



# Free-living snakes as a source and possible vector of *Salmonella* spp. and parasites

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**Abstract** Free-living snakes may carry a broad range of pathogens that differ considerably by geographical locations and source species. The biological role of wildlife snakes for transmission of bacteria and helminths is hardly explored. The purpose of the study was a snapshot on the zoonotic agents load in free-living snakes found dead in a landscape park in central Poland. A total of 16 dead free-living snakes identified as European grass snake (*Natrix natrix*, N=15) and a smooth snake (*Coronella austriaca*, N=1) were tested. Abdominal organs were used for bacteriological testing, whereas, parasites were searched for in the remaining carcasses. Fourteen (87.5 %) individuals were positive for *Salmonella* spp., whereas, trematodes were found in 13 out of 16 samples (81.3 %). A total of 33 isolates representing 11 *Salmonella* serovars or antigenic forms were observed with *Salmonella enterica* subsp. *diarizonae* (IIIb) 38:r:z being the most frequent. The trematodes were recognized as *Alaria alata* mesocercariae (*Distomum musculorum suis*, DMS). Additionally, unidentified nematodes were detected in four samples. No *Yersinia* spp. and *Mycobacterium* spp. were found. The results indicate huge burden of *Salmonella* spp. and muscle parasites carriage by snakes present in the natural environment and indicate possible zoonotic and

epidemiological impact. It justifies our concept of free-living snakes as possible source and vector of pathogens, especially for omnivores and scavengers foraging on snake corpses. Finding of rare and diverse *Salmonella* serovars and unidentified nematodes proves the need for in-depth studies and systematic approach to reveal the role of free-living reptiles in epidemiology of infectious agents.

**Keywords** *Salmonella* spp · *Alaria* spp · Wildlife · European snakes · Vector-borne diseases · Epidemiology

## Introduction

Epidemiology of infectious and parasitic diseases includes extensive transmission cycles with well-defined sources, vectors, and routes of spread of causative agents. Numerous research continuously provide new data for upgrading of state of the art. They usually focus on details in well-known areas, but less affordable or inconvenient elements of epidemiological puzzle remain unrecognized. To exemplify, *Salmonella* occurrence in reptiles and their role in pathogen transmission to humans are commonly known, but the vast majority of research efforts is focussed on *Salmonella* epidemiology in farm animals.

A few snakes occur in the natural environment of Poland. All species, including European grass snake (*Natrix natrix*) and smooth snake (*Coronella austriaca*) are under protection. That fact hampers research efforts unless the clinical threats occur (Mihalca et al. 2010; Sting et al. 2013). Definitely, wildlife snakes carry a broad range of bacteria and parasites that differs considerably by geographical locations and source species (Borkovcová and Kopriva 2005; Fitzgerald et al. 2013; Hacıoglu and Tosunoglu 2014; Lukac et al. 2015; Scheelings et al. 2011; Schmidt et al. 2014; Shimalov and Shimalov

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2000). *Salmonella* spp., being considered natural component of reptile gut flora, is one of the most frequently noted (Goupil et al. 2012; Kuroki et al. 2013; Prapasarakul et al. 2012; Richards et al. 2004; Scheelings et al. 2011). Wildlife snakes are often reported as host of new parasites or record their new geographical location (Borkovcová and Kopřiva 2005; Halajian et al. 2013; Santoro et al. 2011; Shimalov and Shimalov 2000; Yildirimhan et al. 2007).

The biological role of free-living snakes for transmission of bacteria and helminths is hardly explored. One might presume them as paratenic hosts (Duscher et al. 2015; Halajian et al. 2013; Mohl et al. 2009), although public health consequences of direct contact with carrier reptile itself or its' contaminated environment shall not be neglected (Mohl et al. 2009; Zając et al. 2013).

Since there are considerable gaps in evidence-based knowledge of European grass and smooth snakes in transmission of infectious and parasitic agents (Schmidt et al. 2014), the purpose of the study was a snapshot on the zoonotic agents load in free-living snakes found dead in a landscape park in central Poland.

