BioMed Research International

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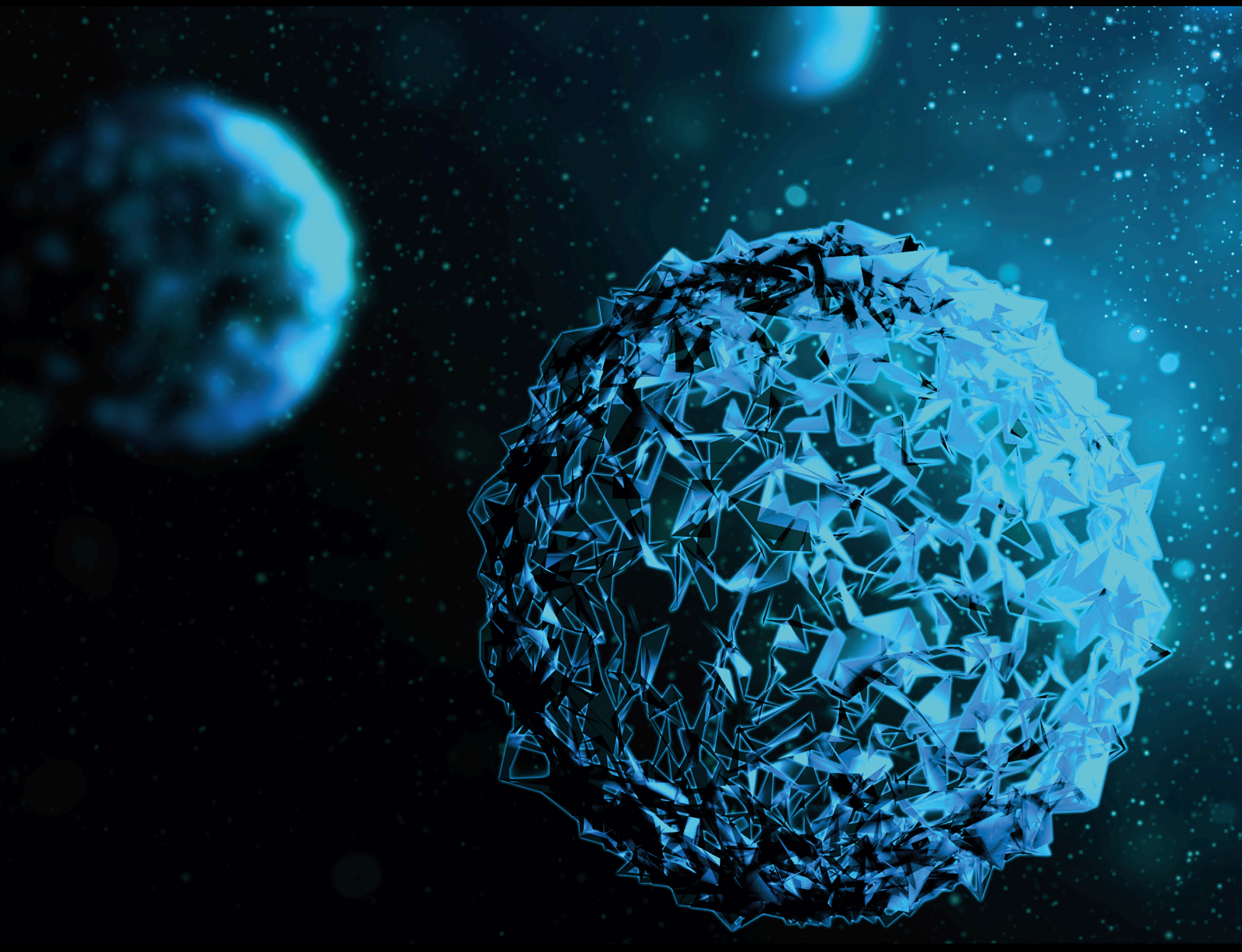

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Contents

Hindawi
BioMed Research International
Volume 2020, Article ID 8936591, 6 pages
https://doi.org/10.1155/2020/8936591

Research Article

Bioserotypes, Virulence Markers, and Antimicrobial Susceptibility of *Yersinia enterocolitica* Strains Isolated from Free-Living Birds

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Received 21 November 2019; Revised 28 February 2020; Accepted 5 March 2020; Published 17 March 2020

Academic Editor: Pengjun Shi

The risk of meat contamination with *Yersinia enterocolitica* poses a threat to consumers and persons who come into contact with bird carcasses. The occurrence of *Y. enterocolitica* in the vast majority of migratory game species, the capercaillie, and the black grouse has never been studied in Poland, Europe, or in the world. The material for the study consisted of cloacal swabs obtained from 143 Eurasian coots, 50 mallards, 30 pochards, 27 greylag geese, 22 white-fronted geese, 22 bean geese, 20 green-winged teals, and 10 tufted ducks, as well as fecal swabs obtained from 105 capercaillie and 18 black grouse. Bacteriological examinations of 894 samples taken from 447 birds led to the isolation of 20 strains with the biochemical features characteristic of the genus *Yersinia*. All 20 strains were molecularly examined, and the genes characteristic of *Y. enterocolitica* were detected in 8 strains. The isolated strains harbored amplicons whose size corresponded to *ystB* gene fragments. Four strains belonged to bioserotype 1A/NI, one strain was identified as bioserotype 1B/O:9, and one as 1A/O:9. The prevalence of *Y. enterocolitica* was determined at 1.4% in green-winged teals, at 5.0% in Eurasian coots, and at 4.8% in capercaillie. All strains were resistant to amoxicillin with clavulanic acid, ampicillin, and cefalexin. The strains isolated from migratory birds were also resistant to kanamycin and streptomycin, and they were characterized by resistance or intermediate resistance to cefotaxime, ceftazidime, chloramphenicol, gentamycin, and tetracycline, to which the strains isolated from the capercaillie were susceptible. *Yersinia enterocolitica* was not detected in the remaining bird species. The presence of *Y. enterocolitica* in green-winged teals, Eurasian coots, and capercaillie indicates that these birds could be carriers, potential reservoirs, and sources of infection for humans. They can also be regarded as reliable bioindicators of *Y. enterocolitica* in their respective habitats.

