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Screening of crows and waterfowls for *Salmonella* and *Listeria monocytogenes* infection

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Abstract

Background and Aim: Wild birds can carry a number of potential human and animal pathogens. These birds can intrude into human habitats giving the opportunity to transmission of such infection. Therefore, the current study was designed to investigate the role of crows and waterfowls as vectors of *Salmonella* and *Listeria monocytogenes* and to compare cultivation methods with direct polymerase chain reaction (PCR) for the detection of infection.

Materials and Methods: A total of 200 fecal dropping samples were collected. *Salmonella* was cultivated by three step method (Universal Pre-enrichment Broth [UPB], Rappaport-Vassiliadis Broth, and Xylose Lysine Desoxycholate agar). The recovered isolates were characterized by biotyping, serotyping, and PCR detection of enterotoxin (*stn*) gene. The antibiogram pattern of isolates against a panel of 8 antibiotics was recorded. *L. monocytogenes* was cultivated on UPB, then on Listeria Oxford Agar and Listeria CHROMagar. The recovered isolates were characterized by biotyping and PCR detection of listeriolysin (*hylA*) gene.

Results: The percentages of *Salmonella* infection in crows and waterfowls were 10 and 20, respectively. The most frequently recovered serovars were Typhimurium, Potengi, Enteritidis, and Kentucky. Antimicrobial susceptibility analysis of *Salmonella* isolates showed that the resistance rates for gentamicin were the highest (92%), followed by amoxicillin (88%) and cefixime (60%). Resistance to 8 antibiotics was recorded in 60% (15/25) of *Salmonella* isolates. The percentages of *L. monocytogenes* infection in crows and waterfowls were 1.3 and 2, respectively. Direct PCR applied to UPB revealed that 12% and 3% of samples were positive for *Salmonella* and *L. monocytogenes*, respectively, and there was no significant difference between direct PCR and cultivation method for the detection of infection.

Conclusion: The present findings indicate that wild birds can harbor zoonotic enteric pathogens and this necessitates monitoring the epidemiologic status of these pathogens among wild birds and humans and applying the appropriate intervention measures to prevent the spread of infection.

Keywords: antibiotic susceptibility, crows, *hylA* and *stn* genes, *Listeria monocytogenes*, *Salmonella*, waterfowl.

Introduction

Numerous wild bird species serve as reservoirs and/or mechanical vectors for foodborne pathogens of veterinary and public health concern [1,2]. The burden of foodborne diseases is substantial; every year, almost one in 10 individuals fall ill and 33 million healthy life years are lost [3]. *Salmonella* is one of the four key global causes of diarrheal illness [3]. Most cases are mild; however, it can be life-threatening. *Salmonella* can be spread by the direct fecal-oral route and by ingestion of contaminated food and water [4]. The serotypes of Typhimurium and Enteritidis are renowned causes of illness in human and wild birds. The prevalence of salmonellae in the environment is related to the degree of contamination by fecal material from infected hosts. Furthermore, most serotypes can survive for a long time in the environment which would be sufficient to maintain their presence from one year to the next [5].

Listeria monocytogenes is a foodborne pathogen that has a worldwide distribution. It has been isolated from numerous mammalian and avian species [6,7]. Most human infections are acquired by ingestion of contaminated food or by direct contact with infected animals and birds [8,9]. Its annual incidence is 0.1-10 cases per 1 million people depending on the region of the world. Although the annual number of cases of listeriosis is small, the high rate of deaths associated with this disease makes it a remarkable public health concern [10]. There are two types of listeriosis illness: A non-invasive form and an invasive form. The non-invasive form is mild febrile gastroenteritis that affects mainly healthy people. An invasive form is a severe form of the disease that affects high-risk individuals; these include pregnant women, immunocompromised patients, older adults, and infants. The latter form is characterized by severe symptoms: Meningitis,

meningoencephalitis, abortion, or stillbirth and a high case fatality rate that reaches 20-30% [8,9].

It is worth mentioning that the emergence of resistance to antibiotics has become a global public health problem [10-12]. The presence of antibiotic-resistant bacteria in wildlife may reflect the impact of human activities on natural ecosystems. Wild bird species acquire and disseminate resistant enteric bacteria by the fecal-oral route. They may also act as reservoirs, carriers, or sentinels of resistant bacterial pathogens [1].

Despite the zoonotic importance of *Salmonella* and *L. monocytogenes*, local studies about their prevalence in wild birds are limited. Hence, this study aimed at the detection and identification of *L. monocytogenes* and *Salmonella* serovars by biochemical, antibiogram typing, and molecular detection of *Salmonella* enterotoxin (*stn*) gene and listeriolysin (*hylA*) gene. Comparing standard microbiological methods with direct PCR for detection of these pathogens in the examined wild birds' samples was also carried out.

Materials and Methods

Ethical approval

The study protocol was approved by The Department of Animal Hygiene and Zoonoses Council, Faculty of Veterinary Medicine, Suez Canal University, Egypt.

