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RESEARCH ARTICLE

Isolation and Characterization of *Salmonella* spp. in Sheltered Wild Birds in Taiwan

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Salmonella are important zoonotic pathogens manifest mostly gastro-enteritis in humans and many animal species. This study investigated the presence of *Salmonella* in wild birds that were rescued and admitted to the Wildlife First Aid Station of the Endemic Species Research Institute from March 2011 to February 2012 in Taiwan. Fecal samples from the wild birds were collected and cultivated for *Salmonella*. *Salmonella* isolates were further subjected to serotype, antimicrobial resistance, and DNA fingerprint analyses. Of the 237 fecal samples, 23 (9.7%) were positive for *Salmonella* using the method described in ISO 6579. Twenty-four *Salmonella* isolates were collected as two isolates were simultaneously obtained from one *Accipiter trivirgatus*. *Salmonella* Albany at 54.2% was the most isolated serotype, followed by *S*. Newport (12.5%) and *S*. Montevideo (8.3%). Other identified serotypes included *S*. Schwarzengrund, Weltevreden, and Itami. Multiple-drug resistance was detected in 62.5% of the isolates; meanwhile, only 20.8% of the isolates were susceptible to five classes of all tested antibiotics. The DNA fingerprints derived from pulse-field gel electrophoresis revealed a high level of similarity among *S*. Albany isolates. This is the first study to report the isolation and antimicrobial resistance of *Salmonella* in sheltered wild birds in Taiwan.

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INTRODUCTION

Salmonella is a microorganism that poses a risk to public health due to its ability to infect a wide range of animal species, including humans (Hoelzer *et al*., 2011). *Salmonella* spp. cause severe disease in food animals, leading to great economic losses. Food animals are considered *Salmonella* hosts and sources of contamination to humans (Foley and Lynne, 2008; Jing *et al*., 2014). Humans may be infected with *Salmonella* through direct contact with the sick or carrier animals or by the ingestion of contaminated food products (Foley and Lynne, 2008; Hilbert *et al*., 2012). In recent years, a plethora of studies have reported a varied rate in isolation of *Salmonella* in pet, exotic, and even wild animals (Awad-Alla *et al*., 2010; Taylor and Philbey, 2010; Molina-Lopez *et al*., 2011). A broad distribution of antibiotic-resistant *Salmonella* in domestic and wild animals further indicates a potential threat of *Salmonella* to public health (Hoelzer *et al*., 2011; Molina-Lopez *et al*., 2011; Kanwal *et al*., 2015; Molina-Lopez *et al*., 2015).

Taiwan is an island that spans tropical and subtropical regions. Its warm and humid weather conditions breed a wealth of animals and plants, of which there are 565 species of wild birds that are classified into 85 families, including resident birds, migratory birds, and vagrants (Council of Agriculture, Endemic Species Research Institute, 2014). Until now, information regarding the presence of *Salmonella* in wild birds in Taiwan was limited. The objective of this study was to determine if *Salmonella* spp. were present in wild birds rescued and temporarily sheltered at the Wildlife First Aid Station of the Endemic Species Research Institute in Taiwan. The serotype, antimicrobial susceptibility, and DNA fingerprints of these isolates were also analyzed.

MATERIALS AND METHODS

Sample collection: Cloacal swabs or feces were collected from 237 wild birds that were rescued/captured and admitted to the Wildlife First Aid Station of Endemic Species Research Institute between March 2011 and February

2012 in Taiwan. Cloacal swabs were collected from birds that appeared healthy; otherwise, feces were collected from birds that appeared weak or in critical condition.

***Salmonella* Isolation, identification, and serotyping:** *Salmonella* were isolated using procedures described in ISO 6579:2002. Briefly, a cloacal swab or 1 g of feces was mixed with 10 ml of buffered peptone water and incubated at 37°C overnight. One hundred microliters of sample was transferred to 10 ml of Rappaport-Vassiliadis broth (Acumedia®) and incubated at 42°C for 18-24 h. A one-milliliter of bacterial suspension culture was inoculated into 10 ml of tetrathionate broth and incubated at 37°C for 18-24 h. A loopful of sample was streaked onto xylose lysine deoxycholate agar (Acumedia®) and brilliant green agar (Acumedia®) plates and incubated at 37°C for 18-24 h. Two to five colonies with typical *Salmonella* characteristics were selected and confirmed by PCR, as described previously (Oliveira *et al*., 2002). Serotypes of the *Salmonella* isolates were determined using multiplex-PCR (Akiba *et al*., 2011; Su *et al*., 2011), followed by agglutination tests using sera specific to the O and H antigens and based on the Kauffmann-White scheme (Chiou *et al*., 2006; Grimont and Weill, 2007).

