in Northern Italy (Ho et al., 2021) and the Catalan Tarragona Province in Spain (Marquèsand Domingo, 2022) provided detailed accounts of the potential effects of both short- and long-term exposure to air pollution on COVID-19 risk and mortality rates. While there was notable county-level variability, Zhu et al. (2021) found compelling evidence that wildfires in the USA amplified the effects of short-term exposure to PM-2.5 on COVID-19 cases and fatalities.

Gaseous pollutants and COVID-19: A study conducted across 66 administrative districts in Italy, Spain, France, and Germany explored the relationship between COVID-19 and the distribution of tropospheric NO_2. The results revealed that 78% of the 4,443 death cases occurred in five regions of northern Italy and central Spain, which also had the highest NO_2 concentrations and downward airflow, impeding the effective dispersion of air pollution (Ogen, 2020). In another study, researchers examined the correlation between pollution levels of SO2, CO, NO_2, and ozone, and COVID-19 mortality. They found that a 10 μg/m^3 increase in NO_2 and ozone was associated with a 6.94% (95% CI: 2.38 to 11.51) and a 4.76% (95% CI: 1.99 to 7.52) rise in daily confirmed COVID-19 cases, respectively. Additionally, a 1 μg/m3 increase in CO levels corresponded to a 15.1% (95% CI: 0.44 to 29.77) increase in daily confirmed COVID-19 cases. Conversely, a 10 μg/m^3 increase in SO2 concentration was negatively correlated with COVID-19 cases, leading to a 7.79% decrease (95% CI: -14.57 to -1.01) in confirmed cases of the virus (Srivastava, 2021; Yang et al., 2020). These effects are illustrated in Figure 2.

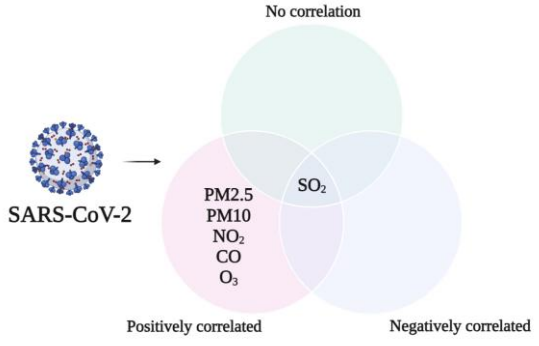

Figure2. Relationship between various pollution parameters with the number of COVID-19 cases

These findings suggest that both short- and long-term exposure to air pollution, including PM and gaseous pollutants, may increase COVID-19 transmission, severity, and mortality. Air pollution could act as a carrier

for viral particles and exacerbate respiratory vulnerability, emphasizing the need for effective air quality management. Reducing emissions, improving urban ventilation, and enforcing pollution control measures could mitigate the health impacts of current and future respiratory viral outbreaks. Overall, these results highlight the critical role of environmental factors in shaping pandemic outcomes and the importance of integrating air pollution mitigation into public health strategies.

Weather conditions and COVID-19: Numerous studies, conducted worldwide, have shown that different climate characteristics, such as temperature, humidity, sunshine, etc., substantially impact the number of coronavirus cases and deaths (Table 1).

Table 1 Effect of various meteorological parameters on the number of COVID-19 cases and mortality

Parameter	Country	Relationship and result
Temperature	China (10 affected provinces)	Temperature and COVID-19 Asymmetric Nexus: Some trends are mixed, some indicate positive results, while a few show negative results (Shahzad et al., 2020)
	USA (New York)	Number of COVID-19 cases drastically declines as average and minimum temperatures rise (Bashir et al., 2020)
	China (Wuhan)	No evidence of a significant temperature increase to stop or delay the COVID-19 infections (Iqbal et al., 2020)
	Italy	A 1 °F increase in daily temperature on average resulted in a 6.4 case reduction each day (Sobral et al., 2020)
	Iran	Temperature and COVID-19 do not significantly correlate (Ahmadi et al., 2020)
	China (17 different cities)	The drop in daily confirmed case numbers was correlated with an increase in ambient temperature of 1 °C (Liu et al., 2020)
	Turkey	The more COVID-19 cases there are on a given day, the lower the temperature is that day (Şahin, 2020)
	Indonesia (Jakarta)	The number of COVID-19 cases is highly correlated with temperature (Tosepu et al., 2020)
	China	The frequency of COVID-19 may be positively impacted by both lower and higher temperatures (Shi et al., 2020)
Humidity	USA (New York)	The number of instances or the overall number of cases is not greatly affected by average humidity (Bashir et al., 2020)
	Iran	Humidity and the rate of a virus outbreak are inversely related (Ahmadi et al., 2020)
	China (all provincial capitals)	Absolute humidity was highly correlated, and an increase in AH of 1 g/m3 was significantly linked to a reduction in the number of confirmed cases (Liu et al., 2020)
	Turkey	A rise in humidity is accompanied by a decline in the number of cases (Şahin, 2020)
	General	The COVID-19 morbidity and mortality are inversely associated with air humidity (Biktasheva, 2020; Martinez et al., 2020)
Rain Fall	USA	Rainfall and COVID-19 dissemination are inversely and sporadically connected (Bashir et al., 2020)
	Italy	Disease transmission increased after a rainstorm. There was an increase of 56.01 instances per day for every average inch per day (Sobral et al., 2020)
	Iran	There is no connection between the frequency of COVID-19 cases and rainfall (Ahmadi et al., 2020)
	Indonesia (Jakarta)	Rainfall and COVID-19 did not significantly correlate (Tosepu et al., 2020)
Wind speed	USA	Wind speed has a negligible impact on the dissemination of the virus (Bashir et al., 2020)
	Iran	Significant outbreak occurs at low wind speeds (Ahmadi et al., 2020)
	Turkey	More cases occur when the wind is blowing faster (Şahin, 2020)
Solar Radiation	Iran	Survival of the virus is threatened by solar radiation. Infection exposure rates were higher in regions with low sun radiation values (Ahmadi et al., 2020)

Temperature and COVID-19: Research aimed at establishing the link between temperature and COVID-19 cases yielded highly unusual results. The majority of the relationships were facility- and location-specific (Srivastava, 2021). There is an asymmetrical relationship between temperature and COVID-19, according to a study done in the top 10 impacted provinces of China. According to Shahzad et al. (2020), five of the 10 provinces showed mixed trends between temperature and COVID-19 instances, with three showing positive and two negative trends. Furthermore, the average and minimum temperatures were found to significantly correlate with COVID-19 instances in a different study carried out in New York (Bashir et al., 2020). An additional study conducted in Wuhan, China, disproves the findings of numerous other studies that suggested temperature played a key effect in limiting the spread of COVID-19. The findings did not support the idea that raising the temperature would help to contain or decrease COVID-19 infections (Iqbal et al., 2020). Meanwhile, in a different Italian investigation, it was discovered that a 1 °F rise in the daily average temperature resulted in a 6.4 per day decrease in the number of cases. However, in some instances, COVID-19 mortality did not exhibit a statistically significant correlation with temperature (Ahmadi et al., 2020; Sobral et al., 2020). A previous study conducted across 17 Chinese cities found that an increase of 1°C in ambient temperature and the diurnal temperature

range was linked to a decrease in the number of daily confirmed COVID-19 cases (Liu et al., 2020). In contrast, a study from Turkey suggested that on days with higher numbers of COVID-19 cases, temperatures were generally lower (Şahin, 2020). However, a study in Jakarta, Indonesia, found no significant correlation between temperature and the number of reported cases (Tosepu et al., 2020).