Study area

The study was carried out in 2017 at El Qantara city which is located in the western side of the Suez Canal with a latitude of 30° 51′ 0″ N and a longitude of 32° 18′ 36″ E. Source: http://dateandtime.info/citycoordinates. It is located in the Egyptian governorate of Ismailia, 160 km northeast of Cairo, and 50 km south of Port Said. The city is a well-known destination for hunting and selling live wild birds.

Birds

Overall, a total number of 200 wild birds were examined which included 150 house crows (*Corvus splendens*) and 50 hunted wild waterfowls; the mallard (*Anas platyrhynchos*, n=25) and the spoonbill duck (*Spatula clypeata*, n=25).

Crows

The study area was divided into districts. Each sampling site was visited only once. Fecal droppings were collected by spreading large (3 m×3 m) clean nylon sheets in areas where crows are congregated, especially, where hunters capture wild waterfowl. Birds were lured to the site using grain-based food baits. The birds were observed at a distance, and they were allowed to freely eat all the sprinkled food without any interruption. After all foods were consumed, the fecal droppings were collected using sterile swabs into tubes containing 5 ml of sterile saline.

Waterfowls

We visited wild bird markets. Wild birds' hunters gather the captured birds in cages until selling them. We identified the species of birds. Then, fresh excreted fecal samples were collected from the floor of cages using sterile swabs into tubes containing 5 ml of sterile saline to be transported to the laboratory in an ice chest containing frozen ice packs.

In the laboratory, thorough mixing of the samples by vortexing was done. 1 ml of the fecal dropping homogenate was aseptically pipetted into each of two sterile tubes containing 9 ml of Universal Preenrichment Broth (UPB) (7510 Acumedia, USA) [13]. The two UPB tubes were incubated at 37°C for 24 h and 30°C for 48 h for the isolation of *Salmonella* and *L. monocytogenes*, respectively.

Isolation and identification of *Salmonella*

Three-step method was used. 1 ml of the incubated UPB tubes was aseptically inoculated into 9 ml of Rappaport-Vassiliadis (R-V) broth (LAB M, UK), incubated at 40°C for 24 h. A loopful of R-V broth was streaked onto Xylose Lysine Desoxycholate (LAB M, UK), incubated at 40°C for 24 h. Suspected colonies were identified by Gram stain and biochemical tests according to the USFDA [14].

Serological identification of *Salmonella* isolates

A 24-h-old nutrient agar slant culture tube of the suspected organism was clearly labeled and put in an ice box with surrounding refrigerant and was sent to the Food Analysis Center (Faculty of Veterinary Medicine, Benha University) and to the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute (Dokki, Egypt), for serologic identification by determination of somatic (O) and flagellar (H) antigens using *Salmonella* antiserum **(**Denka Seiken Co., Japan).

Antibiogram susceptibility pattern of *Salmonella*

Genotypically confirmed *Salmonella* isolates (n=25) were screened against a panel of eight antibiotics using the Kirby–Bauer disc diffusion method on Mueller-Hinton agar following the guidelines of Andrews [15]. This test was not applied to *L. monocytogenes* isolates because they lost their viability during preservation.

The antibiotic discs (Oxoid) used were amoxicillin (AML, 10 µg), gentamicin (CN, 10 µg), cefixime (CFM, 5 µg), cefotaxime (Ctx, 30 µg), doxycycline (Do, 30 µg), cephalothin (KF, 30 µg), novobiocin (NV, 30 µg), and sulfamethoxazole/trimethoprim (SXT, 25 µg). These antibiotics were chosen because they are commonly prescribed in human and veterinary medicine.

Calculation of multiple antibiotic resistance (MAR) index

MAR index for *Salmonella* isolates was calculated using the formula: MAR = a/b, where a is the number of antibiotics to which the isolate was resistant, and b is the number of antibiotics to which the isolate was subjected [16]. The intermediate category was considered resistant [17]. MAR indexing is an indicator to identify high-risk contamination source

that may represent a public health hazard. MAR index values which are higher than 0.2 are considered to have originated from high-risk contamination sources where antibiotics are commonly used [16]. Different strains were assigned to different phenotypic patterns according to their resistance trend to the tested antibiotics.

Isolation and identification of *L. monocytogenes*

Two loopfuls of the incubated UPB tube were simultaneously streaked onto Listeria Oxford Agar (Lab M Limited, UK) and Listeria CHROMagar (CHROMagar, Paris, France), incubated at 37°C for 48 h. Listeria Oxford Agar contains esculin and ferric citrate. Based on esculin hydrolysis, colonies of *Listeria* are concave surrounded by black/brown zone. Concerning listeria CHROMagar, it contains the chromogenic substrate5-bromo-4-chloro-3-indoxyl-beta-D-glucopyranoside for the detection of beta-d-glucosidase, which gives the colony blue color and utilizes cleavage of L-alpha-phosphatidylinositol by the virulence factor phosphatidylinositol-phospholipase C, forming a white precipitation zone around the colony [18]. Five typical colonies were identified by Gram stain and biochemical tests according to the USFDA [14].