Antimicrobial susceptibility test: Antimicrobial susceptibility was tested using the disk diffusion method, as described by Wang *et al*. (2010). The tested antibiotics included ampicillin (AMP), amoxicillin-clavulanic acid (AMC), chloramphenicol (C), florfenicol (FFC), gentamicin (CN), amikacin (AK), cephalexin (CL), ceftiofur (EFT), cefotaxime (CTX), ceftazidime (CAZ), and sulphamethoxazole-trimethoprim (SXT), *Escherichia coli* (ATCC 25922) served as a control. Multidrug-resistance was defined as a bacterium resistant to at least three different classes of antimicrobial agents.

Molecular typing by pulse field gel electrophoresis (PFGE): PFGE was performed accordingly using described methods (PulseNet International, 2009). Briefly, bacterial genomic DNA was digested with the restriction enzyme *Xba*I and analyzed in a 1% pulse field-certified agarose gel using the CHEF Mapper XA System (Bio-Rad Laboratories). *Salmonella* Braenderup H9812 served as a molecular weight control. DNA fingerprints were submitted to a GelCompar II program (Applied Maths NV, Sint-Martens-Latem, Belgium), and the unweighted pair group method with arithmetic averages (UPGMA) and the dice similarity coefficient were used to generate a dendrogram of the DNA fingerprints, which were interpreted according to the guidelines described elsewhere (Tenover *et al*., 1995).

Statistical analysis: The data were analyzed using SAS 9.2 and SPSS 16.0. A P-value <0.05 was considered statistically significant.

RESULTS

***Salmonella* isolation and serotyping:** A total of 237 sheltered wild birds were studied, including 113 raptors and 124 non-raptors. The overall incidence of *Salmonella* isolation was 9.7% (Tables 1 and S1). The isolation rate in raptors was 11.5%, which did not differ significantly from that in non-raptors (8.1%) (P>0.05). A total of 24 *Salmonella* isolates were collected, as two serotypes of *Salmonella* were simultaneously sampled from one *Accipiter trivirgatus* (Table 1). Except of 3 unclassifiable isolates, six serotypes were identified, of which *S*. Albany was the most prevalent (54.2%) (Table 2). Other serotypes obtained from raptors were *S*. Montevideo, Newport, Schwarzengrund, and Weltevreden. An isolate of *S*. Itami was identified in non-raptors (Tables 1 and 2).

Table 1: *Salmonella enteirca* isolates, serotypes, and antimicrobial resistance in sheltered wild birds.

Species	No. sampled	No. positive (%)	Serotype	Antibiotic resistance[a]
Raptors	113	13 (11.5)		
Otus bakkamoena	57	7 (12.3)	Albany	AMP, FFC, C, SXT
			Albany	AMP, FFC, C,SXT
			Albany	AMP,FFC, C, SXT
			Montevideo	SXT
			Weltevreden	None
			Newport	None
			Unclassifiable	SXT
Accipiter trivirgatus	32	4 (12.5)	Albany	AMP, FFC, C, SXT
			Schwarzengrund[b]	AMP, C, CN, SXT
			Newport[b]	AMP, C, SXT
			Newport	None
			Unclassifiable	None
Milvus migrans	2	1 (50)	Montevideo	CTX, SXT
Ninox scutulata	3	1 (33.3)	Albany	AMP, FFC, C, CTX, SXT
Others[c]	19	0 (0)		
Non-raptors	124	10 (8.1)		
Centropus bengalensis	1	1 (100)	Albany	AMP, FFC, C, SXT
Cuculus saturatus	1	1 (100)	Albany	AMP, FFC, C, SXT
Gorsachius melanolophus	17	2 (11.8)	Albany	AMP, FFC, C, CTX, SXT
			Itami	None
Dicrurus macrocercus	1	1 (100)	Albany	AMP, FFC, C, CTX, SXT
Caprimulgus affinis	12	1 (8.3)	Albany	AMP, AMC, FFC, C, SXT
Gallinula chloropus	7	4 (57.1)	Albany	AMP, FFC, C, SXT
			Albany	AMP, FFC, C, SXT
			Albany	AMP, FFC, C, SXT
			Unclassifiable	SXT
Others[c]	85	0 (0)		
Total	237	23 (9.7)		

