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Oral and cloacal aerobic bacterial and fungal flora of free-living four-lined snakes (*Elaphe quatuorlineata*) from Croatia

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ABSTRACT

The aim of this study was to identify oral and cloacal aerobic flora of four-lined snakes (*Elaphe quatuorlineata*) from Croatian islands Cres and Olib to get better insight into bacteria and fungi potentially harmful to both the animals and the people getting in contact with those animals. Oral and cloacal swabs were taken from a total of 20 snakes and analyzed by standard microbiology and by Real-Time PCR method for *Chlamydia* spp. identification. Neither *Salmonella* spp. nor *Chlamydia* spp. were detected, but some potentially human pathogens, such as *Aeromonas hydrophila*, *Escherichia coli*, *Serratia marcescens* and *Stenotrophomonas maltophilia*, were isolated. Some of fungi detected, such as *Candida albicans*, *Aspergillus flavus* and *Cladosporium* sp., have already been described as secondary causative disease agents in reptile collections. To the author's knowledge this study is the first survey of aerobic microflora of four-lined snakes.

Key words: four-lined snakes, oral cavity, cloaca, bacteria, fungi

Introduction

Information related to gastrointestinal microflora of snakes is scarce. Most of the investigations studied bacteria from the oral cavity of venomous snakes and complications of snake bites (JHO et al., 2011; LIU et al., 2012). Non-venomous snakes may also harbor a wide range of bacteria in their oral cavity able to complicate the bite wounds (DIPINETO

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et al., 2014; YAK et al., 2015) so the information on their microflora should not be neglected. Secondary bacterial infections, such as subcutaneous abscess or tetanus, are possible complications of snake bites, either venomous or non-venomous snakes (HABIB, 2002; GARG et al., 2009). In addition to bites, humans can be infected during manipulation of animals (RABINOWITZ et al., 2007; DEKKER and FRANK, 2015) or via infected equipment (FOSTER and KERR, 2005). The popularity of snakes as pets and the models of biological and veterinary research increased the risk for a public health due to the zoonotic potential of these animals.

Many fungal species were isolated from reptiles, including snakes (ROSENTHAL and MADER, 1996; NICHOLS et al., 1999; CHEATWOOD et al., 2003). Although most of the fungi are normal residents of reptile gastrointestinal tract, they can cause secondary infections under suboptimal conditions and can play an important role as disease-causing agents in reptiles including snakes (JACOBSON, 1980; HERNANDEZ-DIVERS, 2001; MILLER et al., 2004; OROS et al., 2004).

There is no information regarding microflora and potential pathogens from gastrointestinal system of Croatian autochthonous snakes. Therefore, oral cavity and cloacal swabs were taken from a total of 20 four-lined snakes (*Elaphe quatuorlineata*) at Croatian islands Cres and Olib, to get more insight into physiological microflora, potential pathogens and opportunistic organisms from oral cavity and cloaca of free-living snakes.

Materials and methods

Animals. A total of 20 four-lined snakes (*Elaphe quatuorlineata*) from islands Cres and Olib, 10 animals from each island, were sampled at their natural habitats. Biological characteristics and behavior of animals and their habitats are described in Table 1. All of the animals were captured during May and June 2013.

Capture and clinical examination. Animals were captured by hands, and manually restrained using leather gloves, at various locations of islands Cres (44°57’36’’N, 14°24’29’’E) and Olib (44°22’39’’N, 14°46’41’’E), mostly in forests, olive-groves and dry walls. Each animal was immediately examined by visual inspection of the eyes, nostrils, cloaca and skin. The complete body surface was also inspected for the presence of injuries or deformations. After opening by a sterile forceps, oral cavity was inspected for the presence of stomatitis or mechanical injuries.

Sampling. Following appropriate marking of swab tubes, the sampling was carried out as follows: one person fixed the snake’s head, the other one opened its orifice laterally to avoid oral cavity injury and took two swabs from both cloaca and oral cavity (Figs. 1 and 2). Swabs taken with transport media (Amies transport swabs Aptaca, Italy) were used for standard microbiology, and those without transport media (Copan plain swab, Italy) were used for molecular diagnostic. The animals were then released at the sites of their capture. The swabs were stored at 4 °C until examination.

