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SALMONELLA INFECTION IN HEALTHY PET REPTILES: BACTERIOLOGICAL ISOLATION AND STUDY OF SOME PATHOGENIC CHARACTERS

FABRIZIO BERTELLONI[1], MARIANNE CHEMALY[2], DOMENICO CERRI[1], FRANÇOISE LE GALL[2] and VALENTINA VIRGINIA EBANI[1]*

[1]Department of Veterinary Science, University of Pisa, Pisa, Italy
[2]Anses, Ploufragan/Plouzané Laboratory, Hygiene and Quality of Poultry and Pork Products Unit, France

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The fecal samples from 213 captive reptiles were examined, and 29 (13.61%) *Salmonella enterica* isolates were detected: 14/62 (22.58%) from chelonians, 14/135 (10.37%) from saurians, and 1/16 (6.25%) from ophidians. The isolates were distributed among 14 different serotypes: Miami, Ebrie, Hermannsweder, Tiergarten, Tornov, Pomona, Poona, Goteborg, Abaetetube, Nyanza, Kumasi, Typhimurium, 50:b:z_6, 9,12:z_{29}:1,5, and a non-motile serotype with antigenic formula 1,4,[5],12:-:-. *Salmonella typhimurium* and 50:b:z_6 isolates showed the *spv* plasmid virulence genes, responsible of the capability to induce extra-intestinal infections. In some cases, pulsed field gel electrophoresis revealed different profiles for the strains of the same serotypes, showing different origins, whereas a common source of infection was supposed when one pulsotype had been observed for isolates of a serovar. Twenty-seven (93.10%) isolates showed resistance to one or more antibiotics. Ceftazidime was active to all the tested isolates, whereas the highest percentages of strains were no susceptible to tigecycline (93.10%), streptomycin (89.66%), and sulfonamide (86.21%).

Keywords: *Salmonella*, pet reptiles, antimicrobial resistance, PFGE, virulence genes, serotype 1,4,[5],12:-:-

Introduction

Salmonellosis is known to be one of the most important causes of public health concern worldwide. In Europe, during 2013, a total of 82,694 confirmed cases of human salmonellosis were reported, with 59 deaths due to non-typhoidal *Salmonella* [1].

*Corresponding author; E-mail: valentina.virginia.ebani@unipi.it

Most of the human cases resulted from consumption of foods of animal origin contaminated with salmonellae. However, several cases of infection due to salmonellae shed from animals, including pet reptiles, are reported.

In the past years, exotic animals, mainly reptiles, were more often searched as companion pets, resulting in a great trade and movement of non-conventional species all around the world. Recent data revealed that Europe is the first reptiles' importer in the world [2]. Reptiles are often asymptomatic carriers of some important pathogenic bacteria [3–5]. In particular, several studies showed that cold-blooded animals are direct or indirect source of salmonellae in human infection outbreaks [6–11].

Salmonellae isolated from wild and captive reptiles mainly belong to *Salmonella enterica* subsp. *salamae, arizonae, diarizonae, indica, houtenae*, and *Salmonella bongori*, even though cases of infection by *S. enterica* subsp. *enterica* are reported in these animals [12].

Some *Salmonella* serovars are probably commensal organisms in reptiles and retain their pathogenicity for warm-blooded animals [13].

The aims of the present investigation were: (1) to determine the prevalence of *Salmonella* spp. excreted in feces of clinically healthy pet reptiles, (2) to verify the presence of plasmid virulence genes in the isolates, (3) to study genetic profiles of the isolates by PFGE, and (4) to determine the susceptibility of the isolates to antibiotics.

Materials and Methods

Bacteriological examinations

During the period 2011–2012, 213 feces samples from captive reptiles (135 saurians, 62 chelonians, and 16 ophidians) were collected in a pet shop. All tested animals have been imported during the last 4 weeks before sampling; they had no contact with each other in the pet shop, but no data were available about their conditions during the travel.