[a]Abbreviation of antibiotics: AMP, ampicillin (10 μg); AMC, amoxicillin-clavulanic acid (20/10 μg); C, chloramphenicol (30 μg); FFC, florfenicol (30 μg); CN, gentamicin (10 μg); AK, amikacin (30 μg); CL, cephalexin (30 μg); EFT, ceftiofur (30 μg); CTX, cefotaxime (30 μg); CAZ, ceftazidime (30 μg); SXT, trimethoprim-sulphamethoxazole (1.25/23.75 μg). [b]: Two *Salmonella* strains were isolated from one bird. [c]: Details are listed in Table S1.

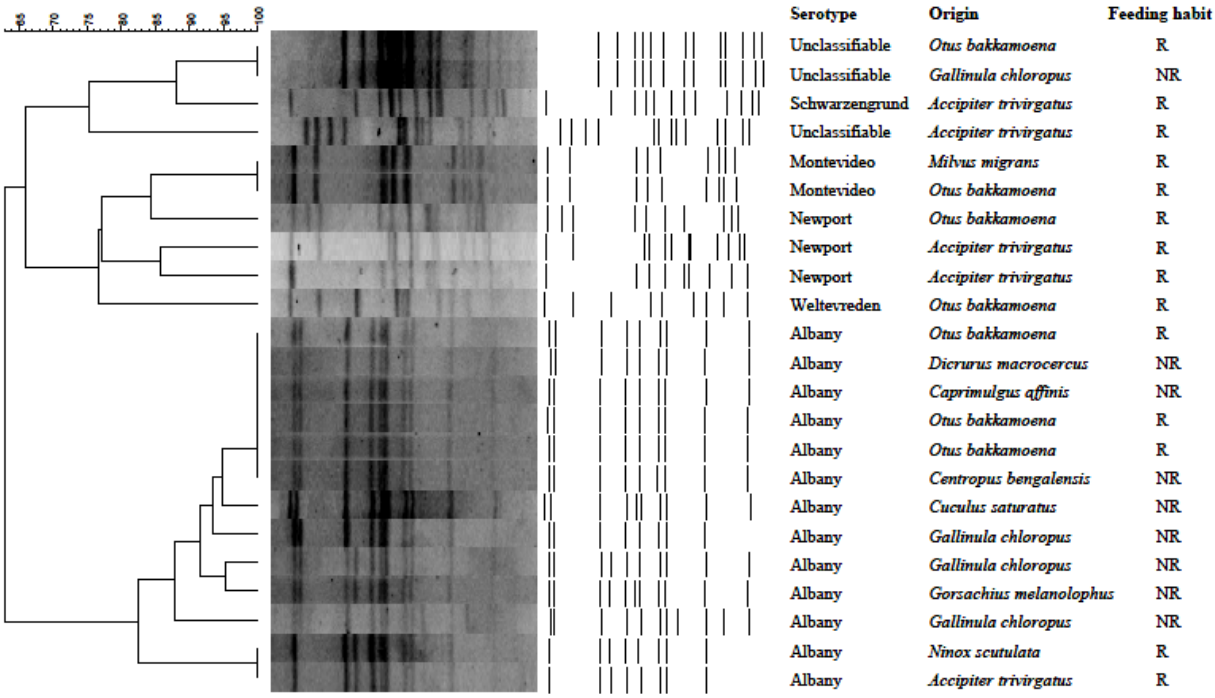

Fig. 1: PFGE dendrogram showing the similarity (%) between *Xba*I-digested chromosomal DNA of *Salmonella* isolates (optimization: 0.5%, position tolerance: 1.5%). R: raptor; NR: non-raptor.

Table 2: Serogroups and serotypes of *Salmonella* isolated from sheltered wild birds.