Table 1. Biological characteristics, behaviour and habitat description of captured four-lined snakes from islands Cres and Olib

Island Cres				
Age	Gender	Behaviour	Habitat description	No.
Adult	Female	Resting in shade	Wood surrounded with dry walls	1
Adult	Male	Resting in shade	Wood surrounded with dry walls	2
Adult	Male	Basking	Open karst terrain with a little bit of vegetation	1
Juvenile	Female	Basking	Open karst terrain with a little bit of vegetation	1
Juvenile	Female	Resting in shade	Wood surrounded with dry walls	2
Juvenile	Male	Basking	Open karst terrain with a little bit of vegetation	3
Total				10
Island Olib				
Adult	Female	Basking	Macchia surrounded with dry walls	1
Adult	Female	Basking	Karst terrain with a low vegetation	1
Adult	Female	Basking	Holm oak wood surrounded with dry walls	1
Adult	Male	Basking	Karst terrain with a low vegetation	2
Adult	Male	Resting in shade	Bushes with rocks	1
Subadult	Female	Basking	Holm oak wood surrounded with dry walls	2
Subadult	Male	Hiding	Karst terrain with a low vegetation	1
Subadult	Male	Escaping	Karst terrain with a low vegetation	1
Total				10

Fig. 1. Oral cavity swabbing of the four-lined snake

Fig. 2. Cloacal swabbing of the four-lined snake

Microbiological procedures. The samples (40 in total) were processed in the bacteriological laboratory at the Department of Poultry Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb. For aerobic bacteria identification standard methods described by BROWN (2005) were used. Samples were plated directly on the Nutrient agar (Difco Nutrient Agar, Becton, Dickinson and Company, SAD) and selective Brilliant green agar (BGA) (Brilliant green agar - modified, Oxoid, England) and incubated under aerobic conditions for 24 hrs at room temperature. Results were read after 24 and 48 hrs, depending on the growth of bacterial colonies. Bacterial colonies were then randomly selected, recultivated to obtain the pure bacterial culture and examined microscopically after Gram-staining (Gramova otopina, Gram-Mol d.o.o., Croatia). Isolated bacteria were further identified morphologically, by oxidase (Oxidase strips, Oxoid, Great Britain) and catalase (vodikov peroksid, Kemika, Croatia) tests, and biochemically by the Analytical profile index (API) (API E and API NE System, Bio Merieux S.A., France). For fungal identification, swabs were plated on Sabouraud dextrose agar (Sabouraud dextrose agar, Oxoid, England) and incubated at room temperature for up to five days. Fungi were identified based on their morphological characteristics and microscopically by lactophenol staining (Lactophenol blue solution, Sigma-Aldrich, France).

Salmonella spp. detection. Cloacal swabs were enriched in Selenite cysteine broth (Becton-Dickinson and Company, France), incubated for 24 hrs at 37 ^{o}C, plated on BGA and Xylose lysine deoxycholate agar (XLD) (Merck, Germany), and incubated 24 hours at 37 ^{o}C and then 24 hrs at room temperature.

Chlamydia spp. detection. Pooled samples of oral cavity and cloaca of each snake were analysed in the Laboratory for Chlamydia at the above Department. The DNA was extracted using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, USA) according to the manufacturer's instructions and examined by *Chlamydiaceae*-specific Real-Time PCR described by EHRICHT et al. (2006). Each sample was run in duplicate, with positive (*C. psittaci* strain) and negative (ultrapure water) controls, also run in duplicate. Samples were analysed by the Mx3005P (Stratagene, USA) instrument with TaqMan system for replicated segments identification under the following conditions: 95 °C 10 min, 50 replication cycles, denaturation for 15 sec at 95 ºC, primer annealing at 60 ºC for 60 sec, when fluorescence was recorded.

Statistics. The results were analysed by the Fischer's exact test using MedCalc, version 10.4.0.0., MedCalc Software bvba, Mariakerke, Belgium.

Results

Clinical examination did not reveal any deformations or pathological changes on animal's eyes, nostrils or skin. The oral cavities of all animals were clean and free of any pathological changes.

Oral and cloacal aerobic flora was analyzed in a total of 20 four-lined snakes from Croatian islands Cres and Olib. Bacteria and/or fungi were detected in cloacal swabs of all animals and in oral cavity swabs of eight animals from each island.

Table 2. Bacterial species isolated from oral cavity and cloaca of four-lined snakes from islands Cres and Olib

Bacterial species	Number of positive findings in 10 animals from each island	
	Cres	Olib
	Oral cavity	
Aeromonas hydrophila	1	
Aeromonas salmonicida	2	3
Escherichia coli		1^{B}**
Proteus mirabilis	1	
Pseudomonas fluorescens	4	1
Serratia marcescens	2	
Stenotrophomonas maltophilia	3	1
Micrococcus luteus		1
Bacillus cereus	2	
Bacillus sp.	2	3
Staphylococcus sp.	3	3
	Cloaca	
Aeromonas hydrophila	4	
Escherichia coli	5	6^{A}
Pseudomonas fluorescens	1	
Serratia marcescens	1	
Stenotrophomonas maltophilia	1	
Proteus sp.	1^{a}**	9^{b}
Bacillus cereus	3	
Micrococcus luteus	1	
Staphylococcus sp.	1	1
Bacillus sp.	8^{a}*	1^{b}

Different lowercase superscript letters indicate differences between Cres and Olib animals, while different uppercase superscript letters indicate difference between oral cavity and cloaca of the whole group of animals; *P = 0.005, **P = 0.001 (Fischer's exact test).

