STUDY OF DIAGNOSIS, ASSOCIATED RISK FACTORS AND IN-VITRO ANTIBIOTIC SUSCEPTIBILITY OF*SALMONELLA* ISOLATED FROM OSTRICH (*STRUTHIO CAMELUS*)

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ABSTRACT

The ostrich meat is considered healthy as it contains low cholesterol and fat levels. Foodborne diseases caused by the bacterium *Salmonella*are a significant public health concern. The study was conducted to determine the prevalence, the risk factors involved, and antimicrobial susceptibility patterns of *Salmonella* isolates recovered from captive ostrich feces in the district of Lahore, Pakistan. There is not a single research on this issue in Pakistan up to date in ostrich. A total of 100 ostrich fecal samples were collected- and specifically cultured on Salmonella Shigella agar. The antimicrobial susceptibility was checked for different antibiotics throughthe Kirby-Bauer disc diffusion method. The 21% fecal samples were found positive for *Salmonella*. The disease was found to besignificantly($p<0.05$) associated with risk factors; water type($p=0.027$), bird's capacity($p=0.042$), cage hygiene($p=0.024$), age of bird($p=0.041$) andother bird species around ($p=0.031$) while enclosure size and diet were found non-significant. The bacterium was found highly susceptible to Amoxicillin followed by Chloramphenicol, Gentamicin, Cefotaxime, andEnrofloxacin respectively.Further *Salmonella* species-level diagnostic studies along with susceptibility checks of more therapeutic agents are suggested to future researchers.

Keywords:*Salmonella***,** Susceptibility, In-vitro, Ostrich, Chloramphenicol.

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INTRODUCTION

The ostrich plays a significant role in livestock businesses in underdeveloped countries, raising ostriches is a profitable way to earn foreign currency by selling meat and skins (Abbas et al., 2018; Abbas et al., 2018b; Fujihara*et al.,* 2004). Ostriches have been identified as a potential bacterial reservoir. Salmonellosis is an infectious zoonotic disease caused by *Salmonella spp.* and is one of the most serious problems for humans, animals, and birds (Abbas et al., 2018c; Oludairo *etal.,* 2013). It can be transferred to humans by the consumption of contaminated food products obtained from food-producing animals. The declining productivity, higher mortality, and the associated risk of chicken products for the safety of human food, enteric illnesses are major concerns of salmonellosis in the poultry industry (Rabsch *et al.,* 2001; Collard *et al.,* 2008; Ramya *et al.,* 2012).A significant public health concern is caused by the approximately 2600 serovars of *Salmonella* (Namata *et al.,* 2009).

In ostrich chicks,*Salmonella*is the major cause of mortality.Commercial ostrich farming began in Pakistan in 2012 as an alternative to livestock and grain production. Ostrich leather, meat, eggs, feathers, and other by-products are in high demand all over the world(Ambreen *et al.,* 2021).To develop ways to lower the risk of human infection, it is essential to identify and characterize*Salmonella* in ostriches and their surroundings. The risk factors linked to ostriches contracting salmonella infection have been the subject of several investigations. For instance, South African research on ostrich farms discovered that the incidence of *Salmonella* was greater. This finding suggests that overcrowding may be a risk factor for *Salmonella* infection.The use of antibiotics in ostrich feed was cited in another Iranian study as a risk factor for the emergence of *Salmonella* strains that are resistant to antibiotics. Poor hygiene and sanitation procedures, insufficient

ventilation, and the presence of infected wildlife or other animals close to the farm are some other possible risk factors for *Salmonella* infection in ostriches. The risk of *Salmonella* transmission can also rise when birds are moved between farms, especially if they are not thoroughly checked for infection before being introduced to a new flock. The diseases caused by *Salmonella* require antibiotic therapy. *Salmonella* and other foodborne diseases are of extreme concernfor the rise of antimicrobial resistance in animals, birds, and humans. The World Health Organization has highlighted the rise of antibiotic-resistant non-typhoid *Salmonella* strains(Cha *et al.*, 2013).

