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Original article

Gut microbiota isolated from the European pond turtle (*Emys orbicularis*) and its antimicrobial resistance

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Abstract

The aim of the study was to isolate cultivable gut microbiota from European pond turtles kept at the Lithuanian Zoo and to determine antimicrobial resistance of the isolates.

The study subjects included 8 elderly turtles living at the Lithuanian Zoo for about 50 years as well as their offspring – 24 young individuals (1-2 years old) that were hatched at the same zoo. Animals were not exposed by treatment with antimicrobials during the last 3 years.

Gut samples were taken from the cloaca and inoculated onto universal media. Isolates then were identified using sequence analysis of 16S rRNA.

The antimicrobial susceptibility testing was performed using the agar diffusion method according to Kirby-Bauer. Clinical breakpoints according to CLSI whenever possible, were used for interpretation of susceptibility. Bacterial isolates resistant to at least three antimicrobials of different classes were treated as multi-resistant.

Fifty-two bacterial isolates were obtained and identified from turtle gut samples. The most prevalent genera included *Aeromonas, Chryseobacterium* and *Citrobacter.* Fifty percent of the isolates obtained from elderly turtles (CI 95% – 19.01-80.99) and 54.8% (CI 95% – 39.75-69.85) of the isolates from young animals were identified as multi-resistant. The most common resistance rates of the isolates from both groups of the turtles were observed toward ampicillin (86.6%), ciprofloxacin (61.5%) and gentamicin (40.4%). The lowest number of resistant isolates were detected toward combination of sulfamethoxazole-trimethoprim (26.9%). The study revealed that European pond turtles kept in captivity are carriers of multi-resistant bacteria however, further studies need to be performed to investigate whether the resistant microorganisms are natural microbiota for this species or they were acquired in the zoo.

Key words: *Emys orbicularis*, turtles, antimicrobial resistance, microbiota

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Introduction

Lithuania is at the northern border of the European pond turtle (*Emys orbicularis*) habitat. This species has a high risk of extinction, and it is under legal protection and covered under the Natura 2000 project (Nowakiewicz et al. 2015). In Lithuania, the European pond turtle has been under legal protection since 1976 and is listed as an endangered species in the Lithuanian Red Book. The Lithuanian population decreased between 1975 and 2010, but has increased significantly since 2010. One of the reasons of successful restoration of the population was implementation of the programme for preserving European pond turtles by keeping adult as well as young – 1-2 years old individuals taken from the nature with further release them into natural environment.

All aquatic or semi-aquatic turtle species live in warm water and thus they have a high risk of contamination by different bacteria and parasites. Moreover, the turtle immune system is sensitive to environmental stress and bacterial infections. These are frequent – particularly in individuals kept in breeding centers (Glazebrook and Campbell 1990, Oros et al. 2005). Although water-dwelling species might have intrinsic immunity against various pathogens, turtles are carriers of different bacterial species including pathogenic ones (Nowakiewicz et al. 2015). The most prevalent bacterial species found in free-living European pond turtles in neighboring Poland were *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus* spp., i.e. bacteria that are common in many avian and mammal species including humans (Nowakiewicz et al. 2015).

European pond turtles have been kept in captivity at the Lithuanian Zoo since 1938. Some individuals are more than 50 years old. Other wild species of turtles and other reptiles taken from the wild are kept in this zoo as well. Since 2010, young European pond turtles are taken from the Regional Park of Meteliai and delivered to Lithuanian Zoo for further growth to increase their viability. Those turtles are then released back into their natural environment. The old turtles living in Lithuanian Zoo were successfully bred as well. The offspring of those turtles still live in the zoo and are not released. Although there are data suggesting that the variety of microbiota in European pond turtles kept in breeding centers are different from natural conditions, we suggest that it may strongly depend on diet, hygienic conditions, the other animals present, etc.

Antimicrobial treatment may also influence the microbiota – particularly in treated animals because infections in turtles are quite frequent (Di Ianni et al. 2015). In veterinary medicine, reptiles are treated as minor species, and there are no indications or determined doses of antibiotics for their treatment. Moreover, clinical material from turtles is rarely studied in clinical laboratories for determination of infectious agent and antibioticograms. Therefore, under-dosing or overdosing of those drugs is assumed based on the cascade usage of antimicrobials. Such inappropriate using of antibiotics might select gut microbiota for antimicrobial resistance. The aim of this study was to isolate cultivable gut microbiota from European pond turtles kept at the Lithuanian Zoo and to determine the antimicrobial resistance of the isolates.

