ORIGINAL ARTICLE

Phenotypic and Genotypic Characterization of *Salmonella enterica* in Captive Wildlife and Exotic Animal Species in Ohio, USA

L. F. P. Farias[1], C. J. B. Oliveira[1,2], J. J. Medardus[3], B. Z. Molla[1,2], B. A. Wolfe[3] and W. A. Gebreyes[2,3]

[1] Department of Animal Science, College of Agricultural Sciences, Federal University of Paraiba, Areia, Brazil
[2] Veterinary Public Health and Biotechnology Global Consortium (VPH-Biotec), The Ohio State University, Columbus, OH, USA
[3] Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, OH, USA

Impacts

- *Salmonella enterica* commonly occurs among mammalian wild animal species in captivity.
- Multidrug-resistant strains of public health important serovars including penta-resistant *S.* serovar Heidelberg and serovars Typhimurium were found to be common.
- Using genotyping of isolates by Pulsed-Field Gel Electrophoresis, we confirmed that *Salmonella* of wildlife origin can survive in the environment and is an important concern to public health via occupational or through contact via wildlife and petting zoos.

Keywords:
Salmonella enterica; wild animals; environmental contamination; antimicrobial resistance; genotyping

Correspondence:
W. A. Gebreyes. Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, 1920 Coffey Rd. Columbus, OH 43210, USA. Tel.: +1 614 292 9559; Fax: +1 614 292 4142; E-mail: Gebreyes.1@osu.edu

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Summary

The purpose of this study was to investigate the occurrence, antimicrobial resistance patterns, phenotypic and genotypic relatedness of *Salmonella enterica* recovered from captive wildlife host species and in the environment in Ohio, USA. A total of 319 samples including faecal (n = 225), feed (n = 38) and environmental (n = 56) were collected from 32 different wild and exotic animal species in captivity and their environment in Ohio. Salmonellae were isolated using conventional culture methods and tested for antimicrobial susceptibility with the Kirby–Bauer disc diffusion method. *Salmonella* isolates were serotyped, and genotyping was performed using the pulsed-field gel electrophoresis (PFGE). *Salmonella* was detected in 56 of 225 (24.9%) faecal samples; six of 56 (10.7%) environmental samples and six of 38 (15.8%) feed samples. *Salmonella* was more commonly isolated in faecal samples from giraffes (78.2%; 36/46), cranes (75%; 3/4) and raccoons (75%; 3/4). *Salmonella enterica* serotypes of known public health significance including *S.* Typhimurium (64.3%), *S.* Newport (32.1%) and *S.* Heidelberg (5.3%) were identified. While the majority of the *Salmonella* isolates were pan-susceptible (88.2%; 60 of 68), multidrug-resistant strains including penta-resistant type, AmStTeKmGm (8.8%; six of 68) were detected. Genotypic diversity was found among *S.* Typhimurium isolates. The identification of clonally related *Salmonella* isolates from environment and faeces suggests that indirect transmission of *Salmonella* among hosts via environmental contamination is an important concern to workers, visitors and other wildlife. Results of this study show the diversity of *Salmonella* serovars and public health implications of human exposure from wildlife reservoirs.

Introduction

Salmonellosis remains one of the leading foodborne pathogens worldwide. While infections in humans are often associated with food consumption, various risk factors including exposure to wild fauna remain an important concern. This is mainly because of the fact that *Salmonella* is ubiquitous with broad host range and its ability to survive

in adverse environments. The majority of studies on the public health implications of salmonellosis in wild animal species focus on *Salmonella* in reptiles and amphibians (Stirling et al., 2008; Erdozain et al., 2013). The role of wildlife mammals and associated environment in the epidemiology of *Salmonella* is an area that has rarely been investigated. This is particularly important as there is exposure to humans directly (mainly occupational) or indirectly through wild birds and rodents as documented before (Andrés-Barranco et al., 2014).