## Materials and methods

### Sampling

Sixteen dead free-living snakes were collected in “Gostynińsko-Włocławski Park Krajobrazowy” (a landscape park in central Poland) in autumn 2014. The location of animal collection (15.8-km long asphalt forest road), injuries, and the state of the corpses indicated on the cause of death due to traffic accidents that happened within few (fresh blood) or several hours (dried body tissues). The corpses were submitted for laboratory testing on two occasions: 5th Oct. (10 animals) and 12th Oct. (6 animals). The animals were identified as European grass snake (*Natrix natrix*, N=15) and a smooth snake (*Coronella austriaca*). Due to the severe damages of the bodies, full-scale necropsy was not feasible, and two types of samples were collected for laboratory diagnostics. Abdominal organs were used for bacteriological testing, whereas, parasites were searched for in the remaining carcasses.

### Bacteriological testing

Fragments of internal organs were homogenized and incubated with oxalic acid. Following a cycle of saline flushes, the sample was streaked on Stonebrink and Petraghani media and cultured for *Mycobacterium* spp.

The remaining fragments of organs were pooled with the intestines and homogenized in buffered peptone water (BPW, 1:10 w/v ratio). One milliliter of the homogenate was inoculated in peptone sorbitol bile broth (PSB, 10 ml, incubation in

5 °C for 2 weeks) and further on cefsulodin-irgasan-novobiocin agar (CIN, 28 °C, 48 h) for *Yersinia enterocolitica* isolation.

The remaining volume of BWP homogenate was cultured for *Salmonella* spp. according to EN ISO 6579:2002/A1:2007. Besides EU official methodology, preincubated culture was streaked on chromogenic RAPID *Salmonella* agar (Bio-Rad). Up to five presumptive colonies from each selective medium plate were confirmed for *Salmonella* with PCR targeting *invA* and serotyped according to White-Kauffmann-Le Minor scheme (Grimont and Weill 2007). From each sample, a single isolate showing distinct serological properties was used for further analyses. These included *Salmonella* subspecies identification with multiplex PCR and antimicrobial susceptibility testing (minimal inhibitory concentration, Sensititre®, Trek D. S.) as previously described (Zając et al. 2013).

### Parasitological testing

Muscle samples were mechanically extracted from unskinned snake's carcasses, although in a few cases, the whole dried carcass was tested. The individual samples were chopped and digested using pepsin and hydrochloric acid by the magnetic stirrer method (MSM) followed by microscopy as required by reference *Trichinella* detection method (Commission Regulation (EC) No 2075/2005). The found parasites were preserved in 96 % ethanol for further species identification with PCR assay. Nematode DNA extract (DNA IQ System, Promega) was used to amplify two fragments of 18S ribosomal DNA (rDNA) gene as described by Blaxter et al. (1998). Both amplicons were sequenced, aligned (Geneious v. 7.1.0), and compared with the GenBank database.

## Results

Of the 16 snakes tested, 14 (87.5 %) were positive for *Salmonella* spp., 13 (81.3 %) carried *Distomum musculorum suis*, and larvae of unrecognized helminths were noted in four cases (Table 1). No *Yersinia* spp. and *Mycobacterium* spp. were found.

A total of 33 isolates representing 11 *Salmonella* serovars or distinct antigenic forms were observed with *Salmonella enterica* subsp. *diarizonae* (IIIb) 38:r:z being the most frequent (9 *N. natrix*). Up to four different *Salmonella* serovars were found in a single animal. The serovars belonged to subspecies *diarizonae* (N=9) and *enterica* (N=2). Twenty-four out of 26 *Salmonella* showed microbiological resistance to streptomycin (MIC range from 32–128 mg/L), whereas, the isolates were susceptible to all tested  $\beta$ -lactams and cephalosporins, phenicol, quinolones and fluoroquinolones,

**Table 1** Summary findings of *Salmonella* spp, *Alaria alata* mesocercariae, and unidentified nematodes in tested free-living snakes