1. Introduction

Free-living birds are a highly interesting and insufficiently investigated group of animals. Game birds play an important role in public health as a potential source of infection with pathogens dangerous for human health. In Poland, 13 species of free-living birds can be hunted: pheasant (*Phasianus colchicus*), green-winged teal (*Anas crecca*), tufted duck (*Aythya fuligula*), greylag goose (*Anser anser*), white-fronted goose (*Anser albifrons*), bean goose (*Anser fabalis*), pochard (*Aythya ferina*), wood pigeon (*Columba palumbus*), hazel grouse (*Tetrastes bonasia*), mallard (*Anas platyrhynchos*), partridge (*Perdix perdix*), Eurasian coot (*Fulica atra*), and Eurasian woodcock (*Scolopax rusticola*). Most of these birds are migratory species that travel considerable distances during migrations and use natural water resources, which underscores their importance in the epidemiological context. The capercaillie (*Tetrao urogallus*) and the black grouse (*Tetrao tetrix*) are classified as endangered species in Poland [1]. However, despite the progressing decrease in the size of capercaillie and black grouse populations, these species are regarded as game birds in some countries (e.g., Belarus,

Russia, Sweden, Finland, and Norway) and are hunted for meat and trophies [1]. Unlike other game birds, the capercaillie and the black grouse do not migrate and inhabit specific territories.

The genus *Yersinia* of the family *Enterobacteriaceae* is composed of 19 species, three of which (*Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*) are pathogenic for humans and animals [2, 3]. Although yersiniosis is not highly prevalent and dangerous for animals, animals are often carriers and sources of *Yersinia* infections for humans. In Europe, *Y. enterocolitica* is the most important etiological factor of human yersiniosis, whereas *Y. pseudotuberculosis* infections rarely cause yersiniosis [4]. Raw or undercooked pork is the main source of human *Y. enterocolitica* infections, and pigs are the main reservoir of bacteria [5, 6]. However, *Y. enterocolitica* was also detected in various species of wild animals. In Poland, the pathogen has been identified in wild boars, roe deer, red deer, and fallow deer [7–9]. In the group of game birds, only pheasants and mallards have been tested for the presence of *Y. enterocolitica* in Poland [10]. The risk of meat contamination with *Y. enterocolitica* poses a threat to consumers and persons who come into contact with bird carcasses. The occurrence of *Y. enterocolitica* in the vast majority of migratory game species, the capercaillie, and the black grouse has never been studied in Poland, Europe, or in the world.

Yersinia enterocolitica strains are classified into six biotypes (1A, 1B, and 2-5) and more than 70 serotypes. Pathogenic strains harbor the *Yersinia* virulence plasmid (pYV) and *ail* and *ystA* chromosomal genes which encode the production of attachment invasion locus (Ail) protein and *Yersinia*-stable toxin A (YstA), respectively [2]. Biotype 1A strains that do not harbor pYV and *ail* and *ystA* genes are usually considered nonpathogenic [2, 11, 12]. However, in recent years, *Y. enterocolitica* biotype 1A strains have also been isolated from clinical cases of yersiniosis presenting with diarrhea [6, 13, 14]. Although biotype 1A strains very rarely produce YstA enterotoxin, they usually harbor the *ystB* gene which encodes the production of YstB enterotoxin. The minimal lethal dose of YstB is much lower in comparison with YstA enterotoxin [15, 16]. Extremely rare strains of biotype 1A harbor the *ystC* gene which encodes the production of YstC enterotoxin [16], and they have never been isolated from clinical cases of yersiniosis.

The aim of this study was to identify the bioserotypes, virulence markers, and antimicrobial susceptibility of *Y. enterocolitica* strains isolated from the different species of game birds in Poland, as well as from the capercaillie and black grouse.

2. Materials and Methods

2.1. Materials. The material for the study consisted of 286 cloacal swabs obtained from 143 Eurasian coots, 100 cloacal swabs obtained from 50 mallards, 60 cloacal swabs obtained from 30 pochards, 54 cloacal swabs obtained from 27 greylag geese, 44 cloacal swabs obtained from 22 white-fronted geese, 44 cloacal swabs obtained from 22 bean geese, 40 cloacal swabs obtained from 20 green-winged teals, and 20 cloacal swabs obtained from 10 tufted ducks. Two samples were collected from each bird and cultured in two different media. The samples from free-living birds were collected during the hunting seasons specific for each species, between autumn 2016 and spring 2019, in hunting districts throughout Poland.

Two hundred and ten fecal swabs from 105 capercaillie fecal samples and 36 fecal swabs from 18 black grouse fecal samples were also collected. Most capercaillie were kept as a part of the EU LIFE Project entitled "Active protection of lowland populations of capercaillie in the Bory Dolnośląskie and the Augustowska Primeval Forest", authorization no. DZP-WG.6401.03.100.2016.km of 31 May 2016, granted by the General Directorate of Environmental Protection. Some samples were collected from wild capercaillie in the Kirov region (Russia) and the Wildlife Park in Kadzidłowo (Poland). All samples were collected from aviaries in breeding centers in 2016-2017. Black grouse samples were collected in June 2017 in the Jedwabno State Forest District which features 5 black grouse preservation zones.

2.2. Biochemical Identification. A total of 894 swabs collected from 447 birds were examined. One swab was placed on the ITC (irgasan, ticarcillin, and potassium chlorate—warm culture) medium, and the other swab was placed on the PSB (peptone, sorbitol, and bile salts—cold culture) medium. Swabs on ITC were incubated at 25°C for 48 h, and swabs on PSB were incubated at 4°C for 3 weeks to determine the ability of *Yersinia* to grow at low temperatures. Next, 0.5 ml of each culture was transferred to 4.5 ml of 0.5% KOH in 0.5% NaCl for 20 s, and a loopful was streaked onto a CIN (cefsulodin, irgasan, and novobiocin) plate and incubated at 30°C for 48 h. Colonies typical for *Yersinia* were transferred to MacConkey agar, incubated at 30°C for 24 h and biochemically analyzed using API 20E strips (bioMérieux, France) according to the manufacturer's instructions.

2.3. DNA Isolation. Genomic DNA was isolated with the Genomic Mini Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. DNA was stored at -20°C for further analyses.

2.4. Molecular Confirmation of Y. enterocolitica Strains. Four chromosomal genes—*ail*, *ystA*, *ystB*, and *ystC*—were amplified in one reaction. The primers for *ail*, *ystA*, *ystB*, and *ystC* were described previously [7, 17]. Multiplex PCR was performed using HotStarTaq Plus DNA Polymerase (Qiagen GmbH, Hilden, Germany) and HotStarTaq Plus Master Mix Kit (Qiagen). The reaction mixture of 20 μl contained approximately 120 ng of isolated DNA (1 to 3 μl), 10 μl of the HotStarTaq Plus Master Mix 2x, 2 μl of CoralLoad Concentrate 10x, and 0.1 μl of each primer (final concentration of 0.5 μM), and it was supplemented with up to 20 μl of RNase-free water. The applied reaction conditions included a preliminary denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 60 s, and elongation at 72°C for 45 s. The last reaction was followed by extension at 72°C for 10 min. Amplicon size was evaluated by comparison with the standard mass of

GeneRuler 100 bp Ladder Plus (Fermentas UAB, Vilnius, Lithuania). The following amplicons were searched: *ail* gene fragments with the size of 356 bp, *ystA* gene fragments with the size of 134 bp, *ystB* gene fragments with the size of 180 bp, and *ystC* gene fragments with the size of 284 bp. The specificity of all amplicons was confirmed by purification with the CleanUp kit (A&A Biotechnology, Gdynia, Poland) and sequencing (Genomed, Warsaw, Poland).