Materials and Methods

Animals and samples

Study subjects included 8 old turtles living in Lithuanian Zoo for about 50 years (born in about 1966; group I) as well as their offspring – 24 young individuals (1-2 years old; group II) that were hatched and kept at the same zoo. The sampling was performed in December 2015, i.e. 1-2 years after young turtles were hatched. The study was approved by the Environmental Protection Agency (under the Ministery of Environment of the Republic of Lithuania, Protocol N59, 7 October, 2015). Samples were collected directly from cloaca during prophylactic inspection using thin sterile cotton swabs with transport medium (Transwab, MWe, UK) in December 2015. Collected material was delivered to the laboratory during 2 hours. According to the analysis of records on drug usage for turtle treatment it was stated that no antimicrobials were used for those animals during last 3 years.

Microbiological and molecular testing

Samples were inoculated onto Columbia Agar supplemented with sheep blood (E&O Laboratories, Scotland) and incubated for 72 hours at +30°C. Predominant type of colonies were selected for further purification with the aim to obtain pure cultures from each individual sample. In case of mixed microbiota (two or more types of colonies were dominant) up to three different types of well isolated colonies were taken with the aim to obtain variety of bacterial species. DNA material for molecular testing was obtained after bacterial lysis according to the extraction protocol prepared by the Community Reference Laboratory for Antimicrobial Resistance with slight modifications. Briefly, the isolates were inoculated onto Mueller Hinton Agar (Oxoid, UK) and incubated

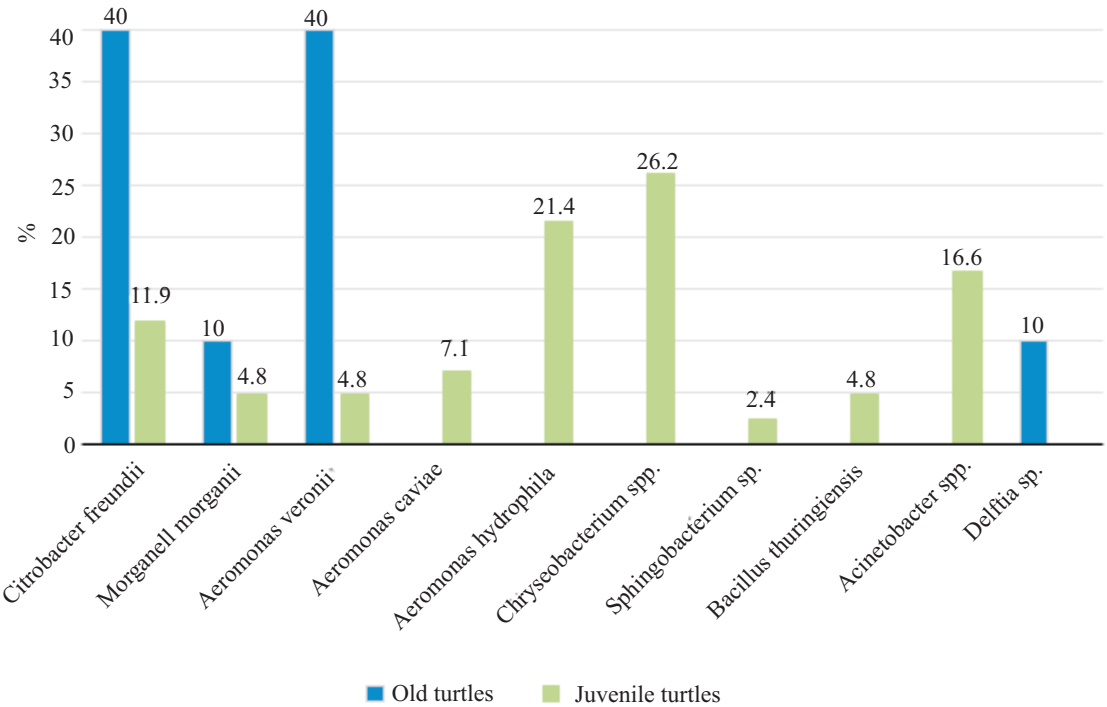


Fig. 1. Species/genus distribution among bacteria isolated and identified from old and juvenile turtes (%).

for 48 hours at +30°C. Thereafter, few colonies were transferred to phosphate buffered saline (pH 7.3). The content was centrifuged for 5 min. Then the supernatant was discarded and the pellet was re-suspended in Tris-EDTA (TE) buffer. The suspension was heated using Biosan (Latvia) thermomixer in 100°C degrees for 10 minutes. Boiled suspension was transferred directly on ice and diluted 1:10 in TE.

