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Salmonella serovar spectrum associated with reptiles in Poland

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Abstract

This study aimed to evaluate the incidence of *Salmonella* isolates from a wide variety of reptiles in Poland. A total of 374 faecal samples from chelonians, lizards and snakes were collected between 2009 and 2012. The nested, two-step PCR and multiplex PCR were performed to access the incidence and to characterize *Salmonella* isolates. *Salmonella* strains were found in 122 of 374 samples (32.6%). Among the different reptilian species, *Salmonella* strains were found in 58 samples from lizards (38.9%), 31 samples from snakes (28.7%) and 33 samples from chelonians (28.2%). Of the total of 122 strains, 72 belonged to the species *Salmonella enterica* subsp. *enterica*, 20 to the species *S. enterica* subs. *salamae* or *S. enterica* subs. *houtanae*. The incidence of *S. enterica* subs. *diarizonae* and *S. enterica* subs. *indica* was low, constituting less than 3.5% of the examined population. The findings show that reptiles can be considered as a reservoir for *Salmonella* and hence could pose a zoonotic hazard. In addition, multiplex PCR assay is a rapid, specific and easy-to-perform method and might be applied for rapid screening of large numbers of *Salmonella* samples.

Chelonians, snake, lizard, reptile-associated salmonellosis, zoonotic agent, PCR

Salmonella is an important zoonotic pathogen of economic significance both in humans and animals. Salmonellosis has declined significantly in the EU by almost one half over the period of five years (2004–2009). Moreover, it was reported by European Food Safety Authority (EFSA) that confirmed cases of human salmonellosis in 2011 decreased by 5.4% compared to 2010. Nevertheless, in 2011 this organism was still the second most frequently reported zoonotic disease in humans, accounting for 95,548 reported cases. The serovars of *Salmonella* associated most frequently with human illness were *S.* enteritidis and *S.* thyphimurium (EFSA 2013). The continued decreasing trend in human cases reflects the impact of *Salmonella* control programmes (e. g. in poultry) put in place by EU Member States and the European Commission. However, these control programmes do not apply to reptiles. These animals may harbour a wide variety of *Salmonella* serotypes in their intestine and can be well adapted to reptilian species with mostly asymptomatic infections (Johnson-Delaney 1996; Woodward et al. 1997; Willis et al. 2002). The increasing popularity of these animals as pets may lead to an increasing number of reptile-associated human salmonellosis cases. Clinical cases of reptile-associated salmonellosis have been reported previously (Weinstein et al. 1995; Friedman et al. 1998; Lakew et al. 2013). *Salmonella* illness remains a public health problem in the United States and these reptiles are a well-established source of human salmonellosis. It was reported that 6% of all human *Salmonella* infection during 1996–1997 in the U.S. were associated with reptiles or amphibians (Mermin et al. 2004). For instance, recently the U.S. Center for Disease Control and Prevention (CDC) published information about eight multistate outbreaks of human *Salmonella* infections linked to small turtles. A total of 473 persons from 41 states were infected (CDC 2013). The investigations indicated exposure to turtles or their environments (e.g., water from a turtle habitat) was the source of these eight outbreaks

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(CDC 2013). Little is known about the epidemiological situation regarding *Salmonella* spp. in reptiles in Poland. The survey done in 1999 in Poland which incorporated over 300,000 animal and food samples showed that the most common serovar of *Salmonella* was *S.* enteritidis and the percentage of positive results, in other than poultry animals (which include turtles), was 3.6% (Hoszowski and Wasyl 2002). In addition, Zajac et al. (2013) have shown that carnivore reptiles can carry multidrug and high level ciprofloxacine resistant *S.* Kentucky type ST198 and those animals should be taken into account as a possible vector of human infection.

This study aimed to evaluate the incidence of *Salmonella* spp. and characterize the isolates from wide variety of reptiles in Poland by means of PCR assay.