2.5. Y. enterocolitica Serotype and Biotype Identification. The molecularly confirmed *Y. enterocolitica* strains were serotyped in the slide agglutination test according to a previously described methodology [7] with use of somatic antigens O:3, O:5, O:8, O:9, and O:27 (Sifin, Berlin, Germany). The strain was classified as nonidentified (NI) in the absence of agglutination with any of the sera. The biotype was determined based on pyrazinamidase and Tween esterase activity, esculin hydrolysis, indole production, and salicin, xylose, and trehalose fermentation according to the PN-EN ISO 10273 standard.

2.6. Antimicrobial Susceptibility of the Isolated Y. enterocolitica Strains. Yersinia enterocolitica strains were tested for susceptibility to amoxicillin with 30 μg of clavulanic acid, 10 μg of ampicillin, 30 μg of cefotaxime, 30 μg of ceftazidime, 30 μg of cefalexin, 5 μg of ciprofloxacin, 30 μg of chloramphenicol, 10 μg of gentamycin, 30 μg of kanamycin, 30 μg of nalidixic acid, 10 μg of streptomycin, sulfamethoxazole/trimethoprim 19 : 1, and 30 μg of tetracycline (Oxoid, Thermo Scientific). These analyses were performed by the standard disc diffusion technique after 24 h incubation on Mueller-Hinton (Oxoid, Thermo Scientific) agar plates at 30°C, and they were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [18].

3. Results

Bacteriological examinations of 894 samples collected from 447 birds led to the isolation of 20 strains with biochemical features characteristic of the genus *Yersinia*. Seven strains were classified as *Y. kristensenii*, 5 strains as *Y. frederiksenii*, 7 strains as *Y. enterocolitica*, and 1 strain as *Y. frederiksenii*/*Y. intermedia* with the use of API 20E. All 20 strains were molecularly examined, and the genes characteristic of *Y. enterocolitica* were detected in 8 strains. Six strains were biochemically identified as *Y. enterocolitica*, and the remaining two strains were identified as *Y. frederiksenii*. One strain, initially classified as a *Y. enterocolitica* with API 20E, was not molecularly confirmed.

The prevalence of *Y. enterocolitica* in green-winged teals was determined at 1.4%. Two strains were isolated from the samples collected in the Małopolska voivodeship. The strains harboring amplicons of *ystB* gene fragments originated from the warm culture (ITC). They belonged to biotype 1A and were serotyped as NI (nonidentified) due to the absence of agglutination with any of the analyzed sera. The results of the biochemical and molecular analyses and the biotypes and serotypes of the isolated *Y. enterocolitica* strains are presented in Table 1.

All *Y. enterocolitica* strains were resistant to amoxicillin with clavulanic acid, ampicillin, cefalexin, kanamycin, and streptomycin. They were intermediately resistant to cefotaxime, ceftazidime, chloramphenicol, and tetracycline. All *Y. enterocolitica* strains were susceptible only to sulfamethoxazole with trimethoprim, and they differed in susceptibility to the remaining antimicrobials. The antimicrobial susceptibility of *Y. enterocolitica* strains is presented in Table 2.

The prevalence of *Y. enterocolitica* in Eurasian coots was determined at 5.0%. Similarly to green-winged teals, one strain was isolated from the sample collected in the Małopolska voivodeship. This strain was *ystB*-positive, and it originated from the cold culture (PSB) and was bioserotyped as 1A/NI (Table 1). The antimicrobial susceptibility test revealed that the isolated *Y. enterocolitica* strain was resistant to amoxicillin with clavulanic acid, ampicillin, cefalexin, cefotaxime, kanamycin, and streptomycin. The strain was susceptible to nalidixic acid and sulfamethoxazole with trimethoprim, and it was intermediately susceptible to the remaining antibiotics. Detailed characteristics are presented in Table 2.

The prevalence of *Y. enterocolitica* in capercaillie fecal samples was determined at 4.8%. Five strains were isolated from the fecal material collected in the Wildlife Park in Kadzidłowo, and the prevalence of *Y. enterocolitica* in these samples reached 62.5%. All strains harbored amplicons whose size corresponded to *ystB* gene fragments. Three strains originated from the ITC culture, and 2 strains originated from the PSB culture. Four strains belonged to biotype 1A, and only one strain was identified as biotype 1B. Serotyping revealed that 1 of the 5 isolated *Y. enterocolitica* strains represented the O:9 serotype, and the remaining strains were not identified. Two *Y. enterocolitica* strains with different bioserotypes (1A/O:9 and 1A/NI) were isolated from a fecal sample collected from the same female. Detailed results of the biochemical and molecular analyses and the biotypes and serotypes of the isolated *Y. enterocolitica* strains are presented in Table 1. All *Y. enterocolitica* strains were resistant to amoxicillin with clavulanic acid, ampicillin, and cefalexin. One strain was additionally resistant to sulfamethoxazole with trimethoprim, and one strain was also resistant to kanamycin. Two of the tested strains were intermediately resistant to chloramphenicol. All *Y. enterocolitica* strains were susceptible to the remaining antibiotics. The antimicrobial susceptibility of *Y. enterocolitica* strains is presented in Table 2.

The bacteriological examinations of 36 fecal swabs from black grouse led to the isolation of 1 strain with biochemical features characteristic of the genus *Yersinia*. This strain was biochemically classified as *Y. kristensenii*. The molecular analysis confirmed that the strain did not harbor amplicons of *yst* genes characteristic of *Y. enterocolitica*.

One strain with biochemical features characteristic of the genus *Yersinia* was also isolated from white-fronted geese. The molecular analysis confirmed that this strain harbored amplicons of the *ail* gene. However, the *ail* gene never occurs alone in *Y. enterocolitica* strains, and it is always accompanied by one of the *yst* genes. In biochemical tests involving API 20E, this strain revealed features characteristic of *Y. frederiksenii*/*Y. intermedia*.

Table 1: Characteristics of *Y. enterocolitica* strains isolated from free-living birds.

