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Prevalence, Potential Risks, and Drug Resistance in *Staphylococcus aureus* and *Salmonella* sp. Infesting Captive Houbara Bustard

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Key words
Houbara bustard, *Staphylococcus aureus, Salmonella*, Prevalence, Risk factors, Drug resistance

ABSTRACT

Defining the enteric flora of clinically healthy bacterial captive houbara bustards as well as their antibiotic resistance is a critical step to understand the epidemiology of bacterial diseases. An investigation was carried out to identify the intestinal *Staphylococcus aureus* and *Salmonella* sp. associated with houbara bustards in captivity and to determine if they are resistant to the currently available antimicrobials. A total of 105 bacterial isolates were recovered by culture-based methods, and bacterial identification revealed that 55.23% (58/105) of them were *S. aureus*. There were 40.95 % (43/105) samples that contained *Salmonella* sp. Trends in potential risk factor analysis found male birds (OR=3.18), open environment (OR=1.14), poultry feed (OR=3.12), and winter season (OR=4.23) to be potential risks for getting *Salmonella* infection. On the other hands, male birds (OR=1.26), natural environment (OR=1.98), poulty feed (OR=5.77), and winter season (OR=1.05) were potential risk factors associated with *S. aureus* infection. As a result of antimicrobial susceptibility tests, strains displayed multidrug resistance phenotypes against a variety of antimicrobials. The current study showed *Staph. aureus* to be highly sensitive to Septran (trimethoprim + sulphamethoxazole) (80%), enrofloxacin (70%), ciprofloxacin (70%), fusidic acid (60%), amikacin (60%), and cefoxitin (60%). Trends in relative percentage of resistant strains were higher in case of *Salmonella* while in case of *Staph. aureus*, intermediate and sensitive strains showed higher relative changes in percentages. The strength of variation on overall basis in terms of percentages was higher in *Salmonella* compared to that of *S. aureus*. The study thus concluded increasing prevalence, decreasing window of potential effects of antibiotics, and increasing potential risks which invites to keep stern preventive measures intact.

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INTRODUCTION

Houbara bustard (*Chlamydotis macqueenii*) are a prominent avian that are some of the oldest land birds. Several factors have contributed to the listing of the houbara bustard on the IUCN red list for threatened species. Several populations of this species exist in North Africa, the Middle East, and Western Asia (Combreau *et al*., 2011). Humans are in close contact with these animals in recent years due to efforts to conserve and rehabilitate them. The microbes transmit virulence and antimicrobial resistance (AMR) genes to migrating birds as well as transmitting emerging infectious diseases, including salmonellosis (Bailey *et al*., 2000a, b; Silvanose *et al*., 2001).

Most of the research conducted was intended to focus on ecological, biomedical, and captive breeding issues. In contrast, very little attention has been directed at their normal bacterial flora and analyses of antimicrobial resistance. Wild birds, especially houbara bustards (Stiévenart and Mohammed, 2004), have been isolated with bacterial strains that are potentially pathogenic and zoonotic (Dobbin *et al*., 2005). Globally, *Salmonella* are among the most important pathogens causing gastrointestinal infections and septicemia in humans and animals. There is a general variation in *Salmonella* prevalence among animal species (Gopee *et al*., 2000). A high prevalence of *Salmonella* has been reported in wild and captive reptiles both healthy and diseased (Hidalgo *et al*., 2007). Similarly, *Staph. aureus* has emerged as ubiquitous pathogen of animals (Liu *et al*., 2022; Ahmed *et al*., 2022), birds and humans causing variety of ailments by expressing a wider range of pathogenic strains. Infections of the urinary tract, joint inflammation, and mastitis are among the diseases caused by *Staph. aureus* in animals, on the other hands (Akbar and Anal, 2013). It is equally important to probe prevalence, risk assessment and drug resistance of enteric pathogens.