Several studies have consistently shown the transmission of *Salmonella* from reptiles to human subjects, particularly children (Pees et al., 2013; Sauteur et al., 2013; Middleton et al., 2014). Reptiles and amphibian exposures are estimated to cause more than 70 000 salmonellosis cases in humans in the United States alone (Mermin et al., 2004). A recent report in Germany showed that bearded dragons were important sources of *Salmonella* infections to children (Pees et al., 2013). A diverse array of serovars of high public health significance including serovar Typhimurium and Newport, and multidrug-resistant strains were identified from reptiles, deer, turtles, wild birds and water samples from various coastal areas in the United States (Gorski et al., 2011; Gruszynski et al., 2014). Contamination of fresh produce and vegetables such as Tomatoes by these strains corroborated by genotyping has recently been documented (Gruszynski et al., 2014). Genotypically related isolates of emerging serovar Kentucky, of public health significance, in snakes, chameleons, lizards and their associated environments were also reported in the United States recently (Zając et al., 2013). Outbreaks of salmonellosis in humans that originated from pet turtles have also been reported in the United States during the past years (CDC, 2012a). Limited previous studies have reported the same strains of *Salmonella* serovars isolated from humans and wildlife species, further confirming that wild animals serve as reservoirs for *Salmonella* infections in humans (Kapperud et al., 1998; Handeland et al., 2002). However, there is paucity of information on the epidemiology of *Salmonella enterica* in wild and exotic mammals raised in captivity.

The genus *Salmonella* includes more than 2600 different serovars (Guibourdenche et al., 2010). Only a limited number of serovars are commonly attributed to outbreaks and sporadic infections (Baggesen et al., 2000). Another major concern in *Salmonella* is the increasing rate of antimicrobial resistance and the dissemination of multidrug-resistant (MDR) strains. Studies on the incidence of MDR bacteria from environmental samples have increased during the last few decades (Li et al., 2010). Ultimately, differential host immune system function, varying pathogenicity of *Salmonella* and the extent of environmental exposure influence the occurrence of *Salmonella* in wild animal species that could be a public health hazard.

The aim of this study was to determine the occurrence, serovar distribution, antimicrobial susceptibility patterns and the phenotypic and genotypic relatedness of *Salmonella* isolates recovered from faecal samples from wildlife and exotic carnivorous and herbivorous mammals in captivity. In addition, we investigated the occurrence of phenotypic and genotypically similar isolates from petting zoo located within the premise of the wildlife captivity.

Materials and Methods

Study design and samplings

A serial cross-sectional study was performed in wildlife and exotic animals in captivity in Ohio, USA. To determine the prevalence and the phenotypic and genotypic relatedness of *Salmonella enterica*, faecal (n = 225), environmental (n = 56) and feed (n = 38) samples were collected from wild animals in Ohio from January to June 2008.

A total of 319 samples were collected from 31 animal species, environment and feed (Table 1). Samples were collected from the following species in captivity: banteng [*Bos javanicus*] (n = 13), bison [*Bison bison*] (n = 1), blue sheep [*Pseudois nayaur*] (n = 2), cheetah [*Acinonyx jubatus*] (n = 15), common crane [*Grus grus*] (n = 9), white-tailed deer [*Odocoileus virginianus*] (n = 31), eland [*Taurotragus oryx*] (n = 15), goose [*Anser* spp.] (n = 7), giraffe [*Giraffa camelopardalis*] (n = 50), goral [*Naemorhedus goral*] (n = 22), hog [*Sus scrofa*] (n = 1), onager [*Equus hemionus*] (n = 11), oryx [*Oryx* spp.] (n = 15), Przewalski's horse [*Equus ferus przewalskii*] (n = 2), rabbit [*Oryctolagus cuniculus*] (n = 1), raccoon [*Procyon lotor*] (n = 4), Indian [*Rhinoceros unicornis*] and white rhino [*Ceratotherium simum*] (n = 19), sable antelope [*Hippotragus niger*] (n = 7), tahr [*Nilgiritragus hylocrius*] (n = 6), takin [*Budorcas taxicolor*] (n = 8), wild dog [*Lycaon pictus*] (n = 6) and zebra [*Equus zebra*] (n = 8). Additional samples were collected from medical barns, and petting zoo including domestic sheep [*Ovis aries*] (n = 4), goat [*Capra aegagrus hircus*] (n = 14), llama [*Lama glama*] (n = 8), pony [*Equus ferus caballus*] (n = 4), ram [*Ovis* spp.] (n = 5), reindeer [*Rangifer tarandus*] (n = 5), unspecified small ruminant (n = 6) and wolves [*Canis lúpus*] (n = 3).