Humidity and COVID-19: Numerous studies conducted worldwide have highlighted the significant role that humidity plays in COVID-19-related morbidity and mortality. A study in New York found that average humidity had no effect on the overall number of cases (Bashir et al., 2020). Conversely, research in Iran indicated a negative correlation between humidity and the rate of virus outbreaks, although high virus transmission was observed in two humid regions of the country (Ahmadi et al., 2020). A study examining all of China's provincial capitals revealed that Absolute Humidity (AH) significantly reduced the number of confirmed cases in four cities. Additionally, a meta-analysis by Liu et al. (2020) showed that each 1 g/m3 increase in AH was notably associated with a decrease in confirmed cases. In Turkey, there was a strong correlation between humidity and daily case numbers, with the overall trend indicating that as humidity increased, the number of cases decreased (Şahin, 2020). Furthermore, another Chinese study found no correlation between COVID-19 frequency and AH (Shi et al., 2020). A notable study found that as of March 10, 2020, South Korea, Japan, Iran, and Northern Italy experienced the highest levels of Covid-19 community transmission. Despite varying relative humidity (ranging from 44% to 84%), these regions consistently showed low specific humidity (3–6 g/kg) and AH (4–7 g/m^3) levels (Sajadi et al., 2020).

Rainfall and COVID-19: There have been few studies examining the relationship between COVID-19 and rainfall. Bashir et al. (2020) conducted a study in the USA and found a sporadic and negative association between rainfall and disease spread, with higher transmission rates in areas experiencing more rainfall. Sobral et al. (2020) observed an increase of 56 cases per day for each inch of average daily rainfall. However, a separate study by Ahmadi et al. (2020) in Iran found no link between rainfall and COVID-19 cases. Similarly, research in Indonesia by Tosepu et al. (2020) also reported no significant relationship between rainfall and the spread of the virus.

Wind speed and COVID-19*:* Limited research has been conducted on the significance of wind speed in COVID-19 transmission. While generally not considered a significant factor, a study in the USA by Bashir et al. (2020) suggests that wind speed may have a modest yet noteworthy impact on virus spread. Conversely, an Iranian study by Ahmadi et al. (2020) found a notable increase in outbreaks during periods of low wind speed. Interestingly, a study in Turkey identified a strong correlation between the number of cases and average wind speed over 14 day (Şahin, 2020). It suggests that this timeframe is crucial for assessing correlations accurately, emphasizing the importance of considering wind speed over this duration when analysing COVID-19 transmission dynamics.

Solar radiation and COVID-19: Research on the connection between COVID-19 and solar radiation remains limited. An Iranian study suggested that solar radiation poses a threat to the virus's survival, with regions experiencing lower sun radiation exhibiting higher rates of illness exposure (Ahmadi et al., 2020). Figure 3 illustrates the relationship between different meteorological parameters and the number of COVID-19 cases.

The reviewed studies indicate that meteorological factors, including temperature, humidity, rainfall, wind speed, and solar radiation, may influence COVID-19 transmission, but the results are highly variable and location-specific. While some studies suggest higher temperatures and humidity reduce case numbers, others report no significant correlations, highlighting inconsistencies across regions and methodologies (Srivastava, 2021; Bashir et al., 2020; Şahin, 2020). Limited research on rainfall, wind speed, and solar radiation further complicates understanding of their roles in viral spread. These inconsistencies reveal critical research gaps, including the need for standardized, multi-location, longitudinal studies that account for confounding factors such as population density, human mobility, and public health interventions. Addressing these gaps would improve the predictive value of meteorological models and inform public health strategies for mitigating COVID-19 and other respiratory virus outbreaks under varying environmental conditions.

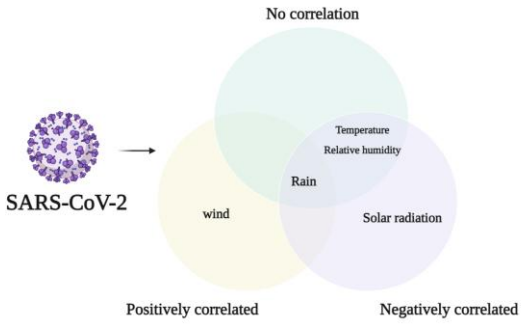

Figure 3. Relationship between various meteorological parameters with number of COVID-19 cases

Positive link between outdoor air pollution and COVID-19 incidence and severity

Recent continental and national studies: A variety of studies have been carried out globally, especially in countries heavily impacted by COVID-19, to explore how different air pollution factors influence mortality rates and case numbers related to the virus, as shown in Table 2.

Table 2. Overview of the findings from recent studies on the impact of air pollution on SARS-CoV-2 transmission and COVID-19 outcomes.