Each sample was collected into an aseptic tube from individual cages and pools and delivered refrigerated, as soon as possible, to the Bacteriology Laboratory of the Department of Veterinary Science, University of Pisa. Samples were kept at 4 °C for a maximum of 4 h before processing in the laboratory.

Salmonella spp. isolation was carried out as previously described [14]. Briefly, about 3 g of feces was incubated in 10 ml of buffered peptone water at 37 °C for 24 h. About 1 ml of this culture was transferred to 10 ml of Selenite broth

(Difco, Becton Dickinson, Sparks, MD, USA) and 10 ml of Rappaport-Vassiliadis broth (Difco) and the tubes were incubated at 42 °C for 24 h. One loopful from each broth culture was streaked onto duplicate plates of Brilliant Green Agar (Difco) and Salmonella-Shigella Agar (Difco), and the plates were incubated at 37 °C for 24 h. Suspected colonies were inoculated into tubes containing Triple Sugar Iron Agar (Oxoid Ltd., Basingstoke, UK) which were incubated at 37 °C for 24 h.

Isolates with biochemical profile reportable to *Salmonella* spp. were serotyped according to the Kaufmann–White *Salmonella* serotyping scheme [15] with polyvalent and monovalent somatic (O) and flagella (H) antisera (Bio-Rad, Marnes la Coquette, France).

Pulsed field gel electrophoresis (PFGE) typing

To verify if the isolates of the same serotype belonged to the same strain, PFGE was performed. PFGE was carried out with a CHEF-DR III system (Bio-Rad) on DNA of each *Salmonella* isolate previously treated with *Xba*I restriction endonuclease, according to the standardized Salmgene and PulseNet protocol [16, 17]. Gels were stained with Gel-Red; DNA-banding patterns were visualized under UV light and captured using the Gel Doc Eq system and Quantity One software (Bio-Rad). DNA patterns were analyzed with BioNumerics software (V 4.1, Applied Maths, Kortrijk, Belgium). Algorithms available within the program were used to compare the patterns. Dendrograms were produced, using the Dice coefficient and the unweighted pair group method with arithmetic averages, with a 1% tolerance limit and 1% optimization.

Detection of plasmid virulence genes

The presence of plasmid virulence genes (*spvC*, *spvB*, and *spvR*) was determined using PCR assays which amplified fragments of 235 bp, 717 bp, and 202 bp, respectively [18, 19].

DNA was extracted from each *Salmonella* isolate using the DNeasyBlood and Tissue Kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's instructions. Each PCR assay was performed in a reaction volume of 50 µl consisting of 200 µM each dATP, dTTP, dCTP, and dGTP, 0.5 µM of each primer, 1.25 U of Taq polymerase (Qiagen), 5 µl of 10× Qiagen PCR buffer, and 2 µl of extracted DNA. All the amplification products were analyzed by electrophoresis on 1.5% agarose gel at 100 V for 45 min; gel was stained with ethidium

bromide and observed. GelPilot 100 bp Plus Ladder (Qiagen) was used as DNA marker.

Presence of Phase I and Phase II flagella genes

Five non-motile isolates with the antigenic formula 1,4,[5],12:-:- were submitted to PCR assays to verify the presence of *fli*C gene, encoding for Phase I flagella, *fljB* gene, encoding the Phase II flagella [20].

Antimicrobial susceptibility test

Salmonella isolates were submitted to the standard disk diffusion method of Kirby-Bauer [21] on Mueller Hinton Agar (Oxoid). The following antimicrobial molecules (Oxoid) were tested: amoxicillin-clavulanic acid (AMC; 30 µg), ampicillin (AMP; 10 µg), amikacin (AK; 30 µg), cephalothin (KF; 30 µg), cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), colistin (CT; 10 µg), enrofloxacin (ENR; 5 µg), florfenicol (FFC; 30 µg), gentamicin (CN; 10 µg), kanamycin (K; 30 µg), nalidixic acid (NA; 2 µg), nitrofurantoin (F; 300 µg), streptomycin (S; 10 µg), sulfamethoxazole-trimethoprim (STX; 25 µg), sulfonamide (S3; 300 µg), tetracycline (TE; 30 µg), tigecycline (TGC; 15 µg), tobramycin (TOB; 10 µg), and trimethoprim (W; 5 µg).