PCR using universal primers 27F and 515R was performed as described previously (Ruzauskas et al. 2014). Obtained PCR products were then purified using DNA Clean and Concentrator-5 Kit (Zymo Research, USA). Identification of the isolates was performed by sequencing of 16S rRNA using the same universal primers and purified PCR products. Sequences were analysed using Molecular Evolutionary Genetic Analysis software (MEGA, version 6). Basic local alignment search tool (BLAST) was used for comparison of obtained sequences with sequences presented in the database of National Centre of Biotechnology Information (NCBI, USA).

Testing for antimicrobial resistance

Antimicrobial susceptibility testing was performed using the agar diffusion method according to Kirby-Bauer. Discs with the following antimicrobials (µg) were used: ampicillin (10), gentamicin (10), tetracycline (30), ciprofloxacin (5), sulfamethoxazole/trimethoprim (25), cefpodoxime (10) and chloramphenicol (30). Interpretation of the results for Enterobacteriaceae and *Aeromonas* spp. was carried-out using the Clinical & Laboratory Standards Institute clinical breakpoints. Interpretation for *Acinetobacter* spp. whenever possible, was carried-out using the CLSI clinical breakpoints set for *Acinetobacter*. As there are no interpretation criteria set for the other genera of bacteria isolated in this study, the interpretation was performed according to inhibition zones set by CLSI for Enterobacteriaceae. The results were presented in metric units (sterile zones in mm) as well. Bacterial isolates resistant to at least three antimicrobials of different classes were treated as multi-resistant isolates.

Data analysis

Antimicrobial resistance rates were given as numbers/percent of resistant isolates per total number of each taxonomic unit. Prevalence of multi-resistant isolates in both animal groups was assessed using logistic regression analysis. Statistical analysis was performed using „IBM SPSS Statistics 20" package. Results were considered statistically significant if $p<0.05$.

