Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.227

RESEARCH ARTICLE

Infection Dynamics of *Salmonella enterica* in Houbara Bustard and Resistance Modulation through Nanocomposites Coupled Antibiotics

Afshan Muneer[1], Syed Qaswar Ali Shah[1*], Amjad Islam Aqib[2#], Muhammad Muddassir Ali[3] and Abdul Ghaffar[1]

[1]Cholistan Institute of Biological Sciences, Cholistan University of Veterinary and Animal Sciences Bahawalpur 63100, Pakistan;
[2]Department of Medicine, Cholistan University of Veterinary and Animal Sciences Bahawalpur 63100, Pakistan;
[3]Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences Lahore 54000, Pakistan
*Corresponding authors: *syedqaswaralishah@cuvas.edu.pk, #amjadislamaqib@cuvas.edu.pk

ARTICLE HISTORY (25-547)

Received: June 10, 2025
Revised: July 23, 2025
Accepted: July 30, 2025
Published online: August 26, 2025

Key words:
Antibiotic resistance
Houbara Bustard
Molecular characterization
Risk factors
Salmonella enterica

ABSTRACT

The emerging infection dynamics of *Salmonella enterica* with special context to Houbara bustard require studies on pattern of prevalence, risk factors, molecular dynamics, drug resistance, and alternative to antibiotics. The study found that 37% (74/200) of cloacal samples from Houbara bustard tested positive for *S. enterica* with a significant association between assumed risk factors and prevalence ($p < 0.05$). Regression analysis revealed 6-12 months of birds' age, natural living environment, birds on mixed type of feeding system, occasional use of antibiotics, and use of non-beta lactam antibiotics as potential risk factors for prevalence of *S. enterica*. The antibiogram showed 70% resistance against florfenicol, 60% against ciprofloxacin and 40% against both amoxicillin and penicillin. Nanocomposite coupled antibiotics showed a concentration-dependent increase in the zone of inhibition (ZOI). Nanocomposite coupled ciprofloxacin at 10 & 0.5mg/mL and nanocomposite coupled oxytetracycline at 0.25mg/mL produced highest ZOI. Blood profiles of *S. enterica*-positive birds showed values beyond normal ranges. Phylogenetic analysis revealed genetic similarity between the study isolates and previously reported strains. Nucleotide motif analysis revealed a major deletion in nucleotide sequence while, protein motif analysis suggested that the protein remained stable and well-structured. The study thus concluded that there is a rising percentage of drug-resistant *S. enterica*, a significant association of assumed risk factors, notable impacts on blood profiles, divergence in molecular make up but still holding sensitivity to alternative treatments. The findings of study thus require implementation of stern measures to tackle spreading of antibiotic-resistant bacteria through birds and other hosts.

To Cite This Article: Muneer A, Shah SQA, Aqib AI, Ali MM and Ghaffar A 2025. Infection dynamics of *Salmonella enterica* in Houbara bustard and resistance modulation through nanocomposites coupled antibiotics. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2025.227

INTRODUCTION

Houbara bustards have long been a traditional game species for falconers in Arabia and hold significant cultural and conservation value. Numerous conservation initiatives have been undertaken to protect both the species and the traditions associated with it. The bird's population experiences decline making it stand among threatened species regarding conservation point of view. Trade and hunting might not be the only reasons for decreased population but health may be considered as a significant risk among others. The major reasons for poor health might be poor husbandry, overcrowding during transportation, domestic bird's exposure, and inadequate food/water supply (Bailey *et al.,* 2000). Houbara bustard has been mostly focused so for on aspects related to ecological, biomedical, and captive breeding while studies on analysis of antibiotic resistance are scarce despite of the fact that birds are significant source of spread of zoonotic pathogens (Dobbin *et al.,* 2005). The closer contact of humans with animals especially through conservation and rehabilitation efforts has also resulted in transfer of antibiotic resistance genes to the microbiota of populations of migratory birds. Among these microbes, *Salmonella* species are particularly concerning due to their high genetic variability and ability to infect both birds and humans (Silvanose *et al.*, 2001).

Salmonella has emerged as a major pathogen of poultry (Durrani *et al.,* 2024), farm animals and reptiles

(Krumova-Valcheva *et al.*, 2024). *Salmonella* is a major bacterial genus responsible for foodborne illnesses, often resulting in thousands of deaths worldwide each year (Alzwghaibi *et al.*, 2018). Increased drug resistance in these pathogens has led to increased hospitalization, longer hospital stays, and higher medical expenses due to reduced effectiveness of antibiotic therapy. Since the emergence of multidrug-resistant *Salmonella* clones during 1990-2000, their global spread among humans, domestic animals, and wildlife has become widespread (Niki *et al.*, 2017). Antibiotics are used to control infectious diseases in both animals and humans (Islam *et al.,* 2024). However, they are often used inappropriately as preventive measures (Ibrahim *et al.,* 2023). Moreover, limited efficacy of clinically important drugs like fluoroquinolones and third-generation cephalosporins, has been reported (Angelo *et al.*, 2016) thus narrowing down the therapeutic options. The expression of virulent determinant of *Salmonella enterica* has been noted in the form of exotoxins, biofilm production, endotoxin, hemagglutinins, fimbriae, adhesins, and invasions (Sabbagh *et al.*, 2010; Feng *et al.,*2023).

The use of antibiotics resulted in antibiotic resistance issues, and this need to investigate the alternative methods for replacing the role of antibiotics (Hamid *et al.,* 2025). It is also equally important to focus on alternative approaches like use of nanoparticles, probiotics (Rashid *et al.,* 2023), prebiotics, and phytochemicals. Recent studies on nanoparticle coupled antibiotics against *Staphylococcus aureus* and *Escherichia coli* have proved to be effective alternative of antibiotics (Muneer *et al.*, 2022; Murtaza *et al.*, 2023). Moreover, tungsten-based nanoparticles have exhibited significant antibacterial properties, offering novel insights into potential treatments for multidrug-resistant pathogens (Hetta *et al.*, 2023). The current study was thus designed to investigate the prevalence of *Salmonella enterica* in cloacal samples of Houbara bustards, identify associated risk factors, assess the antibiotic resistance profile, characterize molecular patterns, and evaluate the efficacy of tungsten oxide nanocomposite-coupled antibiotics.

MATERIALS AND METHODS

Prevalence of *Salmonella enterica:* Cloacal samples (n=200) were collected from the Houbara bustards from Houbara Foundation International, Pakistan. The collected samples were processed for biochemical analysis as per guidelines of Bergey's Manual of Determinative Bacteriology, and pooled information led to the confirmation of *Salmonella.* Association of assumed risk factors, majorly from bird and housing management, were analyzed based on information obtained from questionnaire filled during sampling.

The samples positive for *Salmonella* were further investigated for molecular identification targeting *inVA* gene with forward primer 5′AAATTATCGCCACGTTCGGG 3′ and *inVA* reverse primer 5′ACTCATCTGTTTACCGGGCA 3′ adopting method as described by Pal *et al.* (2017). Positive amplicons were sent for sequencing in order to further process for molecular characterization and *in-silico* analysis. The study pathogen has been submitted to GenBank and can be accessed under accession numbers PV533628 and PV533629.

***In-silico* analysis of type III secretion system export apparatus protein InvA (*invA*) gene of *Salmonella enterica* isolates**

Translation of nucleotide sequences: Nucleotide sequences from the study isolates, along with reference sequences, were translated into amino acid sequences as a prerequisite for amino acid alignment, motif analysis, three-dimensional protein modeling, and Ramachandran plotting to determine protein structural conformation. Sequence translation was carried out using the Translate tool ExPASy.