According to our knowledge, there is not a single research study on this issue in Pakistan yet. The objective of the current study was to determine the prevalence of *Salmonella* in ostriches being kept for either commercial or domestic or display purposes in district Lahore along with the risk factors associated with the occurrence of disease and to check the susceptibility of the selected drugs against *Salmonella* in ostriches.

MATERIALS AND METHODS

Sampling Strategy: A total of 100 fecal samples from ostrich having diarrhea, weight loss, and anorexia were collected from public and private ostrich rearing sites in district Lahore by usinga convenient sampling method.The fecal samples were collected either directly from the rectum of ostriches or freshly passed feces. The fecal samples were placed in a sterile container. The samples were transported in a cooler/box containing ice packs immediately to the Postgraduate Laboratory, Department of Veterinary Medicine, UVAS Lahore forfurther processing.

Bacteriological examination: Pre-enrichment of the fecal sample was done using buffer peptone water (1g in 250 ml buffer peptone water) then incubated overnight at 37^{o}C. After incubation 0.1ml of buffer peptone water was inoculated on 9ml tetrathionatebroth(selective enrichment broth) and then incubated at 42^{o}C for 24 hours. A loopful from each selective enrichment broth (after incubation) was streaked onto the following selective agar media Salmonella shigella agar by streak plate method to observe the colony morphology of *Salmonella*. *Salmonella* isolates were further confirmed by Gram staining and biochemical Tests (Indole Red Test, Methyl Red Test, VogesProskauer, and Citrate Utilization Test (IMVIC).

Risk factors analysis: Data regarding risk factors; animal age (adult, sub-adults, chicks), diet (balanced, not balanced), water for drinking (filtered, tab, pond), enclosure type (small, medium, large), Bird capacity (not crowded, over-crowded), Cage hygiene (good, poor) and Bird species around (yes, no) was collected through questionnaire filled on spot while sample collection from different farms.

Assessment of antimicrobial susceptibility: Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion technique using Mueller-Hinton agar according tothe Clinical Laboratory Standards guidelines (CLSI) manual (Rincón-Gamboa *et al.*, 2021).The antimicrobial agents tested and their corresponding concentrations were as follows: Amoxicillin (AMX: 30 mu/g), Enrofloxacin (ENF: 5 μ/g), Gentamycin (GM: 10 μ/g), Cefotaxime (CTX: 30 μ/g) and Chloramphenicol (CH: 30 μ/g). After incubating the inoculated plate overnight at 37^{o}C the susceptibility of the *Salmonella* to each antimicrobial agent wasmeasured and the results were interpreted followingthe CLSI manual.When the minimuminhibitory concentration (MIC) of the *Salmonella* isolates for a given antimicrobial comes in the intermediate to sensitivity range, *Salmonella*was considered to be not resistant against that antimicrobial agent.

Statistical Analysis: The risk factors were analyzed by using Chi-square ($\chi 2$) and thedata on antibiotic susceptibility was analyzedthrough descriptive statistics. The whole data analysis was carried out through SPSS version 21.0.

RESULTS

Diagnosis of Salmonella: On Salmonella Shigella agar, opaque, translucent colorless, smooth round colonies with a black center appeared as shown in Figure 1. While examination of the stained microscopic slide revealed rod-shaped gram-negative bacteria as shown inFigure 2.

Prevalence ofSalmonella: Out of 100 samples,21%were found confirmed positivefor *Salmonella* on selective media.Among the total positive, following clinical signs with percentages of ostrich were observed;anorexic (57.14%), suffering fromdiarrhea (80.95%),weight loss (38.09%),depressed(85.71%)and history of retarded growth(28.57%).