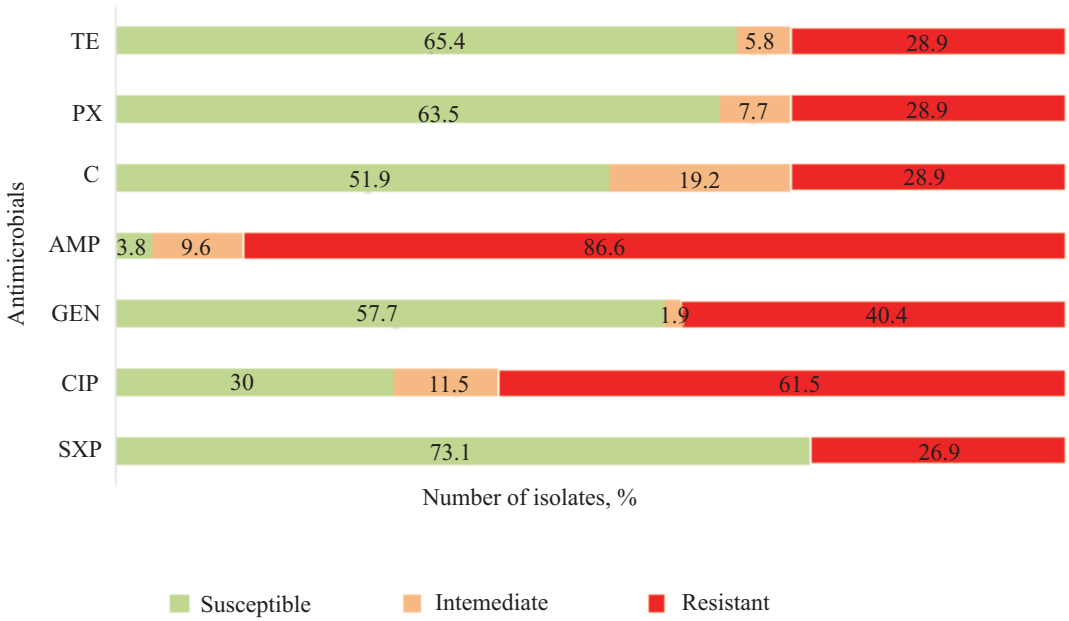
Results

Fifty two bacterial isolates were obtained and identified from gut samples of the turtles. Taxonomic distribution of the isolates is presented in Fig. 1. The most prevalent genera included *Aeromonas* (32.7%),

Table 1. Antimicrobial susceptibility of the isolates and species identified in I and II groups of the turtles.