It is evident that antimicrobial resistance is emerging topic at current for animals and humans which is multifactorial majorly because of lavish use of antibiotics in human, veterinary, and agricultural practices. This situation has created selective pressures that have led to enhanced microbial drug resistance (Levy, 2002). The presence of antibiotic-resistant bacteria has also been reported in wild animals living in remote regions that have been exposed to little or no antibiotics (Bartoloni *et al*., 2004). When captive raised animals are released into wild populations, there is increasing concern about disease and antibiotic resistance transmission (Woodford, 2000). This study aimed to determine the patterns of prevalence, potential risks, and drug resistance profile of *Staph. aureus* and *Salmonella* isolated from captive Asian houbara bustard.

MATERIALS AND METHODS

Sample collection

Sampling units for this study were Houbara bustard kept at the Houbara Foundation International, Lal Sohanra Park, Bahawalpur, Pakistan, a non-government organization for conservation of Houbara bird. The selected birds for study were adults, 22–26 inches long while 53–67 inches across the wings. Briefly, 105 cloacal samples were taken at repeated time intervals from birds early in the morning in sterile swab tubes (El-Shahawy and Abou Elenien, 2015). For further testing, the samples were transported in sterile containers (4°C) to the Central Diagnostic Laboratory of Cholistan University of Veterinary and Animal Sciences, Bahawalpur.

Risk factor estimation

A proforma with predetermined necessary information at time of sampling was filled in to assess association of risk factors with spread of *Staph. aureus* and *Salmonella.* The information in the proforma included age, sex, feeding systems, gastrointestinal parasites, and previous treat etc. Chi square test was applied to assess association of different factors with *Staph. aureus* and *Salmonella* while regression analysis was performed to find potential risk factor. Any level of factor presenting more than 1 Odd's ratio was considered as potential risk factors.

Isolation of Staph. aureus *and* Salmonella

For the initial incubation, samples were incubated at 37°C for 24h in sterile nutrient broth. Incubated material was dipped in sterile cotton swabs which were spread onto blood agar homogenously. Typical colonies appeared on blood agar were further spread over mannitol salt agar and SS (*Salmonella* Shigella) agar and incubated at 37°C for 24h (Sarwar *et al*., 2021). Colonies were picked up using a sterilized platinum loop and were subjected to series of biochemical tests, while pooled information was used to confirm *Staph. aureus* and *Salmonella*. All the obtained data was expressed as percentages which were calculated by multiplying the number of positive samples by 100 (Thrusfield, 2007).

Antibiotic susceptibility of Staph. aureus *and* Salmonella

The clinical and laboratory standards institute (CLSI) guidelines were followed in the application of the disc diffusion method to find susceptibility responses of *Staph. aureus* and *Salmonella*. Ten antibiotic discs *viz a viz* enrofloxacin (ENR 5µg), fusidic acid (FA 10µg), ciprofloxacin (CIP 5µg), septran (S*T 25µg), amikacin (AK 30µg), chloramphenicol (C 30µg), vancomycin (VAN 30µg), gentamicin (CN 10µg), linezolid (LNZ 30µg), cefoxitin (C*T 30µg), cephazolin KZ (30µg), and oxacillin OX(5) were aseptically applied at an equal distance from each other on activated growth of 1-1.5x10^8 CFU/mL. As per CLSI guidelines, zones of inhibition (mm) were measured following 24h of incubation at 37°C and classified as resistant, intermediate, and sensitive.