Nucleotide and amino acid sequence alignment and motif construction: Nucleotide and amino acid sequence alignments were performed to identify nucleotide substitutions that could lead to variations in the amino acid sequence and, ultimately, alterations in the three-dimensional structure and function of the protein. Alignments were conducted using the Clustal Omega tool under default ClustalW parameters with character count enabled. Additionally, nucleic acid and amino acid motifs were analyzed using the MEME Suite (Multiple EM for Motif Elicitation), with five motifs generated for each sequence.

Three-dimensional modeling and secondary structure conformation analysis: Swiss model software was used to determine the 3D structure of the protein. The amino acid sequences of template PBP2a and beta-lactamase proteins were retrieved in PDB format from the Protein Data Bank. Ramachandran plots were designed to visualize the possible conformation of amino acid residues in the protein using the PDBsum tool of bioinformatics. The secondary structure comparison (Alpha helix, Beta turn, Random coil) of study isolates was performed with the help of SOPMA (Self-Optimized Prediction Method with Alignment). Moreover, the physicochemical properties of type III secretion system export apparatus protein InvA (*invA*) were evaluated by ProtParam.

Response of *Salmonella enterica* against antibiotics: Drug susceptibility of *S. enterica* was assessed against antibiotics of different classes using disc diffusion method as per guidelines of Clinical and Laboratory Institute (CLSI, 2018). Multiple drug-resistant isolates were further put to evaluation of antibacterial potential of tungsten oxide nanocomposite coupled antibiotics.

Tungsten oxide nanocomposites (WNC; Tungsten oxide W, nanocomposite NC) composed of WO_3/g-C_3N_4—a combination of tungsten oxide (WO_3) and graphitic carbon nitride (g-C_3N_4)—(WNC) were synthesized and coupled with ampicillin, penicillin, oxytetracycline, and ciprofloxacin. Antibacterial activity was evaluated using the well diffusion method by applying different concentrations (10, 1, 0.5, and 0.25 mg/mL) of treatments on 1–1.5 × 10^8 CFU/mL fresh bacterial culture as per method discussed by Anwar *et al.* (2020).

Blood profile of Houbara bustard birds: Blood samples (n=28) were collected from Houbara bustards under

management of Houbara Foundation International, Pakistan. These samples were processed for liver function tests (LFT), renal function tests (RFT), and complete blood count (CBC). From the collected samples, n=20 were randomly selected from both of *S. enterica* positive and negative birds, regardless of co-infection with other bacteria.

Statistical analysis: The qualitative data was analyzed through descriptive statistics, chi square and regression analysis while quantitative data were processed through t-test and ANOVA using SPSS and/or Minitab statistical software at 5% probability, where applicable.

RESULTS

Prevalence and potential risk determinants of *Salmonella enterica:* The current study found a 37% (74/200) prevalence of *Salmonella* in cloacal samples collected from Houbara bustards, as confirmed through phenotypic and biochemical tests. A statistically significant association was observed between several assumed risk factors and the prevalence of *S. enterica* (Table 1). Male birds exhibited higher odds of infection compared to females, suggesting gender as a potential risk factor. Birds aged 6 to 12 months showed greater susceptibility compared to those older than one year. Exposure to the natural environment was associated with 2.44 times higher odds of infection compared to birds housed in pens. Similarly, birds kept exclusively on poultry feed had 2.953 odds of getting *Salmonella* infection compared to those on a mixed diet (poultry feed and scavenging).

There was significant association of weather with prevalence of *Salmonella.* The odd ratio (OR) for spring was less than 1 while winter showed a statistically significant association with *Salmonella* prevalence (OR=0.274, P=0.001), when summer was kept as constant. The study also showed the significant association (P<0.05) between antibiotics use and the prevalence of *Salmonella* infection. Birds receiving occasional antibiotic treatments had higher odds of infection (OR=1.615, P=0.188) compared to those receiving frequent or no antibiotic use. Additionally, birds exposed to antibiotics other than beta-lactam showed 1.28 times greater odds of infection. The presence of gastrointestinal (GI) issues was also significantly associated with *Salmonella* infection (OR=0.366, P=0.001).

Molecular Characterization

Phylogenetic Tree: The phylogenetic analysis revealed an evolutionary relationship between the two test isolates; H241010-002 E04 1 RP.ab1 442 and H241010-002 G04 2 RP.ab1 442 and various strains of *Salmonella enterica* subsp. *enterica* (Fig. 1). The isolate E04 1 RP.ab1 442 clustered closely with *S. enterica* serovar London and an unspecified *S. enterica* strain indicating a high degree of genetic similarity. Furthermore, shorter length of branches is indicative of a noticeable sequence similarity, supporting the close evolutionary relationship between E04 and the London serovar group. On the other hand, G04 2 RP.ab1 442, falls within a different clade showing higher genetic similarity with the *S. enterica* serovar Montevideo and Schwarzengrund. This indicates that G04 has a different evolutionary origin compared to E04, despite their occurrence within the same sub species group. Several other serovars such as TyPhi, Uganda, Infantis, Anatum, Heidelberg, and Enteritidis share broader relationships through their distribution across various clades. However, some serovars such as the Typhi and Typhi Mumbai cluster within a same clade possibly due to commonly shared serotype. In contrast, other serovars like Enteritidis and Heidelberg fall in outgroups suggestive of significant genetic divergence.