Salmonella isolation and serotyping

Salmonella isolation was performed using conventional microbiological approaches as described before (Gebreyes et al., 2004). Briefly, samples were pre-enriched in 2% buffered peptone water (BPW) (Becton Dickinson, Sparks, MD, USA), at 37°C for 16–18 h. The suspension (100 µl) each was transferred to 9.9 ml Rappaport–Vassilliadis broth (RV) (Becton Dickinson) selective enrichment broth and incubated at 42°C for 24 h. A loopful of the suspension

Table 1. Prevalence of *Salmonella enterica* grouped by source and host species

Source	No. of samples (%)	*Salmonella* prevalence (%)
Faeces	**225 (70.5%)**	**56 (24.9%)**
Giraffe [*Giraffa camelopardalis*]	46 (20.4%)	36 (78.2%)
Crane [*Grus grus*]	4 (1.8%)	3 (75%)
Raccoon [*Procyon lotor*]	4 (1.8%)	3 (75%)
Cheetah [*Acinonyx jubatus*]	10 (4.4%)	6 (60%)
Takin [*Budorcas taxicolor*]	6 (2.7%)	3 (50%)
Goral [*Naemorhedus goral*]	16 (7.1%)	3 (18.8%)
Deer [*Odocoileus virginianus*]	26 (11.5%)	2 (7.7%)
Others[a]	113 (50.2%)	0 (0%)
Environment	56 (17.5%)	6 (10.7%)
Rhino barn[b]	3 (5.3%)	3 (100%)
Crane environment[b]	3 (5.3%)	3 (100%)
Others[c]	50 (89.2%)	0%
Feed	38 (11.9%)	6 (15.8%)
Rhino feed	6 (15.8)	6 (100%)
Others[d]	32 (84.2%)	0%
Total	319 (100%)	68 (21.3%)

[a]Others include Llama [*Lama glama*] (n = 5), blue sheep [*Pseudois nayaur*] (n = 1), wild dog [*Lycaon pictus*] (n = 2), bantang [*Bos javanicus*] (n = 11), onager (n = 10), oryx [*Oryx* spp.] (n = 11), eland [*Taurotragus oryx*] (n = 13), tahr [*Nilgiritragus hylocrius*] (n = 3), zebra [*Equus zebra*] (n = 3), rhino [*Rhinoceros unicornis*] (n = 10), rabbit [*Oryctolagus cuniculus*] (n = 1), wolf [*Canis lúpus*] (n = 2), domestic sheep [*Ovis aries*] (n = 2), goat [*Capra aegagrus hircus*] (n = 9), unspecified small ruminant (n = 4), ram [*Ovis* spp.] (n = 2), reindeer [*Rangifer tarandus*] (n = 2), sable antílope [*Hippotragus niger*] (n = 7), hog [*Sus scrofa*] (n = 1), geese [*Anser* spp.] (n = 7), bison [*Bison bison*] (n = 1), p-horse [*Equus ferus przewalskii*] (n = 2) and pony [*Equus ferus caballus*] (n = 4).

[b]Rhino barn and crane environment include water from the barn.

[c]Wild dog (n = 4), cheetah (n = 3), onager (n = 1), ram (n = 2), buck (n = 2), llama (n = 2), reindeer (n = 2), small ruminant (n = 1), domestic sheep (n = 2), goat (n = 2), deer (n = 2), goral (n = 4), giraffe (n = 3), wolf (n = 1), eland (n = 1), takin (n = 1), tahr (n = 2), oryx (n = 2), bantang (n = 1), zebra (n = 2) and medical barn (n = 10).