Patterns in variation of susceptible strains of *Staph. aureus* compared to *Salmonella* against different antibiotics were calculated to present comparative strength of antibiotics being effective on relative term basis. Percentage resistant strains of *Staph. aureus* against each antibiotic were compared with percentage resistant strains of *Salmonella* against same antibiotic on relative terms basis were executed. Similarly, variation in percentages of resistant strains of *Salmonella* against a particular antibiotic were compared with resistant strains of *Staph. aureus* against same antibiotics on relative terms basis. The same protocol was adopted for intermediate and sensitive strains against antibiotics. Following formulae were applied:

$$\text{Relative variation (\%) in susceptible strains of } \textit{Salmonella with respect to Staph. aureus} = \frac{(\text{\% susceptible strains of } \textit{Salmonella} - \text{\% susceptible strains of } \textit{Staph. aureus}}{\text{\% susceptible strains of } \textit{Salmonella}} \times 100$$

$$\text{Relative variation (\%)in susceptible strains of } \textit{Staph. aureus with respect to Salmonella} = \frac{(\text{\% susceptible strains of } \textit{Staph. aureus} - \text{\% susceptible strains of } \textit{Salmonella}}{\text{\% susceptible strains of } \textit{Staph. aureus}} \times 100$$

Statistical analyses

Analyses were conducted using non-parametric tests (percentage calcualtion, Chi square, and regression analysis). Statistical significance was declared at $p<0.05$. Prevalence was calculated by formula described by (Thrusfield, 2018).

$$\text{Prevalence (\%)} = \frac{\text{No of positive sample}}{\text{Total No of samples tested}} \text{ X } 100$$

RESULTS

Patterns of prevalence

The current study comprised of 105 swab samples in different time periods that further consisted of 33.33% male and 66.67% female birds. The study found 85.71% of cloacal samples presenting growth of bacteria on general purpose media. Prevalence of *Salmonella* and *Staph. aureus* mixed bacteria was noted to be 28.57% (30/105) while prevalence of *Salmonella* alone and *Staph. aureus* alone were 12.38% (13/105) and 26.67% (28/105), respectively. Overall prevalence of *Salmonella* (both alone and in mixed form) were noted to be 40.95% (43/105) and for that of *Staph. aureus* was 55.23% (58/105).

Trends in risk factors

Birds in winter season, having intestinal parasites, exposed to use of antibiotics, eating poultry feed, and having exposed to antibiotics were also significantly associated with spread of *Staph. aureus*. Gender, age, housing system and season did not show significant ($p>0.05$) association as analyzed by chi square analysis (Table I). The current study found gender, feeding system, season, gastrointestinal parasites, antibiotic exposure, and type of antibiotic to be significantly ($p<0.05$) associated with *Salmonella* prevalence in Houbara bustards. However, there was no significant association between age and housing system ($p>0.05$) (Table II).

Regression analysis of assumed risk factors for *Staph. aureus* showed male bird showing 1.26 odds of getting infection but these odds were non-significant when it comes to the comparison with female bird. Age groups in this study did not show higher odds than to the birds greater than 1 year age (Table I). Feeding mere poultry feed presented 5.77 odds of getting infection compared to that of mixed feed and this trend was found highly significant ($p<0.001$). Similarly, winter season showed 1.059 odds of getting infection compared to that of summer season.

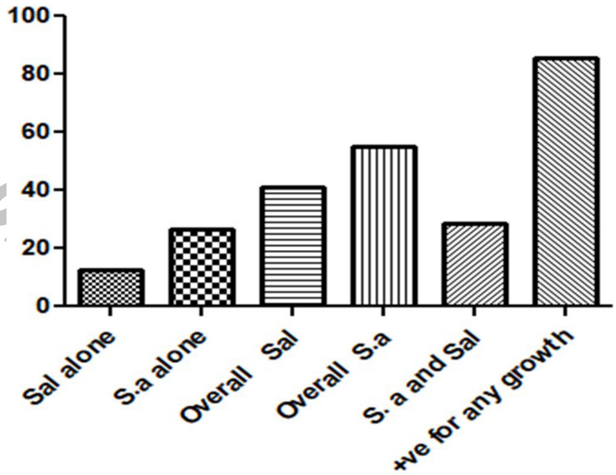


Fig. 1. Patterns of prevalence of *Staph. aureus* and *Salmonella*.
(Sal alone=Samples positive for *Salmonella* only, S.a alone= Samples positive for *Staph. aureus* only, Overall Sal=Samples containing *Salmonella* alone and *Salmonella* in mixture with other bacteria; Overall S.a= Samples containing *Staph. aureus* alone and *Staph. aureus* in mixture with other bacteria; S.a and Sal=Samples containing both S.a and *Salmonella;* +ve for any growth=Samples positive for any growth of bacteria on blood agar.