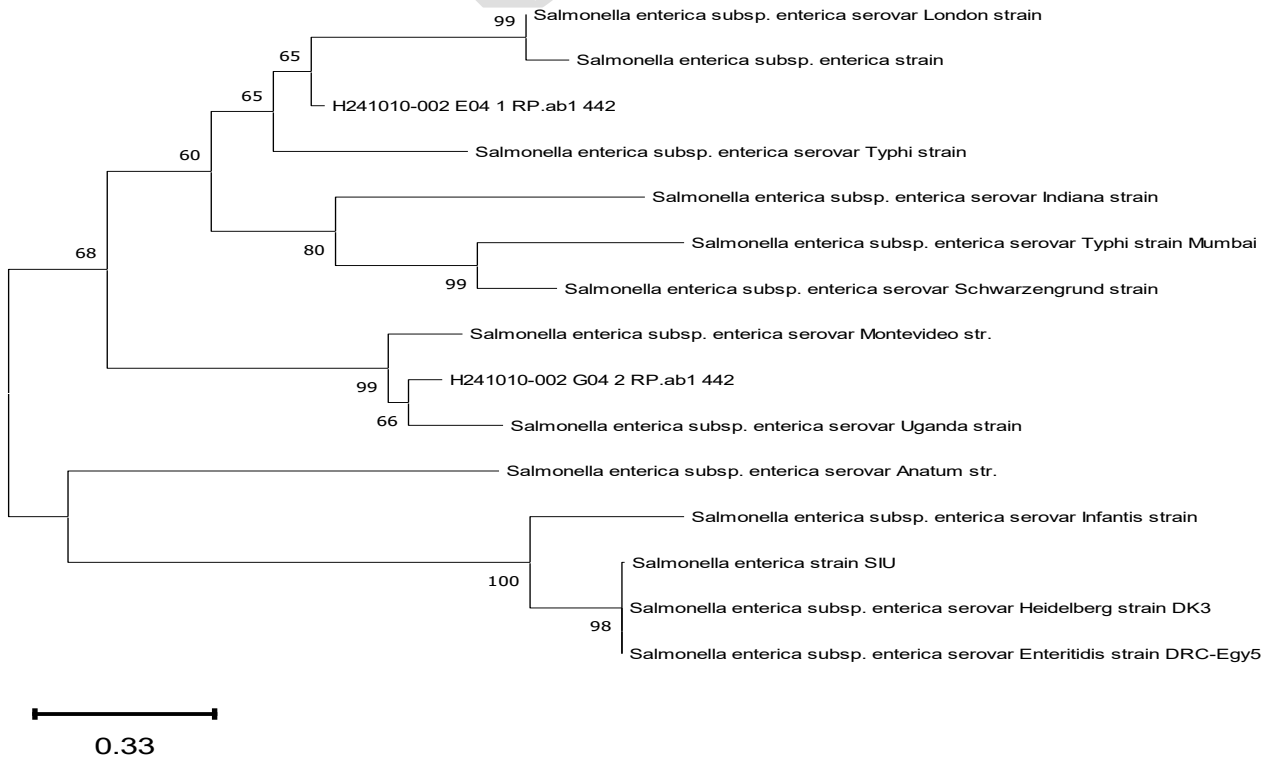

Fig. 1: Phylogenetic tree showing evolutionary relationships among *Salmonella enterica* subsp. *enterica* strains based on genetic similarity. Samples H241010-002 E04 and G04 cluster with London and Montevideo serovars respectively, indicating distinct lineages.

Table 1: Analysis of Risk factor association with prevalence of *Salmonella enterica* isolated from Houbara bustard

Variables	Levels	Screened	Positive	Prevalence (%)	P-value	Regression analysis odd ratio	CI 95%	P value
Gender	Male	80	18	22.50	0.001	3.014	1.596-5.691	0.001
	Female	120	56	46.67		-	-	-
	Upto 6 months	50	10	20.00	0.002	3.826	1.707-8.574	0.001
Age	7-12 months	70	20	28.57		1.913	0.972-3.766	0.061
	>12 months	80	44	55.00		-	-	-
Housing system	Natural environment provision	124	36	29.03	0.003	2.444	1.350-4.426	0.003
	Pen	76	38	50.00		-	-	-
Feeding system	Poultry feed	76	17	22.37	0.001	2.953	1.549-5.627	0.001
	Poultry feed plus Scavenger	124	57	45.97		-	-	-
Season	Spring	80	26	32.50	0.001	0.774	0.382-1.565	0.476
	Winter	50	29	58.00		0.274	0.125-0.583	0.001
	Summer	70	19	27.14		-	-	-
GI Parasites	Yes	88	44	50.00	0.001	0.366	0.203-0.661	0.001
	No	112	30	26.79		-	-	-
Use of antibiotics	Frequent	40	24	60.00	0.002	0.359	0.169-0.763	0.008
	Occasional	60	15	25.00		1.615	0.791-3.300	0.188
	No use	100	35	35.00		-	-	-
Type of antibiotic used	Beta-lactam	70	37	52.86	0.003	0.382	0.199-0.733	0.004
	Other than beta lactam	40	10	25.00		1.286	0.552-2.996	0.560
	NA	90	27	30.00		-	-	-

Nucleotide and amino acid sequence alignment and motif construction Nucleic acid alignment revealed significant similarity between the local isolates and reference sequences. The identity matrix ranged from 44.52 to 100% across different regions of the sequences. Nucleotide motif analysis indicated that the third motif (light blue) was absent in the study isolates, suggesting a major deletion in the nucleotide sequence (Fig. 2). Protein sequence alignment of the type III secretion system export apparatus protein revealed substantial variation between the study isolates and reference sequences. Most amino acid variations were observed at the initiation sites of the study isolates (RP1 and RP2). Protein motif analysis further revealed that all conserved motifs exhibited variability in both the study isolates and reference sequences.

Three-dimensional modeling and secondary structure conformation analysis: The 3D models of the protein provided valuable insights into the structural basis of its function (Fig. 3). The three-dimensional models for the type III secretion system export apparatus protein from the study isolates were identical to each other. A search of the Protein Data Bank (PDB) identified templates for each of our isolates. Specifically, a protein with PDB ID: 2X49 (at a resolution of 1.5 Å) was selected as the template for the study isolates. These isolates showed 100% sequence identity with the selected templates, with an E-value of 8.001e-48. Ramachandran plots indicated good stereochemical quality of the model, with the majority of residues falling within the alpha-helical (α) and beta-sheet (β) regions, consistent with the known secondary structure of the type III secretion system export apparatus protein. Most residues were located within the allowed regions, suggesting that the protein is stable and well-structured (Fig. 4). Furthermore, the Ramachandran plot favored the 3D model of the type III secretion system export apparatus protein InvA (*invA*) at 100%. Secondary structure comparison revealed a predominance of alpha helices and random coils in the study isolates. MOL probity score, Clash score, Ramachandran favoured, Ramachandran outliers, Rotamers outliers, C-beta Deviations, Bad bonds, and Bad angles were 0.50, 0.00, 100.00%, 0.00%, 0.00%, 0.00, 0/577, and 3/776, respectively.

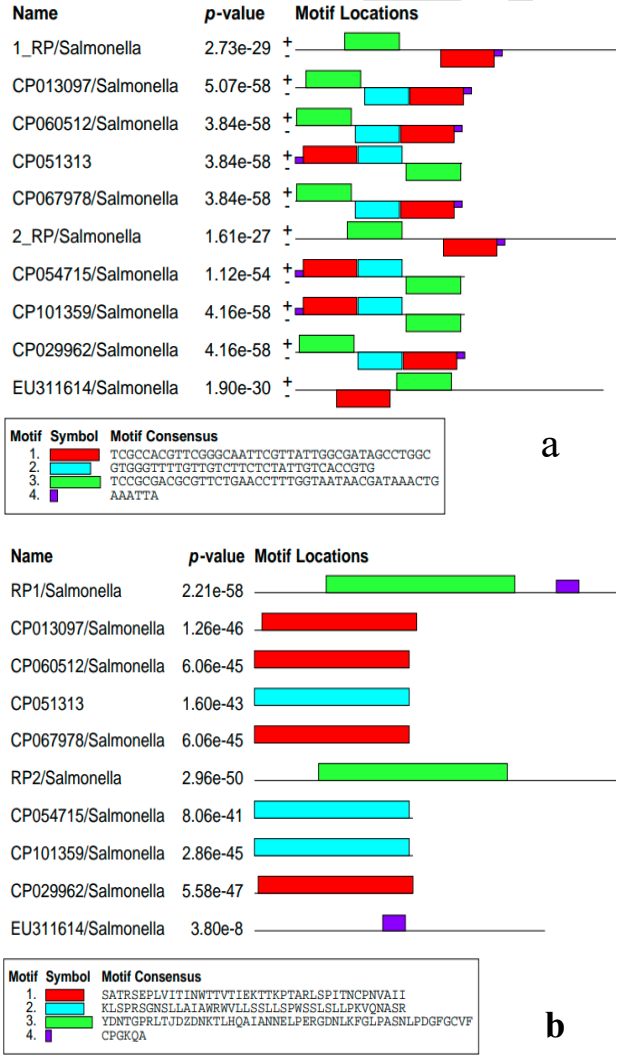

Fig. 2: Nucleotide sequence motif (a) and protein sequence motif (b) of *Salmonella enterica*.