For each isolate, the zone of inhibition around each disk was measured, after incubation at 37 °C for 24 h. Results were interpreted following EUCAST breakpoint tables and, where not possible, according to NCCLS indications [22, 23]: the isolates were classified as susceptible (S), intermediate (I), and resistant (R).

Results

Bacteriological examinations

From the 213 fecal samples, 29 (13.61%) *S. enterica* isolates were detected. In particular, 14/62 (22.58%) of the chelonians, 14/135 (10.37%) of the saurians, and 1/16 (6.25%) of the ophidians were positive.

The 29 *S. enterica* isolates were distributed among 14 different serotypes. Table I shows the results in relation to species and number of animals examined.

Table I. *Salmonella enterica* serotypes isolated from feces samples of reptiles in relation to animal species

Category	Animal species	Number of examined animals	Number of positive animals (%)	*Salmonella enterica* subspecies and serotypes (number of isolates)
Chelonians	*Chrysemys concinna*	1	0	
	Chrysemys picta	1	0	
	Geochelone carbonaria	1	0	
	Pseudemys concinna	1	0	
	Testudo hermanni hermanni	30	13 (43.33)	I Miami (5) I 1,4,5,12:-:- (4) I Hermannsweder (2) I Goteborg (1) II 9,12:z_{29}:1,5 (1)
	Testudo hermanni boettgeri	12	1 (8.33)	I 1,4,5,12:-:- (1)
	Trachemys scripta elegans	14	0	
	Trachemys scripta scripta	2	0	
Saurians	*Eublepharis macularius*	1	0	
	Gerrhosaurus major	1	0	
	Iguana iguana	96	7 (7.29)	I Ebrie (3) I Abaetetube (1) I Nyanza (1) I Kumasi (1) II 50:b:z_6 (1)
	Oplurus cyclurus	2	0	
	Paroedura pictus	4	0	
	Physignathus cocincinus	13	5 (38.46)	I Tiergarten (2) I Pomona (2) I Tornov (1)
	Pogona vitticeps	5	1 (20.00)	I Tornov (1)
	Tiliqua scincoides	1	0	
	Tupinambis merianae	3	0	
	Chamaeleo verrucosus	9	1 (11.11)	I Poona (1)
Ophidians	*Boa constrictor*	1	0	
	Elaphe guttata	3	0	
	Lampropeltis triangulum	1	0	
	Python molurus	2	0	
	Python regius	9	1 (11.11)	I Typhimurium (1)
	Total	213	29 (13.61)	

Note: I = *Salmonella enterica* subsp. *enterica*, II = *Salmonella enterica* subsp. *salamae*.

The serovars most frequently encountered were Miami (5 isolates from tortoises *Testudo hermanni hermanni*), 1,4,[5],12:-:- (4 isolates from *Testudo hermanni hermanni* and 1 from *Testudo hermanni boettgeri*), Ebrie (3 isolates from *Iguana iguana*), Hermannsweder (2 isolates from *Testudo hermanni hermanni*),

Pomona (2 isolates from *Physignathus cocincinus*), Tiergarten (2 isolates from *Physignathus cocincinus*), and Tornov (1 isolate from *Physignathus cocincinus* and 1 from *Pogona vitticeps*).

One strain of Typhimurium was isolated from a *Python regius*.

PFGE typing

PFGE analysis revealed dissimilar situations for the different serotypes.

The five 1,4,[5],12:-:- isolates showed the same profile. The five Miami isolates presented three different pulsotypes, with three isolates identical and the other two with different profiles.