Strain no.	Bird species	Biochemical identification API 20E	Molecular examination *ail*	*ystA*	*ystB*	*ystC*	Molecular identification	Bioserotype
30PSB	Capercaillie	*Y. enterocolitica*	-	-	+	-	*Y. enterocolitica*	1A/NI
31PSB	Capercaillie	*Y. frederiksenii*	-	-	+	-	*Y. enterocolitica*	1A/NI
35ITC	Capercaillie	*Y. frederiksenii*	-	-	+	-	*Y. enterocolitica*	1A/NI
35PSB	Capercaillie	*Y. enterocolitica*	-	-	+	-	*Y. enterocolitica*	1A/O:9
36ITC	Capercaillie	*Y. enterocolitica*	-	-	+	-	*Y. enterocolitica*	1B/NI
116ITC	Eurasian coot	*Y. enterocolitica*	-	-	+	-	*Y. enterocolitica*	1A/NI
117ITC	Eurasian coot	*Y. enterocolitica*	-	-	+	-	*Y. enterocolitica*	1A/NI
1GWPB	Green-winged teal	*Y. enterocolitica*	-	-	+	-	*Y. enterocolitica*	1A/NI

Table 2: Antimicrobial susceptibility of *Y. enterocolitica* strains isolated from free-living birds.

Antimicrobial	Strain no. 30 PSB	31 PSB	35 ITC	35 PSB	36 ITC	116 ITC	117 ITC	1GW PSB
Amoxicillin+clavulanic acid 30 μg	R[a]	R	R	R	R	R	R	R
Ampicillin 10 μg	R	R	R	R	R	R	R	R
Cefotaxime 30 μg	S[b]	S	S	S	S	I	I	R
Ceftazidime 30 μg	S	S	S	S	S	I	I	I
Cephalexin 30 μg	R	R	R	R	R	R	R	R
Ciprofloxacin 5 μg	S	S	S	S	S	I	S	I
Chloramphenicol 30 μg	I[c]	S	S	I	S	I	I	I
Gentamycin 10 μg	S	S	S	S	S	R	I	I
Kanamycin 30 μg	S	R	S	S	S	R	R	R
Nalidixic acid 30 μg	S	S	S	S	S	R	I	S
Streptomycin 10 μg	S	S	S	S	S	R	R	R
Sulfamethoxazole/trimethoprim 19 : 1	R	S	S	S	S	S	S	S
Tetracycline 30 μg	S	S	S	S	S	I	I	I

[a]R: resistant; [b]S: susceptible; [c]I: intermediately resistant.

Strains with biochemical features characteristic of the genus *Yersinia* were not identified in bacteriological analyses of the samples collected from mallards, pochards, greylag geese, bean geese, and tufted ducks.

4. Discussion

Yersinia enterocolitica is one of the most important etiological factors of human diarrhea [4]. Research into the prevalence of *Y. enterocolitica* in various animal species is vital because products of animal origin are the main sources of infection for humans [19]. The prevalence of *Y. enterocolitica* in wild animals has been studied in Poland, Europe, and in other regions of the world. However, some species of free-living birds have never been investigated. In Poland, mallards and pheasant were the only bird species to be tested for *Y. enterocolitica*. Molecularly confirmed *Y. enterocolitica* strains were isolated from 5 out of 45 mallards (11.1%) and were not detected in any of the tested pheasants [7].

In Europe, Levrè et al. [20] examined intestinal loops from 217 birds of 17 species. Thirty-seven strains belonging to the genus *Yersinia* were isolated from 26 birds. Most of the isolates were identified as *Y. enterocolitica* (28 strains), 3 isolates as *Y. frederiksenii*, 3 isolates as *Y. intermedia*, and 1 isolate as *Y. pseudotuberculosis*. However, molecular tests were not conducted. More recently, Foti et al. [21] tested 218 fecal swabs and 21 internal organs from different migratory birds. Eight *Y. enterocolitica* strains (3.35%) were detected in analyses involving bacteriological examinations and API 20E, but molecular tests for *Y. enterocolitica* were not carried out.

The prevalence of *Y. enterocolitica* in wild birds was also studied outside Europe. In Japan, Kato et al. [22] detected *Y. enterocolitica* strains in 2 pheasants, 1 domestic pigeon, 1 tree sparrow, 5 crows, 5 blue magpies, 4 bulbuls, and 2 grey starlings, which accounted for 4% of the tested samples. Shayegani et al. [23] found that 3.3% of 576 wild birds examined in the USA were positive for *Y. enterocolitica*. Bacterial strains were isolated from 2 great horned owls, 1 Canada goose, 2 wild turkeys, 1 mallard, 1 red-tailed hawk, 1 cowbird, 1 starling, 2 grackles, 1 ring-billed gull, 1 ruffed grouse, 2 red-winged blackbirds, 1 sparrow hawk, 1 canvasback duck, 1 long-eared owl, and 1 wood duck. In Malaysia, *Y. enterocolitica* strains were isolated from 32 out of 291 (11.0%)

examined wild ducks and from 13 out of 180 (7.2%) examined wild geese [24].

In our study, *Y. enterocolitica* strains were detected in 1.4% of green-winged teals, 4.8% of capercaillie, and 5.0% of Eurasian coots. Our findings do not differ significantly from those reported by other authors, but none of the cited authors relied on molecular methods which are much more accurate and reliable. In our study, the results of the analysis conducted with API 20E did not fully correspond to the results of the molecular test for *Y. enterocolitica* virulence markers. Four strains were identified as *Y. enterocolitica* using API 20E, but *ystB* gene fragments were detected in 3 strains only. However, 2 strains identified as *Y. frederiksenii* with API 20E were molecularly classified as *Y. enterocolitica*. The above findings indicate that molecular examinations are essential for proper detection and classification of the isolated strains.

Interestingly, the strains identified in the capercaillie were isolated from a single location. The above could be attributed to the fact that *Y. enterocolitica* is easily transmitted. The pathogen is transferred between animals when the carriers are present in the environment [25]. The high percentage of *Y. enterocolitica* strains isolated in the Wildlife Park in Kadzidłowo could result from pathogen transmission in aviaries in breeding centers. The above could pose a threat for humans who come into direct contact with infected birds.

In our study, 1A/NI was the predominant bioserotype. The presence of single *Y. enterocolitica* strains belonging to bioserotypes 1A/O:9 and 1B/NI was also demonstrated. In a study by Kato et al. [22], *Y. enterocolitica* strains were also classified as biovar 1 (biotype 1) with different serotypes (O:3; O:4; O:4,32; O:5A; O:6,30; O:7,8; and O:14). Shayegani et al. [23] classified 12 out of 19 *Y. enterocolitica* strains into nine serogroups (O:4,33; O:5,27; O:6,31; O:7,14; O:8,14; O:16; O:30,31; O:31; and O:34), and the remaining strains were not identified. In our study, 2 *Y. enterocolitica* strains with different bioserotypes were isolated from one fecal sample. Nikolova et al. [26] isolated different *Y. enterocolitica* strains from the same animal, which could indicate that the gastrointestinal tract can be colonized by more than one type of *Y. enterocolitica* strains.