Genotypic confirmation of *Salmonella* and *L. monocytogenes*

DNA extraction from isolates

Biochemically identified colonies (*Salmonella* [n=37] and *L. monocytogenes* [n=13]) were streaked onto nutrient agar, incubated at 37°C for 24 h. A single colony was used for DNA extraction using DNA kit (Bio Basic Inc., Canada) according to the manufacturer's instructions.

DNA extraction from UPB

Standard culture methods commonly used to detect *Salmonella* and *Listeria* in clinical samples are expensive, laborious, and time-consuming. The direct polymerase chain reaction (PCR) assay can overcome these obstacles in terms of speed and sensitivity. Nevertheless, it is important to select enrichment broth that contains low inhibitory substances and to dilute them to the lowest possible concentration [19,20]. The Boiling-centrifugation as described by Soumet *et al.* [21] was used. 1 ml aliquots of UPB broth were centrifuged at 13,000× g for 3 min. The pellets were resuspended in 100 µl of sterile MilliQ water, heated to 95°C in a dry block for 10 min, cooled in ice, and centrifuged at 13,000× g for 3 min. These supernatants were used for PCR assay.

Both the quality and quantity of extracted DNA were measured [22]. The quality of the extracted DNA was estimated by electrophoresis in 1% agarose gel containing ethidium bromide in 1× TBE buffer for 30 min at 100 V along with a 100 bp DNA ladder. DNA bands were viewed under UVI pro Silver Gel Documentation System. If DNA appears as a clear single band in the agarose gel, this indicates that it is not degraded. To determine DNA purity and concentration, total extracted DNA was quantified using ND-1000 spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA). The DNA was preserved at −70°C until use.

PCR conditions for the detection of *Salmonella*

The test was applied to confirm the identity of the suspected *Salmonella* isolates (n=37) as well as for rapid detection of *Salmonella* from enrichment broth (n=200) within 30 h of sample processing. PCR was implemented to detect *Salmonella* enterotoxin (*stn*) gene that is widely distributed among *Salmonella* irrespective of the serovars and source of isolation. The reaction mixture was adjusted at 25 µl using 5 µl of DNA, 1 µl of each primer (30 pmol), 12.5 µl of master mix (GeneDirex, USA and Taiwan), and 5.5 µl of sterile MilliQ water. Primers were designed using the program "Primer 3" targeting *Salmonella* enterotoxin gene (*stn*) of *Salmonella* Typhimurium (Accession: L16014.1 GI: 295215). The following primers (Eurofins Genomics, Brussels, Belgium) were used for the amplification of *stn* gene, left primer 5′- AGACTTTCTCACGCACCTGA-3′ and *stn* right primer 5′ GTATTGAGGGTAAAGGCGCG-3′. The following parameters were used following the guidelines of Murugkar *et al.* [23] with modification: (1) Initial denaturation at 95°C for 4 min and (2) thirty cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The program was ended by a final extension at 72°C for 5 min, and the product was kept at 4°C until analysis. 8 µl of PCR product were loaded into 1.5% agarose gel containing ethidium bromide. The amplified products were then run along with a 100 bp DNA ladder (GeneDirex, USA and Taiwan) in 1× TBE buffer for 30 min at 100 V and then viewed using the UVI pro-Silver Gel Documentation System, UK. The gel picture was analyzed using computer software (SynGene-GeneTools - File version: 4.03.05.0, Synoptics Ltd, Cambridge, UK). *Salmonella* spp. gave specific band at 218 bp.

PCR conditions for the detection of *L. monocytogenes*

The test was performed according to Bansal [24] with modification to confirm the identity of 13 *L. monocytogenes* isolates as well as for rapid detection of *L. monocytogenes* from UPB. Primers (Eurofins Genomics, Brussels, Belgium) LF (5'- CAA ACG TTA ACA ACG CAG TA-3') and LR (5'-TCC AGA GTG ATCGAT GTT AA-3') were applied for species confirmation of *L. monocytogenes* strains. The total reaction volume was 25 µl consisting of: 5 µl of template DNA, 1 µl of each primer (30 pmol), 12.5 µl of master mix (GeneDirex, USA & Taiwan) and 5.5 µl of sterile MilliQ water. The PCR was run in Master Cycler Gradient (Eppendorf, Germany). The thermal profile consisted of an initial denaturation step at 95°C for 1 min, followed by 35 cycles of 94°C denaturation

for 30 s, 51°C annealing for 20 s, and 72°C elongation for 30 s. At the end of amplification, the mixture was subjected to a final extension at 72°C for 8 min. A total of 8 µl of amplified products were analyzed by electrophoresis in 2% agarose gel (Caisson, USA) stained with 0.5 µg/ml ethidium bromide along with a 100 bp DNA ladder (GeneDirex, USA and Taiwan) in 1× TBE buffer and visualized under UV light (UVI pro gel imaging, UK). The gel picture was analyzed using computer software (SynGene-GeneTools - File version: 4.03.05.0). *L. monocytogenes* yielded a 750 bp product.