Materials and Methods

A total of 374 samples were collected from different species of reptiles (chelonians, n = 117; snakes, n = 108, lizards, n = 149). The list of reptiles tested is shown in Table 2. Faecal samples were collected between 2009 and 2012 from reptiles which were housed in zoos and with private keepers. The detection of *Salmonella* isolates was performed according to the ISO standard no. 6579:2003/A1:2007. Afterwards, three colonies per plate with characteristics of *Salmonella* were spread onto nutrient agar (Nutrient Agar, Merck, Germany) for 24 h at 37 °C and further identified biochemically. For DNA extraction, all strains were plated on trypticase soy agar (Becton Dickinson) with overnight incubation at 37 °C. Bacterial genomic DNA was then extracted after growth overnight in Luria-Bertani broth (Difco Laboratories, Detroit, MI, USA) by using the Genomic DNA Prep Plus® kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. DNA was quantitated by spectrophotometry (BioPhotometer, Eppendorf) and stored at 4 °C.

DNA was extracted as described above and then amplified by nested, two-step PCR using the primers 11L (AACCATTGCTAAATTGGCGCACAACC) and 15L (GAAATTCCCAGCGGGTACTGGGTACTG), and the nested primers ST12 (CGTCCGGCATGACGATGGTA), and ST13 (ATTCACCGGAACGGCGCCGT) (Aabo et al. 1993; Haedicke et al. 1996). PCR reactions contained 50 ng template DNA, 2.5 U Green *Taq* polymerase, 10 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs (Fermentas, Vilnius, Lithuania), and 20 pmol of 11L and 15L primers (Genomed, Warszawa, Poland) at a final volume of 25 µl. Reaction conditions were as follows: initial denaturation 5 min at 94 °C, 40 cycles of 1 min at 70 °C, 30 s at 94 °C and 3 min at 72 °C. For the nested PCR, 5 µl of the first round product and ST12 and ST13 primers (Genomed, Warszawa, Poland) were used. The following cycling conditions were performed: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 55 °C for 30 s, 72 °C for 1 min and 94 °C for 30 s, and a final extension at 72 °C for 3 min. Samples which were

Table 1. Oligonucleotide primers and expected band patterns of each *Salmonella* species or subspecies.

Primer	Sequence (5`–3`)	Target gene	Product size (bp)	*Salmonella* species or subspecies						
				I	II	IIIa	IIIb	IV	VI	V
fljB1	GACTCCATCCAGGCTGAAATCAC	*fljB*	848	d	d		+		+	+
fljB2	CGGCTTTGCTGGCATTGTAG									
mdcA 7	GGATGTACTCTTCCATCCCCAGT	*mdcA*	728		+	+	+			
mdcA 8	CGTAGCGAGCATCTGGATATCTT									
gatD P5	GGCGCCCATTATTATCCTATTAC	*gatD*	501	+	+				d	+
gatD P6	CATTTCCCGGCTATTACAGGTAT									
stn f1	CGATCCCTTTCCCGCTATC	*stn*	179	+	+	+	+	+	+	
stn r1	GGCGAATGAGACGCTTAAG									
STM4057 f1	GGTGGCCTCGATGATTCCCG	STM4057	137	+						
STM4057 r1	CCCACTTGTAGCGAGCGCCG									
INVA-1	ACAGTGCTCGTTTACGACCTGAAT	*invA*	244	+	+	+	+	+	+	+
INVA-2	AGACGACTGGTACTGATCGATAAT									

d - differs among strains

positive for *Salmonella* were then examined by multiplex PCR amplification based on formula described previously by Lee et al. (2009). The list of primers used in multiplex PCR which give unique results for each subspecies of *Salmonella* strains are shown in Table 1. The PCR reactions were set up as 25 µl reaction containing 50 ng template DNA, 1 U Green Taq polymerase, 10 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP and 20 pmol of each primers using the following PCR conditions: denaturation, 1 min at 95 °C; annealing, 1 min at 60 °C; and extension 1 min at 72 °C for 40 cycles, followed by a final extension at 72 °C for 15 min. The DNA amplification products were resolved on a 1.5% agarose gel stained with ethidium bromide and visualised using the Gel-Doc UV transilluminator system (Biorad, Warszawa, Poland) with Quantity-One software (Biorad, Warszawa, Poland).