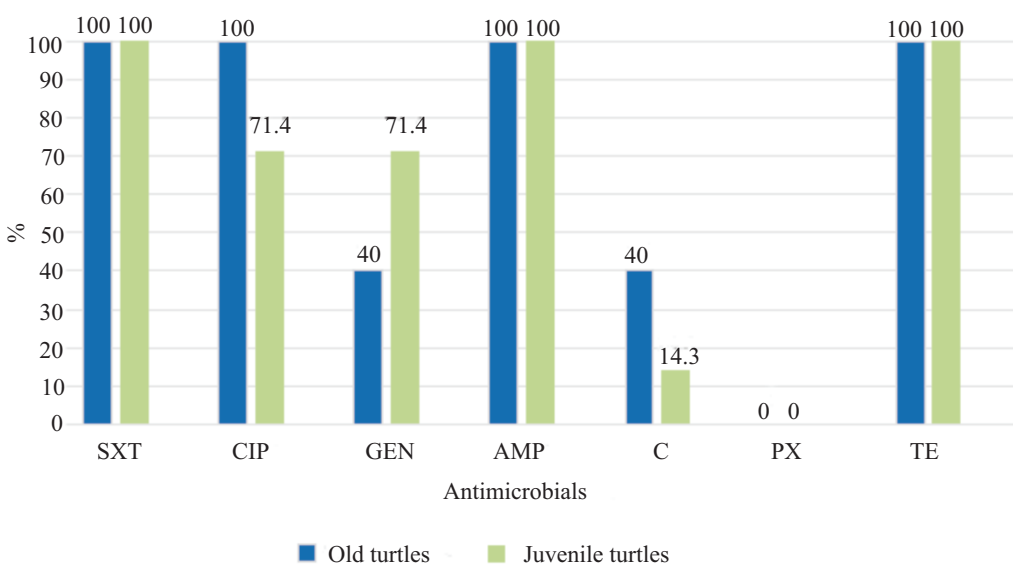
Turtle number	Isolate number	Antimicrobial agents, susceptibility and inhibition zones in mm							Taxa (genus, species)
		SXT	CIP	GEN	AMP	C	PX	TE	
I group (elderly turtles)									
1	V15	R(6)	R(7)	R(6)	R(6)	I(17)	S(33)	R(6)	*Citrobacter freundii*
2	V49	S(27)	I(16)	S(19)	R(6)	S(32)	S(40)	R(10)	*Aeromonas veronii*
3	V50	R(6)	R(6)	S(18)	R(6)	S(21)	I(18)	R(6)	*Citrobacter freundii*
3	V51	S(25)	R(6)	S(16)	I(16)	S(18)	I(18)	S(21)	*Delftia*
4	V52	R(6)	R(6)	S(19)	R(6)	S(30)	S(30)	R(6)	*Citrobacter freundii*
5	V39	S(25)	I(17)	S(20)	R(6)	S(32)	S(40)	I(12)	*Aeromonas veronii*
6	V39	S(25)	I(17)	S(20)	R(6)	S(32)	S(40)	I(12)	*Aeromonas veronii*
6	V17	R(6)	R(6)	S(20)	R(6)	R(11)	S(30)	R(8)	*Citrobacter freundii*
7	V11	R(6)	R(6)	R(11)	R(6)	R(6)	S(31)	R(6)	*Morganella morganii*
8	V9	S(28)	I(17)	S(20)	R(6)	S(38)	S(35)	R(10)	*Aeromonas veronii*
II group (juvenile turtles)									
9	V10	S(24)	R(6)	I(13)	R(10)	R(6)	I(20)	S(17)	*Acinetobacter*
10	V7	S(22)	R(8)	S(20)	I(15)	S(20)	I(19)	S(20)	*Acinetobacter*
11	V8	S(26)	R(6)	R(9)	R(6)	R(9)	R(6)	S(18)	*Chryseobacterium*
12	V32	S(30)	R(6)	R(6)	R(6)	R(8)	R(6)	S(23)	*Chryseobacterium*
13	V18	S(30)	I(16)	S(16)	R(6)	I(17)	S(36)	S(33)	*Aeromonas veronii*
13	V31	R(6)	R(8)	R(6)	R(6)	S(23)	S(30)	R(6)	*Citrobacter freundii*
14	V16	S(22)	R(6)	R(10)	R(6)	I(13)	R(6)	S(30)	*Chryseobacterium*
14	V27	S(29)	R(6)	R(8)	R(6)	R(8)	R(6)	S(20)	*Chryseobacterium*
15	V22	S(30)	R(15)	R(6)	S(29)	S(31)	S(38)	S(40)	*Sphingobacterium*
16	V23	R(6)	S(24)	S(21)	R(6)	R(6)	S(35)	R(6)	*Morganella morganii*
16	V44	R(6)	R(6)	R(6)	R(6)	I(14)	S(30)	R(6)	*Citrobacter freundii*
16	V45	S(28)	R(6)	R(8)	R(6)	R(6)	R(6)	S(20)	*Chryseobacterium*
17	V19	S(31)	R(6)	R(8)	R(6)	R(8)	R(6)	S(21)	*Chryseobacterium*
18	V20	S(26)	S(26)	S(23)	R(6)	S(32)	S(38)	S(40)	*Aeromonas hydrophila*
18	V28	S(30)	R(6)	R(6)	R(6)	R(8)	R(6)	S(25)	*Chryseobacterium*
18	V30	R(6)	R(8)	R(6)	R(6)	S(25)	S(31)	R(8)	*Citrobacter freundii*
19	V33	S(30)	R(6)	R(11)	S(18)	I(17)	S(21)	S(28)	*Acinetobacter*
20	V47	S(25)	S(24)	S(21)	R(6)	S(30)	S(31)	S(32)	*Aeromonas hydrophila*
21	V48	R(6)	R(8)	R(6)	R(6)	S(26)	S(26)	R(6)	*Citrobacter freundii*
22	V5	S(17)	S(28)	S(21)	R(6)	S(33)	S(35)	S(31)	*Aeromonas hydrophila*
22	V6	S(18)	S(41)	S(28)	R(6)	S(27)	R(6)	S(23)	*Bacillus thuringiensis*
23	V46	R(6)	R(6)	R(6)	R(6)	S(25)	S(30)	R(6)	*Citrobacter freundii*
23	V42	S(30)	R(6)	S(16)	I(16)	I(16)	S(21)	S(24)	*Acinetobacter*
23	V25	S(24)	R(15)	S(21)	R(6)	S(18)	R(6)	S(18)	*Aeromonas caviae*
24	V21	S(16)	S(25)	S(22)	R(6)	S(35)	S(33)	S(44)	*Aeromonas caviae*
24	V41	S(26)	R(6)	S(16)	I(16)	I(16)	I(20)	S(29)	*Acinetobacter*
24	V43	S(23)	I(16)	S(21)	R(6)	S(35)	S(38)	S(16)	*Aeromonas caviae*
25	V24	S(30)	S(26)	S(23)	R(6)	I(13)	S(37)	S(18)	*Aeromonas hydrophila*
26	V12	S(17)	S(25)	S(20)	R(6)	R(12)	S(33)	I(13)	*Aeromonas hydrophila*
27	V13	S(23)	R(15)	S(20)	R(6)	S(35)	S(42)	R(11)	*Aeromonas veronii*
27	V14	S(25)	R(6)	R(10)	R(6)	R(6)	R(6)	S(17)	*Chryseobacterium*
28	V1	R(6)	R(6)	R(6)	R(6)	R(6)	R(6)	S(19)	*Chryseobacterium*
28	V3	S(25)	S(22)	S(20)	R(6)	S(28)	S(35)	S(30)	*Aeromonas hydrophila*
29	V2	S(26)	R(6)	S(20)	R(6)	I(16)	I(19)	S(25)	*Acinetobacter*
29	V26	S(30)	S(23)	S(24)	R(6)	S(24)	S(30)	S(36)	*Aeromonas hydrophila*
30	V29	S(26)	S(25)	S(25)	R(6)	S(35)	S(38)	S(38)	*Aeromonas hydrophila*
30	V34	R(6)	S(22)	S(20)	R(6)	S(30)	S(26)	R(6)	*Morganella morganii*
30	V37	S(30)	R(6)	R(6)	R(6)	R(6)	R(8)	S(25)	*Chryseobacterium*
31	V35	S(25)	R(6)	R(6)	R(6)	R(6)	R(6)	S(22)	*Chryseobacterium*
31	V38	S(27)	S(26)	S(21)	R(6)	S(26)	S(36)	S(31)	*Aeromonas hydrophila*
32	V36	S(27)	R(6)	R(6)	I(16)	I(16)	R(6)	S(25)	*Acinetobacter*
32	V4	R(6)	S(27)	S(21)	R(6)	S(22)	R(6)	S(22)	*Bacillus thuringiensis*
Total number (n) of resistant, intermediate and susceptible isolates									
Resistant		14	32	21	45	15	15	15	
Intermediate		0	6	1	5	10	5	3	
Susceptible		38	14	30	2	27	32	34	