The antimicrobial susceptibility analysis revealed that all tested isolates were resistant to amoxicillin with clavulanic acid, ampicillin, and cefalexin. It should also be noted that the strains isolated from migratory birds (green-winged teals, Eurasian coots) and those obtained from birds living in a specific territory (capercaillie) differed in resistance to chemotherapeutics. The strains isolated from migratory birds were also resistant to kanamycin and streptomycin, and they were resistant and intermediately resistant to cefotaxime, ceftazidime, chloramphenicol, gentamycin, and tetracycline, to which the strains isolated from the capercaillie were susceptible. The fact that these strains were susceptible to only one or two chemotherapeutics gives serious cause for concern because migratory birds use public drinking water sources. Our results cannot be compared with the findings of Foti et al. [21] who studied antimicrobial susceptibility but did not report specific results for *Y. enterocolitica* strains.

5. Conclusions

The presence of *Y. enterocolitica* in green-winged teals, Eurasian coots, and capercaillie indicates that these birds could be carriers, potential reservoirs, and sources of infection for humans. These birds can also be regarded as reliable bioindicators of *Y. enterocolitica* in their respective habitats.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

This study was funded by the KNOW (Leading National Research Centre) Scientific Consortium "Healthy Animal–Safe Food," decision of Ministry of Science and Higher Education No. 05-1/KNOW2/2015. This publication was funded by the Minister of Science and Higher Education in the range of the program entitled "Regional Initiative of Excellence" for the years 2019-2022, Project No. 010/RID/2018/19, amount of funding 12,000,000 PLN.

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Hindawi
BioMed Research International
Volume 2020, Article ID 7594136, 7 pages
https://doi.org/10.1155/2020/7594136

Research Article

Salmonella spp. in Wild Free-Living Birds from Atlantic Forest Fragments in Southern Bahia, Brazil

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Received 20 November 2019; Revised 13 January 2020; Accepted 6 February 2020; Published 2 March 2020

Guest Editor: Aleksandra Platt-Samoraj

Wild animals have an ecological function and can serve as sentinels to identify infectious agents and as indicators of environmental health. Among the zoonotic pathogens, *Salmonella* spp. deserve special attention due to their high worldwide prevalence and their ubiquity of hosts. With the aim of investigating the presence of *Salmonella* spp. in wild birds from the Atlantic Forest in southern Bahia, Brazil, we collected 114 fecal samples of wild birds (14 families) between 2016 and 2017. Fecal samples were collected by means of cloacal swab and subjected to microbiological culture to isolate and serotype *Salmonella* spp. specifically. Antibiotic susceptibility was determined using the disk diffusion test protocol. Only one bird, *Ceratopipra rubrocapilla*, tested positive for *Salmonella enterica* subsp. *enterica* serotype Agona, which is the first record for this bird species. This isolate exhibited intermediate sensitivity to amikacin and gentamicin and sensitivity to the other 13 antibiotics tested. Results may indicate environmental preservation since the studied areas had minimal human activity and good sanitary quality. Despite the low prevalence, it is necessary to monitor wildlife and establish disease control and surveillance systems, especially for zoonotic diseases.

1. Introduction

The Atlantic Forest is the second largest forest in South America. Despite its extremely high levels of endemism (up to 40% for plants and 60% for amphibians), this biodiversity hotspot is also the most endangered biome of Brazil, as only 8% of its original area remains. The Brazilian population has been historically concentrated along the Southeastern coast, which resulted in more than 60% of the country's population (more than 100 million inhabitants) occupying the areas originally covered by the forest. This proximity to the effervescent development of the country's major capitals of the country leads to the extended cycles of land exploitation for the intensive production of commodity exports, particularly wood, sugarcane, and coffee. Additionally, the expansion of urban limits and country houses, illegal logging, subsidized soybean plantation, pine and eucalyptus production, palm heart extraction, wildlife poaching, and hydroelectric dams were additional factors that lead to the profound transformation of this landscape. The unprofitable areas of mountain ranges, marshes, and mangroves were the only areas spared from the devastation, alongside with a few small protected areas [1–3].

Currently, this biome accounts for less than 10% of the original native forest of the south of Bahia (Brazil) [3]. Even with deforestation, these remaining forest fragments are considered the centers of endemism of this ecosystem [4]. Human actions, such as irregular forest occupation and deforestation, may alter and influence the epidemiology of zoonosis by creating conditions for the dissemination of infectious agents

and even the resurgence of emerging diseases [5]. Consequently, these ecosystems should be monitored regarding the presence of pathogens that are important for public health, to ensure the early implementation of control strategies.

Wild animals have an ecological function and can serve as sentinels to identify infectious agents and as indicators of environmental health [6]. Among the zoonotic pathogens, *Salmonella* spp. deserve special attention due to their high worldwide prevalence and their ubiquity of hosts. This bacterium is often associated with outbreaks and food infections and it is one of the world's three main food-borne diseases [7] with high morbidity and mortality rates [8]. *Salmonella* spp. are usually found in the intestinal tract of humans and animals, and they are transmitted through feces that eliminate the agent and contaminate water and food [9].

Studies indicate that *Salmonella* Typhimurium is the most prevalent serovar in wild birds [10, 11] with reports of outbreaks of septicemia and deaths in wild birds in the United Kingdom, Canada, Sweden, Switzerland, and Norway [12–16]. Wild birds affected by *Salmonella* spp. can present specific symptoms, such as pneumonia, anorexia, diarrheal stools, and neurological disorders or even sudden death [17, 18]. On the other hand, one of the main characteristics of *Salmonella* is the latent carriers. Latency corresponds to a condition in which the individual does not present clinical symptoms but remains intermittently eliminating the agent in the feces [19]. However, there is a period in which the etiological agent remains hidden in an intracellular compartment and is not eliminated and therefore may mask laboratory results. Thus, these latent carriers become natural reservoirs and therefore maintain the pathogen in both the food chain and nature [20].

Studies on the health of wildlife, especially on the role of species as carriers of *Salmonella* spp., can help identify the elements involved in the dissemination of the bacterium in the environment and the association with health factors and epidemiological variables. Additionally, such studies can help detect species that are more vulnerable to the agent and correlate the presence of the bacterium to anthropic factors such as deforestation and human occupation of environmental areas.

Thus, the aim of this study was to investigate the presence of *Salmonella* spp. in wild birds of the Atlantic Forest biome, in the mesoregion of southern Bahia, and characterize it phenotypically regarding serotype and antimicrobial susceptibility. These data can complement information on epidemiology, control, and prevention of this agent and, consequently, help maintain the quality of public health.

2. Material and Methods

2.1. Study Area. The animals were sampled in three collection points in three municipalities of southern Bahia (Ilhéus, Una, and Uruçuca) (Figure 1), in areas of the Atlantic Forest biome. Climate in this region is typically humid tropical with an annual average temperature of 24°C and an annual rainfall of around 1300 mm [21].