[d]Ram (n = 1), buck (n = 1), llama (n = 1), reindeer (n = 1), small ruminant (n = 1), crane (n = 2), blue sheep (n = 1), goral (n = 2), giraffe (n = 1), zebra (n = 3), cheetah (n = 2), deer (n = 3), bantang (n = 1), oryx (n = 2), eland (n = 1), takin (n = 1) and tahr (n = 1) and medical barn (n = 7) feed.

was streaked onto xylose-lactose-tergitol 4 (XLT4) (Becton Dickinson) plates and incubated at 37°C for 24 h. Selected presumptive *Salmonella* colonies were then inoculated onto triple sugar iron (TSI) agar (Becton Dickinson) and urea slants (Becton Dickinson) and incubated at 37°C for 24 h. All presumptive *Salmonella* positive isolates were then submitted to the National Veterinary Services Laboratories (USDA-NVSL, Ames, IA, USA) for serotyping.

Antimicrobial susceptibility testing

The antimicrobial resistance profiles of *Salmonella enterica* isolates were tested to a panel of 12 antimicrobials (BD Diagnostics, Sparks, MD, USA) using the Kirby–Bauer disc diffusion method, according to Clinical and Laboratory Standards Institute (CLSI, 2002). The following antimicrobials and their respective disc potencies were used: ampicillin (Am; 10 μg), amoxicillin-clavulanic acid (Ax; 30 μg), ceftiofur (XNL; 30 μg), ceftriaxone (Ce; 30 μg), cephalothin (Ch; 30 μg), chloramphenicol (Cl; 30 μg), ciprofloxacin (CIP; 5 μg), gentamicin (Gm; 10 μg), kanamycin (Km; 30 μg), streptomycin (St; 10 μg), sulfisoxazole (Su; 250 μg) and tetracycline (Te; 30 μg). *Escherichia coli* ATCC 25922 was used as a reference control strain. Isolate showing resistance to three or more classes of antimicrobials was classified as multidrug resistant (MDR).

Genotyping using pulsed-field gel electrophoresis (PFGE)

Representative isolates (n = 24) including one isolate from each species and type of sample were systematically selected and genotyped using PFGE. PFGE was performed according to the PulseNet PFGE protocol for *Salmonella* used by the Center for Disease Control and Prevention (CDC), as previously described (Ribot et al., 2006). Briefly, DNA digestion was performed using *Xba*I restriction enzyme. The PulseNet 'universal' standard marker strain, *Salmonella enterica* serovar Braenderup H9812 was used as a molecular reference marker. After staining with ethidium bromide, DNA fragments were visualized under UV trans-illumination (Gel Doc 2000, Bio-Rad Laboratories, Hercules, CA, USA) . Gel images were photo documented using the Quantity one 1D analysis software (Bio-Rad Laboratories). PFGE gels were analysed using BIONUMERICS software V. 4.61 (Applied Maths NV, Keistraat, Belgium) using Dice coefficient similarity index and unweighted pair group average (UPGMA) cluster analysis.

Results

Salmonella enterica was detected in 56 of 225 (24.9%) faecal samples; 6 of 56 (10.7%) environmental samples and 6 of 38 (15.8%) feed samples (Table 1). Seven of the 31 mammalian host species were found to carry *Salmonella* at different frequencies. *Salmonella* was more frequently isolated in faecal samples from giraffes (78.2%; 36/46), cranes (three of four) and raccoons (three of four).

The frequency of *S. enterica* serovars according to type of sample and animal species is shown in Table 2. The most frequent isolates were *S.* Typhimurium (64.3%), *S.* Newport (32.1%) and *S.* Heidelberg (5.3%), all among the

predominant serovars of public health significance. In addition to the relatively higher occurrence of *Salmonella* in faecal samples, we also found more serovar diversity among faecal samples (five serovars) dominated mainly by two serovars; Typhimurium (64%) and Newport (32%) as compared to environment (only serovar Heidelberg) and feed (two serovars; Heidelberg and Agbeni) samples.