Risk factors associated with the occurrence of Salmonella in ostrich : Theanalysis of risk factors revealed that out of 7 variables, 5 were identified as potential risk factors ($p<0.05$) for *Salmonella* in ostriches (Table 1). These variables werewater source, bird's capacity, cage hygiene condition, access of other species to farm, and age.While enclosure type and diet type were non-significantly associated risk factors.

Antimicrobial susceptibility: The susceptibility of all antibiotics for isolated salmonella was determined as shown in Figure 3. After biochemical confirmation, 21 isolates of *Salmonella* were used for antibiotic sensitivity pattern and the results are shown in Table 2 and Figure 4.

The study showed that *Salmonella* has the highest susceptibility toward Chloramphenicol. *Salmonella* showed the highest resistance towards Amoxicillin and Enrofloxacin.

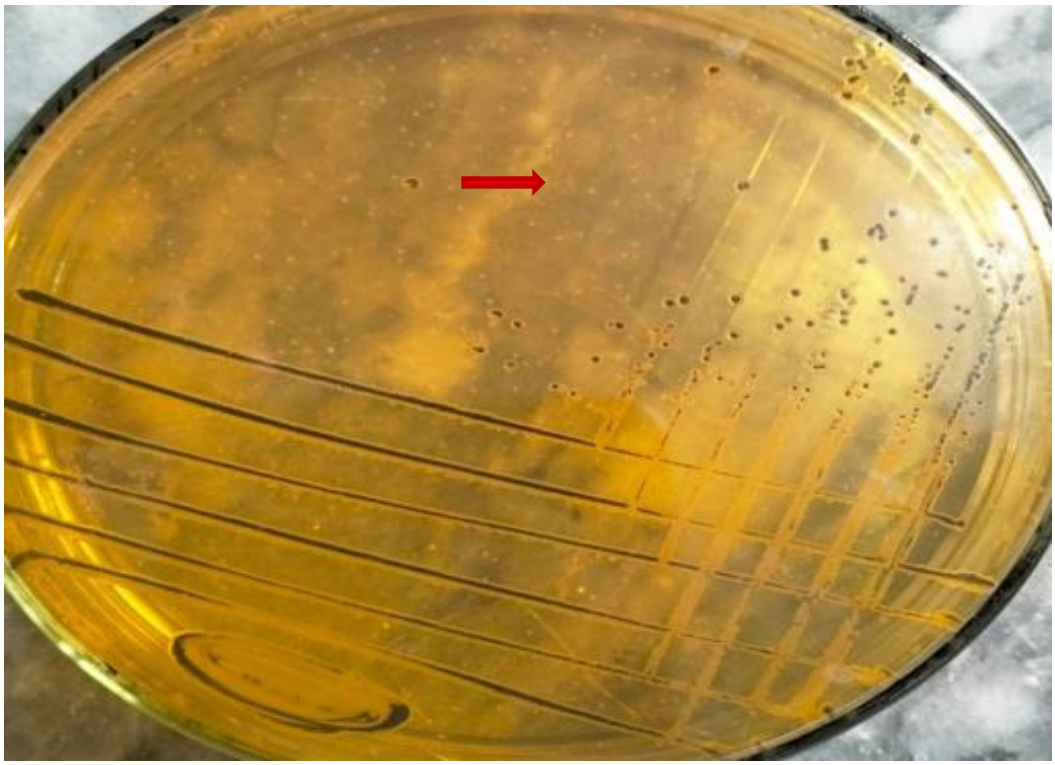

Figure 1:Appearance of *Salmonella* as small, round, translucent, and colorless colonies with a black center on Salmonella Shigella agar.