The collection points (P1, P2, and P3) have the following geographic coordinates: P1 (municipality of Uruçuca in the *Fazenda Matinha* Area: 14°35′58.3″S, 39°16′33.7″W), P2 (municipality of Ilhéus, at the UESC: 14°47′52.06″S, 39°10′33.366″W), and P3 (municipality of Una at the *RPPN Nova Angélica* Estate: 15°14′59.0″S, 39°04′41.0″W). Collection point P1 is a fragment of the Cabruca agroforestry system, which is a reforested area with cocoa plantations. The other collection points (P2 and P3) are fragments of native Atlantic Forest.

2.2. Sampling of Animals. In all, 114 birds were caught using fog nets for about 10 hours per day in each area. In the municipality of Uruçuca (P1), samples were collected from May to June 2017 in a single sampling. The nets were inspected every 40 minutes for 4 consecutive days, applying the sample effort of 32400 $h \cdot m^2$. The site in Ilhéus (P2) was sampled in November 2016 and April and June 2017. In Una (P3), samples were collected in November 2016 and March and August 2017. In P2 and P3, nets were inspected every 40 minutes for 5 consecutive days, applying a sampling effort of 40500 $h \cdot m^2$ in each collection period. In P1, P2, and P3, the nets were controlled 15 times a day with the interval of 40 minutes. The number of leaks and losses was 17, and there were 8 deaths in total.

For the acquisition of fecal samples by means of cloacal swab, the birds were kept in paper boxes for about 1 hour. Sterile swab samples were collected from the cloacal region. After this procedure, the samples were identified and placed in sterile microtube containing 1 mL of Buffered Peptone Water (APT) culture medium under temperature conditions (15-25°C), to be transported to the facilities of the Santa Cruz State University Veterinary Hospital, where they were analyzed for the presence of *Salmonella* spp.

Once captured, the specimens were removed from the nets, identified, and classified by order and family [22]. Then, they were numbered in increasing order, photographed, marked temporarily with non-toxic ink, and released in the sampled site. Bird capture was carried out under authorization No. 53000-1, issued by the Chico Mendes Institute for Biodiversity Conservation (ICMBio), and approval No. 014/2014 of the Ethics Committee on Animal Use of the State University of Santa Cruz (UESC).

2.3. Isolation and Identification of Salmonella spp. Cloacal swab was incubated in 1 mL of Buffered Peptone Water–APT (Liofilchem) at 37°C/24 hours, for pre-enrichment. Then, selective enrichment was performed in 1 mL of Rappaport-Vassiliadis (RV) broth (Oxoid Ltd.) at 43°C/18-24 hours. On the third day, isolation was performed in xylose-lysine-deoxycholate-XLD agar (Neogen Corporation-Acumedia) and Hektoen-HE enteric agar (Becton, Dickinson and Company Sparks), both incubated in an incubator at 37°C/18-24 hours. The suspected colonies (red colonies with or without black center on XLD Agar and green to bluish-green colonies with or without black center in HE agar) were inoculated in 1 mL of Tryptone Soya Broth (TSB; HiMedia Laboratories Pvt. Ltd.) and incubated at 37°C/18-24 hours. Presumptive biochemical identification was performed using Triple Sugar Iron Agar (TSI; HiMedia Laboratories Pvt. Ltd.), Lysine Iron

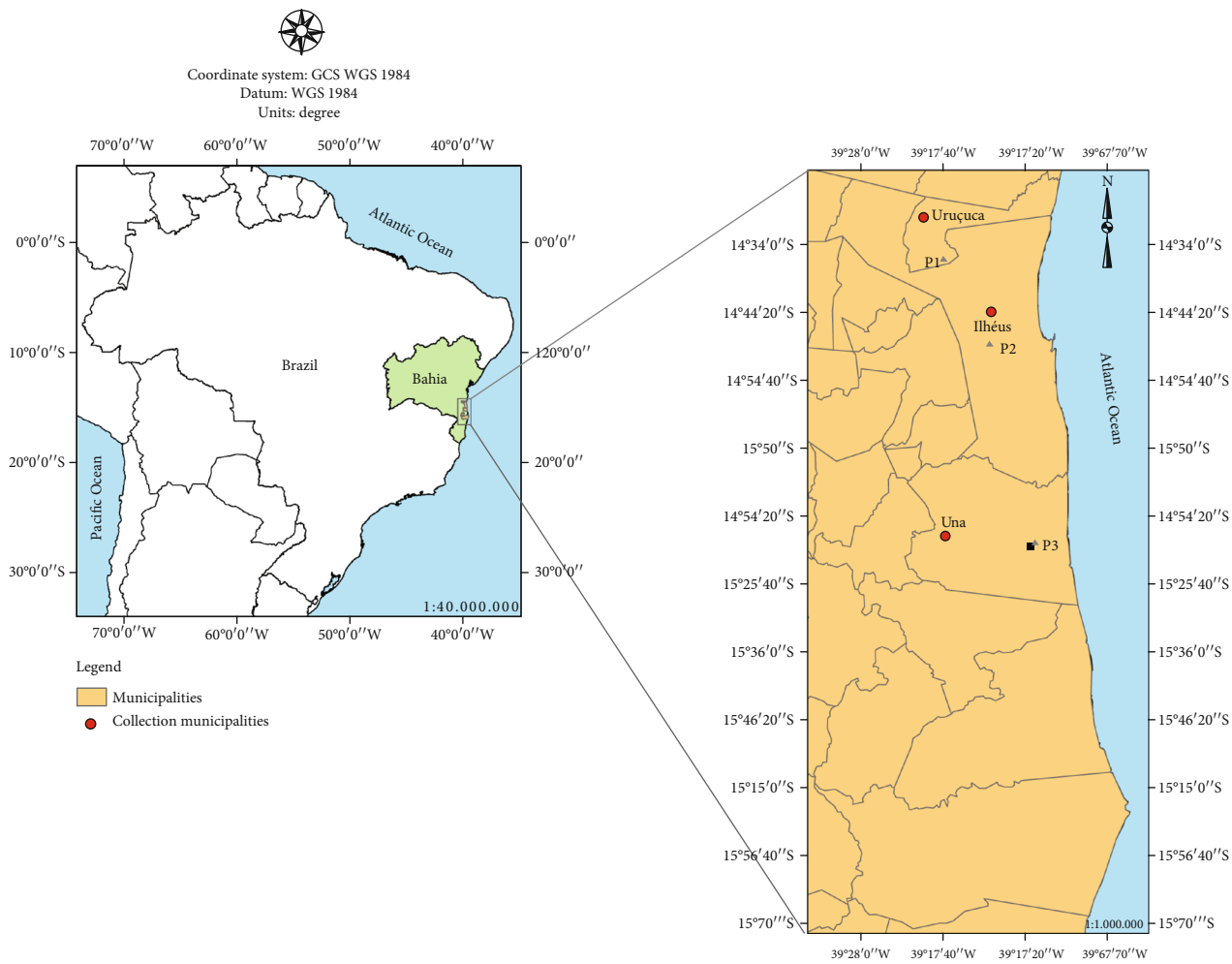

Figure 1: Bird collection sites in southern Bahia, Brazil.