Drug resistance profile of *Salmonella enterica*: The current study observed an increasing trend in antibiotic resistance among *Salmonella* isolates. The antibiogram revealed the highest resistance to florfenicol (70%) followed by ciprofloxacin (60%) and both amoxicillin and

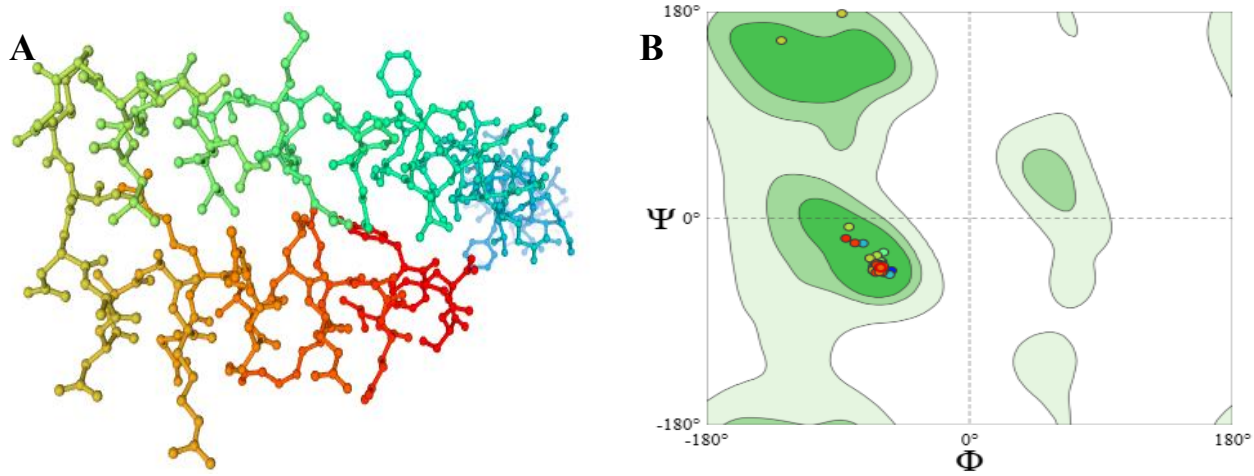

Fig. 3: 3D Modelling of Type III secretion system export apparatus protein (a) and Ramachandran plots of *Salmonella enterica*.

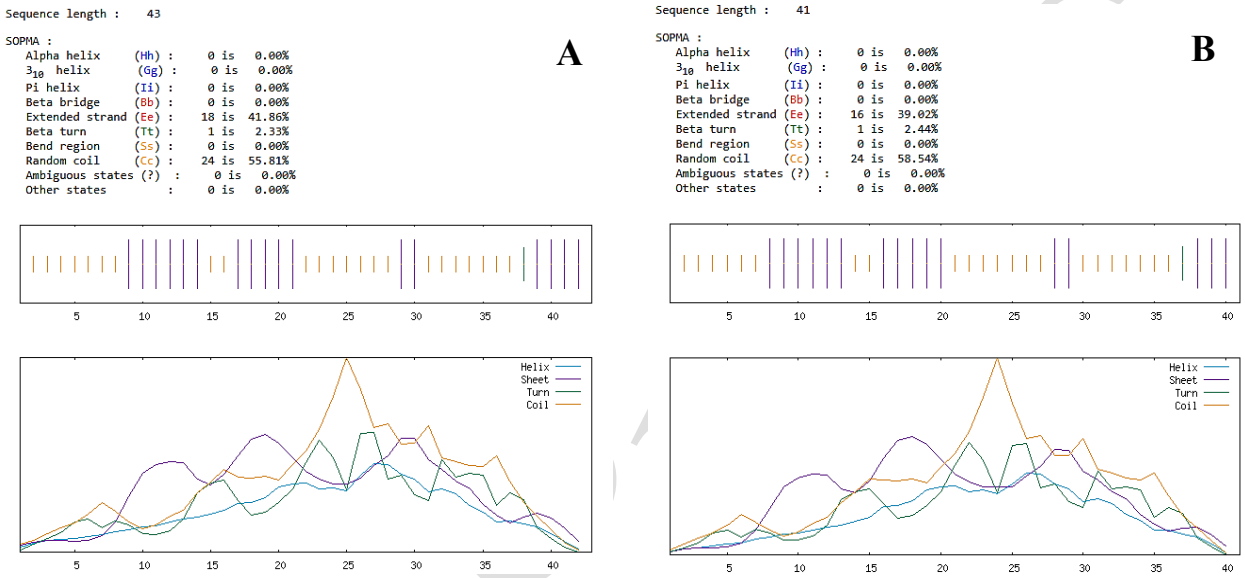

Fig. 4: Secondary structure prediction, a) at 41 sequence length, b) at 43 sequence length using SOPMA

Penicillin (40%) (Table 2). Resistance to doxycycline, enrofloxacin, colistin and neomycin was observed in the 30% of the isolates while amikacin, and tilmicosin was 20%. On the other hand, amikacin exhibited the highest efficacy with 80% of *Salmonella* isolates found to be sensitive. Doxycycline and tilmicosin demonstrated 70% sensitivity, while amoxicillin, enrofloxacin, and penicillin showed 60% sensitivity. Additionally, the study found that 10-40% of isolates were intermediate-susceptible to enrofloxacin, tilmicosin, neomycin, florfenicol, ciprofloxacin and colistin.

Response of *Salmonella enterica* against nanocomposite (WO_3/g-C_3N_4) coupled antibiotics: The comparative response of WNC (tungsten oxide W, nanocomposite NC) consisting of WO_3/g-C_3N_4 and coupled with antibiotics showed significant differences in most cases (Table 3). At a concentration of 10mg/mL, WNC coupled with ciprofloxacin exhibited the highest zone of inhibition (ZOI) (21.43 ± 5.38mm), while at lower concentrations like 1, 0.5, and 0.25 mg/mL, the highest ZOIs were found in case of WNC coupled with ampicillin (13.00 ± 5.63mm), WNC coupled with ciprofolaxcin (10.00 ± 6.00mm), and WNC coupled with oxytetracycline (8.714 ± 1.89mm).

Table 2: Antibiotic susceptibility pattern of *Salmonella* against different antibiotics

Antibiotics	Resistant (%)	Intermediate (%)	Sensitive (%)
Amikacin	20	0	80
Amoxicillin	40	0	60
Doxycycline	30	0	70
Florfenicol	70	20	10
Enrofloxacin	30	10	60
Colistin	30	40	30
Neomycin	30	20	50
Tilmicosin	20	10	70
Penicillin	40	0	60
Ciprofloxacin	60	30	10

Table 3: Zone of inhibition between different treatments at different concentration

Treatment	10mg/mL	1mg/mL	0.5mg/mL	0.25mg/mL
WNC+Ciprofloxacin	21.43±5.38^{a}	12.00±4.00^{a}	10.00±6.00^{a}	6.86±2.79^{a}
WNC+Pencillin	16.414±4.41^{ab}	7.714±1.799^{a}	5.71± 3.15^{a}	6.86±4.14^{a}
WNC+Ampicillin	17.29±8.34^{ab}	13.00±5.63^{a}	9.00±3.51^{a}	7.571±1.988^{a}
WNC+Oxytetracycline	10.57±3.21^{b}	9.429±1.813^{a}	9.286±1.254^{a}	8.714±1.890^{a}
WNC	13.00±3.87^{b}	10.86±4.45^{a}	7.00±3.11^{a}	5.71±3.55^{a}

WNC=Tungsten oxide (W), nanocomposite (NC), consisting of WO_3/g-C_3N_4; Different superscripts within column indicate a significant difference ($P<0.05$).