Results

The incidence of *Salmonella* in different species of reptiles is shown in Table 2. *Salmonella* strains were found in 122 of 374 samples (32.6%). Among the different reptilian species *Salmonella* strains were found in 58 samples from lizards (38.9%), 31 samples from snakes (28.7%) and 33 samples from chelonians (28.2%). Of the total of 122 strains, 72 belonged to the species *Salmonella enterica* subsp. *enterica* (19 of 33 chelonians carriers, 19/31 snakes and 34/58 lizards) and as a result this serotype predominated in all of the reptiles tested (59.02%). The second most frequently reported serotypes were *S. enterica* subs. *salamae* and *S. enterica* subs. *houtanae* (both of 16.39%). However, we found differences in incidence between those serotypes. *S. enterica* subs. *salamae* was recorded in 8 of 31 snake carriers (25.81%) compared to chelonians and lizards (18.18% and 10.34%, respectively) whereas the percentage of *S. enterica* subs. *houtanae* samples was higher in lizards (25.86%) compared to chelonians (15.15%) and, interestingly, this species was not detected in snakes (Fig. 1). The incidence of *S. enterica* subs. *diarizonae* and *S. enterica* subs. *indica* was low, constituting less than 3.5% of the examined population. Five strains were non-typeable (NT).

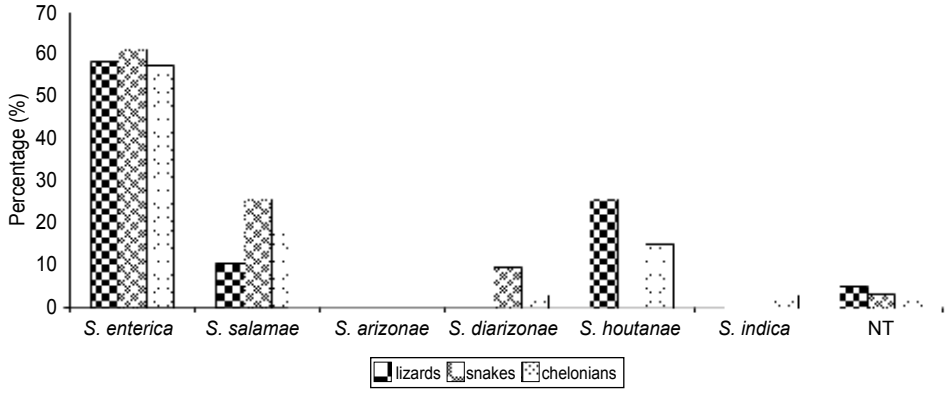

Fig. 1. The incidence of *Salmonella* subspecies in reptiles
NT - non-typeable

Discussion

Reptiles are very common carriers of *Salmonella* and can be infected without showing any symptoms, thus, posing a hazard in terms of a zoonosis. The increasing popularity of these animals as pets worldwide may lead to an increased number of reptile-associated human salmonellosis cases. This survey has confirmed moderate to high prevalence of *Salmonella* strains in reptiles in Poland, ranging from around 28.0% in snakes and chelonians to almost 39% in lizard. In Central Europe (Germany and Austria), *Salmonella*

Table 2. Incidence of *Salmonella* spp. in different species of reptiles.