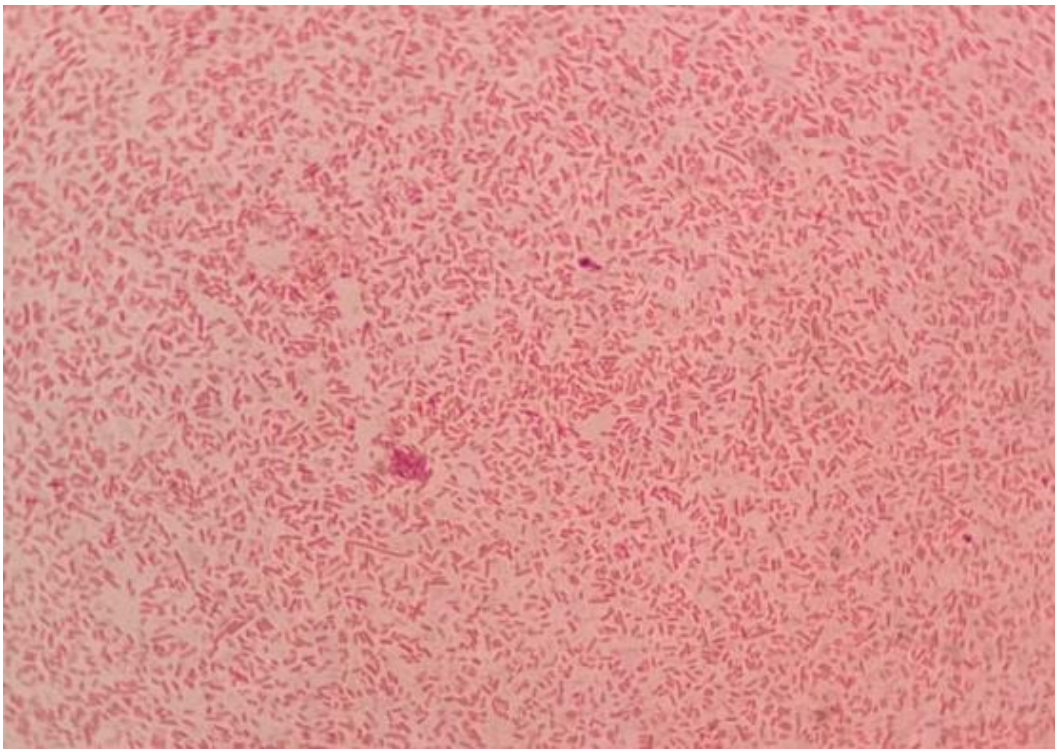

Figure 2:*Salmonella* bacteria are rod-shaped (bacilli) and appear as Gram-negative cells under Gram's staining

Table 1: Risk factor analysis associated with *salmonella*infection in ostrich in Lahore

variables	Total Responses (n=100)	Percentage Positivity (%)	p-value
Water			
Filtered	22	6%	0.0271
Tab	68	10%	
Pond	10	5%	
Enclosure			
Small	48	13%	0.070*
Medium	22	6%	
Large	30	2%	
Birds capacity			
Not crowded	27	2%	0.042*
Overcrowded	73	19%	
Cage hygiene			
Good	55	7%	0.024*
Poor	45	14%	
Bird species around			
Yes	37	12%	0.031*
No	63	9%	
Diet			
Balanced	41	6%	0.192
Not balanced	59	15%	
Age			
Adult	41	6%	0.041
Sub-adult	30	11%	
Chicks	29	4%	

*= p<0.05 shows a significant association.