Sequencing [25]

The test was applied to confirm the identity of some isolates that were recovered on Listeria Oxford Agar but could not be identified. A pure single colony was grown on nutrient broth for 24 h. DNA was extracted using Bio Basic kit, Canada. We used bacteria-specific primers 27F paired with the universal primer 1492R that amplifies bacterial 16S rRNA genes. PCR was performed on a GeneAmp PCR system 9600 (Applied Biosystems), using 25 µl AmpliTaq Gold (Applied Biosystems), 1 µl each of 10 pM concentrations of each primer, forward and reverse primers (primers 27F-P GAGTTTGATCCTGGCTCAG, 1492R-P GGTTACCTTGTTACGACTT), 18 µl sterile Milli-Q water, and 5 µl DNA template, for a total volume of 50 µl. The PCR condition is initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1.5 min, final extension at 72°C for 5 min, and holding at 4°C. PCR products were visualized through electrophoresis on a 1% agarose gel with ethidium bromide. Clones with correctly sized vectors (ca. 1500 bp) were sequenced unidirectionally using the forward primer (Solgent Co. Ltd., Korea). Sequence files were quality scored and edited using Phred and Greengenes and compared to the GenBank nucleotide database using the Basic Local Alignment Search Tool.

Statistical analysis

A statistical comparison between infections rates was analyzed by Chi-square test, and also different antimicrobial susceptibility patterns were analyzed by Fisher's exact test. p-value was set at ≤0.05 (SPSS version 20).

McNemar matching coefficient was used to compare cultivation method (gold standard) and direct PCR.

The gold standard is the best single test (or a combination of tests) that is considered the current endorsed method of diagnosing a particular disease (X) [26]. All other methods of diagnosing X need to be challenged against this "gold" standard.

Sensitivity is the ability of a test to correctly classify an individual as "diseased."

Sensitivity = (True positive)/(True positive + False negative) × 100

Specificity is the ability of a test to correctly classify an individual as "disease free".

Specificity = (True negative)/(True negative + False positive) × 100.

Results

The prevalence of *Salmonella* infection in wild birds as per serological and PCR confirmation was 12.5% (25/200); the prevalence in crows and waterfowls was 10% and 20%, respectively. This difference was non-significant (χ^2=2.575, p=0.1085).

In crows, the frequency of isolation of different *Salmonella* serovars as related to the total number of *Salmonella* isolates was 20% for *S.* Typhimurium (1,4,[5], 12, i: 1,2), 13.3% for *S*. Potengi (18, Z-), and 6.7% for each of *S*. Papuana (6,7 r: e, n, Z15), *S*. Inganda (6,7 z10: 1,5), and *S.* Enteritidis (1, 9, 12 g, m: -), while 46.7% were untypable (Table-1).

For waterfowls, the frequency of isolation of different *Salmonella* serovars as related to the total number of *Salmonella* isolates was 20% for *S*. Kentucky and 10% for each of *S*. Bloomsburry (3,10 g,t 1,5), *S*. Potengi, *S*. Virginia (8, d: 1,2), *S*. Muenchen (6,8 d: 1,2 [z67]), *S*. Ferruch (8: e, h 1, 5), *S*. Enteritidis, *S*. Wingrove (6, 8 c: 1, 2), and *S*. Molade (8,20 Z10 Z6) (Table-1).

Antimicrobial susceptibility analysis of a total of 25 *Salmonella* isolates showed that all the examined isolates were resistant to Ctx, Do, KF, SXT, and NV. The resistance rates for CN were 92%, AML 88%, and CFM 60% (Table-2). The difference between the resistance rates of different antibiotics was non-significant (Fisher's exact test=52.572, p=0.980). *Salmonella* isolates were lied into five phenotypic patterns with MAR index that ranged between 0.75 and 1 (Table-3).

The prevalence of *L. monocytogenes* infection in wild birds was 1.5% (3/200). The prevalence of infection as detected on Listeria Oxford Agar, Listeria CHROMagar, and by PCR confirmation of isolates in crows was 8.7%, 4%, and 1.3%, respectively, while the prevalence of infection in waterfowls in the same sequence was 24%, 14%, and 2%, respectively (Table-4).

Direct PCR performed on UPB revealed that 12% (24/200) and 3% (6/200) of the examined wild birds' samples were positive for *Salmonella* (*stn*) gene and *L. monocytogenes* (*hylA*) gene, respectively (Tables-5 and 6, Figures-1 and 2).

The sensitivity and specificity of direct PCR to detect *Salmonella* were 72% and 96.6% and for *L. monocytogenes* were 66.7% and 98%, respectively.

Matching of cultivation method with direct PCR method using McNemar matching coefficient revealed that the difference between the two methods was non-significant. For *Salmonella*, p=1, and *Listeria*, p=0.3711.

Other potential human pathogens that were detected on Listeria Oxford Agar and were confirmed by sequencing were *Staphylococcus sciuri* and

Table-1: Percentages of *Salmonella* infection in wild bird species.