Study objectives	Key outcomes and summary	Reference
To investigate how PMs may have played a role in the COVID-19 outbreak in cities across Italy	An increase in COVID-19 infection is associated with both short-term and prolonged exposure to high amounts of contaminants. To confirm the varying susceptibility to infection between PM-exposed and unexposed cells, COVID-19 infection should be assessed with angiotensin-converting enzyme 2 (ACE2) expression after PM exposure.	(Comunian et al., 2020)
Exploring potential linkages: Air quality and SARS-CoV-2 spread in India's affected regions	In environments with moderate-to-high humidity, polluted conditions can increase the transmission rate of SARS-CoV-2.	(Manoj et al., 2020)
Impacts of air pollution on COVID-19 spread and mortality	The susceptibility to infection and mortality from COVID-19 may be increased by exposure to air pollution, particularly NO_2 dioxide and PM-2.5.The prognosis of individuals with SARS-CoV-2 infection can be negatively impacted by air pollution.	(Ali and Islam, 2020)
Studying the transmission and lethality of COVID-19 influenced by air pollution	Chronic air pollution exposure has a significant impact on the spread and fatality of COVID-19. Compared to PM-10, PM-2.5 and NO_2 had a stronger correlation with COVID-19.	(Copat et al., 2020)
Correlation between COVID-19 and ambient air pollution level	Exposure to PM may impair immune function and cause dysregulation, making it more difficult to fend off viral invasion. The invasion of the virus may be accelerated by ACE2 overexpression brought on by PM exposure. SARS-CoV-2 transmission distance may increase due to airborne PM.	(Wang et al., 2020a)
To calculate the percentage of COVID-19 deaths that can be attributed to exposure to ambient fine particle air pollution over a lengthy period	The risk of COVID-19 death is significantly increased by air pollution.	(Pozzer et al., 2020)
To provide a summary of SARS-CoV-2 transmission pathways	The research suggests that certain persistent factors influence the environmental behaviour and longevity of SARS-CoV-2. Outdoor risk factors, such as PM and aerosolized particles from wastewater treatment, should be closely examined because they may serve as carriers for the virus.	(Senatore et al., 2021)
How exposure to outdoor pollution may impact the pathogenesis of COVID-19 and the SARS-CoV-2 viral life cycle	PM, NO_2, and ozone exposure may increase the risk of COVID-19-associated immunopathology in exposed people by intensifying tissue inflammation and damage caused by the virus.	(Woodby et al., 2021)
Research from laboratory, animal, and human studies on how outdoor air pollution affects COVID-19	Air pollution exposure, both short-termand prolonged, may, through a variety of mechanisms, be a significant aggravating factor for the transmission of SARS-CoV-2 as well as the severity and fatality of COVID-19.	(Bourdrel et al., 2021)
Possible relationship between air pollution and COVID-19 mortality and occurrence	Air pollution, whether short-term or prolonged, may significantly contribute to the spread of SARS-CoV-2 through the air and could exacerbate the severity of COVID-19. Contact with NO_2 and PM-2.5 was linked to COVID-19 cases and deaths more often than exposure to PM-10.	(Ali et al., 2021b)
The part played and possible correlation between air pollution, particularly PM pollution, and the spread of COVID-19	The distribution of COVID-19 seems to be positively correlated with atmospheric PM pollution. According to certain research, PM acts as a carrier of viruses, encouraging their airborne spread. Population resistance to infection may be weakened by exposure to ambient PM.	(Maleki et al., 2021)
Potential COVID-19 transmission pathways and various virus mutations through environmental media	SARS-CoV-2 may be spread via PM. Further research concentrating on the virus's environmental transmission channels is necessary to help avoid and manage the COVID-19 pandemic.	(Shao et al., 2021)
The relationship between the frequency, prevalence, severity, and mortality of COVID-19 and acute and chronic exposure to air pollution	The most consistent contributors to COVID-19 include both short- and prolonged exposure to PM-2.5, as well as prolonged exposure to NO_2. ozone exposure seems to have a connection with the occurrence of new cases only. Research evaluating the consequences of acute exposures showed significant bias risks.	(Katoto et al., 2021)
To recap the function of PM in the transmission of COVID-19 and the connection between COVID-19, PM, and ACE2	There is scientific proof that PM levels and the SARS-CoV-2 spread are related. ACE2 is crucial to the COVID-19 pandemic.	(Khan et al., 2021)
COVID-19 is impacted by air pollution and climatic indices.	The rate at which COVID-19 cases spread and their severity are influenced by air pollution and meteorological factors. These processes may encompass several factors such as air pollution-induced comorbidities, damage to the respiratory system, increased pulmonary epithelial permeability, disruptions in immune and inflammatory responses, changes in metabolic pathways, and pollution-driven elevation of ACE-2 receptor expression.	(Zhao et al., 2021)

Table 2. (Continued).

Study objectives	Key outcomes and summary	Reference
Possible connections between PM and COVID-19 and several fatal human disorders	PM exposure may aid in COVID-19 transmission and SARS-CoV-2 spread. It is believed that oxidative damage and inflammatory responses are the main processes responsible for PM's detrimental effects.	(Zhu et al., 2021)
To examine the combined impact of SARS-CoV-2 transmission and ambient PM-2.5 exposure on worsening cardiopulmonary outcomes	Exposed patients to air pollution are more vulnerable to contracting COVID-19, which puts them in a pre-inflammatory state. Air pollution impacts cardiovascular and respiratory health, and the existence of respiratory and cardiovascular comorbidities affects COVID-19 mortality and prognosis. Chronic air pollution exposure increases inflammation, making certain populations more susceptible to contracting COVID-19	(Lai et al., 2021)
Explore the research on SARS-CoV-2 transmission during the COVID-19 pandemic, focusing on why airborne transmission has been less impactful from an environmental standpoint.	One reason for the reduced attention on airborne transmission could be the lower quantity of viruses in smaller droplets compared to larger ones. SARS-CoV-2 in small droplets might bind or mix with existing PM, thereby allowing PM composition to influence their behaviour and eventual outcome.	(Ram et al., 2021)

China: To explore potential links between environmental factors and COVID-19 cases and deaths in Wuhan and Xiao Gan, Li et al. (2020) investigated various weather elements, the air quality index (AQI), and four pollutants (PM-2.5, PM-10, CO, and NO_2). Their research suggests PM-2.5 and NO_2 may influence the spread of COVID-19 and found a correlation between disease frequency and temperature. In a similar study, Jiang et al. (2020) examined the potential links between air pollution, meteorological conditions, and daily COVID-19 case numbers in Wuhan, Xiao Gan, and Huan gang. The study focused on air pollutants such as PM-2.5, PM-10, SO_2, CO, NO_2, and ozone. The findings indicated that COVID-19 risk was associated with both humidity and PM-2.5 levels, while lower risks of COVID-19 were observed in relation to temperature and PM-10.

Lin et al. (2020) examined the influence of meteorological elements and air quality across mainland China to understand the factors significantly affecting SARS-CoV-2 transmissibility. From January 21, 2020, to April 3, 2020, they analyzed meteorological variables and levels of PM-2.5, PM-10, CO, SO_2, NO_2, and ozone, correlating them with the COVID-19 basic reproductive ratio. Their findings highlighted that higher ambient CO levels posed a risk for increased SARS-CoV-2 transmissibility in provinces with high flow, while higher temperatures, atmospheric air pressure, and effective ventilation lowered transmissibility. The impacts of meteorological variables and air pollutants varied regionally, with daily maximum temperature and 24-hour average NO_2 concentration inversely associated with the basic reproductive ratio. In contrast, X. Zhang et al. (2021b) conducted a study analyzing time series data from December 1, 2019, to April 6, 2020, to assess the correlation between daily confirmed COVID-19 cases and various environmental factors. Their research examined concentrations of PM-2.5, PM-10, CO, NO_2, SO_2, and ozone, alongside meteorological variables. The study revealed significant positive correlations between daily new confirmed cases and short-term exposure to PM-2.5, PM-10, and NO_2, indicating a strong link between air pollution and the spread of the virus.

Italy: Italy was one of the hardest-hit countries in Europe during the initial phase of the current pandemic. Consequently, many studies investigating the relationship between air pollution levels and the spread of COVID-19 have focused on Italy. For instance, Coccia (2020) conducted a study to identify factors contributing to the spread of COVID-19. They found a strong association between the rapid and widespread diffusion of COVID-19 in Northern Italy and air pollution levels in cities, particularly those beyond the limits set for ozone or PM-10. The study analyzed data from 55 district capitals in Italy, focusing on cases of infection up until April 7, 2020. In addition, Leonardo Setti et al. (2020a) examined 34 outdoor PM-10 samples collected from an industrial area in Bergamo Province, the epicenter of Italy's COVID-19 outbreaks, between February 21 and March 11, 2020. Their goal was to explore the potential role of PM in the spread of COVID-19 in Northern Italy. The findings suggested that SARS-CoV-2 could be present on outdoor PM, and that the virus might associate with PM-10 under stable meteorological conditions and high PM levels, thereby increasing its persistence in the atmosphere. In a subsequent study, Setti et al. (2020b) investigated whether air pollution could have a "boost effect" on the COVID-19 outbreak, potentially contributing to rare "super-spreader events." This Italian observational study, the first of its kind, examined the early spread of the virus across 110 provinces and found a significant relationship between daily PM-10 exceedances and the geographic spread of the virus.

