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Estimation of Salmonella spp. Prevalence and Diversity among Free-Living Turtles and a Zoo Collection

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Abstract. **Salmonellosis infection is caused by bacteria of the genus *Salmonella*. There are many pathways for the pathogenic bacteria to spread which is through contaminated food, water, and contact with animals. The research was conducted to detect *Salmonella* spp. carriage in free-living aquatic turtles and zoo turtles. Turtles are frequent inhabitants of zoos and the owners of a large number of bacteria in their outer skin and shell surfaces that under adverse conditions can multiply and lead to the development of infection. However, they are known to be a frequent source of *Salmonella* infection without showing signs of disease. The risk of human infection exists if personal hygiene is not followed after contact with a turtle. Identification of bacteria of the genus *Salmonella*, the main causative agent of the disease was determined by the standard method on chromogenic *Salmonella* LAB-Agar™ (BIOMAXIMA, Poland) agar. The inoculation was incubated under aerobic conditions at 37 ± 1°C for 72 h. Serotype (D serotype) was determined by serotyping in 11 from 16 (68.75%) Zoo inhabitants but not found in 25 free-living turtles. Our present research is of theoretical and practical value for the study of free-living turtles as well as from the collections of zoos under protection in Latvia and Europe. It is necessary to remember the possibility of infection with pathogenic bacteria.**

Keywords: bacteria, Chromogenic Agar, infection, salmonellosis, turtle.

I. Introduction

Salmonella spp. species are motile Gram-negative facultative anaerobes of the *Enterobacteriaceae* family that can survive for several weeks in a dry climate and several months in water [1]. Most of the described species are pathogenic. More than 90% of reptiles carry *Salmonella*, sometimes strains that are highly invasive and virulent to humans [2]. *Salmonella* spp. is divided into 60 serogroups and over 2400 serotypes [3-4].

Salmonellosis, one of the most common infectious diseases affecting humans and animals, is widespread worldwide. Non-typhoidal *Salmonella* spp causes 93.8 million cases of gastroenteritis and 155 000 deaths in humans every year [5]. Subspecies cause more than 99% of human *Salmonella* spp. cases I serotypes and about half by *Typhimurium* and *Enteritidis* serotypes [6]. Humans often become infected with salmonellosis through contaminated animal faeces or contaminated soil. Infectious disease can also be spread indirectly through human clothing that has been in contact with reptiles or through animal bites and scratches [7-9]. Most *Salmonella* spp. pathogenic infections in humans result in a mild, self-limiting illness characterized by diarrhoea, fever, nausea, and abdominal cramps [1,10]. However, the infection can circulate in the bloodstream, bone

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marrow, or nervous system, leading to sepsis and even death. These serious complications can be hazardous for infants, children, pregnant women, and older adults with weakened immune systems [11]. Recently, reports of salmonellosis in free-living reptiles have attracted increasing attention. Wild and captive reptiles, particularly lizards, snakes, and turtles, are considered natural reservoirs for a wide variety of *Salmonella* subspecies and serovars readily colonized by vertical and horizontal transport [12-13]. *Salmonella* spp. in reptiles is generally limited to the intestinal tract without invasion of extraintestinal tissues. Only under stress conditions, such as parasitism and trauma, can cause infectious pathological processes [14]. Most *Salmonella* spp. isolates that cause disease in mammals are *Salmonella enterica* subsp. *enterica*, which shed these bacteria in the faeces [15-16]. Reptiles are responsible for somewhere about 6% of sporadic cases of human salmonellosis [17]. Despite the presence of *Salmonella* spp. as a reptile pathogen and its significant zoonotic potential, there is a lack of information on the colonization of *Salmonella* spp. in free-living turtles and zoological collections. Therefore, the purpose of the present study was to reveal *Salmonella* spp. from the intestines of different turtle species in the group of the local Latgale Zoo and the territory of the Silene Nature Park NATURA2000 (Latvia) (N 55.690835°; E 26.788760°) (Fig. 1).