Male birds, on the other hands, to get infected with *Salmonella* were 3.187 odds compared to that of female birds with significant strength. Age of bird from 7-12 months showed 1.37 odds of getting *Salmonella* compared to the reference value i.e., above year old birds (Table II). Feeding poultry feed presented 3.127 odds of getting infection with significant effect in this study. Natural environment presented 1.114 odds of getting *Salmonella* while taking winter season into account, there were 4.23 odds of getting *Salmonella* infection at significant effect compared to the reference entries.

Antibiotic susceptibility of Staph. aureus *and* Salmonella

The study found highest resistant strains as 40% against oxacillin and 30% against cefoxitin and vancomycin while the percentage resistant strains of *Staph. aureus* against all other antibiotics remained between 10-20% (Table III). Percentage intermediate susceptible strains against gentamicin and linezolid were 40% while 30% against chloramphenicol and all other percentages in this category remained between 10-20%. The percentage sensitive strains against gentamicin were 40% while all other percentages of sensitive strains were greater than 50% indicating a better window of efficacy of antibiotics in current scenario. The percentage susceptible strains of *Salmonella* against different antibiotics were comparatively less towards sensitive category compared to that of *Staph. aureus*. There were 50% of *Salmonella* strains

Table I. Risk factor analysis of *Staph. aureus* isolated from enteric source of Houbara bustard.

Variable	Chi square analysis				Regression analysis					
	Screened	Positive	Prevalence (%)	p value	CI 95% ratio		OR	CI 95% for OR		p value
					Lower	Upper		Lower	Upper	
Gender										
Male	35	18	51.42	0.57	35.57	67.01	1.26	0.55	2.84	0.57
Female	70	40	57.14		45.48	68.06	-	-	-	-
Age										
0-6 month	15	8	61.66	0.43	30.11	75.19	0.88	0.27	2.75	0.81
7-12 month	36	23	63.88		47.58	77.53	0.56	0.23	1.34	0.19
Above 1 year	54	27	50		37.11	62.89	-	-	-	-
Housing system										
Natural environment provision	67	33	49.25	0.10	37.65	60.93	1.98	0.87	4.52	0.104
Pen	38	25	65.78		49.89	78.79	-	-	-	
Feeding system										
Poultry feed	38	11	28.95	<0.01	17.01	44.76	5.77	2.40	13.84	<0.001
Poultry feed plus scavenger	67	47	70.15		58.35	79.77	-	-	-	-
Season										
Spring	40	25	6.25	0.11	47.03	75.78	0.63	0.25	1.59	0.33
Winter	30	15	50		33.15	66.85	1.05	0.39	2.81	0.91
Summer	35	18	51.42		35.57	67.01	-	-	-	-
Gastro intestinal parasites										
Yes	44	38	86.36	<0.01	73.29	93.59	0.07	0.02	0.21	<0.001
No	61	20	32.78		22.34	45.28	-	-	-	-
Exposure of antibiotics										
Frequent	25	21	84		65.35	93.6	0.08	0.02	0.27	<0.001
Occasional	30	22	73.33	<0.01	55.55	85.81	0.15	0.05	0.42	<0.001
No use	50	15	30		19.1	43.75	-	-	-	-
Type of antibiotic used										
Beta-lactam	50	39	78		19.1	43.75	0.12	0.04	0.29	<0.001
Other	10	4	40	<0.01	16.82	68.73	0.64	0.15	2.61	0.53
NA	50	15	30		19.1	43.75	-	-	-	-

p<0.05 indicate significant association, NA means they do not use antibiotics or they use other than antibiotic or record is not available.
OR, odd ratio

Table II. Risk factor analysis of *Salmonella* isolated from enteric source of Houbara bustard.