However, Zoran et al. (2020) explored the link between surface air pollution and the high rates of SARS-CoV-2 infection, rapid spread, and mortality in the Milan metropolitan area. Their study, conducted from January to April 2020, investigated how common gaseous air pollutants like ozone and NO_2, along with weather factors, influenced the spread of SARS-CoV-2. They observed a positive link between air ozone levels and a negative link between NO_2 levels and the number of reported COVID-19 cases, daily new infections, and overall mortality rates. The researchers suggested that air pollutants might impact COVID-19 transmission and severity by causing respiratory issues and weakening the immune system. Zoran et al. (2020) also highlighted the significant influence of atmospheric PM-10 and PM-2.5 on the rise of COVID-19 cases in Milan. They suggested that exposure to these PM, in combination with potential bacterial or viral carriers, could impair the immune system, potentially exacerbating the spread and severity of COVID-19 cases.
In the Lombardy region, Dragone et al. (2021) investigated the relationship between air pollution, alongside meteorological patterns, and the SARS-CoV-2 illness spread. Their findings suggested that both air pollution and

climatic factors could potentially facilitate the spread of infectious virus particles. Similarly, Coccia (2021) analyzed statistical data from cities in Northern Italy, indicating support for the dynamic spread of SARS-CoV-2. Specifically, low wind speeds were identified as a potential factor prolonging the persistence of viral particles like SARS-CoV-2 in contaminated air. However, it's important to note that the spread of infectious diseases is influenced by various factors, making this conclusion tentative. On a related note, Accarino et al. (2021) examined the relationship between COVID-19 metrics (prevalence and mortality) and short-term exposure to PM-2.5, PM-10, and NO_2 during the first quarter of 2020. Their findings indicated that an increase in the number of days with PM-10, PM-2.5, and NO_2 levels surpassing annual limits was strongly correlated with higher COVID-19 prevalence, mortality, and lethality rates. In Italy, PM-2.5 and PM-10 had more substantial associations with these rates compared to NO_2. Similarly, Filippini et al. (2021) observed a positive, non-linear relationship between increased NO_2 levels in the troposphere and higher COVID-19 fatality rates in 16 provinces in Northern Italy that were heavily impacted by the pandemic

In contrast, De Angelis et al. (2021) employed an ecological approach to investigate the effects of prolonged exposure to PM-2.5, PM-10, and NO_2 on COVID-19 prevalence and all-cause mortality in Lombardy from March to April 2020. Their study, which accounted for demographic, social, and meteorological factors, found that a 10 $\mu g/m^3$ increase in the annual average levels of PM-2.5 and PM-10 from previous years correlated with a 58% and 34% increase in COVID-19 prevalence, respectively. Furthermore, a 10 $\mu g/m^3$ rise in annual PM-2.5 concentration was associated with a 23% increase in all-cause mortality. However, they observed an inverse relationship between NO_2levels and both COVID-19 prevalence and mortality. Stufano et al. (2021) also investigated the short-term association between air pollution and SARS-CoV-2 susceptibility in Lombardy, factoring in climate effects. They found that short-term exposure to ozone, PM-10, and PM-2.5 was linked to higher COVID-19 prevalence, but they concluded that air pollution and climate were not key drivers in SARS-CoV-2 transmission. This connection might reflect increased host susceptibility, potentially due to immune system vulnerabilities or exacerbated conditions tied to severe COVID-19 infections.

Additionally, Ho et al. (2021) investigated the impact of PM-2.5, PM-10, NO_2, SO_2, and ozone on COVID-19 incidence, mortality, and fatality rates, both short-term and prolonged, in Lombardy and Veneto over eight years (January 2013–May 2020). They found that exposure to SO_2 significantly contributed to the COVID-19 pandemic by causing systemic and respiratory inflammation. Other pollutants had effects similar to those reported in earlier Italian studies.

Studies from China and Italy have consistently shown positive associations between air pollutants—particularly PM-2.5, PM-10 and NO_2 and COVID-19 incidence and severity; however, these associations should be interpreted cautiously due to ecological study designs, limited time frames, and insufficient control for confounding factors such as meteorological conditions, mobility restrictions, and socioeconomic differences. Overall, the evidence suggests that air pollution may increase population susceptibility to respiratory infections rather than directly enhance viral transmission. Future studies should employ longitudinal or case-control designs with individual-level exposure assessments, high-resolution spatiotemporal modeling, and mechanistic analyses to clarify causality and inform targeted air-quality interventions for disease prevention.

Fecal-transmission of SARS-CoV-2

Viruses can be transmitted to humans through direct or indirect contact with contaminated fluids, such as surface water, food, and fomites (De Graaf et al., 2017; Radin; D. Xu et al., 2005). Another possible route of transmission is through feces, suggesting that SARS-CoV-2 may spread via this pathway as well (Figure 4). Recent data on COVID-19 has shown the presence of SARS-CoV-2 in the stool of affected patients. For example, an RT-PCR test conducted on a patient in Washington, USA, detected SARS-CoV-2 RNA in a stool sample taken on the seventh day of illness, even though serum samples tested negative (Holshue et al., 2020). Similar findings have been reported in other studies.(Y. Chen et al., 2020; Ling et al., 2020; A. N. Tang et al., 2020b; Y. Wu et al., 2020e; Yang et al., 2020; J. Zhang et al., 2020a; Y. Zhang et al., 2020b). Even after respiratory viral RNA tests negative, viral RNA can persist in feces for up to 33 day. Several studies have highlighted the potential for fecal transmission of SARS-CoV-2. For example, Ong et al. (2020) found that samples taken from a restroom used by a COVID-19-infected patient tested positive for the virus on surfaces such as the inside of the sink, the door handle, and the toilet bowl, even after cleaning, though post-cleaning samples tested negative. Similarly, van Doremalen et al. (2020) reported that viable virus particles could survive in aerosols for at least 3 hours and on plastic and stainless-steel surfaces for up to 2 or 3 days. Additionally, Zhang et al. (2020b) observed that the median duration of viral shedding in respiratory tract swabs was 10 days, while it could persist for up to 22 days in feces. Unhygienic environments, such as public restrooms, may promote the fecal-oral spread of the virus when individuals touch their mouth, nose, or eyes with contaminated hands (Ong et al., 2020; van Doremalen et al., 2020; Zhang et al., 2020b). However, the precise mechanism of SARS-CoV-2 transmission via the fecal-oral route remains uncertain (Xu et al., 2020), despite the prolonged shedding of the virus from the digestive system compared to the respiratory tract.