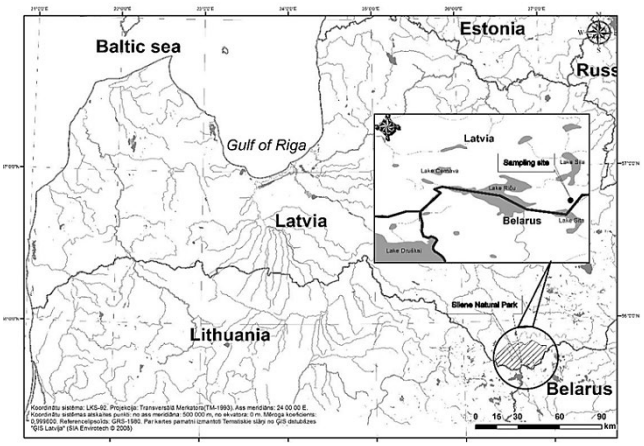

Fig. 1. Sampling site. Silene Nature Park NATURA2000 (Latvia) (N 55.690835°; E 26.788760°).

II. MATERIALS AND METHODS

Sample Collection

During the study period, 41 samples of cloacal swabs were taken from different species of turtles, namely 16 reptiles that were in zoological exposition - *Testudo* (*Agrionemys*) *horsfieldii*, *T. graeca*, and quarantined turtles *T.* (*Agrionemys*) *horsfieldii*, *Trachemys scripta*, *Emys orbicularis* (L.), living in the Latgale Zoo, as well as 25 samples of free-living tortoises – *Emys orbicularis*, *Trachemys scripta* from Silene Nature Park were tested for the presence of *Salmonella* spp. (Fig.1). Before sampling, the reptiles were examined and weighed by a veterinarian. Wild free-living animals, as well as from the zoo's collections, were subjected to physical immobilization. To collect material, the tips of sterile bacteriological swabs (CliniswabTS transport swabs) made of soft viscose fiber were carefully inserted into the cloaca to a depth of 2.5 cm and rotated for 10 s to obtain material from the cloaca. Next, the tubes were transferred to a preservative medium. Before taking a smear, the area around the cloaca was treated with sterile distilled water to prevent sample contamination. All tested reptiles showed no symptoms associated with the presence of *Salmonella*. Cloacal swab samples (CliniswabTS sterile transport swabs) from reptiles were collected immediately to avoid contamination with environmental materials and processed within 24 hours. All turtles were considered healthy at the time of sampling based on daily observations by zookeepers.

Cultivation conditions and identification of *Salmonella* spp. isolates

The collected material was delivered to the laboratory within 2 hours and inoculated on a differential medium: Chromogenic *Salmonella* LAB-Agar™ (BIOMAXIMA, Poland). All cultures were incubated for 72h at 37 ± 1°C under aerobic conditions. Based on studies carried out by the classical method of cultivating microorganisms [18-19], it was noticed that after incubation of the inoculations on the differentiated Chromogenic *Salmonella* Lab-AgarTM medium, the growth of bacterial colonies was visible after three days, which were a red colour, medium size, and round with smooth edges [20,30]. The *Salmonella* spp. isolates were stored at -20°C for further serotyping analysis. Individual colonies were selected, and Gram microscopy was performed. Gram-negative bacteria were isolated.

Serotyping of *Salmonella* spp. isolates

Serotyping is a subtyping procedure that separates strains of different microorganisms into various groups based on their antigenic composition [21]. *Salmonella* spp. serotyping methods are based on the determination of the phenotypic characteristics of microorganisms that are simple, fast, economical, and informative. For research, a pure *Salmonella* spp. culture was used, and incubation occurred for 18-24 hours on nutrient slant agar at a temperature of 37±1ºC. A drop of dissolved serum was applied to the glass with a pipette, and an entire loop of the studied culture was taken from the nutrient agar and placed at a distance of 2-3 mm from the drop. To determine the O-antigen, a culture should be taken from the upper part of the slant agar, and to determine the H-antigen, condensate from the lower growth area (the most mobile individuals). The culture was emulsified in serum for 1 min. The agglutination reactions were interpreted in accordance with the Kauffmann-White protocol [22]. The Kauffman-White scheme is a serological classification of *Salmonella* spp. according to antigenic formulas of *Salmonella* spp. serotypes is formed based on the immunoreactivity of two surface structures of *Salmonella*: the O-antigen and the H-antigen. The reptile

was marked *Salmonella* positive if collected samples were tested positive.