The pattern of comparison of ZOI across different concentrations was similar for both WNC alone and WNC coupled ampicillin. A significant difference in ZOIs was observed at 10mg/mL when compared with 0.5, and 0.25 mg/mL concentrations. No significant difference (P>0.05) of ZOI was observed between 10 and 1 mg/mL, and same goes among 1, 0.5, and 0.25 mg/mL concentrations (Fig. 5). A similar pattern of ZOI response across different concentrations was observed for nanocomposites (WNC) coupled with ciprofloxacin and penicillin. A significant difference (P<0.05) of ZOIs was observed at 10mg/mL when compared with 1, 0.5, and 0.25mg/mL. However, the differences among 1, 0.5, and 0.25 mg/mL concentrations were not statistically significant (P>0.05). NC coupled with the oxytetracycline showed only significant differences *(*P<0.05) of ZOI at 10 mg/mL compared with that of 0.5 mg/mL while comparison among 1, 0.5, and 0.25 mg/mL remained non-significant *(*P<0.05) (Fig. 5).

Impact of *Salmonella enterica* on blood profile: The current study found distinct variations in liver function tests (LFT), renal function tests (RFT), and complete blood count (CBC) between *S. enterica*-positive and negative Houbara bustards. LFT values were higher than the normal ranges for most parameters in both females and males. However, some individuals in both groups (either positive or negative for *S. enterica*) exhibited an opposite trend, as indicated by the higher standard deviation values for certain parameters. Specifically, values for ALT, ALP, total protein, and AST were elevated above the upper normal limit in *S. enterica*-positive birds, while the birds negative for the pathogen showed average and some higher values within the normal range. RFT results revealed creatinine levels to be higher than the upper normal range in *Salmonella* positive male and female birds, while *Salmonella* negative male bird showed slightly higher than upper value. Blood urea nitrogen (BUN) levels were found to be normal in *Salmonella* negative birds but lower in the positive groups of birds (Table 4).

The CBC profile of *S. enterica*-positive Houbara bustards showed lower-than-normal values for hemoglobin (Hb), white blood cells (WBC), mean corpuscular hemoglobin (MCH), and platelet count, while the reverse trend was observed in red blood cells (RBCs), hematocrit (HCT), mean corpuscular volume (MCV). In case of monocytes, and neutrophils all values of positive and negative birds group found normal or in range with the normal value. On the other hand, the birds negative for *S. enterica* exhibited values within the normal range, except for platelet count, which was lower than the normal value (Table 5).

Table 4: Effect of *Salmnella enterica* on liver, and kidney functioning tests of Houbara bustard birds

Parameters	Gender	*Salmonella positive* (Mean±SD)	*Salmonella Negative* (Mean±SD)	P-*value*	Normal Value
Liver function test (LFT)					
ALT	Female	86±7.549	31.7142±18.154	0.263	9.50-37.2U/L
(µL)	Male	71±7.438	35.16±17.04	0.384	
ALP	Female	2943.6±945.347	525.8±185.704	0.011	339-736µL
(µL)	Male	2660.66±698.783	396.25±129.134	0.225	
Total Protein	Female	6.05±0.479	3.33±1.24	0.089	3.27 - 4.40g/dL
(g/dL)	Male	6±0.98	3.885±0.622	0.335	
AST	Female	345.33±32.578	149.33± 25.58	0.491	110 - 220µL
(µL)	Male	355.66±28.98	196.14±56.85	0.279	
Renal function test (RFT)					
Creatinine	Female	1.22±0.46	0.26 ±0.181	0.139	0.10 and 0.40mg/dL
	Male	1.48± 0.19	0.925±0.419	0.287	
BUN	Female	2.1±1.68	6±0.89	0.053	4.40 and 6.38 mg/dL
	Male	1.5± 0.707	5.571±1.397	0.174	

Normal values quoted in/taken from Fitri *et al.*, 2021, Reference values may vary in poultry at other areas, BUN=blood urea nitrogen

Table 5: Effect of *Salmonella enterica* on complete blood count (CBC) of Houbara bustard

Parameters	Gender	*Salmonella positive* (Mean±SD)	*Salmonella Negative* (Mean±SD)	P-*value*	Normal Value
Hb	Female	3.666±1.51	9.271±3.91	0.263	7-13g/dl
	Male	3.7±0.98	10.675±2.449	0.079	
WBC	Female	3.7±2.65	14±2.16	0.591	12-30 x 10^3µL
	Male	3.38±1.77	16.43±5.69	0.012	
RBC (g/dL)	Female	9.7±0.644	2.425±1.090	0.280	2.5-3.5 x106µL
	Male	9.528±1.453	2.3±0.98	0.220	
HCT	Female	40.94±8.880	24.2±4.086	0.129	22% and 35%
	Male	42.028±9.078	24±1	0.222	
MCV	Female	108.25±19.259	45.516±11.212	0.206	1.6–89.1fL
	Male	103.33±11.930	46.728±6.370	0.128	
Platlets	Female	761±106.587	554.428±196.533	0.350	150,000
	Male	677.666±95.510	394±113.76	0.945	
MCH	Female	15.06±0.403	26 ±2.160	0.078	27.2 and 28.9
	Male	15.012±0.622	30±2.828	<0.001	
MCHC	Female	17.866±3.557	33.57±8.544	0.564	26–36.2%
	Male	32.24±10.556	28.14±0.95	<0.001	
Monocytes	Male	0.775±0.403	3.33±0.516	0.248	2-8%
	Female	5.133±4.628	5 ±0.816	<0.001	
Neutrophils	Male	20±8.406	38.33±8.334	0.949	20-50%
	Female	34.333±13.67	26.75±0.95	0.022	

Normal values taken/quoted in/from Odunitan *et al.*, 2018 and Horhoruw & Kewilaa, 2024, Reference values may vary in poultry at other areas