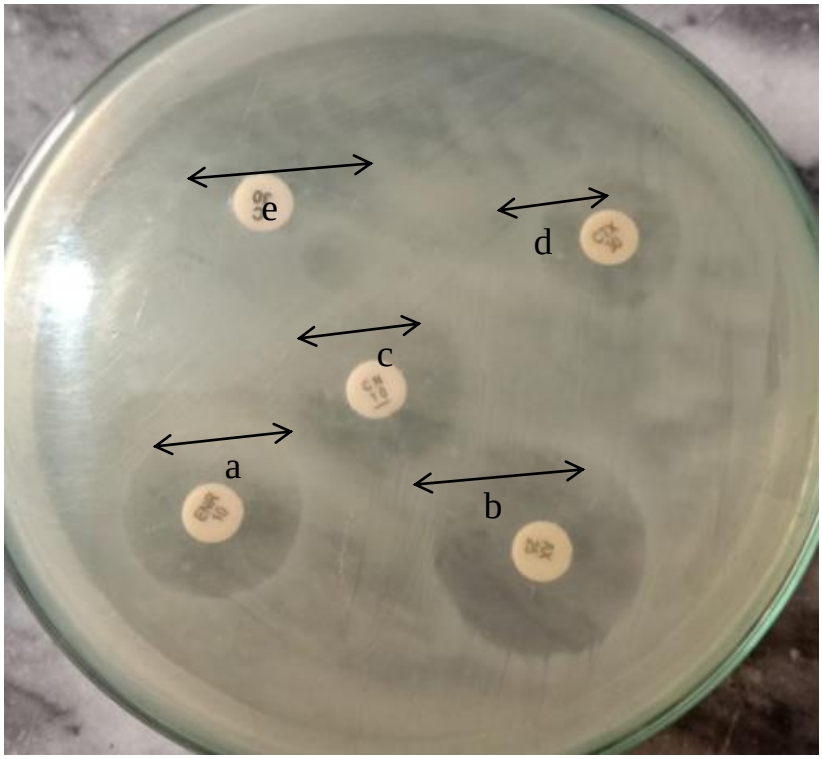

Figure 3: Mueller Hilton agar, with antibiotic disc embedded for *Salmonella* Isolates, showing zone of inhibitions, 'a' of Enrofloxacin, 'b' of Amoxicillin, 'c' of Gentamycin, 'd' of Cefotaxime, and 'e' of Chloramphenicol.

Table 3:*In-vitro* drug susceptibility of antibiotics against *Salmonella* isolates of ostrich.

Antibiotic discs	Disc Concentration	Isolates of Salmonella n=21		
		Sensitive	Intermediate	Resistance
Amoxicillin	25ug	11(52.38%)	4(19.04%)	5(23.80%)
Enrofloxacin	10ug	12 (57%)	6 (28.5%)	3 (14.2%)
Gentamicin	10ug	15 (71.4%)	4 (19.04%)	2(9.5%)
Cefotaxime	30ug	17 (80.95%)	2 (9.5%)	2 (9.5%)
Chloramphenicol	30ug	19 (90.4%)	2 (9.5%)	0

DISCUSSION

Salmonellosis is a food-borne and zoonotic disease infecting a wide range of vertebrate hosts. When humans come into close contact with the bacterium from wild animals or ingest meat products, they might become infected with *Salmonella*. Because there is a lack of knowledge about prevalent *Salmonella* serotypes, monitoring of farms is essential to controlling and preventing the spread of *Salmonella* to people, particularly in the case of the ostrich business (Oludairo *et al.,* 2013). In this study 21% percentage positivity of *Salmonella*was observed.This finding was almost in agreement with (Hameed *et al.*,2019) who isolated *Salmonella* from ostriches and recorded a prevalence of 28.57%. Despite disagreeing with (Metawea, 2013) who discovered *Salmonella* in an ostrich farm with an incidence of 8.6%. According to MA *et al.,* 2016 *Salmonella* were present in 20.8% and 24.4% of all samples collected from chick yards and adult yards, respectively. Oliveira *et al.,* 2006 discovered that none of the 80 samples of ostrich droppings from different age groups collected in the southeast Brazilian region had *Salmonella*. In the Ismailia, it was discovered that Salmonella was present in faces, cloacal swabs, and internal organs of ostrich flocks older than three months, but not in flocks younger than two months (Effata *et al.,* 2003). In the intestines and caeca of ostriches; more *Salmonella spp*. were identified with overall percentage positivity 18.5% (46/248) (Vanhooser *et al.,* 1995). According to research by Gaedirelwe and Sebunya, 2008 in Botswana;*Salmonella* was isolated from 51.6% (16/31) of the ostriches. The difference in *Salmonella spp.*prevalence may be due to variation in housing type, hygienic condition, and species susceptibility variations. Variation in the percentage positivity of ostrich for salmonellosis may be due to variations in climatic and rearing conditions of each geographical area.