III. RESULTS AND DISCUSSION

Prevalence of *Salmonella* from cloacal samples of turtles

Salmonella species are widely distributed in domestic and wild animals and those living in captivity and most infections in humans are acquired from eating contaminated foodstuffs [16]. According to the Quality Control certificate, it was noticed that after incubation of the inoculations on the differentiated Chromogenic *Salmonella* Lab-AgarTM medium, the growth of bacterial colonies was visible after 72h, which were red in color, medium size, and round with smooth edges. Based on the culture medium Chromogenic *Salmonella* Lab-AgarTM certificate, it was noted that *Salmonella* was identified in samples (Table 1). The isolated cultures of *Salmonella* were identified with sera corresponding to serotypes: serotype B and serotype D. The results were summarized by O- serotyping, the serovar was determined according to the Kauffmann-White scheme. The isolated cultures have given a positive agglutination reaction with serum O:9. It has been receiving antigenic formula O:9, which denoted *Salmonella enterica* subsp. serotype (D serotype). Results revealed that *Salmonella* spp. was isolated from 11 turtles (68.75%) from Zoo turtle species but was not found in free-living specimens (Fig. 2).

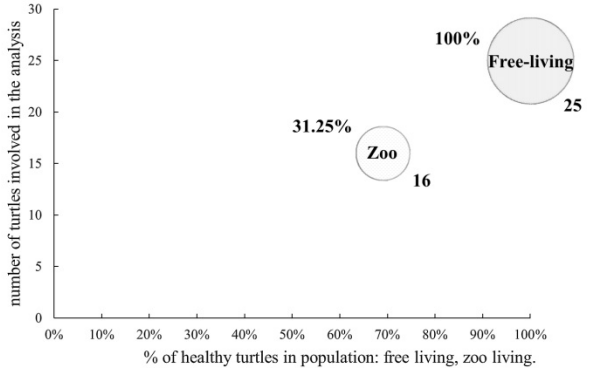

Fig. 2. % of healthy turtles in population: free-living, Zoo-living.

Potentially, there is a risk of *Salmonella* spp. infection from reptiles, so conducting a study on their carriage made sense. Overall, there are more than 2300 *Salmonella* spp. serotypes, and virtually all of them are considered pathogenic [23].In the present study, an isolate from turtles, *Salmonella* species *enterica* (serotype D), was presented using bacteriological and serological analyses. Serotypes differ from each other in the presence of particular antigens: O (thermostable somatic), Vi (thermolabile capsular), and H (flagellar) antigens [24].

The relatively high prevalence of *Salmonella* spp. in the zoological collection can be explained by the high population density in the exposure, minimal exposure to unsanitary conditions, and, possibly, contact with contaminated food. Fresh foods can also become a source of infection. Researchers [25] demonstrated a low prevalence of *Salmonella* spp. in captive turtles, with only one asymptomatic carrier out of 14 studied.

Infected animals often show no signs of illness. It is estimated that 90% of all reptiles carry and excrete *Salmonella* spp. in their feces [26]. The findings highlight the possible potential zoonotic risk when handling reptiles in a Zoo.

The distribution of *Salmonella* spp. in reptiles can reflect their environment [27]. Species of *Salmonella* are ubiquitous and persistent bacteria that can survive for several weeks in a dry climate and several months in water. *Salmonella* species survive well in environmental conditions and persist for up to 10 months. This persistence allows *Salmonella* spp. to be transmitted through environmental surfaces long after the reptile has been returned to its cage [4]. Thus, turtles are kept in aquariums with water that can be contaminated with *Salmonella*, which poses increased risks for infection transmission. Moreover, recent reports indicate that lizards and snakes, compared with turtles, maybe the most common source of reptile-associated salmonellosis in humans [26]. Based on a large number of literary sources and scientific studies, the following assumptions can be made: any clinically healthy animal (reptile) should be considered a potential source of salmonellosis. This highlights the importance of maintaining the strictest hygiene measures in zoological gardens and personal hygiene when in contact with the animal and its microenvironment. Transmission of *Salmonella* spp. from reptiles can occur through environmental pollution. The high temperature and humidity of the reptile house are likely to contribute to the growth and survival of *Salmonella* spp. in the environment [28]. Reptiles can also become infected by ingesting arthropod prey [29].