Bird species	Examined N	Positive culture n (%)	Positive serology and PCR n (%)	Serotypes recovered n (%)											
				Bloomsburry	Potengi	Virginia	Muenchen	Ferruch	Kentucky	Typhimurium	Enteritidis	Wingrove	Molade	Papuana	Inganda	Untypable
Crows	150	24 (16)	15 (10)	-	2 (13.3)	-	-	-	-	3 (20)	1 (6.7)	-	-	1 (6.7)	1 (6.7)	7 (46.7)
Waterfowls	50	13 (26)	10 (20)	1 (10)	1 (10)	1 (10)	1 (10)	1 (10)	2 (20)	-	1 (10)	1 (10)	1 (10)	-	-	-
Total n (%)	200	37 (18.5)	25 (12.5)	1 (4)	3 (12)	1 (4)	1 (4)	1 (4)	2 (8)	3 (12)	2 (8)	1 (4)	1 (4)	1 (4)	1 (4)	7 (28)

χ^2=2.575, *P*=0.1085 (non-significant)

Lysinibacillus massiliensis which were isolated from crows and waterfowls, respectively. Their prevalence was not calculated.

Discussion

Some wild bird species are carriers of zoonotic enteric bacteria. These wild birds may have the potential to transmit the infection to domestic animals, birds, and humans [12,27]. Moreover, they can be used as sentinels to understand the relative risk of these zoonotic pathogens [2].

The current study is an attempt to address the importance of crows and waterfowls as vectors of two important foodborne pathogens (namely, *Salmonella* and *L. monocytogenes*) in the study area.

Salmonella is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family. More than 2500 different serotypes have been identified. These serotypes can cause gastroenteritis, which is often uncomplicated and self-limiting, but the disease can be severe, especially, in infants and elderly and in patients with weakened immunity [10].

L. monocytogenes is widely distributed in nature. It can be found in soil, water, vegetation, and feces of some animals and birds, and it can contaminate food [6,7,28]. It has been implicated in foodborne outbreaks leading to serious illness and high fatalities [8,9,29].

The birds under scrutiny in the current study were crows and wild waterfowl spp. Crows are one of the most widespread bird species in the world. Crows are omnivorous. They will eat other birds, fruits, nuts, insects, seeds, eggs, nestlings, mice, and carrions. On the one hand, these birds help to clean up the environment by scavenging carrions and garbage [30]. On the other hand, they strew trash and transfer diseases to humans, animals, and birds [11,12].

Waterfowls have a cosmopolitan distribution. The tradition of waterfowl hunting is a culmination of several activities (food, sport, selling, and earning money). On the negative side, these wild birds may contribute to microbial water contamination and can serve as vectors of antimicrobial resistance genes between sources of fecal wastes and agricultural and aquatic environments [31].

Prevalence of *Salmonella* in crows

The prevalence of *Salmonella* infection in crows was 10% as confirmed by serology and PCR.

The most frequently isolated serotype was *S.* Typhimurium.

Salmonella prevalence in crows in the present study is nearly in the same line with Krawiec *et al.* [31] who found *Salmonella* in 16.7% (1/6) of the examined rook (*Corvus frugilegus*) samples in Poland.

Conversely, the current result is higher than that reported in Norway (2%, 1/52) and Tanzania (8%, 8/100) by Refsum *et al*. [5] and Katani *et al*. [12]. However, in Bangladesh [11], a much higher *Salmonella* prevalence has been reported (65%, 26/40). Albeit, other

Table-2: Antibiogram susceptibility patterns of *Salmonella* serovars.