Variable	Chi square analysis				Regression analysis					
	Screened	Positive	Prevalence (%)	p value	CI 95% ratio		OR	CI 95% value		p value
					Lower	Upper		Lower	Upper	
Gender										
Male	35	8	22.85714	<0.01	0.12	0.39	3.18	1.27	7.98	0.01
Female	70	34	48.57143		0.37	0.60	-	-	-	-
Age										
0-6 M	15	8	53.33	0.41	0.30	0.75	0.60	0.19	1.90	0.38
7-12 M	36	12	33.33		0.20	0.49	1.37	0.57	3.31	0.47
Above 1 Y	54	22	40.74		0.28	0.54	-	-	-	-
Housing system										
Natural environment provision	67	26	38 .81	0.74	0.28	0.50	1.14	0.51	2.58	0.74
Pen	38	16	42.11		0.27	0.57	-	-	-	-
Feeding system										
Poultry feed	38	9	23.68	0.01	0.12	0.39	3.12	1.28	7.60	0.01
Poultry feed plus Scavenger	67	33	49.25		0.37	0.60	-	-	-	-
Season										
Spring	40	18	45.00	0.02	0.30	0.60	1.29	0.52	3.21	0.57
Winter	30	6	20.00		0.09	0.37	4.23	1.39	12.90	0.01
Summer	35	18	51.43		0.35	0.67	-	-	-	-
Gastro intestinal parasites										
Yes	44	31	70.45	0.009	0.55	0.81	0.09	0.03	0.23	<0.01
No	61	11	18.03		0.10	0.29	-	-	-	-
Exposure of antibiotics										
Frequent	25	16	64.00	<0.01	0.44	0.79	0.15	0.05	0.45	<0.01
Occasional	30	15	50.00		0.33	0.66	0.28	0.10	0.75	0.01
No use	50	11	22.00		0.12	0.35	-	-	-	-
Type of antibiotic used										
Beta-lactam	50	28	56.00	0.002	0.42	0.68	0.22	0.09	0.53	0.01
Other	10	3	30.00		0.10	0.60	0.65	0.14	2.98	0.58
NA	50	11	22.00		0.12	0.35	-	-	-	-

p<0.05 indicate significant association, NA means they do not use antibiotics or they use other than antibiotic or record is not available.
OR, odd ratio

resistant against cephazolin and the same percentage was resistant against vancomycin while 40%, 30%, and 30% of *Salmonella* were resistant against oxacillin, amikacin, and fusidic acid, respectively (Table III). Percentage of *Salmonella* resistant strains against all other antibiotics were between10-20% while in case of intermediate susceptible strains, the percentages were not more than 10-20% against antibiotics except against gentamicin (40%), amikacin (30%), and linezolid (30%). Highest percentage sensitive strains were against septran (80%) followed by ciprofloxacin (70%), cefoxitin (70%), enrofloxacin (60%), and chloramphenicol (60%). All other percentage sensitive strains against antibiotic were between 30-40% (Table III).

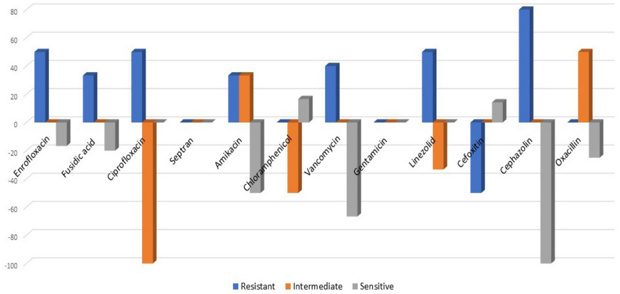
Patterns in variation of susceptible strains against different antibiotics

Comparative percentage changes of susceptibile strains (resistant, intermediate, sensitive) in *Staph. aureus*