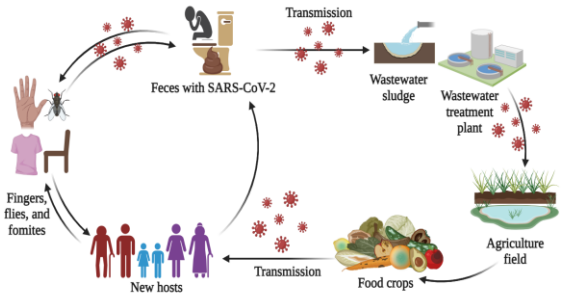

Figure 4. Fecal transmission of SARS-CoV-2.

Based on the research mentioned, while SARS-CoV-2 has been detected in the feces of infected individuals, there is no evidence supporting its presence in urine. Wang et al. (2020c) reported that no studies have confirmed the virus's presence in urine samples. Ling et al. (2020) suggested that

the rare transmission via urine or blood may be due to the low frequency of positive findings in patients.

Detection of SARS-CoV-2 RNA in fecal samples and on contaminated restroom surfaces indicates the potential for fecal-oral transmission; however, current evidence remains largely indirect and based on RNA detection rather than isolation of infectious viral particles. The prolonged presence of viral RNA in feces, even after respiratory samples test negative, suggests possible gastrointestinal persistence, yet the absence of consistent findings of viable virus limits definitive conclusions. Environmental studies demonstrating surface contamination and viral stability on fomites support the plausibility of this pathway, but do not confirm its epidemiological significance. To clarify this potential route, future research should focus on isolating infectious virus from fecal samples, assessing viral viability under varying environmental conditions (temperature, humidity, pH), and conducting epidemiological investigations linking sanitation infrastructure and wastewater exposure to infection risk. Standardized protocols for sampling, detection, and viability testing, along with experimental models assessing gastrointestinal infectivity, are essential to establish whether fecal-oral transmission contributes meaningfully to SARS-CoV-2 spread.

Presence of SARS-CoV-2 in WW sewage sludge, and surface water: Considering the initial reports of SARS-CoV-2 detection in feces (Holshue et al., 2020; Wölfel et al., 2020; Wu et al., 2020b), sewage should be recognized as a potential reservoir for a significant number of infectious virions. Various sources contribute to the presence of SARS-CoV-2 in household and hospital WW, including sputum, handwashing, and feces from infected individuals. Numerous studies, as listed in Table 3, have explored the presence of SARS-CoV-2 in river water, sewage, and sludge. However, it's important to note that some researchers have focused on detecting the virus rather than quantifying it. Additionally, reported virus levels are often expressed in different units, posing challenges for comparisons between studies. This underscores the need for standardized methods and units to facilitate meaningful comparisons in future research efforts.

In untreated WW, studies have reported varying rates of positive SARS-CoV-2 RNA detection, ranging from 13.3% to 100%, with concentrations in some cases exceeding 10^6 copies per liter. SARS-CoV-2 RNA has been consistently detected in WW in multiple studies. For example, in the Netherlands, the first detection of SARS-CoV-2 in sewage was reported, where 24-hour flow-dependent composite samples exhibited concentrations ranging from 2.6 x 10^3 to 2.2 x 10^6 copies per liter (Medema et al., 2020). The identification of SARS-CoV-2 RNA was carried out using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) with CDC N1, N2, and N3 assays, and viral concentration was achieved through ultrafiltration. Similarly, in Massachusetts, United States, F. Wu et al. (2020b) reported SARS-CoV-2 loads ranging from 10^3 to 10^5 copies per liter in composite raw sewage samples, using ultracentrifugation and polyethylene glycol precipitation methods with a sample volume of 40 mL. In France, Wurtzer et al. (2020b) successfully recovered SARS-CoV-2 from WW samples using ultracentrifugation, with virus concentrations identified by the E gene RT-qPCR assay ranging from 5 x 10^4 to 3 x 10^6 copies per liter, showing a trend of increasing viral concentrations during the exponential rise in coronavirus cases.

Table 3. Occurrence of SARS-CoV-2 in sludge, WW, and river water.

Nation	Sample category	Positive sample rates, number (%)	Amount of SARS-CoV-2 (copies per liter)	Reference
Spain				
	Raw WW	4(66.7)	$7.5x10^3$- 15×10^3	(Balboa et al., 2020)
	Primary sludge	9(100)	10^4–4×10^4	
	Biological sludge	9(100)	$7.5\ x10^3$-$10\ x10^3$	
	Raw WW Secondary effluent	35(83.3) 2(11.1)	2.5×10^5	(Randazzo et al., 2020b)
	Raw WW	13(86.7)	5.22–5.99 $\log^{10}$	(Randazzo et al., 2020a)
Japan	Raw WW	7(25.9)	$2.1x10^4$ – 4.4×10^4	(Hata et al., 2020)
	WW	1(20)	$1.4x10^2$–$2.5x10^3$	(Haramoto et al., 2020)
Turkey	Raw sewage	5(71.4)	$2.89x10^3$–$1.80x10^4$	(Bilge Alpaslan et al., 2020b)
	Sewage sludge	9(100)	$1.17x10^4$ –$4.02x10^4$	(Bilge Alpaslan et al., 2020a)
USA	Raw WW	18(81.8)	Average 42.7×10^3	(Hyatt et al., 2020)
	Primary sludge	36(100)	$1.7x10^6$- 4.6×10^8	(Jordan et al., 2020)
	Raw WW	10(71.4)	10^4 – $>2 \times 10^5$	(F. Wu et al., 2020a)
	Raw WW	7(100)	$>3x10^4$	(Nemudryi et al., 2020)
	Raw WW	2(13.3)	3.2 $\log_{10}$	(Sherchan et al., 2020)
	Raw WW	120(60.6)	10^2–10^5	(Gonzalez et al., 2020)
	Raw WW	126(61)	66–390	(Weidhaas et al., 2021)
Australia	Raw WW	2(22.2)	19–120	(Ahmed et al., 2020a)
France	Raw WW	23(100)	$5x10^4$–$3x10^6$	(S Wurtzer et al., 2020a)
Germany	Raw WW Secondary effluent	9(100) 4(100)	$3.0\ x10^3$–20×10^3 2.7–$37\ x10^3$	(Westhaus et al., 2021)
	Influent	44(86)	$2.0\ x10^3$ –3.0×10^6	(Agrawal et al., 2021)
India	Raw WW	2(100)	$0.78x10^2$–$8.05x10^2$	(Kumar et al., 2020)
	Raw WW	30(100)	$3.08\ x10^4$–$2.19\ x10^5$	(Hemalatha et al., 2020)
	Raw WW	6(35.3)	N.A	(Sudipti et al., 2020)
Italy	Raw WW	6(50)	N.A	(La Rosa et al., 2020)
	Raw WW	4(50)	N.A	(Rimoldi et al., 2020)
	River waters	3(75)	N.A	

Table 3. (Continued).