Salmonella **serotypes** **Antibiotic tested**	**Bloomsburry S(%) I(%) R(%)**	**Potengi S(%) I(%) R(%)**	**Virginia S(%) I(%) R(%)**	**Muenchen S(%) I(%) R(%)**	**Ferruch S(%) I(%) R(%)**	**Kentucky S(%) I(%) R(%)**	**Typhimur -ium S(%) I(%) R(%)**	**Enterit -idis S(%) I(%) R(%)**	**Wingrove S(%) I(%) R(%)**	**Molade S(%) I(%) R(%)**	**Papuana S(%) I(%) R(%)**	**Inganda S(%) I(%) R(%)**	**Untypable S(%) I(%) R(%)**	**Total S(%) I(%) R(%)**
Amoxicillin	-	-	-	1 (33.3)	1 (33.3)	-	-	-	-	-	-	-	1 (33.3)	3 (12)
(AML 10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1 (4.5)	3 (13.6)	1 (4.5)	-	-	2 (9.1)	3 (13.6)	2 (9.1)	1 (4.5)	1 (4.5)	1 (4.5)	1 (4.5)	6 (27.3)	22 (88)
Gentamycin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(CN 10)	1 (50)	-	-	-	-	-	-	-	-	-	-	-	1 (50)	2 (8)
	-	3 (13)	1 (4.3)	1 (4.3)	1 (4.3)	2 (8.7)	3 (13)	2 (8.7)	1 (4.3)	1 (4.3)	1 (4.3)	1 (4.3)	6 (26.1)	23 (92)
Cefixime	-	2 (20)	1 (10)	1 (10)	1 (10)	-	-	-	-	-	-	-	5 (50)	10 (40)
(CFM 5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1 (6.7)	1 (6.7)	-	-	-	2 (13.3)	3 (20)	2 (13.3)	1 (6.7)	1 (6.7)	1 (6.7)	1 (6.7)	2 (13.3)	15(60)
Cefotaxime	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Ctx 30)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1 (4)	3 (12)	1 (4)	1 (4)	1 (4)	2 (8)	3 (12)	2 (8)	1 (4)	1 (4)	1 (4)	1 (4)	7 (28)	25 (100)
Doxycycline	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Do 30)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1 (4)	3 (12)	1 (4)	1 (4)	1 (4)	2 (8)	3 (12)	2 (8)	1 (4)	1 (4)	1 (4)	1 (4)	7 (28)	25 (100)
Cephalothin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(KF 30)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1 (4)	3 (12)	1 (4)	1 (4)	1 (4)	2 (8)	3 (12)	2 (8)	1 (4)	1 (4)	1 (4)	1 (4)	7 (28)	25 (100)
Sulfamethoxazole/	-	-	-	-	-	-	-	-	-	-	-	-	-	-
trimethoprim (SXT 25)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1 (4)	3 (12)	1 (4)	1 (4)	1 (4)	2 (8)	3 (12)	2 (8)	1 (4)	1 (4)	1 (4)	1 (4)	7 (28)	25 (100)
Novobiocin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(NV 30)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1 (4)	3 (12)	1 (4)	1 (4)	1 (4)	2 (8)	3 (12)	2 (8)	1 (4)	1 (4)	1 (4)	1 (4)	7 (28)	25 (100)
Total isolates examined	1	3	1	1	1	2	3	2	1	1	1	1	7	25

S=Sensitive. I=Intermediate. R=Resistan. Fisher exact test= 52.572, p value =0.980 (non-significant)

Table-3: Different phenotypic patterns of *Salmonella* isolates.

Pattern	Amoxicillin Penicillin group	Gentamycin Aminoglycoside group	Cefixime Cephalosporin group	Cefotaxime Third generation Cephalosporin group	Doxycycline Tetracycline group	Cephalothin Cephalosporin group	sulfamethoxazole –trimethoprim Sulfonamide- benzylpyrimidines group	Novobiocin Aminocoumarin group	a	MAR index a/b
Type 1	R	R	R	R	R	R	R	R	8	1
Type 2	R	R	S	R	R	R	R	R	7	0.875
Type 3	S	R	S	R	R	R	R	R	6	0.75
Type 4	R	I	R	R	R	R	R	R	8	1
Type 5	R	I	S	R	R	R	R	R	7	0.875

Multiple antibiotic resistance index: MAR index= a/b, a=number of antibiotics to which the isolate is resistant, b=total number of tested antibiotics=8

studies in Poland and Malaysia [31,32] could not isolate *Salmonell*a from crows' samples.

Prevalence of *Salmonella* in waterfowls

For waterfowls, a prevalence of 20% was recorded. The most frequently isolated serotype was *S*. Kentucky. Other studies in the USA [33,34] have reported a lower prevalence (8/342, 2%, and 2/375, 0.5%) of *Salmonella* in free-living waterfowl. Furthermore, in Chile, a prevalence of 6% was reported by Fresno *et al*. [35]. Krawiec *et al*. [31] examined cloacal swabs from dead mallard duck (*A. platyrhynchos*), and the prevalence was 6.61% (8/121). On the contrary, Antilles *et al*. [36] could not isolate *Salmonella* from any of the 318 waterfowls sampled in Spain.

Distribution of *stn* gene among *Salmonella* isolates

All the tested *Salmonella* serovars produced 218 bp DNA fragment specific for *stn* gene. Thus, it is recommended as a target gene for the detection of *Salmonella* infection, a finding previously confirmed by Murugkar *et al*. [23] and Tekale *et al*. [37].

Antimicrobial susceptibility of *Salmonella* isolates

The human health consequences of resistant pathogens include prolonged hospitalization, increased health-care costs, and increased rates of treatment failures and mortalities [38].

Antimicrobial susceptibility of *Salmonella* isolates showed that all of them were resistant to Ctx, Do, KF, SXT, and NV. The resistance rates for CN were 92%, AML 88%, and CFM 60%. Surprisingly, 15/25 (60%) of *Salmonella* isolates were resistant to the eight tested antibiotics. *Salmonella* isolates were lied into five phenotypic patterns with MAR index that ranged between 0.75 and 1 which indicates high-risk source of contamination and antibiotic misuse [16].