Island Cres. Nine bacterial species, belonging to Gram-positive and Gram-negative flora, were identified in the oral cavity. The most represented species was *Pseudomonas fluorescens*, followed by *Staphylococcus* sp., *Stenotrophomonas maltophilia*, *Aeromonas salmonicida*, *Serratia marcescens*, *Bacillus* sp., *Bacillus cereus*, *Aeromonas hydrophila* and *Proteus mirabilis* (Table 2). Only three fungal species, *Cladosporium* sp., *Mucor* sp. and *Candida albicans* were isolated from oral cavity samples (Table 3).

Table 3. Fungal species isolated from oral cavity and cloaca of four-lined snakes from islands Cres and Olib

Fungal species	Number of positive findings in 10 animals from each island	
	Cres	Olib
	Oral cavity	
Candida albicans	2	1
Cladosporium sp.	5	2[B]
Mucor sp.	2	3
	Cloaca	
Aspergillus flavus		1
Aspergillus niger	1	
Candida albicans	1	
Rhodotorula rubra	2	
Cladosporium sp.		1[A]
Mucor sp.	1	

Different superscript letters indicate difference between oral cavity and cloaca of the whole group of animals; P = 0.04 (Fischer's exact test).

Ten bacterial species, mostly Gram-negative, were isolated from cloacal swabs: *Bacillus* sp., *Escherichia coli*, *A. hydrophila*, *B. cereus*, *Proteus* sp., *Staphylococcus* sp., *Micrococcus luteus*, *S. maltophilia*, *S. marcescens* and *P. fluorescens* (Table 2). Four fungal species were isolated from cloacal swabs: *Rhodotorula rubra, Aspergillus niger*, *C. albicans* and *Mucor* sp. (Table 3).

Island Olib. Bacterial flora in oral cavity of snakes from island Olib was also composed of Gram-positive and Gram-negative bacteria, with the predominance of Gram-negatives. Bacteria *Bacillus* sp., *Staphylococcus* sp. and *A. salmonicida* were present, as well as *E. coli, P. fluorescens, M. luteus* and *S. maltophilia* (Table 2). Three fungal species were isolated from oral cavity: *Mucor* sp., *Cladosporium* sp. and *C. albicans* (Table 3).

In cloacal swabs only four bacterial species were identified: *Proteus* sp., *E. coli*, *Staphylococcus* sp. and *Bacillus* sp. *Proteus* sp. was identified in significantly more, and

Bacillus sp. in significantly less cloacal swabs than in Cres animals (Table 2). Two fungal species were isolated from cloacal swabs: *Aspergillus flavus* and *Cladosporium* sp. (Table 3).

All of the oral cavity and cloacal samples from both islands were negative for *Salmonella* spp. and *Chlamydia* spp.

Discussion

The four-lined snake (*Elaphe quatuorlineata*), the biggest European snake (KREINER, 2007), is strictly protected wild taxon in Croatia (ANONYMOUS, 2013) where it inhabits the whole littoral up to the altitude of 600 m, and many islands.

From a total of 20 animals bacteria and/or fungi were isolated from oral cavity of eight snakes from each island. A total of 11 different bacterial species were identified, with a non-significant predominance of Gram-negatives. This finding is in line with the results of YAK et al. (2015) who isolated Gram-negative organisms from oral cavity of 8/10 free-living reticulated pythons (*Python reticulates*) in Singapoore. No significant differences between two islands in the number or type of bacteria were noted. Most of the isolated Gram-negative, opportunistic microflora has already been described as a potential cause of diseases in animals and humans (DRAPER et al., 1981; HARRIS and ROGERS, 2001; CHEN et al., 2011). Considering an increasing popularity of reptiles in Croatia, this aspect of human infection should not be overlooked.

Cloacal bacteria were isolated from all of the animals studied. A total of 10 species were isolated, with predominance of Gram-negative ones, similarly to the situation in the oral cavity. In 50% of Cres and 60% of Olib animals *E. coli* was isolated, compared to 0% and 10% in the oral cavity, respectively. This significant difference indicates that, like in other animal species, the microflora along the snake gastrointestinal tract is different and does not have to be uniform in all of its segments. Significantly more cloacal swabs were positive to *Bacillus* sp. in Cres and to *Proteus* sp. in Olib animals. Both findings may indicate differences in geographic locations of particular snake populations regarding their oral cavity and cloacal aerobic microflora. However, the influence of potential *Proteus* sp. overgrowth due to longer-lasting transportation of samples from Olib than from Cres, should also not be excluded as a factor contributing to higher number of *Proteus* sp. isolates in Olib animals.