Only one pulsotype was identified for each of the serotypes Ebrie, Hermannsweder, and Tornov, respectively. Different profiles were found for the two Pomona and two Tiergarten isolates, respectively.

Detection of plasmid virulence genes

Among the investigated isolates, the strain Typhimurium and the strain 50:b:z6 showed the *spvC*, *spvB*, and *spvR* plasmid virulence genes.

Presence of genes encoding for Phase I and Phase II flagella

PCR assay detected the presence of *fliC* and *fljB* genes encoding the first and the second flagellar phases, respectively, in the five non-motile isolates tested.

Antimicrobial susceptibility test

The results of the antimicrobial susceptibility tests are shown in Table II. Twenty-seven (93.10%) isolates were resistant to one or more antibiotics, and 20 (68.96%) were multiresistant.

Ceftazidime resulted as active to all the tested isolates. High percentages of sensitive *Salmonella* strains were observed with cephalosporins (93.10%–100%), penicillins (93.10%–96.55%), sulfonamide–trimethoprim (89.66%–93.10%), and quinolones (82.76%–89.66%).

On the other hand, considering the isolates classified as resistant and intermediate, the highest percentages of strains resulted non-susceptible to tigecycline (93.10%), streptomycin (89.66%), and sulfonamide (86.21%).

Table II. Number of *Salmonella enterica* isolates susceptible, intermediate, and resistant to the antimicrobial agents tested

Antibiotics	Susceptible		Intermediate		Resistant		Non-susceptible: intermediate+ resistant	
	Number of isolates	%	Number of isolates	%	Number of isolates	%	Number of isolates	%
Quinolones								
NA	24	82.76	2	6.90	3	10.34	5	17.24
CIP	26	89.66	3	10.34	0	0.00	3	10.34
ENR	24	82.76	3	10.34	2	6.90	5	17.24
Penicillin								
AMP	27	93.10	0	0.00	2	6.90	2	6.90
AMC	28	96.55	0	0.00	1	3.45	1	3.45
Cephalosporins								
CTX	27	93.10	2	6.90	0	0.00	2	6.90
KF	28	96.55	0	0.00	1	3.45	1	3.45
CAZ	29	100	0	0.00	0	0.00	0	0.00
Aminoglycoside								
CN	22	75.86	7	24.14	0	0.00	7	24.14
K	8	27.59	21	72.41	0	0.00	21	72.41
S	3	10.34	23	79.31	3	10.34	26	89.66
AK	19	65.52	9	31.03	1	3.45	10	34.48
TOB	18	62.07	8	27.59	3	10.34	11	37.93
Tetracyclines								
TE	7	24.14	14	48.28	8	27.59	22	75.86
TGC	2	6.90	12	41.38	15	51.72	27	93.10
Sulfonamide–trimethoprim								
S3	4	13.79	1	3.45	24	82.76	25	86.21
W	26	89.66	1	3.45	2	6.90	3	10.34
SXT	27	93.10	2	6.90	0	0.00	2	6.90
Others								
CT	27	93.10	1	3.45	1	3.45	2	6.90
F	16	55.17	7	24.14	6	20.69	13	44.83
C	24	82.76	5	17.24	0	0.00	5	17.24
FFC	23	79.31	6	20.69	0	0.00	6	20.69

Note: AMC = amoxicillin-clavulanic acid, AMP = ampicillin, AK = amikacin, KF = cephalothin, CTX = cefotaxime, CAZ = ceftazidime, C = chloramphenicol, CIP = ciprofloxacin, CT = colistin, ENR = enrofloxacin, FFC = florfenicol, CN = gentamicin, K = kanamycin, NA = nalidixic acid, F = nitrofurantoin, S = streptomycin, STX = sulfamethoxazole-trimethoprim, S3 = sulfonamide, TE = tetracycline, TGC = tigecycline, TOB = tobramycin, W = trimethoprim

Discussion

The results obtained during the present investigation confirm that captive reptiles are important reservoirs of salmonellae and represent a source of infection for humans. Chelonians, in particular terrestrial turtles, which are the reptiles most commonly present in domestic environment, seem to be infected most frequently than the other cold-blooded animals. The examined *Testudo hermanni* shed salmonellae belonging to several serotypes: Miami, Hermannsweder, Goteborg, 9,12:z_{29}:1,5, and the non-motile 1,4,[5],12:-:-.