Serotype (serogroup)	Number of isolates (%)		
	Raptor	Non-raptor	Total
Albany (C_3)	5 (35.7)	8 (80)	13 (54.2)
Newport (C_2)	3 (21.4)	0	3 (12.5)
Montevideo (C_1)	2 (14.3)	0	2 (8.3)
Schwarzengrund (B)	1 (7.1)	0	1 (4.2)
Weltevreden (E)	1 (7.1)	0	1 (4.2)
Itami (D)	0	1 (10)	1 (4.2)
Unclassifiable serotype	2 (14.3)	1 (10)	3 (12.5)
Sub-total	14	10	24 (100)

Antimicrobial susceptibility: Antimicrobial analysis revealed that these *Salmonella* isolates at 20.8% were sensitive to all five classes of the testing antibiotics (Tables 1 and 3). All isolates were susceptible to AK, and cephalosporin group of antibiotics (CL, EFT, and CAZ) (Table 3). As many as 54.2% of the isolates, including all of the 13 *S*. Albany, were resistance towards AMP, FFC, C, and SXT (Table 3). A notable 62.5% of the isolates harbored multidrug resistance (MDR) (Table 3).

Molecular typing: The analytical results showed that different serotypes gave rise to distinct PFGE pattern. The DNA fingerprints of *S*. Newport isolates appeared more diverse, while that of 13 *S*. Albany isolates shared high degrees of similarity and appeared as an indistinguishable to closely related cluster (Fig. 1).

DISCUSSION

Many studies have reported variable isolation rate of *Salmonella* in wild birds in other areas or countries worldwide, indicating a region-specific endemic distribution (Awad-Alla *et al*., 2010; Phalen *et al*., 2010; Molina-Lopez *et al*., 2011). Our study demonstrated a 9.7% *Salmonella* isolation rate in sheltered wild birds that appeared clinically healthy, suggesting a state of carrier or subclinical infection in these animals. These findings are consistent with previous observations by others that *Salmonella* could be isolated from wild birds which had no clinical manifestation (Awad-Alla *et al*., 2010; Molina-Lopez *et al*., 2011; Molina-Lopez *et al*., 2015).

Feeding habit may be one of the factors affecting *Salmonella* isolation rate in wild birds. For instance, in parrots and pigeons, which usually consume grain, fruit, or seed, as low as 0-3.9% were *Salmonella* positive (Tanaka *et al*., 2005; Butron and Brightsmith, 2010). Carnivorous and omnivorous birds had a higher *Salmonella* isolation rate that ranged from 9.9 to 17.0% (Awad-Alla *et al*., 2010; Molina-Lopez *et al*., 2011). It is possible that raptors become *Salmonella* carriers or suffer from clinical Salmonellosis after eating *Salmonella*-infected small animals or birds (Tizard, 2004). In our study, the observation of higher *Salmonella* isolation rate found in raptors was in agreement with the findings by others (Molina-Lopez *et al*., 2011). Interestingly, *Salmonella* isolates derived from raptors encompassing 5 serotypes, while only 2 serotypes were identified in non-raptors. A previous review has pointed out that the variable pattern of the prevalent serotypes in wild birds might reflect the nature of infection from multiple sources (Tizard, 2004). We speculated that feeding habit might have accounted for the higher isolation rate and serotype diversity observed in raptors.

Serotypes Montevideo and Newport are the common serotypes that causes fowl paratyphoid (Porter, 1998). Although the pathogenicity of these serotypes towards humans is low, they have been responsible for the incidence of human clinical cases or outbreaks (Centers for Disease Control and Prevention, June 29, 2010 and May 20, 2016). Therefore, appropriate precautious measures have been suggested to the staff at the Wildlife First Aid Station when handling and treating sheltered wild birds.

Table 3: Antibiotic resistance in different serotypes of *Salmonella* isolates (n=24)

Antibiotics	No. of isolates with resistance in each serotype (%)							Total number of resistant isolates (%)
	A (n=13)	N (n=3)	M (n=2)	S (n=1)	W (n=1)	I (n=1)	U (n=3)	
AMP	13 (100)	1 (33.3)	0	1 (100)	0	0	0	15 (62.5)
AMC	1 (7.7)	0	0	0	0	0	0	1 (4.2)
C	13 (100)	1 (33.3)	0	1 (100)	0	0	0	15 (62.5)
FFC	13 (100)	0	0	0	0	0	0	13 (54.2)
CN	0	0	0	1 (100)	0	0	0	1 (4.2)
AK	0	0	0	0	0	0	0	0 (0)
CL	0	0	0	0	0	0	0	0 (0)
EFT	0	0	0	0	0	0	0	0 (0)
CTX	3 (23.1)	0	1 (50)	0	0	0	0	4 (16.7)
CAZ	0	0	0	0	0	0	0	0 (0)
SXT	13 (100)	1 (33.3)	2 (100)	1 (100)	0	0	2 (66.7)	19 (79.2)
MDR[a]	13 (100)	1 (33.3)	0	1 (100)	0	0	0	15 (62.5)

[a]:Isolate resistance to 3 or more classes of antibiotic agents. Abbreviation of *Salmonella* serotype: A, Albany; N, Newport; M, Montevideo; S, Schwarzengrund; W, Weltevreden; I, Itami; U, unclassifiable serotype.