None of the samples in this study were positive to *Salmonella* sp., otherwise very frequent finding in snakes (KUROKI et al., 2013; SCHEELINGS et al., 2011; STING et al., 2013; LUKAC et al., 2015) and a common cause of disease in humans. Low incidence of *Salmonella* was also described by SCHMIDT et al. (2014) who reported 14.3% (8/56) *Salmonella* spp. positive cloacal swabs in free-living snakes and lizards sampled in Germany. However, all of the animals in our study were sampled only once, so that one negative finding does not mean that animals were free of *Salmonella*, probably due to

bacterial intermittent shedding (ACKMAN et al., 1995). Bacteria from *Chlamydiaceae* family, known to cause pneumonia (TAYLOR-BROWN et al., 2015) and hepatitis (HUCHZERMEYER et al., 2008) in reptiles, were also not detected in this study.

Three fungal species were isolated from oral cavity of snakes from both islands. The most frequent was *Cladosporium* sp. Although this is an ubiquitous organism, it was described as a cause of pneumonia in sea turtles (JACOBSON et al., 1979), nephritis and peritonitis in the loggerhead sea turtle (*Caretta caretta*) (DOMICIANO et al., 2014) and pneumonia in humans (METZGER et al., 2010). Opportunistic *C. albicans* was described as a cause of necrosis, bleeding and gastrointestinal edema in the Aldabra giant tortoise (*Geochelone gigantea*) (JUNIANTITO et al., 2009) and pulmonary candidiasis in spur-thighed tortoise (*Testudo greca*) (HERNANDEZ-DIVERS, 2001). Although ENWEANI et al. (1997) described *Candida* spp. and *Mucor* spp. as normal isolates from gastrointestinal system of agamas, these fungi in combination with other microorganisms may cause ulcerative epidermitis (JACOBSON, 1980) and osteomyelitis (GARTRELL and HARE, 2005) in reptiles under suboptimal conditions.

Six fungal species were isolated from cloacal swabs of animals from both islands. *Candida* sp. and *Aspergillus* sp. were described as causes of secondary infections in gopher tortoise (*Gopherus polyphemus*) (MYERS et al., 2009) and both genera can cause various symptoms in humans, such as respiratory, intestinal or cutaneous problems, especially in immunocompromised people (BERNARDESCHI et al., 2015; NOBILE and JOHNSON, 2015). *Rhodotorula rubra* was reported to cause septicemia (EL-TAHAWY and KHALAF, 1999) and meningitis in immunocompromised persons (THAKUR et. al., 2007). There is no information on its effects in reptiles.

In conclusion, the results of this study indicate that four-lined snakes from Croatian islands Cres and Olib harbor numerous fungi as well as Gram-positive and Gram-negative bacteria, with some predomination of Gram-negative ones. Most of the isolated bacteria have already been described as causes of infection in both reptiles and humans. To the author's knowledge this is the first survey of oral cavity and cloacal aerobic microflora of four-lined snakes.

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SAŽETAK

Bakterijske i gljivične bolesti dobro su poznat problem gmazova u zatočeništvu. Mnogo je manje podataka dostupno o fiziološkoj flori probavnog sustava i mogućim patogenima divljih gmazova. Svrha je ovog istraživanja bila identificirati aerobnu floru usne šupljine i kloake kravosasa (*Elaphe quatuorlineata*) s hrvatskih otoka Cresa i Oliba, kako bi se saznalo više o bakterijama i gljivicama koje pod nepovoljnim okolnostima mogu uzrokovati bolest u samih životinja ali i u ljudi koji dolaze u kontakt s tim životinjama. Uzeti su obrisci usne šupljine i kloake od ukupno 20 kravosasa i analizirani standardnim mikrobiološkim metodama, te metodom real-time PCR za dokazivanje bakterija *Chlamydia* spp. U uzorcima nisu bile dokazane ni salmonele ni klamidije, no izdvojene su neke bakterije poznate kao mogući patogeni u čovjeka, poput vrsta *Aeromonas hydrophila*, *Escherichia coli*, *Serratia marcescens* i *Stenotrophomonas maltophilia*. Izdvojene su i neke gljivice, poput vrsta *Candida albicans*, *Aspergillus flavus* i *Cladosporium* sp., koje su već opisane kao sekundarni uzročnici bolesti u kolekcijama gmazova. Prema saznanjima autora, ovo je prvo istraživanje aerobne mikroflore kravosasa.

Ključne riječi: kravosasi, usna šupljina, kloaka, bakterije, gljivice