In this investigation, we discovered 6 percent, 5 percent,and 10 percent percentage positivity of *Salmonella* in ostriches offered filtered, pond, and tab water respectively. Comparatively to the 36% observed in commercial poultry drinking water in Thailand, the isolation rate from drinking water was 21%(Sasipreeyajan *et al.,*1996). The reason is that *Salmonella* infections can be caused by contaminated drinking water, which has also been linked to the spread and persistence of the disease in poultry farms (Adzitey *et al.,* 2011). Nonetheless, our study's isolation rates were also somewhat higher than the reported 3.3% (poultry water) isolation rates from layer farms in Northern India (Singh *et al.,*2013). It was found that higher percentage of positivity (19%) in crowded flocksas compared to less crowded flocks (2%). The reason is that more number of bird per area lead to maintenance of infection as less hygienic conditions and more chances of transmission between birds (Khalilzadeh *et al.,* 2023).According to this study small cage size, less cage hygiene, more bird's species around, less balanced diet, and younger age were responsible for higher prevalence 13%, 14%, 12%, 15%, and 11% respectively. According to another study Ostriches that were clinically and subclinically infected with *Salmonella* were found to be in fence-to-fence contact with other animal species (Brassó *et al.,* 2023). Small cage size leads to overcrowding and causes stress to birds which is responsible for less immunity and higher prevalence in the case of small cage size. Poor hygienic condition and higher exposure to otherbird species leads to the sustainability of infection in the environment for a long duration and are responsible for widespread transmission to other nearby rearing facilities so responsible for high prevalence. At younger ages less immune development is the reason for higher prevalence in younger age.

In the farming of birds; rodents are a significant source of *Salmonella* infection(Carrique-Mas *et al.,* 2009).According to the research, the introduction of flocks infected with *Salmonella* may cause transmission in rodents, which becomes a source of the microbe for subsequent flocks and creates a challenging challenge(de Freitas Neto *et al.*,2009).The differences in frequency between farms may be explained by the sanitary practices used in each farm, the housing arrangement, the water source, the location of the sample, the season, the addition of antibiotics, and the health of the flock. The antibiotic susceptibility of the Salmonella strains was assessed in this investigation. All the isolates were sensitive to Amoxicillin,Chloramphenicol, and Gentamicin. Resistance to Cefotaxime and Enrofloxacin was present. According to Mohammadzadeh *et al.,* 2017 all the isolates were resistant to ampicillin and

tetracycline. According to research by Shetty *et al.,* (2012) and Murugkar *et al.,* (2005), ceftriaxone and ciprofloxacin were the most effective antibacterial medications. According to another research, it was identified that *Salmonella*is resistant to Tetracycline, Penicillin, Chloramphenicol, Erythromycin, Amoxicillin, Gentamicin, Ceftriaxone, Nalidixic Acid, Levofloxacin, and Vancomycin, and susceptible to Azithromycin (Jahan *et al.,* 2017).This variation can result from the varied antibacterial medications that were utilized in various areas.

Conclusion: This study concluded that the prevalence of *Salmonella* in ostriches in district Lahore is 21%. The water source, bird capacity in pens, pen hygiene, different bird species present around the farm, and age of the bird are the significantly associated risk factors for the occurrence of salmonellosis in ostriches. *Salmonella* is highly sensitive to chloramphenicol so we can use it to treat its infection in ostriches.

Recommendations: The current study furnished the baseline data but further *Salmonella* species-level diagnostic studies along with susceptibility checks of more therapeutic agents are suggested for future researchers. There is also a need to study this organism in ostriches on the molecular level (molecular characterization, study on immunogenic and pathogenic genes, and work on its vaccine development due to both veterinary and public health concerns.

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