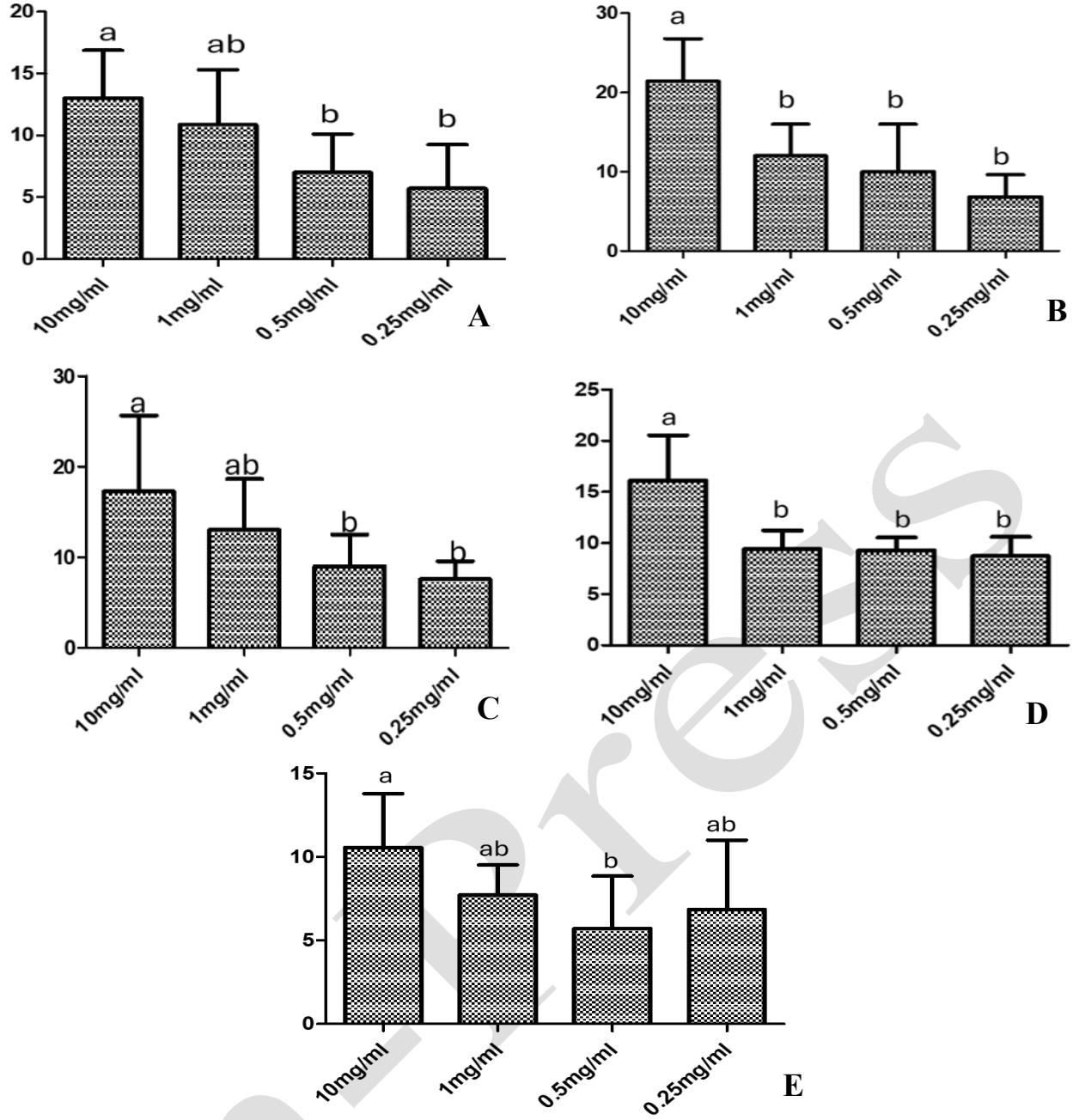

Fig. 5: Comparison of zone of inhibitions (mm) among different concentrations (10, 1, 0.5, and 0.25mg/mL) of nanocomposite coupled antibiotics (A, C, D, E) upon their application on *Salmonella enterica*. Different alphabets on graph indicate significant difference among concentrations. A=WNC {tungsten oxide W, nanocomposite NC; (WO_3/g-C_3N_4)} alone; B=WNC {tungsten oxide W, nanocomposite NC; (WO_3/g-C_3N_4)} coupled ciprofloxacin; C=WNC {tungsten oxide W, nanocomposite NC; (WO_3/g-C_3N_4)} coupled ampicillin; D=WNC {tungsten oxide W, nanocomposite NC; (WO_3/g-C_3N_4)} coupled penicillin; E=WNC {tungsten oxide W, nanocomposite NC; (WO_3/g-C_3N_4)} coupled oxytetracycline.

DISCUSSION

Prevalence and risk determinants: The prevalence of *Salmonella* in the samples studied during the current research was 37%, which contradicts with the findings of Wei *et al.* (2015), who reported a prevalence of 0.93% in migratory birds while consistent with that of Al Baqir *et al.* (2019) who reported 32.6% of *Salmonella* in chickens. Similarly, Maqbool *et al.* (2024) reported 40.95% of *Salmonella* in cloacal samples collected from Houbara bustard. A lower frequency of *Salmonella* isolation was recorded by Leinyuy *et al.* (2022), who reported a prevalence of 18.78% in healthy broilers. Likewise, Callaway *et al.* (2014) reported a lower prevalence of 14.9% from samples collected from migratory birds, including brown-headed cowbirds (*Molothrus ater*), common grackles (*Quiscalus quiscula*), and cattle egrets (*Bubulcus ibis*). However, Begum *et al.* (2024) reported a *Salmonella* prevalence of 30.92% in migratory and captive wild birds, which is consistent with the findings of the present study. Konak and Avdatek (2025) study was inconsistent with current study, as they found 1.3% prevalence of *Salmonella*.

Furthermore, it is important to highlight that migratory birds may disseminate other microbial infections. For instance, the spread of *Campylobacter* by wild birds has been reported in Korea (Wei *et al.*, 2015). The association of assumed risk factors with the prevalence of *Salmonella*

showed variable results when compared with previous studies; some factors aligned with earlier findings, while others were contradictory. These discrepancies may be attributed to differences in bird species, isolation sites, geographical regions, antimicrobial exposure, and other contextual factors. Djeffal *et al.*, (2018) identified age, water source, and housing type as risk factors significantly associated with *Salmonella* prevalence. Similarly, Sharma *et al.*, (2021) found a notable association between feed and *Salmonella*, while Abayneh *et al.*, (2023) demonstrated a statistically significant link between the source of chick feed and *Salmonella* prevalence in breeding farms. These findings highlight multiple potential risk factors contributing to the unchecked spread of pathogenic *Salmonella* in Houbara bustard populations and underscore the need for improved biosecurity and management practices.

Molecular Dynamics: This study focused on the *invA* gene for *Salmonella*, which was detected by using PCR, which is a quick and accurate way to identify pathogens. However, it is important to carefully select primers for *invA* gene because there is a risk of non-specific amplification, particularly with fecal and gut-associated bacteria (Pal *et al.*, 2017). The *invA* virulence gene is widely reported to be highly prevalent among *Salmonella* serovars, reflecting its conserved nature as a key pathogenic factor. In current study, *invA* gene showed highest similarity across the analyzed *Salmonella* serovars comparing to any other related study, hence align with the results of Samanta *et al.* (2014). This high degree of conservation underscores its utility not only for diagnostics but also for tracking evolutionary relationships among serovars. In line with our study, Mwambene *et al.*, (2025) found that single-primer sequencing allowed for the maximum hierarchy of all strains. In another study (Adhikari *et al.*, 2025), a strong genetic relationship was found among bacterial strains isolated at various stages of sampling from broiler pullets.

Impact on blood profiles: The current study revealed distinct variations in LFT, RFT, and CBC parameters between *S. enterica*-positive and -negative Houbara bustards. Consistent with our findings, Soria *et al.* (2015) reported bloodborne *Salmonella* infections to be associated with decreased monocyte counts and total protein levels. The non-significant differences of mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in females was in line with findings of Emennaa *et al* (2019) who found non-statistical difference (p>0.05) in MCH and MCHC between infected and control groups at various time points post-infection. In contrast, males in the current study showed significant difference of MCH and MCHC which was however in contradiction to Emennaa *et al.* (2019). Elevations in ALT have been documented in other studies involving infected chicks (Laptev *et al.*, 2021). Polansky *et al.* (2018) also reported alterations in protein quantity in the liver and serum of chickens infected with *S. enteritidis*, further supporting the impact of infection on hepatic function and systemic physiology. Non-significant difference of AST levels between groups in current study is in contrast to the findings of Emennaa *et al.* (2019), who concluded significant elevations in both AST and ALT (p≤0.05) in infected groups between 7- and 10-days post-infection. This suggests that a substantial physiological impact of *Salmonella* infection may vary depending on the stage or severity of infection. Nevertheless, in alignment with previous findings, our study did observe a significant increase in ALT levels in *Salmonella*-infected birds.