Several serotypes were also detected among saurians, in particular from iguanas *Iguana iguana* and water dragons *Physignathus cocincinus*, which were the most numerous animals tested. Serotypes encountered in these reptiles are not frequently isolated from warm-blooded animals, and a few data about their spreading among humans and animals are available in the literature, except for *Salmonella abaetetuba* which has been previously detected in wild iguanas and geckos [5], and has been associated to cases of human salmonellosis [24, 25].

A range of 14 different *Salmonella* serotypes was detected in the present survey. A predominant serotype was not encountered and host-specific association between certain *Salmonella* serotypes and some reptile species does not seem to exist, in accordance with the results obtained by other authors [26].

Salmonellae isolated during the present survey belonged to serotypes not frequently encountered in other animal species. In contrast with the results reported by other investigations that have commonly associated *S. enterica* subsp. *houtenae* and *S. bongori* with reptiles [27], the highest number of our detected isolates belonged to *S. enterica* subsp. *enterica*. Salmonellae of the subsp. *enterica* are the most frequently isolated from humans and warm-blooded animals and often associated to clinical forms; thus, these results underline the zoonotic risk of reptiles housed as pet animals.

Moreover, a *Python regius* allowed the isolation of *S.* Typhimurium that is considered as one of the most pathogenic serotypes for humans, mammals, and birds. This strain had the investigated virulence genes, confirming its pathogenic potential.

Among the other analyzed isolates, only *S. enterica* subsp. *salamae* 50:b:z6 had the *spv* virulence genes, suggesting that, as Typhimurium, it is maybe cause of intestinal and extra-intestinal infections. In fact, the *spv* virulence locus is required for sustained extra-intestinal infections and clinical disease through macrophage cytotoxicity and destabilization of the cytoskeleton of the eukaryotic cell. The absence of the *spv* virulence genes in the other studied isolates supports the hypothesis that extra-intestinal infections are scarce in reptiles [28].

Five strains, isolated from tortoises, were classified as a non-motile serotype with antigenic formula 1,4,[5],12:-:-.

The 1,4,[5],12:-:-strains were the most prevalent non-motile *Salmonella* spp. circulating in France during the period 2000–2009, even though they have been rarely reported in food, environment, or animals when compared with other serotypes [29, 30].

The genetic studies during the present investigation show that these isolates bring the genes for both Phase I and Phase II flagella, even though they did not express them in any cases. Previous studies considered the 1,4[5,],12:-:- strains as non-motile variant of *S.* Typhimurium. The presence of *fliC* and *fliB* amplicons in the phenotypically non-motile isolates suggests that their expression should be blocked by mutation or deletion in the promoter regions linked to the flagellar phase expression [30].

Another atypical variant of *S.* Typhimurium is the monophasic 1,4,[5],12:i:- strain, lacking the Phase II flagellar antigen, which has become increasingly important since the mid-1990s worldwide, including Italy [31].

The non-motile 1,4,[5],12:-:- strain has been rarely isolated and characterized, and in particular, no data are available in the literature about its presence in Italy. Its detection in captive reptiles confirms that cold-blooded animals, coming from exotic geographic areas, contribute to the introduction and spreading of new pathogens in the country of importation.

Habitat and diet components are generally considered as the origin of intestinal *Salmonella* in reptiles [26]. The origin of the salmonellae isolated in this survey is not clear because it is not possible to determine if the animals have contracted salmonellae in the country of exportation, during the traveling, in the sale center.