We found antimicrobial resistance among the isolates at varying frequencies. The highest frequency of resistance was found against streptomycin (11.8%) and tetracycline (11.8%) followed by ampicillin (8.8%), gentamicin (8.8%) and kanamycin (8.8%). We also found varying degrees of R-type profiles. No resistance was found against amoxicillin–clavulanic acid, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin and sulfisoxazole. Overall, unlike studies in humans and food animals, frequency of resistance, particularly multidrug resistance (MDR), was low. Eight (11.8%) of the isolates were resistant to at least one antimicrobial, and all of these isolates were resistant to streptomycin and tetracycline with StTe R-type. We also found six isolates that exhibited penta-resistance MDR with AmStTeKmGm R-type. It should be noted that all of the penta-resistant isolates belonged to serovar Heidelberg and were recovered from the environment and feed samples but not in faeces. Sixty of the isolates (88.2%) were pan-susceptible to the panel of 12 antimicrobials included in this study. These isolates belonged to Typhimurium ($n = 36$), Newport ($n = 18$), Montevideo ($n = 3$) and Agbeni ($n = 3$). Patterns of antimicrobial resistance according to the *Salmonella* serovars and type of sample are shown in Table 2.

Further genotypic analysis of the 24 representative *Salmonella* isolates using PFGE genotyping resulted in five genotypic clusters (A, B, C, D and E) among the three predominant serovars: Typhimurium, Newport and Heidelberg with additional sporadic clones of Agbeni, Montevideo and the Rough serovars. *Salmonella* Typhimurium and Newport isolates were grouped in two clusters each (Typhimurium- A and B and Newport- D and E) at the 70% genotypic threshold clustering breakpoint (Fig. 1). Clonally identical *S.* Newport isolates (genotypic clusters D and E) were found from more than one host species: goral, takin, crane and raccoon, suggesting horizontal transmission of *Salmonella* among the wildlife reservoirs. We also found identical clones between feed and environment in the penta-resistant serovar Heidelberg (genotypic cluster C).

Table 2. Serovars and R pattern of *Salmonella enterica* isolates grouped by source

Source	Serovar	Serovar frequency (%)	Resistance pattern
Faeces	Typhimurium (36)	64.3	Pan-susceptible (36)
	Newport (18)	32.1	Pan-susceptible (18)
	Montevideo (3)	5.3	Pan-susceptible (3)
	4, 12: Non-motile (1)	1.8	StTe (1)
	Rough_O:fg:- (1)	1.8	StTe (1)
Environment	Heidelberg (3)	5.3	AmStTeKmGm – MDR[a] (3)
Feed	Heidelberg (3)	5.3	AmStTeKmGm – MDR (3)
	Agbeni (3)	5.3	Pan-susceptible (3)

[a]MDR, multidrug resistant.

Discussion

The present findings suggest that the prevalence of *Salmonella* among mammalian wildlife and captive species is relatively high in the study area indicating the importance of mammalian wildlife as potential sources of human infections through occupational or other direct or indirect exposure to humans in contact with wild animals. In this study, the prevalence varied among the host species and also the origin of the samples (Table 1). In addition, most of the serovars that have been implicated frequently in outbreaks or sporadic cases of human illness in the United States were identified. The predominant serovars included Typhimurium and Newport. Both of these serovars were reported by the Center for Disease Control and Prevention in recent years as predominantly identified from humans covering a wide range of states (CDC, 2012b, 2013a,b,c).

In the present study, serovars and antimicrobial resistance (R-type) patterns varied according to the sample sources. Serovar *S.* Heidelberg was found in environmental and feed samples only but not from faeces. The Heidelberg strains were highly multidrug resistant with penta-resistant pattern AmStTeKmGm (8.8%; 6/68) suggesting the significance of this serovar in animal and public health. Serovar Heidelberg has previously been reported from a wide range of domestic animals and humans including studies conducted in swine production systems by our team (Gebreyes and Thakur, 2005; Patchanee et al., 2008). The majority of the isolates were pan-susceptible (88.2%; 60/68).