Drug resistance and its modulation: The drug resistance profile of *Salmonella* in current study showed similarity at various points with the findings of Maqbool *et al.,* (2024). In their study, 30% of *Salmonella* isolated from Houbara bustard were resistant which is 10 points higher compared with findings of current study. Moreover, the percentage of amikacin sensitive isolates in current study was twice the percentage reported by Maqbool *et al.* (2024) i.e., 80% versus 40%. *Salmonella* isolated from sources other than Houbara reported different antibiotic susceptibility patterns against antibiotics. For instance, Thung *et al.* (2018) reported 23% of *Salmonella*, isolated from beef source, resistant to penicillin which is contrary to the findings of current study. Such variations in antimicrobial susceptibility may be influenced by differences in sampling regions, animal species, antimicrobial usage practices, and the genetic background of the isolates. The current study found tungsten nanocomposites coupled ciprofloxacin showing the highest ZOI against *Salmonella* which is in line to Habtemariam & Alemu, (2021). They found a strong antibacterial activity of the synthesized WO_3 nanoparticles both at 250 and 125 mg/mL concentrations against *Salmonella.* In agreement to our study, strong antibacterial activity for Gram negative bacteria has been reported in previous studies (Duan *et al.*, 2019; Baig *et al.*, 2020). Bashir *et al.*, (2024) reported substantial antibacterial efficacy of WO_3-coated antibiotics against *S. aureus* and *E. coli.* Similarly, the findings of Javed *et al.* (2025) align with our results, showing enhanced antibacterial effects. Specifically, when the CeO2/WO3 nanocomposites were used at the same 0.2 mg/L concentration as pure NPs, they were predicted to exhibit excellent antibacterial activity versus the pure NPs against *E. coli*. Zia *et al.*, (2023) found significant antibacterial efficacy of WO_3 coupled antibiotics against single and mixed culture of *E. coli* and *S. aureus,* as evidenced by prominent zones of inhibition.

Conclusions: The current study revealed higher prevalence of *S. enterica* along with notable resistance against commonly used antibiotics. Most of the assumed risk factors were found to be significantly associated with the prevalence of *S. enterica.* Molecular characterization and genome analysis revealed similarity with isolates from other sources indicating infection dynamics involving multiple transmission sources. Molecular characterization and protein analysis showed divergence in makeup, an indication of evolution. LFT, KFT, and CBC revealed that pathogen holds significant impact on blood profile. Despite being multidrug-resistant, the pathogens were still able to respond to tungsten oxide-carbon nanocomposites coupled antibiotics reflecting its potential to accommodate alternative antimicrobial as effective therapeutics. This study thus emphasizes the need for *in-vivo* efficacy and safety trials of these nanocomposites along-with exploration of underlying mechanisms of action. These efforts will support the development of effective control

strategies against multidrug-resistant *Salmonella* infections.

Author's contribution: A.M. conducted the research, collected the data, conducted the data analysis, and prepared the initial draft; S.Q.A.S. conceived idea, did data analysis, revised final draft; A.I.A. conceived the idea, arranged the resources, conducted the research work, collected the data, prepared the final version, and revised the manuscript; M.M.A conducted the research work, analyzed the data, and prepared the final draft; A.G conceived idea, arranged resources, revised final draft. All authors have read and agreed to the published version of the manuscript.

Acknowledgements: Authors are thankful to Houbara Foundation International Pakistan, Lal Sohanra, Bahawalpur while special thanks go to Brig.(R) Mukhtar Ahmad, Major Rahat Afzal and Dr Talha Riaz for supporting this study.

REFERENCES

Abayneh E, Goba H and Shurbe M, 2023. Salmonellosis prevalence and risk factors in chicken breeding farms in and around Arba Minch town, Gamo Zone, Ethiopia. J Infect Dev Ctries 17:226-35.

Adhikari Y, Bailey MA, Kitchens S, *et al.*, 2025. Whole-genome sequencing and phylogenetic analysis of *Salmonella* isolated from pullets through final raw product in the processing plant of a conventional broiler complex: a longitudinal study. Microbiol Spectr 13:e02090-24.

Al-baqir A, Hussein A, Ghanem I, *et al.*, 2019. Characterization of paratyphoid Salmonellae isolated from broiler chickens at Sharkia governorate, Egypt. Zagazig Vet J 47:183-92.

Alzwghaibi AB, Yahyaraeyat R, Fasaei BN *et al.*, 2018. Rapid molecular identification and differentiation of common *Salmonella* serovars isolated from poultry, domestic animals and foodstuff using multiplex PCR assay. Arch Microbiol *200*(7), 1009-1016.

Angelo KM, Reynolds J, Karp BE, *et al.*,2016. Antimicrobial resistance among nontyphoidal *Salmonella* isolated from blood in the United States, 2003–2013. J Infect Dis 214:1565-70.

Anwar MA, Aqib AI, Ashfaq K, *et al.*, 2020. Antimicrobial resistance modulation of MDR *E. coli* by antibiotic coated ZnO nanoparticles. Microb Pathog 148:104450.

Baig U, Gondal MA, Rehman S, *et al.*, 2020. Facile synthesis, characterization of nano-tungsten trioxide decorated with silver nanoparticles and their antibacterial activity against water-borne gram-negative pathogens. Appl Nanosci 10:851-60.

Bailey T, Silvanose CD, Naldo J, *et al.*, 2000. Health considerations of the rehabilitation of illegally traded houbara bustards *Chlamydotis undulata macqueenii* in the Middle East. Oryx 34:325-34.

Bashir A, Khan SR, Aqib AI *et al.*, 2024. Multifunctional integration of tungsten oxide (WO_3) coating: A versatile approach for enhanced performance of antibiotics against single mixed bacterial infections. Microb Pathog 189:106571.

Begum R, Asha NA, Dipu DCC, *et al.*, 2024. Virulence and antimicrobial resistance patterns of *Salmonella* spp. recovered from migratory and captive wild birds. Vet Med Sci 10:e70102.

Callaway TR, Edrington TS and Nisbet DJ, 2014. Isolation of *Escherichia coli* O157:H7 and *Salmonella* from migratory brown-headed cowbirds (*Molothrus ater*), common grackles (*Quiscalus quiscula*), and cattle egrets (*Bubulcus ibis*). Foodborne Pathog Dis 11:791-94.

CLSI, 2018. Methods for antimicrobial susceptibility testing of anaerobic bacteria. Approved standard, 9th Ed. Clin Lab Stand Inst, USA.

Djeffal S, Mamache B, Elgroud R, *et al.*, 2018. Prevalence and risk factors for *Salmonella* spp. contamination in broiler chicken farms and slaughterhouses in the northeast of Algeria. Vet World 11:1102.

Dobbin G, Hariharan H, Daoust PY, *et al.*, 2005. Bacterial flora of free-living double-crested cormorant (P*halacrocorax auritus*) chicks on Prince Edward Island, Canada, with reference to enteric bacteria and antibiotic resistance. Comp Immunol Microbiol Infect Dis 28:71-82.

Duan G, Chen L, Jing Z, *et al.*, 2019. Robust antibacterial activity of tungsten oxide (WO_{3-x}) nanodots. Chem Res Toxicol 32:1357-66.

Durrani RH, Sheikh AA, Humza, *et al.*, 2024. Evaluation of antibiotic resistance profile and multiple antibiotic resistance index in avian adapted *Salmonella enterica* serovar Gallinarum isolates. Pak Vet J, 44(4): 1322 1326. http://dx.doi.org/10.29261/pakvetj/2024.253

Emennaa PE, Eze DC, Chah FK, *et al.*, 2019. Haematological and serum biochemistry profile of cockerels experimentally infected with *Salmonella enterica* serovar Zega. Open J Vet Med 9:171-84.