However, travel and new environments represent cause of stress for reptiles, favoring the fecal excretion of pathogens including salmonellae.

Several strains resulted resistant to one or more antibiotics. Among them, one Poona isolate was resistant to six antibiotics and four isolates (Ebrie, Tornow, Kumasi, 9,12:z_{29}:1,5) to five antibiotics.

Ceftazidime was active to all the tested isolates, and most strains were sensitive to enrofloxacin, which are frequently used against salmonella infections [32].

Different values of resistance to aminoglycoside were observed. In fact, good results were obtained with gentamicin, with 75.86% of susceptible isolates, whereas streptomycin gave the 89.66% of non-susceptible strains. Resistance to streptomycin has been largely documented. Its extensive use, in particular in veterinary medicine, has contributed to the successful spread of resistance genes [33].

During the present study, great variability was found among the different aminoglycoside molecules tested, but this result could be related to the

mechanisms of action of these antibiotics. All aminoglycoside molecules act primarily by impairing bacterial protein synthesis through binding to prokaryotic ribosomes. However, the site of action differs for individual molecules and resistance, associated to a numerous genes, more often develops against single molecules rather than all members of the class [34, 35].

A high percentage (93.10%) of isolates resulted non-susceptible to tigecycline. This is a member of the glycylcycline group of antibiotics, and was registered in the EU in April 2006. It is a bacteriostatic antibiotic active against a broad range of bacteria, with only few naturally resistant exceptions (*Proteus* spp., *Morganella morganii*, *Providencia* spp., and *Pseudomonas aeruginosa*). Specifically, tigecycline is effective against multidrug resistant bacteria, including *Enterobacteriaceae*. Reports of resistance to tigecycline have been rare in naturally susceptible pathogens; however, resistant variants may be encountered [36].

Our results suggest that resistance to tigecycline is not so rare and it represents an emerging problem for the bacterial treatment in veterinary and human medicine, as supposed by other authors [37–39].

A high percentage (75.86%) of isolates resulted non-susceptible to tetracycline too. This result is in agreement with those obtained by other authors, which found reduced efficacy of this antimicrobial against salmonellae, maybe because of the indiscriminate use in humans and animals therapy [40–42].

In some cases, PFGE showed different pulsotypes, when isolates belonging to the same serotype were compared. These results suggest that animals infected by the same *Salmonella* serotype have contracted the infection from different sources. In other cases, one pulsotype has been observed for the isolates of a same serotype (Ebrie, Tornov, and Hermannsweder) showing that they belong to a same strain and suggesting that they have been contracted from the same source.

All the five isolates belonging to the serotype 1,4,[5],12:-:- had the same PFGE profile, whereas the antimicrobial susceptibility test revealed three different resistotypes (S3, TGC-S3, TE-S3); these results suggest that the five non-motile isolates are different strains of a same cluster.

Conclusion

Pet reptiles may be important source of salmonellae for their owners. In particular, children, which share living space with indoor reptiles, result more susceptible than adult developing severe invasive disease [43]. Infected reptiles can transmit salmonellae to other household animals, such as dogs and cats, increasing the risk of exposure for humans. Reptiles are usually considered as

fecal reservoirs of salmonellae able to cause only intestinal infections, but in some cases, they can excrete more pathogenic strains causing extra-intestinal forms.

Moreover, salmonellae excreted by cold-blooded vertebrates could be involved in the antibiotic resistance threat. In fact, these strains can be resistant to one or more antibiotics determining, in some cases, treatment failure; moreover, they can be a source of antimicrobial resistance genes for other bacteria. For this reason, the antimicrobial sensitivity test is essential in determining an appropriate antibiotic therapy that, anyway, should be accompanied by the correction of environmental and nutrition deficiencies.

Conflict of Interest

We declare that we have no conflict of interest.

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