Particular attention should be paid to reptile salmonellosis in zoological gardens where people, especially young children, come into direct contact with reptiles. Thus, systematic monitoring of the prevalence of salmonellosis in reptiles in the zoological garden appears be necessary to reduce the risk of transmission of salmonellosis from reptiles to humans.

If *Salmonella* spp. is found in the culture, objectively evaluating the results is also necessary. Thus, healthy reptiles can be carriers of *Salmonella* spp., so if the animal does not have clinical signs of salmonellosis, it does not need to be treated. The use of antimicrobials in specific regimens can stop the shedding of *Salmonella* spp., but there is no evidence yet of how long the treatment effect will last. The choice of antibiotic is based on bacteriological culture data. The use of antibiotics can cause the development of microbial resistance. Also, re-infection is possible with insufficient sanitation of the environment (terrarium) of the reptile.

TABLE 1. DATA ABOUT INFECTION PREVALENCE, DETENTION CONDITIONS, WEIGHT, AND THE REASON FOR QUARANTINE OF *SALMONELLA* IN TURTLES FROM THE ZOOLOGICAL COLLECTION

Conditions of Detention	Tortoise Species	Weight (kg)	Average Weight (kg)	Reason for Quarantine	*Salmonella spp.*
1. Quarantine	Central Asian tortoise (*Testudo (Agrionemys) holsfieldii)*	1.601	1.011±0.42	coccidiosis, kidney failure	not detected
2. Quarantine	Central Asian tortoise (*Testudo (Agrionemys) holsfieldii*))	0.411		kidney failure	not detected
3. Exposition	Central Asian tortoise (*Testudo (Agrionemys) holsfieldii*)	0.705	0.615±0.06	–	not detected
4. Exposition	Central Asian tortoise (*Testudo (Agrionemys) holsfieldii*))	0.515		–	not detected
5. Exposition	Central Asian tortoise (*Testudo (Agrionemys) holsfieldii*))	0.625		–	detected
6. Quarantine	Long-necked tortoise (*Deirochelys reticularia*)	0.335	0.441±0.08	Avitaminosis A	detected
7. Quarantine	Long-necked tortoise (*Deirochelys reticularia)*	0.574		Avitaminosis A	detected
8. Quarantine	European pond turtle (*Emys orbicularis*)	0.339	0.379±0.03	Avitaminosis A	detected
9. Quarantine	European pond turtle s) *Emys orbicularis*)	0.419		Avitaminosis A	detected
10. Exposition	Greek tortoise (*Testudo graeca*)	0.663	0.684±0.06	–	detected
11. Exposition	Greek tortoise (*Testudo graeca*)	0.935		–	detected
12. Exposition	Greek tortoise (*Testudo graeca*)	0.741		–	detected
13. Exposition	Greek tortoise (*Testudo graeca*)	0.542		–	detected
14. Exposition	Greek tortoise (*Testudo graeca*)	0.605		–	detected
15. Exposition	Greek tortoise (*Testudo graeca*)	0.620		–	detected
16. Quarantine	Pond slider (*Trachemys scripta*)	0.621	0.621	rachitis, avitaminosis D	detected

Salmonella spp. has a high serological diversity and a high zoonotic potential, which may pose a risk to other animals and humans [30].

IV. CONCLUSIONS

This study provides the first set of scientific data on estimating *Salmonella* spp. prevalence and diversity among reptiles in a local Latgale Zoo and the territory of Silene Nature Park NATURA 2000 (Latvia) (N 55.690835°; E 26.788760°). In this study, only the *Salmonella enterica* subsp. (D serotype) was represented and obtained from turtles in Latgale Zoo (Latvia). Hygiene practices would also be recommended to personnel employed in the zoological gardens in order to minimize the risk of infection from turtles to humans. Visitors to the zoological parks should be informed about the potential risks of maintaining these animals. Immunocompromised individuals, pregnant women, and young children should avoid direct and indirect contact with reptiles.

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