Feng Z, Hag ME, Wang N, Qin T, Chen S and Peng D, 2023. Negative regulation of RpoS-mediated STM1703 in biofilm formation of *Salmonella* pullorum. Pak Vet J, 43(1): 25-32. http://dx.doi.org/10.29261/pakvetj/2022.086

Fitri A N, Fitriana I, Rosetyadewi A W *et al.*, 2021. The effect of colistin administration as medicated feed on alanine aminotransferase and creatinine level in broiler infected with *Escherichia coli*. In BIO Web of Conferences (Vol. 33, p. 03002). EDP Sciences.

Habtemariam AB and Alemu Y, 2021. Synthesis of WO_3 nanoparticles using *Rhamnus prinoides* leaf extract and evaluation of its antibacterial activities. Biointerface Res Appl Chem 12:529-36.

Hamid IS, Mahendra I, Kurniawan A, *et al.*, 2025. Recent updates on encapsulated probiotics in poultry: a review. Pol J Vet Sci *28*(2), 345-353.

Hetta HF, Ramadan YN, Al-Harbi AI, *et al.*, 2023. Nanotechnology as a promising approach to combat multidrug resistant bacteria: a comprehensive review and future perspectives. Biomedicines 11:413.

Horhoruw W and Kewilaa, A I. 2024. Hematological Profile in Free-Range Chickens at The Age Of 4, 8, 12 Weeks. Adva Soc Human Res, 2(5), 731-736.

Ibrahim D, Awad A, and Younis G. 2023. Prevalence and characterization of quinolone-resistant *Escherichia coli* isolated from retail raw beef and poultry meat in Egypt. J Adv Vet Anim Res., 10(3), 490.

Islam MA, Bose P, Rahman M Z, *et al.*, 2024. A review of antimicrobial usage practice in livestock and poultry production and its consequences on human and animal health. J Adv Vet Anim Res., 11(3), 675.

Javed K, Kousar S, Begum B, *et al.*, 2025. Green synthesis strategy of CeO_2 nanowires, WO_3 nanoparticles and CeO_2@WO_3 nanocomposites by using *Azadirachta indica* leaf extract: its surface defects, Ce^{3+}/W^{5+} ratio for their enhanced antibacterial activity. J Sol-Gel Sci Technol 113:496-517.

Joshua BI, Luka HS, Elisha IL, *et al.*, 2022. Haematological and serum biochemical parameters of broilers slaughtered at Bukuru live bird market of Jos South Local Government Plateau State Nigeria. Acta Sci Vet Sci 4(6); 05-11.

Konak S, and Avdatek F. 2025. Bacterial flora and antibiogram sensitivity in the preputium samples of healthy rams. Pol J Vet Sci 243-250.

Krumova-Valcheva G, Mateva G, Milanov M, *et al.*, 2024. Occurrence and antimicrobial resistance of *Salmonella* isolates in farrow-to-finish pig farms in Bulgaria. Pol J Vet Sci 397-405.

Laptev GY, Yildirim EA, Ilina LA, *et al.*, 2021. Effects of essential oils-based supplement and *Salmonella* infection on gene expression, blood parameters, cecal microbiome, and egg production in laying hens. Animals 11:360.

Leinyuy JF, Ali IM, Karimo O and Tume CB, 2022. Patterns of antibiotic resistance in *Enterobacteriaceae* isolates from broiler chicken in the West Region of Cameroon: a cross-sectional study. Can J Infect Dis Med Microbiol 2022:4180336.

Maqbool S, Murtaz M, Kumar S, *et al.*, 2024. Prevalence, Potential Risks, and Drug Resistance in *Staphylococcus aureus* and *Salmonella* sp. Infesting Captive Houbara Bustard. Pak J Zool, *57*(3); 1-9.

Muneer A, Kumar S, Aqib AI, *et al.*, 2022. Evaluation of sodium alginate stabilized nanoparticles and antibiotics against drug resistant *Escherichia coli* isolated from gut of houbara bustard bird. Oxid Med Cell Longev 2022:7627759.

Murtaza M, Aqib AI, Khan SR, *et al.*, 2023. Sodium alginate-based MgO nanoparticles coupled antibiotics as safe and effective antimicrobial candidates against *Staphylococcus aureus* of houbara bustard birds. Biomedicines 11:1959.

Mwambene SJ, Mtenga AB, Chengula AA, *et al.*, 2025. Detection and antibiotic resistance of *Salmonella* isolates from selected poultry farms in Dar es Salaam, Tanzania. Access Microbiol 7:000879-v5.

Niki M, Shakeel A, Zahid K, *et al.*, 2017. Prevalence, risks and antibiotic resistance of *Salmonella* in poultry production chain. Curr Top *Salmonella* Salmonellosis, InTechOpen, London, UK, pp:216-34.

Odunitan-Wayas F, Kolanisi U, and Chimonyo M. 2018. Haematological and serum biochemical responses of Ovambo chickens fed Provitamin A Biofortified maize. Braz J Poult Sci, 20, 425-434.

Pal S, Dey S, Batabyal K, *et al*., 2017. Characterization of *Salmonella Gallinarum* isolates from backyard poultry by polymerase chain reaction detection of invasion (*invA*) and *Salmonella* plasmid virulence (*sPvC*) genes. Vet World 10:814.

Polansky O, Seidlerova Z, Faldynova M, *et al*., 2018. Protein expression in the liver and blood serum in chickens in response to *Salmonella Enteritidis* infection. Vet Immunopathol 205:10-16.

Rashid S, Tahir S, Akhtar T, *et al*., 2023. *Bacillus*-based probiotics: an antibiotic alternative for the treatment of salmonellosis in poultry. Pak Vet J., 43(1): 167-173. http://dx.doi.org/10.29261/pakvetj/2023.017

Sabbagh SC, Forest CG, Lepage C, *et al*., 2010. So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. FEMS Microbiol Lett 305:1-13.

Samanta I, Joardar SN, Das PK, *et al*., 2014. Prevalence and antibiotic resistance profiles of *Salmonella* serotypes isolated from backyard poultry flocks in West Bengal, India. J Appl Poult Res 23:536-45.

Sharma S, Fowler PD, Pant D, *et al*., 2021. Prevalence of non-typhoidal *Salmonella* and risk factors on poultry farms in Chitwan, Nepal. Vet World 14:426.

Silvanose CD, Bailey TA, Naldo JL, *et al*., 2001. Bacterial flora of the conjunctiva and nasal cavity in normal and diseased captive bustards. Avian Dis 45:447-51.

Soria MA, Bonnet MA and Bueno DJ, 2015. Relationship of *Salmonella* infection and inflammatory intestinal response with hematological and serum biochemical values in laying hens. Vet Immunol Immunopathol 165:145-53.

Thung TY, Radu S, Mahyudin NAA, *et al*., 2018. Prevalence, virulence genes and antimicrobial resistance profiles of *Salmonella* serovars from retail beef in Selangor, Malaysia. Front Microbiol 8:2697.

Wei B, Cha SY, Kang M, *et al*., 2015. Dissemination of multidrug-resistant Campylobacter in wild birds from South Korea. Int J Antimicrob Agents 45:197-198.

Zia S, Peng S, Bashir A, *et al*., 2023. Resistance modulation of individual and polymicrobial culture of *S. aureus* and *E. coli* through nanoparticle-coupled antibiotics. Biomedicines 11:2988.