ID and snake species	<i>Salmonella</i> spp. <sup>a</sup>	<i>Alaria alata</i>	nematodes
01 <i>N. natrix</i>	IIIb 38:r:z IIIb 47:l,v:z	positive (7) [45 g]	
02 <i>C. austriaca</i>	<i>S. Schleissheim</i> IIIb 40:i:z <sub>53</sub> :z <sub>54</sub> <sup>c</sup>		positive (2) [20 g]
03 <i>N. natrix</i>	<i>S. Sunnycove</i> IIIb 21:l,v: <sup>b</sup> IIIb 38:r:z IIIb 47:l,v:z <sub>57</sub>	positive (21) [42 g]	positive (2) [42 g]
04 <i>N. natrix</i>	IIIb 38:r:z IIIb 48:k:z <sub>53</sub>	positive (10) [40 g]	
05 <i>N. natrix</i>	IIIb 21:l,v: <sup>b</sup> IIIb 38:r:z IIIb 47:l,v:z IIIb 48:k:z <sub>53</sub>		
06 <i>N. natrix</i>	IIIb 38:r:z	positive (7) [40 g]	
07 <i>N. natrix</i>	IIIb 38:r:z IIIb 47:l,v:z	positive (12) [17 g]	positive (1) [17 g]
08 <i>N. natrix</i>	IIIb 38:r:z	positive (45) [40 g]	
09 <i>N. natrix</i>		positive (3) [21 g]	
10 <i>N. natrix</i>	IIIb 21:l,v: <sup>b</sup> IIIb 38:r:z	positive (11) [40 g]	positive (4) [40 g]
11 <i>N. natrix</i>	IIIb 21:l,v: <sup>b</sup> IIIb 48:k:z <sub>53</sub>	positive (15) [49 g]	
12 <i>N. natrix</i>	IIIb 47:l,v:z IIIb 28:z <sub>10</sub> :z IIIb 48:k:z <sub>57</sub> <sup>e</sup> IIIb 47:l,v:z <sub>10</sub>	positive (5) [30 g]	
13 <i>N. natrix</i>	IIIb 21:l,v: <sup>b</sup> IIIb 47:l,v:z <sub>57</sub>		
14 <i>N. natrix</i>	IIIb 28:z <sub>10</sub> :z	positive (12) [36 g]	
15 <i>N. natrix</i>	<i>S. Sunnycove</i> IIIb 28:z <sub>10</sub> :z IIIb 38:r:z	positive (7) [44 g]	
16 <i>N. natrix</i>		positive (22) [32 g]	
total	14 positives, 33 isolates	13 positives	4 positives

Numbers in brackets indicate the (number of larvae) found in [g] of sample

<sup>a</sup> Subspecies designated as: IIIb—*Salmonella enterica* subsp. *diarizonae*, S—*Salmonella enterica* subsp. *enterica* (I)

<sup>b</sup> Atypical serological form

<sup>c</sup> Serovar not listed in White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007)

<sup>e</sup> Serovar already found in Germany in free-living Eurasian adders (Krautwald-Junghanns et al., 2013) and human in Poland (Issenhuth-Jeanjean et al., 2014)

aminoglycosides, tetracycline, sulphonamides, trimethoprim, and colistin.

Thirteen snakes (81.3 %) were infected with mesocercaria of *Alaria alata*, usually named *Distomum musculorum suis* Duncker, 1896 (DMS). The number of trematodes ranged from three to 45 per analyzed sample (Table 1). Additionally, yet unidentified nematodes (up to four per sample, electron microscopy results not shown) were found in four carcasses, including three snakes with DMS co-

infestation. The amplification and sequencing of 18S rDNA have not defined the nematode genus, and the definite identification is still under investigation.

## Discussion

Both tested snake species are under strict protection according to international and national law. Grass snake is the most

common snake found in Poland living in the environments close to the inland waters and preying mainly on amphibians. The smooth snake prefers dry and sunlight areas and feeds mainly on lizards but also amphibians and small rodents. Every year, numerous snakes are killed on road because of their migration and the need for basking. Availability of snake carcasses on roads provides an opportunity of easy meal for omnivores and scavengers, i.e., red foxes or wild boars. Some studies have reported over 50 % of snake carcasses removal from the roads within 8 hours and up to 1 hour from forest roads (DeGregorio et al. 2010). This fact indicates road-killed snakes as a potential source of bacterial infection and parasitic infestation for wild scavengers and omnivores.

Since deliberate capture of protected reptiles is prohibited, the coincidental acquisition of number of dead animals gave a huge opportunity to put some insight on carriage of selected bacteria and parasites in native population. Scientific literature (WoS search, August 2015) on the topic is sparse, and current results might be considered basic and unique.