Agar (LIA; HiMedia Laboratories Pvt. Ltd.), and a urease test (Neogen Corporation-Acumedia).

The presumptive colonies of *Salmonella* spp. identified in the biochemical tests were confirmed through polymerase chain reaction (PCR), based on a previous study [23]. Each 25 μL of the reaction contained 1X PCR (Invitrogen, Carlsbad, CA, USA); 1.25 mM $MgCl_2$ (Invitrogen); 200 μM dNTP; 10 pmol of each initiator ST11 (5′-AGCCAACCA TTGCTAAATTGGCGCA-3′) and ST15 (5′-TTTGCGACT ATCAGGTTACCGTGG-3′), specific for the genus *Salmonella* [24]; 1.25 U of Taq DNA polymerase (Invitrogen); and a suspicious colony of the pathogen. The reaction volume was completed to 25 μL with sterile water free of nucleases. *Salmonella* enteritidis PT1 was used for positive control, the colony being omitted from the reaction for the negative control. Reactions were performed in a ProFlex PCR thermal cycler (Applied Biosystems, Life Technologies, Carlsbad, USA). The amplification cycles consisted of 5 min at 94°C for initial denaturation, followed by 35 cycles (30 sec at 94°C, 30 sec at 62°C, and 1 min at 72° C), and a final extension step of 7 min at 72°C. The PCR product was visualized in 1% agarose gel stained with SYBER-Green (Invitrogen) and examined under UV light.

Positive PCR samples were cultivated in tryptic soy agar (TSA; HiMedia Laboratories Pvt. Ltd.) and sent to the Enterobacteria Laboratory of the Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, for serotyping using serogroup- and serotype-specific antisera.

2.4. Antimicrobial Susceptibility Test. Antibiotic susceptibility was determined using the disk diffusion procedure in Mueller-Hinton agar (HiMedia Laboratories Pvt. Ltd.), with bacterial suspension adjusted to a turbidity of 0.5 McFarland standard, according to the Kirby-Bauer method [25] and following the guidelines by the Clinical Laboratory Standards Institute [26]. The disks used in the test were amikacin (30 μg), amoxicillin/clavulanic acid (20/10 μg), ampicillin + sulbactam (10/10 μg), cefepime (30 μg), cefoxitin (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), gentamicin (10 μg), imipenem (10 μg), lomefloxacin (10 μg), norfloxacin (10 μg), piperacillin-tazobactam (100/10 piperacillin-tazobactam), sulphazotrim (trimethoprim/-sulfamethoxazole 25 μg), tobramycin (10 μg), and trimethoprim (5 μg) (LABORCLIN—Produtos para Laboratórios Ltda, Pinhais—Paraná, Brazil). The culture of *Escherichia coli* ATCC 25922 was used to control test quality.

2.5. Statistical Data Analysis. The signal test, which is a nonparametric statistical method, was applied to verify the agreement of the methodologies tested in the identification of *Salmonella* spp.

3. Results

A total of 114 samples of wild birds were studied: 77 being of undefined sex (13 juveniles and 64 adults), 19 males (7 juveniles and 12 adults), and 18 females (5 juveniles and 13 adults). The samples were of 32 different species, 4 of which were not identified (Table 1). None of the animals showed clinical signs or gross lesions that could be associated with salmonellosis (e.g., diarrhea, anorexia, neurological disorders, polyuria/polydipsia syndrome, and respiratory problems, such as dyspnea and pneumonia) [17, 18].

After biochemical tests, the PCR confirmed only one positive sample for *Salmonella* spp., in a bird of the species *Ceratopipra rubrocapila* (Figure 2). This specimen was an adult female found in the Cabruca system of the UESC site (P2).

In serotyping, this isolate was identified as *Salmonella enterica* subsp. *enterica* serotype Agona (*S.* Agona). This is to the authors' knowledge the first isolation of Salmonella spp. in this bird species in Brazil. This sample was subjected to the antimicrobial susceptibility test and exhibited intermediate resistance to amikacin and gentamicin and sensitivity to the other antimicrobial agents tested.

4. Discussion

Our study showed a low prevalence of *Salmonella* spp. in wild birds caught in the forest in southern Bahia, probably because they are individuals from areas of fragments of native Atlantic Forest, with little human activity. In fact, the only positive specimen for *Salmonella* spp. was found in a fragment close to a urban area (P2). Bird species is usually found in the Atlantic Forest and in the Amazon region, in the south of river Amazonas. It is also found in Bolivia and Peru, particularly in wetlands and capoeira areas [27]. It is a bird of the Passeriformes order of the family Pipridae [22] and, in ecological terms, plays an important role in preserving the forest [28] because it usually feeds on small fruits and, consequently, helps scatter the seeds of different species of flora [29].

The low prevalence of *Salmonella* spp. in wild free-living birds has also been reported in other studies that found few positive results for this bacteria in free-living birds [11, 30]. The research conducted with 62 wild birds, namely 30 *Columba livia*, 19 *Pterodroma baraui*, 10 *Puffinus lherminieri*, and three *Phaethon lepturus*, from industrial sites and rescue centers, and found that only one specimen of two species each tested positive for *Salmonella* spp. [31]. The study with 364 Passeriformes and Piciformes found no positive isolate for *Salmonella* spp. [32]. However, birds from urban areas may exhibit a higher prevalence, as found in a study with 126 Columbiformes of the species *Columbia livia* in an urban area, in which 7.96% tested positive for bacteria of the genus *Salmonella*: 8 were *Salmonella* Typhimurium, one was *Salmonella enterica* subsp. *enterica* serotype 4,12,i and one was *Salmonella enterica* subsp. *enterica* serotype 4,12 [33]. This higher prevalence may be due to birds' access to areas of environmental contamination, e.g., sewage and human waste sites [34].

The prevalence of pathogens is higher when studying captive animals, since infectious agents in the captive environment can be more easily transmitted due to the greater contact between individuals and the plurality of their health status [35, 36]. It analyzed fecal samples and cloacal swabs of 30 Passeriformes of the species *Paroaria dominicana* and 19 of *Paroaria coronata* apprehended in the fight against the illegal wildlife traffic in the state of São Paulo, Brazil. Although the highest prevalence was for *Escherichia coli* (85.7%) and *Klebsiella pneumoniae* (57.1%), two specimens of *Paroaria dominican* were positive for *Salmonella* spp. (4%). The possible cause for the health status of these cardinals was attributed to the stress arising from the conditions of trafficking [37].