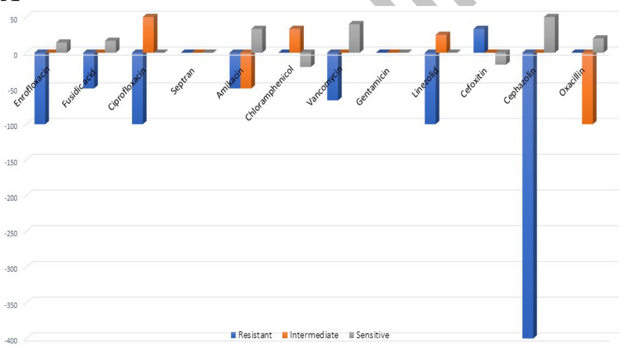
Table III. Antibiotic susceptibility (%) of *Staph. aureus* and *Salmonella* spp. against different antibiotics

Antibiotic name	*Staph. aureus*			*Salmonella* spp.		
	R	I	S	R	I	S
Enrofloxacin (ENR 5 µg)	10	20	70	20	20	60
Fusidic acid (FA 10µg)	20	20	60	30	20	50
Ciprofloxacin (CIP 5 µg)	10	20	70	20	10	70
Septran (S*T 25µg)	10	10	80	10	10	80
Amikacin (AK 30 µg)	20	20	60	30	30	40
Chloramphenicol (C 30µg)	20	30	50	20	20	60
Vancomycin (VAN 30µg)	30	20	50	50	20	30
Gentamicin (CN 10µg)	20	40	40	20	40	40
Linezolid (LNZ 30µg)	10	40	50	20	30	50
Cefoxitin (C*T 30µg)	30	10	60	20	10	70
Cephazolin KZ(30)	10	10	80	50	10	40
Oxacillin OX(5)	40	10	50	40	20	40

R, resistant; I, intermediate; S, susceptible.



A



B

Fig. 2. Percentage variation of susceptibility (resistant, intermediate, sensitive) in *Salmonella* in comparison with *Staph. aureus* (A) and *Staph. aureus* in comparison to *Salmonella* (B).
Formula for A % variation= (% susceptible strains of *Salmonella* - % susceptible strains of *Staph. aureus)*/ % susceptible strains of *Salmonella**100
Formula for B % variation= (% susceptible strains of *Staph. aureus*- % susceptible strains of *Salmonella)*/ % susceptible strains of *Staph. aureus**100.

in comparison with those of *Salmonella* showed downfall trend in resistant category in that-400% change against cephazolin, -100% against enrofloxacin and ciprofloxacin were noted followed by vancomycin (-66.67%), amikacin (-50%), and fusidic acid (-50%). In case of sensitive strains, percentage variation was found in positive numbers except cefoxitin while intermediate susceptible strains showed -100% change against oxacillin, and -50% against amikacin (Fig. 2). The pattern of relative variation in susceptible strains of *Salmonella* in comparison to *Staph. aureus* showed majorly positive values (Fig. 2). Except cefoxitin (-50%), all other antibiotics were found with positive percentages in variation for *Salmonella* relative to that of *Staph. aureus* for resistant strains. Percentages of variation in intermediate susceptible strains of *Salmonella* in comparison to those of *Staph. aureus* showed -100%, -50%, and -33.33% against ciprofloxacin, chloramphenicol, and linezolid, respectively. All other were either positive values or zero change for intermediate category. In case of resistant strains, there were 50% of variations falling in negative values while rest of were either positive or zero. Negative values indicate decrease in susceptibe strains of one bacteria compared to those of other bacteria against perticular rantibiotic.