Nation	Sample category	Positive sample rates, number (%)	Amount of SARS-CoV-2 (copies per liter)	Reference
Ecuador	River water	3(100)	$2.91x10^5$–$3.19x10^6$	(Guerrero-Latorre et al., 2020)
Netherlands	Raw WW	All*	N.A	(Lodder de Roda Husman, 2020)
	Sewage	14(58.3)	$2.6 x10^3$–$30 x10^3$ $7.9 x10^5$–$2.2 x10^6$	(Medema et al., 2020)
Pakistan	Raw WW	21(26.9)	N.A	(Salmaan et al., 2020)
Emirates	Raw WW	33(85)	$2.86x10^2$ –$2.9x10^4$	(Hasan et al., 2021)

N.A: not available

In Spain, Randazzo et al. (2020b) observed similar concentrations of SARS-CoV-2 RNA in untreated and treated WW samples using aluminum flocculation-based techniques and RT-qPCR assays (CDC N1–3), with levels estimated at approximately 2.5 x 10^5 copies per liter. This finding aligns with the research by Westhaus et al. (2021) in Germany, who employed centrifugal ultrafiltration and RT-qPCR targeting the M-gene or RNA-dependent RNA polymerase (RdRp). They reported SARS-CoV-2 RNA levels ranging from 3 x 10^3 to 2 x 10^4 copies per liter in influent and 2.7 x 10^3 to 3.7 x 10^3 copies per liter in effluent from 24-hour, flow-dependent composite samples. In Japan, Haramoto et al. (2020) detected SARS-CoV-2 RNA in secondary-treated WW samples using the electronegative membrane-vortex method and membrane adsorption-direct RNA extraction combined with the N_Sarbeco, CDC-N1, and CDC-N2 assays. Their results indicated approximately 2.5 x 10^3 copies per liter, about half the concentration reported by Randazzo et al. (2020b). In Australia, Ahmed et al. (2020a) used direct RNA extraction from electronegative membranes and ultrafiltration, detecting virus loads ranging from 120 to 19 copies per liter in grab samples and untreated WW composite. Despite the widespread use of WW surveillance for SARS-CoV-2 monitoring, there is a lack of research on the stability and survivability of the virus in water or WW (Tran et al., 2021). While most studies on SARS-CoV-2 quantification and detection in sewage have not investigated viral viability, some research, such as that by Rimoldi et al. (2020) and Wang et al. (2020b) has shown that the virus' infectivity in WW is nonexistent. Meanwhile, Westhaus et al. (2021) evaluated the infectivity of raw WW using a viral outgrowth test and found no infectivity.

In WW, SARS-CoV-2 concentrations are considerably lower than in feces, where levels can peak at 10^8 RNA copies per gram. This is due to significant dilution in the WW system, resulting in a 5-fold reduction in viral load (Foladori et al., 2020). The amount of SARS-CoV-2 in WW depends on the proportion of the population served by the sewer network who test positive for the virus, as well as on daily flow rates, which are estimated to dilute the viral load by approximately 1000 times. Additionally, factors such as rainfall can contribute further to the dilution process (Agrawal et al., 2021). Additionally, variations in pH, temperature, adsorption to solids, and settling, as well as differences in virus populations and infectivity, can occur along the sewer network (Foladori et al., 2020). The observed decrease in SARS-CoV-2 RNA levels in WW in Spain correlates with a decline in virus shedders within the community (Chavarria-Miró et al., 2020). Interestingly, a sudden drop in SARS-CoV-2 RNA in sewage was noted following a large rainfall event, which introduced a significant dilution factor to the virus concentration in WW (Chavarria-Miró et al., 2020). This highlights the importance of considering environmental factors, such as rainfall, when interpreting WW surveillance data. To accurately and reliably characterize the viral content in sewage, the method, timing, and volume of sample collection are crucial. Virus levels may fluctuate significantly throughout the day, making composite samples collected over time preferable and possibly advisable. (Foladori et al., 2020). Studies have found varying quantities of SARS-CoV-2 RNA in sludge, ranging from 7.5 x 10^3 to 4.6 x 10^8 copies/L. Primary sewage sludge was found to contain a range of 1.7 x 10^6 to 4.6 x 10^8 copies/L of SARS-CoV-2 RNA (Peccia et al., 2020). However, secondary and biologically treated sludge showed lower levels of contamination, with ranges of 10^4–4 x 10^4 copies/L and 7.5 x 10^3–10^4 copies/L, respectively (Balboa et al., 2020).These results align with a study by Kocamemi et al. (2020) in Turkey, where SARS-CoV-2 RNA was found in 9% of sludge samples. The detected viral RNA levels ranged from 1.17 x 10^4 to 4.02 x 10^4 copies/L Figure 5.

The inability to isolate infectious SARS-CoV-2 from water environments does not necessarily indicate its absence; rather, it may result from challenges in detection methods (Bogler et al., 2020). The lack of standardized and optimized techniques remains a significant obstacle to the detection and quantification of SARS-CoV-2 in WW samples. According to Ahmed et al. (2020a), the difficulties in isolating the virus from WW samples can be attributed to various factors, including the sampling method, low virus concentration, and the sensitivity of detection techniques, particularly when dealing with low virus levels. Additionally, factors present in WW, such as temperature, pH, solids, disinfectants, and micropollutants, can contribute to the degradation and inactivation of the SARS-CoV-2 genome, potentially leading to virus inactivation (Kitajima et al., 2020). While RT-PCR and RT-qPCR are widely used as the gold standard for SARS-CoV-2 detection, they cannot distinguish between infectious and inactive particles, necessitating the use of cell culture infectivity tests to assess virus viability. Overcoming these challenges is crucial for establishing standardized and reliable techniques for virus quantification and detection (Kitajima et al., 2020; Lodder and de Roda 2020; Tran et al., 2021). Recent research in Ecuador has identified the presence of SARS-CoV-2 RNA in rivers, with concentrations ranging from $2.91x10^5$ to $3.19x10^6$ copies/L. This suggests that contaminated natural water bodies could act as environmental reservoirs for coronaviruses such as SARS-CoV-2, highlighting the importance of taking strict measures to prevent re-infection (Danchin et al., 2020). Particularly in low-income nations with inadequate sanitary infrastructure, concerns about potential dispersion are significant (Guerrero-Latorre et al., 2020).

Despite the presence of bodily fluids with high viral loads, such as sputum and saliva, in greywater discharged from sinks, showers, and drains, it is not considered a primary route for SARS-CoV-2 transmission (Wang et al., 2020c; Wölfel et al., 2020). This may be due to the presence of disinfectants like detergents and soaps in greywater, which can reduce the persistence and infectivity of SARS-CoV-2 (Chin et al., 2020; Kampf et al., 2020). There is a growing need to evaluate the occurrence, persistence, and potential public health risks associated with SARS-CoV-2 in wastewater. Converging evidence could highlight the potential of wastewater-based epidemiology (WBE) to track the spread of SARS-CoV-2 within communities.

Figure 5. SARS-CoV-2 in sewage, sources, and eventual routes for spreading on soils, crops, and communities.

Epidemiological significance of monitoring SARS-CoV-2 in wastewater: The use of SARS-CoV-2 detection in WW as an early warning system to track current and future epidemic trends has gained significant attention, driving interest in WBE. Research has focused on the principles of this approach, with Orive et al. (2020) noting that changes in viral concentrations in WW can signal shifts in disease cases within human populations. This method involves measuring SARS-CoV-2 RNA markers in WW to monitor COVID-19 prevalence and gain insights into disease spread across communities (Holshue et al., 2020).