	Number of samples		*Salmonella*						
	investigated	positive	*enterica* (I)	*salamae* (II)	*arizonae* (IIIa)	*diarizonae* (IIIb)	*houtanae* (IV)	*indica* (VI)	NT
Order Testudines (chelonians)	117	33	19	6	0	1	5	**1**	**1**
Family Chelidae									
Chelus fimbriatus	3	1	0	1	0	0	0	0	0
Family Pelomedusidae									
Pelomedusa subrufa	1	0	0	0	0	0	0	0	0
Family Chelydridae									
Chelydra serpentina	1	1	0	0	0	0	1	0	0
Family Emydidae									
Cuora amboinensis	1	0	0	0	0	0	0	0	0
Trachemys scripta	14	6	3	1	0	1	1	0	0
Family Geoemydidae									
Geoemyda spenglei	2	0	0	0	0	0	0	0	0
Heosemys grandis	1	0	0	0	0	0	0	0	0
Family Testudinidae									
Astrochelys radiata	1	0	0	0	0	0	0	0	0
Chelonoidis nigra	1	0	0	0	0	0	0	0	0
Geochelone denticulata	2	0	0	0	0	0	0	0	0
Geochelone elegans	1	0	0	0	0	0	0	0	0
Geochelone pardalis	1	1	1	0	0	0	0	0	0
Geochelone sulcata	1	0	0	0	0	0	0	0	0
Indotestudo elongata	1	0	0	0	0	0	0	0	0
Kinixys homeana	1	0	0	0	0	0	0	0	0
Kinixys nogueyi	3	0	0	0	0	0	0	0	0
Stigmochelys pardalis	1	0	0	0	0	0	0	0	0
Testudo graeca	4	1	0	0	0	0	1	0	0
Testudo hermanni	25	6	5	0	0	0	1	0	0
Testudo horsfieldi	34	9	6	2	0	0	1	0	0
Testudo kleinamani	14	7	4	1	0	0	0	1	1
Family Carettochelyidae									
Carettochelys insculpta	2	1	0	1	0	0	0	0	0
Family Kinosternidae									
Sternotherus odoratus	2	0	0	0	0	0	0	0	0
Suborder Serpentes (snakes)	108	31	19	8	0	3	0	**0**	**1**
Family Boidae									
Acanthophis dumerilii	1	0	0	0	0	0	0	0	0
Boa constrictor	35	9	6	2	0	0	0	0	1
Epicrates cenchria	6	3	2	0	0	1	0	0	0
Epicrates striatus	2	0	0	0	0	0	0	0	0
Eryx colubrinus	1	1	0	1	0	0	0	0	0
Morelia spilota	1	0	0	0	0	0	0	0	0
Morelia viridis	15	8	5	2	0	1	0	0	0
Python molurus	3	0	0	0	0	0	0	0	0
Python regius	13	4	3	1	0	0	0	0	0
Sanzinia madagascariensis	2	0	0	0	0	0	0	0	0

	Number of samples		*Salmonella*						
	investigated	positive	*enterica* (I)	*salamae* (II)	*arizonae* (IIIa)	*diarizonae* (IIIb)	*houtanae* (IV)	*indica* (VI)	NT
Family Colubridae									
Elaphe guttatus	10	0	0	0	0	0	0	0	0
Elaphe obsoleta	2	0	0	0	0	0	0	0	0
Elaphe radiata	1	0	0	0	0	0	0	0	0
Lamprophis fuliginosus	4	1	0	0	0	1	0	0	0
Bogertophis subocularis	2	1	1	0	0	0	0	0	0
Coluber hippocrepis	2	2	1	1	0	0	0	0	0
Lampropeltis triangulum	1	0	0	0	0	0	0	0	0
Thamnophis marcianus	2	0	0	0	0	0	0	0	0
Ahaetulla mycterizans	1	0	0	0	0	0	0	0	0
Orthriophis taeniurus	3	2	1	1	0	0	0	0	0
Family Viperidae									
Cryptelytrops albolabris	1	0	0	0	0	0	0	0	0
Order Squamata									
Suborder Lacertilia (lizards)	149	58	34	6	0	0	15	**0**	**3**
Family Agamidae									
Agama agama	1	1	0	0	0	0	1	0	0
Gonocephalus chamaeleontinus	3	0	0	0	0	0	0	0	0
Pogona vitticeps	25	13	12	0	0	0	1	0	0
Physignathus lesueurii	1	1	1	0	0	0	0	0	0
Physignathus cocincinus	7	0	0	0	0	0	0	0	0
Uromastyx aegyptia	4	1	1	0	0	0	0	0	0
Family Chamaeleonidae									
Chamaeleo calyptratus	1	0	0	0	0	0	0	0	0
Family Cordylidae									
Cordylus tropidosternum	2	0	0	0	0	0	0	0	0
Family Corytophanidae									
Basiliscus vittatus	2	1	1	0	0	0	0	0	0
Laemanctus longipes	1	1	1	0	0	0	0	0	0
Family Crotaphytidae									
Crotaphytus collaris	3	3	3	0	0	0	0	0	0
Family Geconidae									
Coleonyx elegans	1	0	0	0	0	0	0	0	0
Gekko petricolus	1	0	0	0	0	0	0	0	0
Gekko vittatus	2	2	2	0	0	0	0	0	0
Eublepharis macularius	13	5	4	0	0	0	1	0	0
Hemitheconyx caudicinctus	2	1	0	0	0	0	1	0	0
Homopholis fasciata	1	0	0	0	0	0	0	0	0
Phelsuma madagascariensis	4	3	2	1	0	0	0	0	0
Rhacodactylus auriculatus	1	1	0	0	0	0	1	0	0
Tarentola annularis	2	1	0	1	0	0	0	0	0
Tarentola mauritanica	4	1	0	1	0	0	0	0	0