Previous studies showed different trends of *Salmonella* toward antibiotics. Fallacara *et al.* [33] found that *S*. Typhimurium isolates (n=8) were susceptible to CN and SXT. Lee *et al.* [39] examined nine *Salmonella* Panama isolates in Taiwan. They found high rates of antimicrobial resistance to SXT (66.7%) and CN (44.4%). All isolates were susceptible to CFM. In contrast, de Toro *et al.* [40] reported that AML is among the drugs of choice for treating salmonellosis.

Katani *et al.* [12] revealed that *Salmonella* isolates recovered from Indian house crows were highly resistant to AML (100%), suggesting that it is ineffective for the treatment of infections caused by these serotypes in the study area. Al Faruq *et al*. [11] recorded that the resistance rate of KF was 30-67% from Asian pied starling and house crows that were caught in Bangladesh. Grigar *et al.* [34] isolated *S*. Thompson and *Salmonella* Braenderup, and both isolates were susceptible to all antimicrobial agents tested. Fasaei and Tamai [1] recorded that 42.8% of *Salmonella* isolated from wild animals in Iran were resistant to AML. Even though, all isolates were susceptible to KF, CN and Ctx.

Table-4: Detection of *Listeria monocytogenes* infection in wild birds using different diagnostic tools.

Species	Examined N	Positive	Positive	PCR	Direct PCR
		Oxford agar n (%)	CHROM agar n (%)	Confirmed culture n (%)	Positive n (%)
Crows	150	13 (8.7)	6 (4)	2 (1.3)	2 (1.3)
Waterfowls	50	12 (24)	7 (14)	1 (2)	4 (8)
Total	200	25 (12.5)	13 (6.5)	3 (1.5)	6 (3)

χ^2=0.1128, p=0.7370 (non-significant)

Table-5: Direct PCR versus cultivation for detection of *Salmonella* infection in wild birds.

PCR result	Cultivation (Gold standard)	Cultivation (Gold standard)	Total N
	Positive N	Negative N	
Direct PCR Positive	18 True positive (TP)	6 False positive (FP)	24
Direct PCR Negative	7 False negative (FN)	169 True negative (TN)	176
Total	25 (TP+FN)	175 (TN+FP)	200
Calculation	SE%=(TP)/(TP+FN)×100 SE%=18/(18+7)×100=72%	SP%= (TN)/(TN+FP) × 100 SP%= 169/(169+6)×100=96.6%	

McNemar Chi square=0.00, p=1 (non-significant)

Table-6: Direct PCR versus cultivation for detection of *listeria monocytogenes* infection in wild birds.

PCR result	Cultivation (Gold standard)	Cultivation (Gold standard)	Total N
	Positive N	Negative N	
Direct PCR Positive	2 True positive (TP)	4 False positive (FP)	6
Direct PCR Negative	1 False negative (FN)	193 True negative (TN)	194
Total	3 (TP+FN)	197 (TN+FP)	200
Calculation	SE%=(TP)/(TP+FN)×100 SE%=2/(2+1)×100= 66.7%	SP%=(TN)/(TN+FP)×100 SP%=193/(193+4)×100=98%	

McNemar Chi square=0.800, p=0.3711 (non-significant)

In general, antibiotic resistance is an emerging global problem. A growing list of infections - such as tuberculosis, septicemia, and foodborne diseases - are becoming harder, and sometimes impossible, to treat as antibiotics become less efficient. The problem stemmed from the misuse of antimicrobials. In countries without standard treatment guidelines, antibiotics are often overprescribed by physicians and veterinarians and overused by the public. In these countries where antibiotics can be bought for medical or veterinary use without a prescription, the emergence and spread of resistance are made worse [41]. Therefore, an urgent action plan is needed; otherwise, the world is heading for a post-antibiotic era, in which common diseases and minor injuries could kill again [10,41].

Prevalence of *L. monocytogenes* in crows and waterfowls

The prevalence of *L. monocytogenes* infection in wild birds as confirmed by PCR was 1.5% (3/200); for crows, it was 1.3%, while the prevalence of infection in waterfowls was 2%.

In the current study, we used UPB which allows simultaneous detection of *Salmonella* and *Listeria*. It allows sublethally injured bacteria to resuscitate and proliferate to high levels. Another crucial point, its low carbohydrates formula which prevents the pH of the medium from dropping rapidly in the presence of microorganisms which is a keystone for survival of *Listeri*a spp. [13,42].

Previous studies carried out worldwide had reported different rates of *Listeria* infection among the examined wild bird species. Yoshida *et al.* [7] recorded, in Japan, that crows and green-winged teals carry *Listeria* spp. at the rate of 43.2% (130/301) and 2.6% (3/115), respectively. Moreover, *L. monocytogenes* was recovered from crows at the rate of 1.7% (5/301), while it could not be recovered from green-winged teals and mallards. Hellström *et al.* [6] collected wild birds' feces (n=212) from a municipal landfill site and urban areas in the Helsinki region. The prevalence of *L. monocytogenes* in bird feces was 36%. They could not detect *L. monocytogenes* in the examined crow feces