DISCUSSION

Prevalence of Staph. aureus *and* Salmonella

Prevalence of *Staph. aureus* and *Salmonella* was in contradiction to the findings of Gutiérrez *et al*. (2012) who reported 6.1% of *Staph. aureus* from food contact surfaces. However, in agreement to the findings of current study, were those of Nazia *et al*. (2015) who reported 68.00% prevalence of bacteria in broilers birds. Mulders *et al*. (2010) found 6.9% of n=405 broiler birds were positive for MRSA when sampled from slaughterhouse. A study by Elsohaby *et al*. (2021) revealed that among 37 strains of *Staphylococcus* spp., that were isolated from wild birds around Lake Al-Asfar, there were 9.5% positive for *Staph. aureus*. In another study, Silva *et al*. (2022) reported prevalence of *Staph. aureus* to be 20.9% from trachea and cloacal swabs of nocturnal raptors. It was reported that the prevalence of Spanish birds for bacterial isolates was 8.5% (Millán *et al*., 2004). In another study from Italy, there were 2.2% positivity rates for *Salmonella*. According to Wei *et al*. (2015), migratory birds were detected with 0.93 % *Salmonella*. Telli *et al*., (2022) reported pathogenic strains of *E. coli* and *Salmonella* as major carcass deteriorating pathogens. It is also noteworthy that migratory birds can carry other pathogens including *Campylobacter* which was more prevalent during the same periods in the same species (Wei *et al*., 2015). Consequently, these migratory birds could carry a variety of pathogens, which increases the risk of human and animal disease transmission.

Risk factor

The current study's findings for associated risk factors were in agreement with some of studies while contradicting to other studies that might be because of differences in species of birds, isolation sites, geographicals zones, exposure to antimicrobials and several others. The study of Nacer *et al*., (2022) showed significant association of poultry with season for *Staph. aureus* and *Salmonella*. In an other study there was significant association of animals (cattle) exposure for spread of *Staph. aureus* (Wardynet *al*., 2015).

Antibiotic susceptibility of Staph. aureus *and* Salmonella

In contrast to our findings, Amoako *et al*. (2020) reported 100% sensitivity of *Staph. aureus* to chloramphenicol, while Miranda *et al*. (2008) and Suleiman *et al*. (2013) reported 72.5% sensitivity. Münch *et al*. (2012) revealed lower prevalence of MDR *Salmonella* infections in poultry (61%) than in quails (41%), stone curlews (35%), or bustards (33%). In contrast to our study, Wei *et al*. (2020) study showed lower prevalence of Salmonella in migrating birds, i.e. 0.93%. Shobrak *et al*. (2013) reported *Salmonella* to be highly resistant to tetracycline. The findings of Münch *et al*. (2012) reported *Salmonella* to be detected in birds that were resistant to major antimicrobials. Zhao *et al*. (2007) analyzed antimicrobial resistance in 380 *Salmonella* strains recovered from domestic animals in the USA between 2002 and 2003. They found zero resistance against ciprofloxacin. Similar findings were also reported by Wei *et al*. (2020) in that most of *Salmonella* were resistant to cipfroloxacin. The alternative therapeutics might be required to tackle the rise in antimicrobial resistance (Shnawa *et al*., 2022).

CONCLUSION

Houbara bustard showed increasing prevalence of *Staph. aureus* and *Salmonella* and there were significant association of most of assumed risk factors. Relying only poultry feed, winter season, open environment, and sex of the bird were potential risk factors for bacteria. Currently, a good number of antibiotics were found effective against both bacteria while there was also increasing trend in resistant and intermediate susceptible strains which is able to serious concerns in near future. The *Salmonella* pathogen in reference to *Staph. aureus* showed increased percentage variation in resistant category against antibiotics while overall strength of variations was also inclined towards *Salmonella.* This study thus concludes increased prevalence, higher number of potential risk factors, and emerging resistant strains of pathogens. The further investigations in terms of molecular studies and mechanisms behind the spread of antibiotic resistance and development of alternative therapeutics are the need of the hour.

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Ethical statement

There was no intervention other than collecting swab samples from cloaca which was done as per standard protocols. All other study was done in vitro and no further contact with animal was executed in this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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