	Number of samples		*Salmonella*						
	investigated	positive	*enterica* (I)	*salamae* (II)	*arizonae* (IIIa)	*diarizonae* (IIIb)	*houtanae* (IV)	*indica* (VI)	NT
Family Gerrhosauridae									
Gerrhosaurus flavigularis	1	1	1	0	0	0	0	0	0
Gerrhosaurus nigrolineatus	1	0	0	0	0	0	0	0	0
Gerrhosaurus validus	2	1	0	0	0	0	0	0	1
Zonosaurus maximus	1	0	0	0	0	0	0	0	0
Family Iguanoidae									
Brachylophus fasciatus	1	0	0	0	0	0	0	0	0
Cyclura nubila	1	1	1	0	0	0	0	0	0
Iguana iguana	29	13	3	0	0	0	10	0	0
Sauromalus obesus	1	0	0	0	0	0	0	0	0
Family Lacertidae									
Podarcis sicula	3	0	0	0	0	0	0	0	0
Family Phrynosomatidae									
Sceloporus malachiticus	3	1	0	1	0	0	0	0	0
Family Polychrotidae									
Anolis equestris	2	1	1	0	0	0	0	0	0
Anolis sagrei	1	0	0	0	0	0	0	0	0
Anolis barbatus	1	0	0	0	0	0	0	0	0
Family Scincidae									
Eumeces schneideri	1	0	0	0	0	0	0	0	0
Eumeces schneideri	1	0	0	0	0	0	0	0	0
Trachylepis (Mabuya) quinquetaeniata	1	0	0	0	0	0	0	0	0
Tiliqua rugosa	1	0	0	0	0	0	0	0	0
Tiliqua scincoides	1	1	0	0	0	0	0	0	1
Family Teiidae									
Ameiva ameiva	1	0	0	0	0	0	0	0	0
Family Varanidae									
Varanus exanthematicus	11	3	1	1	0	0	0	0	1
Varanus salvadorii	1	0	0	0	0	0	0	0	0
Varanus salvator	3	1	0	1	0	0	0	0	0
Total	374	122	72	20	0	4	20	1	5