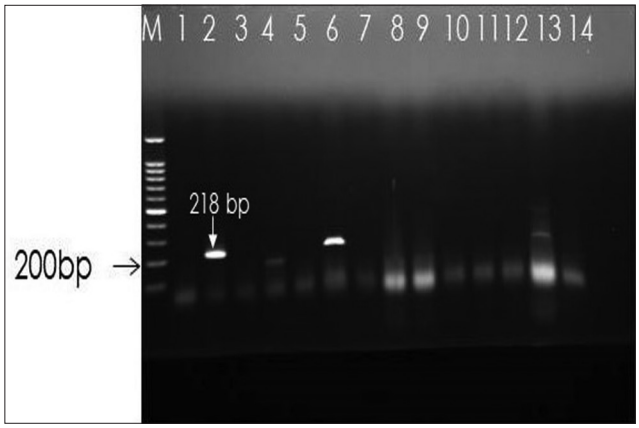

Figure-1: Agarose gel electrophoresis of the polymerase chain reaction products amplifying enterotoxin gene of *Salmonella*, M: 100 bp marker ladder, Lane 1: Negative control, Lanes 2, 6, 13 *Salmonella*-positive samples showed bands at 218 bp.

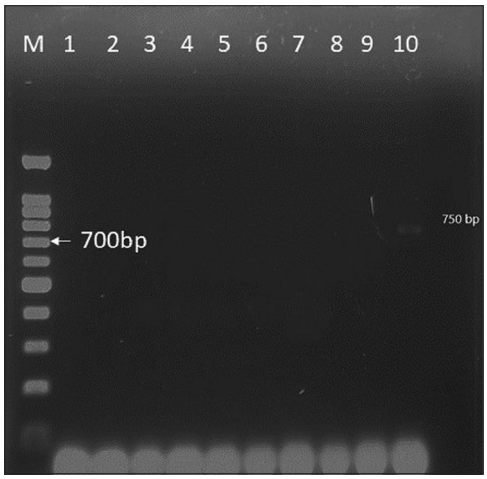

Figure-2: Agarose gel electrophoresis of the polymerase chain reaction products amplifying listeriolysin gene of *Listeria monocytogenes*, M: 100 bp marker ladder, Lane 2: Negative control, Lane 10: *L. monocytogenes*-positive sample showed clear band at 750 bp.

(n=1). Jagtap *et al.* [28] examined 165 fecal samples from 12 peridomestic bird species for *L. monocytogenes*. Virulent *L. monocytogenes* serogroup 4b strain was recovered from 10% (1/10) of crows' droppings.

These variations in the prevalence rates can be attributed to several factors like sampling size, methods of diagnosis, feeding habitat and living environment of the birds themselves. To sum up, handling birds that excrete enteric zoonotic pathogens or their contaminated environment may result in infection in human contacts [4,6,27,31].

Direct PCR versus cultivation

Direct PCR of UPB revealed that 12% (24/200) and 3% (6/200) of the examined wild birds' samples were positive for *Salmonella* and *L. monocytogenes*, respectively. Matching the results of PCR confirmed isolates with direct PCR using McNemar matching coefficient revealed that the difference between the two methods was non-significant. This means that direct detection of these pathogens using PCR is comparable to cultivation method. However, in terms of labor and money, direct PCR has the advantage of rapid detection of *Salmonella* from enrichment broth within 30 h of sampling and for *L. monocytogenes* within 60 h of sampling, with less labor and money. Traditional cultivation and typing methods will be accomplished in about 14 days. The use of boiling technique for DNA extraction helped to save extra expenses.

Accordingly, Freschi *et al.* [19] highly appreciated the speed and low cost of the boiling-centrifugation technique for direct detection of *Salmonella* from broth culture. Schrank *et al.* [20] found that direct PCR technique was more sensitive in detecting infected animals than the standard microbiological procedure. In the same line, Jeršek *et al.* [43] tested different enrichment broths for the detection of *L. monocytogenes* in artificially contaminated food samples. They recorded that only UPB allowed the detection of *L. monocytogenes* in samples after 24 h of incubation. They also found that the PCR-based method gave equal results as a standard cultural method by analysis of food samples. Moreover, PCR-based method briefed the time required for the detection of infection.

We could confirm by sequencing the presence of other potential human pathogens on Listeria Oxford Agar like *S. sciuri* and *L. massiliensis*. *S. sciuri* is a Gram-positive, animal-associated bacterial pathogen, but its clinical pertinence for humans is increasing, since it has been associated with various human infections [44]. *L. massiliensis* is a Gram-negative, endospore-forming bacterium. It has been previously isolated from a patient with cerebellar syndrome [45].

Conclusion

It is evident that wild birds can act as vectors of potential antibiotic-resistant zoonotic pathogens. Given these points, the implementation of national and regional surveillance systems on zoonotic pathogens must be highly appreciated to check up the epidemiologic status of these pathogens among wild birds and humans and to take the appropriate intervention measures.

Authors' Contributions

HMF was responsible for the collection of samples, statistical analysis, performance of the experiments, writing and revising of the manuscript. AA shared in writing and revising the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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