WBE employs a theoretical approach that begins with measuring the concentration of SARS-CoV-2 in municipal WW from a known urban area serviced by a sewer system. This concentration, expressed in copies per cubic meter, is then multiplied by the daily WW flow rate to calculate the daily viral load in copies per day. The daily viral load is compared with the viral copies found in the feces of individuals who tested positive for SARS-CoV-2, aiding in the estimation of the number of positive cases in the urban area (Foladori et al., 2020). Recent studies confirm the effectiveness of this method in monitoring COVID-19 spread. For instance, in Southeastern Virginia, variations in SARS-CoV-2 levels in WW correlated with documented outbreaks over 21 weeks (Gonzalez et al., 2020). Similar patterns have been observed in the Boston metropolitan area, as well as in the USA, Australia, France, and Spain, establishing a link between SARS-CoV-2 RNA levels in WW and clinical case surveillance (Ahmed et al., 2020a; Weidhaas et al., 2021; Wu et al., 2020c; Wurtzer et al., 2020b).

The study by F. Wu et al. (2020b) identified a notable discrepancy between COVID-19 prevalence estimates derived from WW analysis and clinical testing. While clinical tests reported a prevalence of 0.026%, viral levels detected in WW suggested a significantly higher estimate, ranging from 0.1% to 5%. Similarly, research conducted in Hyderabad, India, found that the estimated proportion of infected individuals (6.6%) exceeded the reported active cases (0.4%) (Hemalatha et al., 2020). This variation may be attributed to factors such as underreporting of asymptomatic or mildly symptomatic cases, constraints in testing capacity and accuracy, and delays between symptom onset and viral shedding (Hemalatha et al., 2020; Medema et al., 2020; F. Wu et al., 2020b).

Contrary to previous findings suggesting a threshold for detecting SARS-CoV-2 in WW, Hata et al. (2020) observed its presence even when confirmed cases were less than 1 per 100,000, aligning with Wurtzer et al. (2020a). Moreover, delays in clinical confirmation after symptom onset mean reported cases may not reflect the true infection frequency during the study period. The accuracy of WW monitoring is influenced by factors such as viral load in feces and the sensitivity of detection methods. Efficient virus concentration is crucial for the reliable detection of SARS-CoV-2 in WW. Recent studies (Medema et al., 2020; Nemudryi et al., 2020; Wu et al., 2020b; Wurtzer et al., 2020b) have explored various concentration techniques. Among these, Ahmed et al. (2020b) found that using an electronegative membrane combined with $MgCl_2$ pre-treatment was the most effective method for recovering SARS-CoV-2 from WW, using murine hepatitis virus as a surrogate. In contrast, Sherchan et al. (2020) demonstrated that the ultrafiltration method successfully retrieved SARS-CoV-2 RNA from untreated WW, while Jafferali et al. (2021) preferred the adsorption-elution technique with electronegative membranes. These differences highlight the need for further research to evaluate the effectiveness of existing virus concentration methods in accurately detecting and quantifying SARS-CoV-2 RNA in WW.

Various assays have been developed to detect SARS-CoV-2 by targeting genes encoding the nucleocapsid (N) protein, envelope (E) protein, and RNA-dependent RNA polymerase (RdRp), each with different detection limits. For instance, Corman et al. (2020) introduced a highly sensitive RT-qPCR assay targeting the N gene, capable of detecting as few as five RNA copies per reaction. The N gene is the most commonly used target in RT-qPCR tests (Corman et al., 2020; Shirato et al., 2020). Additionally, paper analytical devices (PADs) have gained attention for detecting viral nucleic acids due to their accuracy, simplicity, sensitivity, speed, and cost-effectiveness (Mao et al., 2020; Tran et al., 2021). These advantages make PADs a promising tool for SARS-CoV-2 detection in water environments (Orive et al., 2020; Mao et al., 2020; Tran et al., 2021). Still, most reports on WBE have focused on short-term studies (Orive et al., 2020).

While WBE approaches hold promise for monitoring COVID-19 outbreaks, systematic evaluation and forecasting of these outbreaks have yet to be established (Polo et al., 2020). The current lack of comprehensive data suggests caution in using them as routine surveillance methods for COVID-19 (Amahmid et al., 2022). Challenges arise in correlating viral levels in WW with clinically confirmed cases, given varied transmission patterns and geographic regions. Addressing these challenges will require further research, particularly in optimizing sampling methods and establishing standardized protocols for viral concentration and detection in WW (Ahmed et al., 2020b; Orive et al., 2020). Despite these limitations, WBE can serve as an early warning system for monitoring SARS-CoV-2 in surface

waters. By monitoring river water at multiple locations near major sewage discharge sites, it becomes possible to detect potential increases in infection rates and subsequently control the spread of the virus (Amahmid et al., 2022; Núñez-Delgado, 2020). However, the full potential of this approach will depend on overcoming technical and logistical challenges and accumulating more robust data to validate its efficacy in real-world settings.

SARS-CoV-2 persistence in soil

Ensuring the health of plants, animals, and humans relies heavily on maintaining healthy soil conditions. Soil-transmitted pathogens, such as viruses, can persist in the soil for extended periods, posing a risk of transmission to hosts via soil particles (Amoah et al., 2017). Human enteroviruses, for instance, have been documented to survive up to 100 d in soil (Duboise et al., 1976; Ekanayake et al., 2023).However, while there has been extensive research on the persistence and transmission of viruses in water, studies examining SARS-CoV-2 persistence in soil environments remain limited.

The soil environment is exposed to various contaminants, including solid waste, sewage from wastewater treatment plants (WWTPs), biosolids from landfills, and airborne particles (Ekanayake et al., 2023). Studies indicate that applying sewage to soil may promote pathogen survival and transport, largely due to the high organic matter content of biosolids (Horswell et al., 2010). Studies have shown that municipal sewage sludge spreading on land can lead to the contamination of soil and water with enteroviruses, which may persist for up to 14 d in soil (Pourcher et al., 2007). Moreover, *Escherichia coli* bacteria have been found to survive for four weeks in leachate produced from laboratory-treated sludge (Ekanayake et al., 2023; Pousada-Ferradás et al., 2012).
Recent studies have detected SARS-CoV-2 RNA in sewage sludge, raising concerns about potential soil contamination. Traces of the virus have been found in primary sludge from municipal WWTPs in New Haven, USA, as well as in waste-activated sludge from WWTPs in Istanbul (J. Peccia et al., 2020b). Furthermore, the presence of SARS-CoV-2 in both treated and untreated sludge suggests that conventional WW sludge treatment methods may not be entirely effective in eliminating the virus (Serra-Compte et al., 2021).