Table S1: Orders and species of *Salmonella*-negative wild birds

Order, species	Number of birds
Raptors (n=19)	
FALCONIFORMES	
Accipiter virgatus	1
Butastur indicus	1
Elanus caeruleus	2
Falco peregrines	2
Falco tinnunculus	3
Pernis ptilorhynchus	1
Spilornis cheela	8
STRIGIFORMES	
Tyto capensis	1
Non-raptors (n=85)	
PASSERIFORMES	
Acridotheres javanicus	1
CORACIIFORMES	
Alcedo atthis	1
GRUIFORMES	
Amaurornis phoenicurus	1
APODIFORMES	
Apus affinis	10
CAPRIMULGIFORMES	
Caprimulgus indicus	1
COLUMBIFORMES	
Chalcophaps indica	2
Columba livia	5
Streptopelia chinensis	6
Streptopelia tranquebarica	9
GALLIFORMES	
Chrysolophus amherstiae	1
Bambusicola thoracicus	1
PASSERIFORMES	
Dendrocitta formosae	1
Hirundo striolata	1
Hirundo tahitica	3
Hypsipetes madagascariensis	2
Motacilla alba	2
Paradoxornis webbianus	1
Passer montanus	9
Pycnonotus sinensis	4
Stachyris ruficeps	1
Urocissa caerulea	1
Zosterops japonica	6
CICONIIFORMES	
Egretta garzetta	1
Ixobrychus sinensis	1
Numenius phaeopus	1
Nycticorax nycticorax	3
Platalea minor	1
Tachybaptus ruficollis	1
PICIFORMES	
Megalaima oorti	4
CHARADRIIFORMES	
Tringa stagnatilis	1
Vanellus vanellus	1
Charadrius alexandrines	1
GRUIFORMES	
Turnix suscitator	1
Total	104

Serotype Albany was the most prevalent isolates in both raptor and non-raptors in our study. Past studies have shown that serotypes Albany, Schwarzengrund, and Enteritidis, are the major serotypes found in chicken and chicken meat in Taiwan (Lin *et al*., 2008; Chen *et al*., 2010), and layers in Japan (Kudaka *et al*., 2006). DNA fingerprint profiles of *S*. Albany isolates from wild birds exhibited indistinguishable or closely related PFGE patterns, suggesting a possible common origin. As for whether *S*. Albany in wild birds and chicken share common origin warrants further investigation.

MDR-harboring *Salmonella* in wild birds has been identified worldwide (Awad-Alla *et al*., 2010; Molina-Lopez *et al*., 2011; Molina-Lopez *et al*., 2015). Consistently, in the present study, a notable 62.5% of *Salmonella* isolates from wild birds exhibited MDR towards antibiotics AMP, FFC, C, and SXT. A similar MDR (towards AMP, NA, SXT, colistin, and tetracycline) pattern has been reported in *Salmonella* isolates from broilers and simulative native chickens (Lin *et al*., 2008). These results indicate that *Salmonella* with MDR are wide-spread not only in chicken but also in wild birds in Taiwan. The possibility of dissemination of MDR between wild birds and from wild birds to the environment requires further studies. The prevalent MDR in *Salmonella* isolates points to the importance of selection of appropriate antibiotic for effective therapy.

Conclusions: This is the first study to report the isolation of *Salmonella* in rescued/sheltered wild birds in Taiwan, with a great percentage of *Salmonella* isolates harboring multidrug resistance. Because these wild birds were sheltered temporarily in the institute and were eventually released into the wild, whether they could become a source of *Salmonella* toward other animals and to the environment would be a concern and require further evaluation.

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Author's contribution: YSH, YCW and CWH performed the experiments. FTC, CHC and KSY assisted with data analysis. YSH, THC and SLH designed the experiments and wrote the paper. All authors read and approved the final manuscript.

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