By associating the frequency of enterobacteria with the taxonomic order of birds, Suphoronski et al. [38] found that *Salmonella* spp. was more prevalent in Passeriformes, while *Escherichia coli* was more prevalent in Columbiformes. Moreover, it was observed that *E. coli* occurred in 82.33% of Columbiformes and *Salmonella* spp. occurred in 46.67% of the Passeriformes.

Salmonella Agona has been isolated from several species of animals. It can affect reptiles, domestic birds (anseriformes and galliformes), non-domestic birds, and mammals. Furthermore, it is one of the most prevalent serotypes isolated from cattle with clinical signs of salmonellosis; however, in pigs, it may occur in sick and asymptomatic animals [39]. This serotype was also isolated from seabirds [40], both in seagulls leaving in the proximity of garbage deposits [34] and in Magellan penguins from the Chilean Patagonia [41].

From an epidemiological perspective, the presence of this serotype in wild birds should not be ignored, despite the low prevalence, since in recent years the number of worldwide outbreaks determined by *S.* Agona has been considerable. From 2014 to July 2018, 147 cases were reported in the United Kingdom, Finland, Denmark, Germany, and Ireland. Of these 147 cases, the United Kingdom was responsible for 129 occurrences. Between 2007 and 2016, in countries of the European Union, *S.* Agona caused 13 outbreaks with 636 human cases and 12 hospitalizations, none of which resulted in deaths. In these countries, between 2004 and 2015, 4144 animals of different species tested positive for *Salmonella* Agona, especially chicken ($n = 3236$), cattle ($n = 322$), pigs ($n = 271$), ewes ($n = 183$), and turkeys ($n = 61$). In the same period, *S.* Agona was isolated from several food sources, mainly meat and its derivatives, especially pork ($n = 512$) and chicken meat ($n = 422$). To a lesser extent, this serotype can be isolated from other food categories, such as eggs and derivatives, fish products, fruits and vegetables, and food for animal consumption [42].

Studies have reported that *S.* Agona is resistant to sulfonamide, tetracycline [24], gentamicin, and chloramphenicol [43]. However, in our study, of the 15 tested antimicrobials, *S.* Agona was intermediately susceptible only to amikacin

Table 1: Taxonomic and geographic identification of wild birds from the Atlantic Forest of southern Bahia, Brazil.

Order	Family	Species	Collection points (number of individuals)
Apodiformes	Trochilidae	*Anthracothorax nigricollis*	P2 (1)
		Glaucis dohrnii	P2 (1)
Columbiformes	Columbidae	*Geotrygon montana*	P3 (1)
		Leptotila rufaxilla	P2 (5)
Passeriformes	Dendrocolaptidae	*Campylorhamphus trochilirostris*	P3 (1)
		Dendrocincla turdina	P1 (3), P2 (3), P3 (1)
		Dendrocolaptes platyrostris	P2 (2)
		Unidentified species	P2 (1)
		Glyphorynchus spirurus	P3 (4)
		Sittasomus griseicapillus	P1 (1)
		Xiphorhynchus fuscus	P1 (2), P2 (2), P3 (4)
	Fringillidae	*Euphonia chlorotica*	P2 (1)
	Furnariidae	*Automolus leucophthalmus*	P3 (1)
	Grallariidae	Unidentified species	P3 (1)
	Onychorhynchidae	*Myiobius barbatus*	P2 (1)
	Passerellidae	*Arremon taciturnus*	P2 (1)
	Pipridae	*Ceratopipra rubrocapilla*	P2 (7*), P3 (5)
		Dixiphia pipra	P3 (9)
		Machaeropterus regulus	P1 (1), P3 (5)
		Manacus manacus	P2 (3), P3 (4)
	Rhynchocyclidae	Unidentified species	P3 (1)
	Thraupidae	*Coereba flaveola*	P2 (1)
		Saltator maximus	P2 (1)
		Tangara palmarum	P2 (2)
		Tangara seledon	P2 (1)
	Turdidae	*Turdus amaurochalinus*	P2 (1)
		Turdus leucomelas	P1 (1), P2 (9), P3 (8)
		Turdus rufiventris	P1 (7), P2 (5), P3 (1)
	Tyrannidae	*Attila rufus*	P1 (2)
		Unidentified species	P3 (1)
		Rhytipterna simplex	P2 (1)
Piciformes	Picidae	*Celeus flavescens*	P3 (1)

*One sample was positive for *Salmonella enterica* subsp. *enterica* serotype Agona.

Figure 2: Exemplary of the *Ceratopipra rubrocapilla* species positive for *Salmonella* Agona. Photo: Josiane Moreira Rocha, 2017.

and gentamicin and sensitive to the others [44]. In our region (southern Bahia, Brazil), sulfonamide-resistant *S.* Agona was reported as the second most prevalent serotype in a study that analyzed 30 giant tegus (*Tupinambis merianae*) born in captivity and was isolated in 27% of asymptomatic adult individuals [24]. Increased growth of multidrug-resistant bacteria poses a potential risk to human and animal health. In wild animals, the prevalence of antibiotic-resistant strains reaches levels that are similar to those observed in humans and in domestic animals. This is explained by the presence of resistance genes, which are expressed in environments in which the selection pressure is a consequence of antimicrobial residues which contaminate the environment and harm the wildlife [44]. Environmental contamination caused by antimicrobials in forest areas may be more evident in perirural areas, because some property owners use these

substances to treat and prevent animal diseases or apply subtherapeutic dosages in feed to foster growth [45]. The indiscriminate use of antimicrobials increases the dissemination of resistant agents in zoonosis and may indicate failures in treatment and misconceptions in the disease control system [46]. All these conditions, isolated or associated with each other, may cause resistance of the pathogens to the most diverse antimicrobial agents to increase or multiply [45]. The identification of these causes, as well as their subsequent control and neutralization, is critical to prevent the development of new resistance profiles [46].

5. Conclusion

The results of this study suggest a low prevalence of *Salmonella* spp. in wild birds from the Atlantic Forest in southern Bahia. However, considering the environmental heterogeneity of the studied sites, the multiplicity of species and niches of the Atlantic Forest, and the increasing anthropization of this biome, it is necessary to periodically monitor forest areas to identify pathogens of importance to public health and evaluate the effectiveness of the already implemented control measures. Some mitigating actions include reducing deforestation, controlling human occupation, and eliminating waste and wastewater in forests, in addition to implementing and maintaining environmental protection areas in rural properties. In parallel, the recorded data should be evaluated together with epidemiological elements and sanitary and environmental variables. This type of investigation is necessary because wild animals play a critical ecological role and may act as a reservoir of *Salmonella* spp., which means they can serve as sentinel species to identify infectious agents, indicate the quality of human and environmental health, and reveal the results of human action in forest areas.

Data Availability

The analyse data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado da Bahia, Brasil (FAPESB; Grant PNED 001/2014) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Grant 306308/2015-0). This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES), Finance Code 001, for the scholarship to Eliege Jullia Eudoxia dos Santos.

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