The presence of organic materials in sludge and the virus's hydrophobic nature may contribute to its affinity for sludge (Conde-Cid et al., 2021). Consequently, soil and crop plants may become contaminated in areas where sewage is used as a soil amendment, potentially leading to the contamination of food products (Núñez-Delgado, 2020). Direct release of disinfected solid waste, application of untreated WW for irrigation, and disposal of medical waste on land further contribute to soil contamination with SARS-CoV-2. In outdoor hospital environments, SARS-CoV-2 has been detected in soil samples, with counts ranging from 205 to 550 copies/g in areas close to hospitals and WW treatment facilities (Zhang et al., 2021). The detection of SARS-CoV-2 in soil samples within two meters of WW treatment tanks suggests that outdoor hospital environments should be regarded as high-risk areas, potentially acting as secondary transmission pathways. Additionally, the improper disposal of PPE equipment , such as face masks and gloves, without adequate decontamination increases the likelihood of viral migration into the soil (Ilyas et al., 2020).

Viable SARS-CoV-2 viruses have been shown to persist on solid waste surfaces, potentially heightening the risk of soil contamination (Li et al., 2020b). Research indicates that SARS-CoV-2 can survive in soil environments for over ten weeks under favorable conditions, highlighting the need to quantify the virus in soil to assess future risks of transmission. This approach is similar to WW surveillance, which serves as an important epidemiological tool for tracking viral spread (Ekanayake et al., 2023).

While there is limited evidence of SARS-CoV-2 infecting humans or spreading through food and soil, precautions should be taken to prevent its migration to other environmental compartments. WW should undergo thorough screening before being applied to soil to mitigate the risk of COVID-19 transmission. Additionally, the disposal of PPEP M equipment and medical waste on land without proper decontamination could increase the risk of soil contamination with SARS-CoV-2 (Ekanayake et al., 2023).

DISCUSSION, IMPLICATIONS, AND FUTURE PERSPECTIVES

The evidence accumulated to date demonstrates that the transmission dynamics of SARS-CoV-2 are influenced by a multifactorial interplay involving environmental, meteorological, and anthropogenic factors. Meteorological variables including temperature, humidity, rainfall, wind speed, and solar radiation have been investigated extensively for their role in shaping COVID-19 incidence. However, the findings remain inconsistent and highly location-specific. While some studies suggest that elevated temperatures and higher humidity may reduce viral transmission, others report weak or non-significant associations, reflecting heterogeneity in regional climatic conditions, population behaviors, and methodological approaches. For instance, research in China and Italy highlighted positive correlations between low ambient temperatures or specific humidity levels and higher COVID-19 cases, whereas studies in Indonesia and New York found negligible or mixed effects. These inconsistencies underscore the complexity of environmental determinants and indicate that meteorological factors alone cannot reliably predict SARS-CoV-2 spread.

Air pollution, particularly exposure to fine PM (PM-2.5, PM-10) and NO_2, has consistently been linked with increased COVID-19 incidence and severity. Evidence from China and Northern Italy indicates that populations exposed to higher levels of air pollutants exhibited elevated case numbers, mortality, and disease severity. The underlying mechanism is likely indirect: air pollutants may exacerbate respiratory and systemic inflammation, impair immune defense, and increase susceptibility to viral infections rather than directly promoting SARS-CoV-2 transmission. These findings reinforce the broader public health importance of environmental quality and air pollution control as complementary strategies in pandemic preparedness. Moreover, regional variations in pollutant types and concentrations highlight the necessity of contextualizing epidemiological analyses within local environmental and demographic settings.

WBE has emerged as a robust and non-invasive tool for monitoring SARS-CoV-2 prevalence at the community level. Detection of viral RNA in untreated and treated WW, primary and secondary sludge, and surface waters has provided valuable insights into infection dynamics,

particularly for asymptomatic or untested individuals. Studies across multiple countries including the USA, Spain, Japan, Germany, and Australia have demonstrated that viral RNA concentrations in WW often precede clinically confirmed cases, making WBE an effective early warning system. However, SARS-CoV-2 RNA quantification in WW is subject to several limitations, including dilution from variable flow rates, temporal fluctuations in viral shedding, rainfall events, and the technical sensitivity of concentration and detection methods. While RNA detection is robust, the infectivity of SARS-CoV-2 in WW appears negligible in most studies, suggesting that WW is primarily a monitoring medium rather than a significant transmission route. Nonetheless, in low-income regions with insufficient sanitation infrastructure, the potential for environmental dissemination warrants careful attention.

Soil and sludge contamination represents an additional potential environmental reservoir for SARS-CoV-2. The persistence of viral RNA in WW sludge, coupled with its application to agricultural land or inadvertent release into the environment, raises concerns about indirect exposure pathways. Evidence suggests that SARS-CoV-2 can remain detectable in soil and sludge for extended periods, particularly in the presence of organic matter, moisture, and favorable physicochemical conditions. While direct transmission from soil to humans or through food remains unproven, improper management of sewage, sludge, and medical waste such as face masks and gloves may facilitate viral deposition in terrestrial ecosystems. These findings highlight the need for rigorous treatment of WW, decontamination protocols for medical waste, and consideration of soil as a potential environmental compartment in viral epidemiology.

The public health implications of these findings are substantial. Integrating environmental and meteorological monitoring with WBE provides a cost-effective and sensitive means of tracking infection dynamics in real time, particularly in regions with limited clinical testing capacity. Furthermore, monitoring air quality and environmental contamination can inform targeted interventions to reduce population susceptibility and prevent secondary exposure events. The potential role of WW and soil as reservoirs emphasizes the importance of robust sanitation infrastructure, proper sludge management, and environmental hygiene to mitigate indirect viral transmission.

Looking ahead, several key areas require attention to strengthen the utility of environmental surveillance in pandemic management. First, there is a critical need for standardized protocols for sampling, viral concentration, and detection in WW, sludge, and soil. Harmonization of methodologies including RT-qPCR targets, sample collection volumes, and timing will improve the comparability and reliability of findings. Second, longitudinal, multi-site studies incorporating diverse environmental, climatic, and demographic contexts are essential to disentangle confounding factors and elucidate causal relationships between environmental variables and SARS-CoV-2 transmission. Third, assessing viral viability, not just RNA presence, is crucial for accurately estimating infection risks associated with WW and environmental matrices. Fourth, integrative modeling approaches that combine meteorological data, air quality, WBE metrics, and epidemiological information can enhance outbreak forecasting and guide public health interventions. Finally, comprehensive risk mitigation strategies including advanced WW treatment, safe sludge management, and controlled disposal of medical waste are imperative to prevent environmental reservoirs from contributing to future outbreaks.

CONCLUSION

In conclusion, SARS-CoV-2 transmission is shaped by a complex, multifactorial interplay between environmental conditions, air quality, human behavior, and sanitation practices. While direct infectivity from environmental compartments such as wastewater or soil appears limited, these media serve as valuable surveillance tools that can provide early warnings and insights into infection prevalence. Addressing methodological gaps, standardizing detection techniques, and expanding longitudinal studies will be critical to fully realize the potential of environmental epidemiology. A holistic, integrated approach that combines meteorological monitoring, air quality assessment, WBE, and soil surveillance will not only enhance pandemic preparedness but also strengthen resilience against future respiratory viral outbreaks.

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Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

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