NT - non-typeable

were detected in 86 of 159 (54.1%) faecal reptile samples, mostly in lizards and snakes. However, it was only detected in one sample from a single turtle out of 38 turtles investigated (Geue and Löschner 2002). In the present study, high percentage of *Salmonella* positive samples in chelonians was shown. This is consistent with the findings from a study by Nowakiewicz et al. (2012) where *Salmonella* were isolated from 15 to 80 (18.75%) tortoises investigated in Poland. Moreover, in Italy *Salmonella* spp. were isolated from 81 of 220 (36.8%) and from 17 of 67 (25.4%) cloacal swabs collected from *T. graeca* and *T. hermanni* tortoises, respectively (Percipalle et al. 2011). These findings confirm that tortoises can be considered a reservoir for *Salmonella.* The high percentage of *Salmonella* among lizards and snakes is consistent with the results of other investigators (Mermin et al. 1997; Kuroki et al. 2013; Krautwald-Junghanns et al. 2013; Sting et al. 2013).

Salmonella was frequently found in the green iguana (*Iguana iguana)* and bearded dragon (*Pogona vitticeps)* (Geue and Löschner 2002), as shown in our study by 13 out of 29, and 13 out of 25, respectively. In Poland, popularity of the green iguana and bearded dragon increases year after year and those two species of reptiles are becoming very popular as pet reptiles. Taking together, this study suggests that the prevalence of *Salmonella* spp. in reptiles in Poland does not vary considerably between the species of reptiles tested but we have found that some species of reptiles may carry the *Salmonella* more frequently (e.g. *Iguana iguana, Pogona vitticeps, Morelia viridis*) than others (*Physignathus cocincinus, Boa constrictor*).

All serotypes detected in this study were non-typhoidal. Those serotypes of *Salmonella* may lack pathogenicity in reptiles but they can be responsible for severe invasive infections, especially in infants, elderly or immunocompromised individuals (Van Meervenne et al. 2009). Almost 60% of *Salmonella* positive samples from reptiles belong to the species *Salmonella enterica* subsp. *enterica*. This serovar of *Salmonella* was also found to be predominant by other investigators (Pedersen et al. 2009; Percipalle et al. 2011; Nowakiewicz et al. 2012; Marin et al. 2013). Pasmans et al. (2005) examined 44 serotypes of *Salmonella enterica* from pet reptiles and found that all were able to invade human intestinal epithelial cells and could cause a disease, depending on the age of individual and their immune status. It was also shown in this study that *S. enterica* subs. *salamae* and *S. enterica* subs. *houtanae* were the second most frequently common serovars of *Salmonella*. Interesingly, *S. enterica* subs. *salamae* was recorded especially in snakes whereas *S. enterica* subs. *houtanae* was detected in lizards and not in snakes. This may reflect different ecological behaviours and feeding habits. However, based on the results obtained by others investigators (Kuroki et al. 2013; Krautwald-Junghanns et al. 2013; Marin et al. 2013; Pasmans et al. 2005; Sting et al. 2013) it is difficult to state exactly what kinds of *S. enterica* subspecies are rarely isolated from reptiles, from which other uncommon *Salmonella* subspecies are often isolated. This huge serovars sectrum of *Salmonella* strains isolated from reptiles may pose a hazard to human health because it is difficult to predict which kind of *Salmonella enterica* may be carried in a particular reptile pet. Moreover, the diagnosis of reptile-associated human salmonellosis may pose substantial challenges for laboratories in those countries where the controls program are not applied as a routine procedure. For instance, Pedersen et al. (2009) have shown that most of the serovars detected are called, usually, exotic serotypes, and many do not have a common name. This study confirmed that multiplex PCR assay is rapid, specific and easy to perform, and could be a simple tool for *Salmonella* detection and characterisation. This assay is therefore well adapted for rapid screening of large numbers of samples.

In conclusion, the detection of *Salmonella* in pet reptiles in Poland confirms that those animals are potential carriers of the bacteria. The incidence of *Salmonella* detected in this study was moderate (32.6%). Nevertheless, all of the serovars are capable of causing clinical infections in human. That is why appropriate hygienic conditions should be recommended for the handling of reptiles and their trade should be monitored. It could also be of importance to apply *Salmonella* control programmes for reptiles in Poland. In addition, multiplex PCR provides a rapid, sensitive and cost-effective detection assay.

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