These findings suggest that pathways of *Salmonella* contamination and shedding by infected wild animals are multifaceted and environmental contamination through direct and indirect sources such as animals, environment, humans and feed sources could play important roles. Salmonellae are known to carry plasmids associated with antimicrobial resistance and virulence and the emergence of multidrug-resistant *Salmonella* strains poses serious risk to public health as well as that of highly valued wild mammalian species (Dobiasova et al., 2013). Three isolates in the present study, *S.* Heidelberg from rhino, environment and feed,

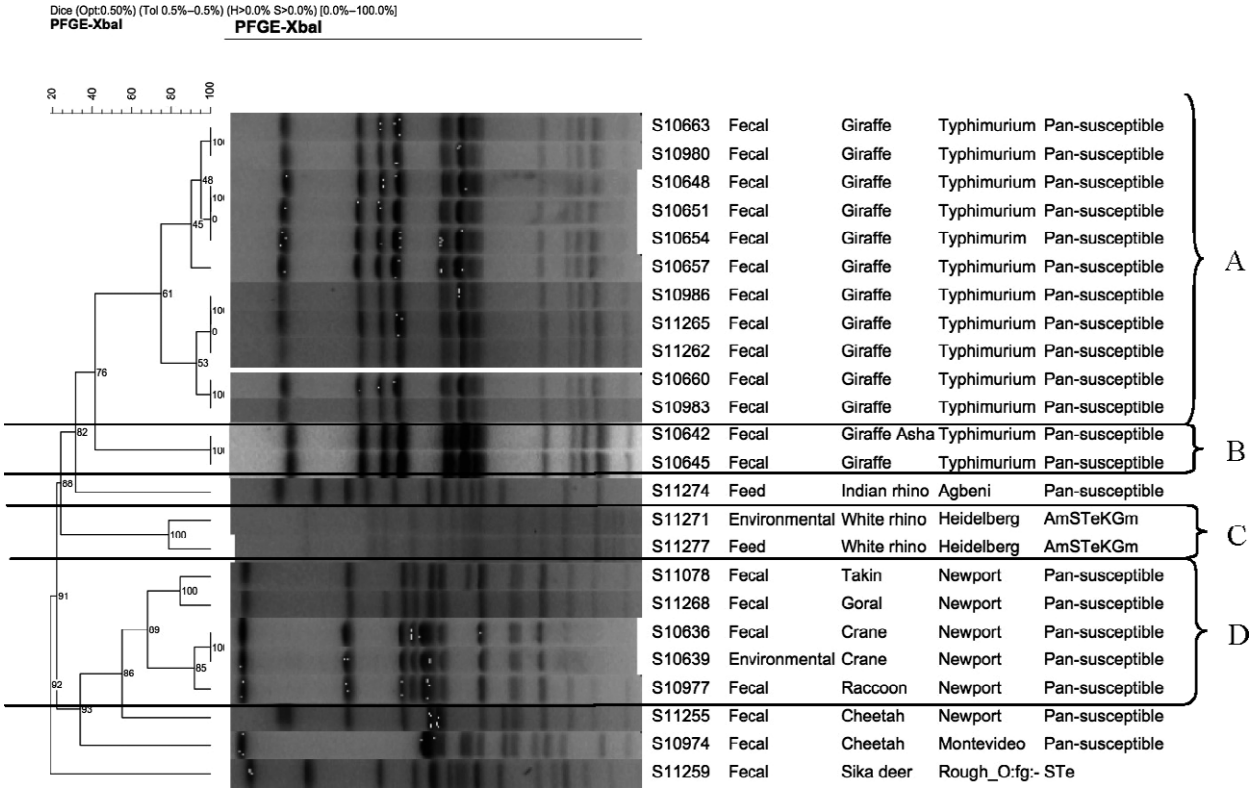

Fig. 1. Dendrogram of pulsed-field gel electrophoresis-*Xba*I fingerprinting, serovar and anti-biograms of *Salmonella enterica* isolates recovered from wildlife animals in Ohio, 2008.

showed a wider spectrum of resistance to multiple classes of antimicrobials. A recent study showed that *Salmonella enterica* serovar Heidelberg is among the top five serovars associated with human salmonellosis and is disproportionately associated with invasive infections and mortality in humans (Han et al., 2012).