The study showed high occurrence of diverse *Salmonella* serovars belonging mostly to *Salmonella* (*S.*) *enterica* subspecies *diarizonae*. This includes the second frequent atypical serological form (21:l,v:-) with missing second phase antigens. It is in line with German study reporting unknown and rare *Salmonella* serovars (Krautwald-Junghanns et al. 2013). The observed frequency of *Salmonella* carriage was higher than found in the literature (Krautwald-Junghanns et al. 2013; Kuroki et al. 2013) but comparable to the studies focused on captive animals (Geue and Loschner 2002; Prapasarakul et al. 2012). Our results clearly confirm free-living snakes as reservoir of numerous *S. enterica* subspecies and serovars (Grupka et al. 2006; Krautwald-Junghanns et al. 2013; Kuroki et al. 2013). The tangible proofs for that are finding of *S. enterica* subspecies *diarizonae* 40:i:z<sub>53</sub>,z<sub>54</sub> (not listed in White-Kauffmann-Le Minor scheme), and *S. enterica* subspecies *diarizonae* 48:k:z<sub>57</sub> already reported in free-living adders in Germany (Krautwald-Junghanns et al. 2013) and humans in Poland (Issenhuth-Jeanjean et al. 2014). The later striking example draws attention to possible epidemiological links and public health consequences of free-living snake-associated salmonellosis.

Although both analyzed snake species were obtained from the same location, *Salmonella* serovars noted in *Coronella austriaca* did not occur in *Natrix natrix*. The same serovars found in several grass snake individuals might suggest their direct contact, i.e., during mating or basking, as main route of *Salmonella* transmission. Research done on lizard population proved that bacteria are transmitted from host to host around the social network, rather than adjacent individuals from some common source (Bull et al. 2012). The observed differences also can result from different habitats and prey preferences of both snakes species. Influence of food intake on *Salmonella* prevalence is hard to estimate, but some studies indicate on

this route as a way of transmission of new serovars for an individual (Fuller et al. 2008).

Antimicrobial resistance testing of bacteria isolated from free-living snakes is seldom reported. Further, reptile-associated *Salmonella* show less resistance compared to the ones from farm animals (Zajac et al. 2013). The observed streptomycin resistance is usually the sole resistance being reported in often pan-susceptible *Salmonella* of snake origin (Kuroki et al. 2013; Schmidt et al. 2014; Smith et al. 2012; Zajac et al. 2013).

Despite non-tuberculosis mycobacteria and *Yersinia* spp. were not found, there are studies reporting those pathogens in reptiles (Mitchell 2012; Soldati et al. 2004).

The study demonstrated high prevalence of DMS, the trematode also found in game animals in Poland (Chmurzyńska et al. 2013). The obtained data suggest that free-living snakes may act as vectors of parasites for wild scavengers and omnivores, i.e., wild boars. High numbers of DMS reported in free-living snakes (Mohl et al. 2009; Shimalov and Shimalov 2000) might be related with their prey on amphibians. Noteworthy, both the prevalence and enumeration of parasites might have been reduced by several factors. These include sample condition (limited volume, dried or decayed carcasses) and methodology. The digestion method was arbitrary chosen based on the assumption that small rodents, a component of snake's diet, are reservoirs of *Trichinella* (Flis 2011). Unexpected *Alaria alata* mesocercariae might have been efficiently detected with migration method, since a percentage of DMS is destroyed by acid concentration and pepsine or stuck on the sieve (Riehn et al. 2010). These, along with the finding of still unidentified nematodes, show the area for further investigation and possible knowledge gaps.

In conclusion, current results indicate huge burden of *Salmonella* spp. and muscle parasites carriage by snakes present in the natural environment. Since clinical consequences might be neglected, the findings draw attention to possible zoonotic and epidemiological impacts. Snake carcasses removal from roads by omnivorous, i.e., wild boars, may lead to high numbers of this animals infected with *Alaria alata* and *Salmonella* spp. The prevalence of *Salmonella* in wild boars in Poland reached 6.0 % (unpublished study), and some infrequent *S. enterica* serovars were noted (i.e., *S. Tripoli*). It might justify our concept of free-living snakes as possible source and vector of both pathogens. Finding of rare *Salmonella* serovars, still unidentified nematodes prove the need for further studies and systematic approach to reveal the role of reptiles in epidemiology of infectious agents.

#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

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