Previous studies have shown that wildlife species could be the main source of *Salmonella enterica* contamination to a variety of samples such as water, wildlife, cattle, pre-harvest lettuce and spinach, and soil and sediment collected in a major produce region of the United States (Gorski et al., 2011). These results are relevant to potential environmental contamination by transport and shedding of *Salmonella* by wildlife and human and vice versa. Besides, indirect transmission through contaminated food and water and persistence in the environment may complicate prevention and control efforts (Hoelzer et al., 2011). *Salmonella enterica* serovar Typhimurium is another important serovar in terms of public health and is frequently involved in human infections, and often exhibits MDR patterns (Gebreyes et al., 2004; Molla et al., 2010). However, in the current study, all Typhimurium isolates were pan-susceptible. In this study, we found all isolates of animal origin to be pan-susceptible while the isolates that originated from environment and feed sources were MDR. This observation was unique to serovars Heidelberg and seems to be clonal. We believe this is due to a recent introduction of the strain via feed contamination. As the design of the study was a cross-sectional, we did not detect it in animals. However, we anticipate a longitudinal study could have shed light on this strains occurrence in the animals.

Genotyping of isolates in the current study showed that the majority of the *Salmonella* isolates within a serovar are clonally related (Fig. 1). Interestingly, clonally related isolates detected in both environment and feed (cluster C) and faeces and environment (cluster E), suggest the spread of *Salmonella* in the barn, probably because of *Salmonella* shedding in the faeces by infected animals. It is difficult to conclude the direction of transmission between feed and environment, as the sampling was performed in a serial cross-sectional design, and isolates were detected at the same temporal sampling stage. However, the high incidence of *Salmonella* in feed reported in other studies (Molla et al., 2010; Jackson et al., 2013) coupled with the current finding emphasizes the role of contaminated feed as an important source of contamination to animals in captivity. Besides, the presence of clonally related isolates in different species of captive wild animals from the same location indicates

that the spread of *Salmonella* among different barns could be due to fomites including farm staff, implements, vehicles, visitors and other species, such as wild birds. Some of those isolates were obtained from environment and feed samples, suggesting that these two sources are reservoirs for dissemination in the region.

While this study is limited in scope, we recommend a longitudinal study in wider and larger spatial and temporal aspects to determine whether the high incidence seen in this study reflects a true prevalence of *Salmonella* in wildlife and whether there are geographic and seasonal differences. Further, longitudinal studies are important for identifying risk factors including the risk of occupational infection in people with regular contact with the wild animals. Only a few research studies have been conducted to investigate *Salmonella* spread in mammalian wildlife species. Based on the findings of this study, it can be inferred that wildlife is asymptomatic carriers of *Salmonella*. The high *Salmonella* contamination in the environment of captive wildlife species and the antimicrobial resistance patterns and diverse set of *Salmonella* serovars detected are findings of public health concern, particularly for humans in contact with those animals in captivity. Understanding the epidemiology and patterns of transmission of *Salmonella* between wildlife in captivity and humans could lead to strategies to prevent and control the introduction and spread of infection.

In summary, we found a large variety of *Salmonella* serovars among various species of wild and exotic animals in captivity. We also noted that within a serovars, isolates were clonally related from the environment and faeces suggesting that indirect transmission of *Salmonella* among hosts via environmental contamination is an important public health concern. The study also underlines the need for an integrated surveillance of *Salmonella* and other zoonotic pathogens in captive wild animals and the environment.

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