R – resistant, I – intermediate, S – susceptible, SXT – sulfamethoxazole/trimethoprim, CIP – ciprofloxacin, GEN – gentamicin, AMP – ampicillin, C – chloramphenicol, PX – cefpodoxime, TE – tetracycline.



TE – tetracycline, PX – cefpodoxime, C – Chloramphenicol, AMP – ampicillin, GEN – gentamicin, CIP – ciprofloxacin, SXT – sulfamethoxazole/trimethoprim.

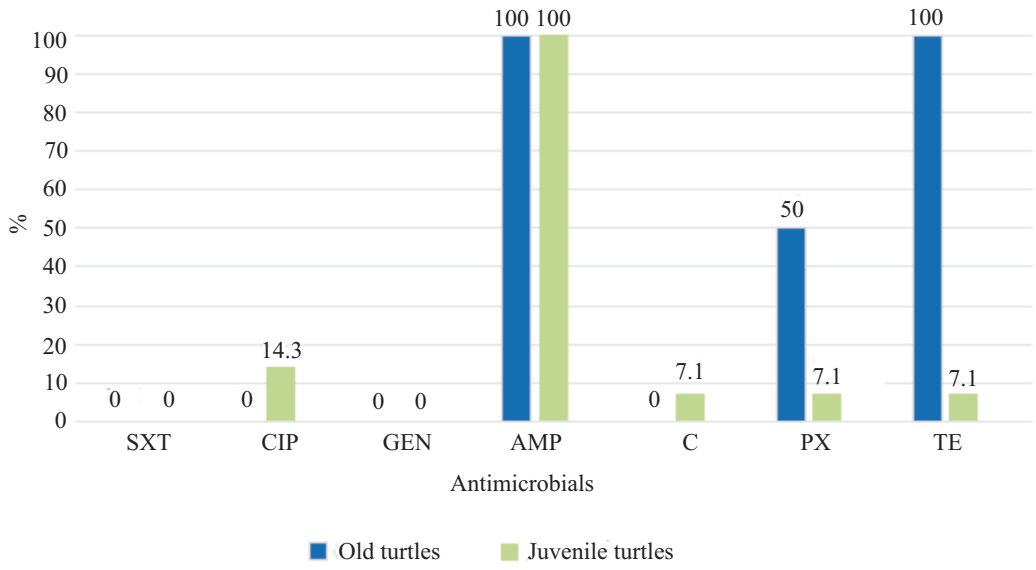
Fig. 2. Antimicrobial resistance rates (%) of all the isolates obtained from the European pond turtles (n = 52).



SXT – sulfamethoxazole/trimethoprim, CIP – ciprofloxacin, GEN – gentamicin, AMP – ampicillin, C – Chloramphenicol, PX – cefpodoxime, TE – tetracycline

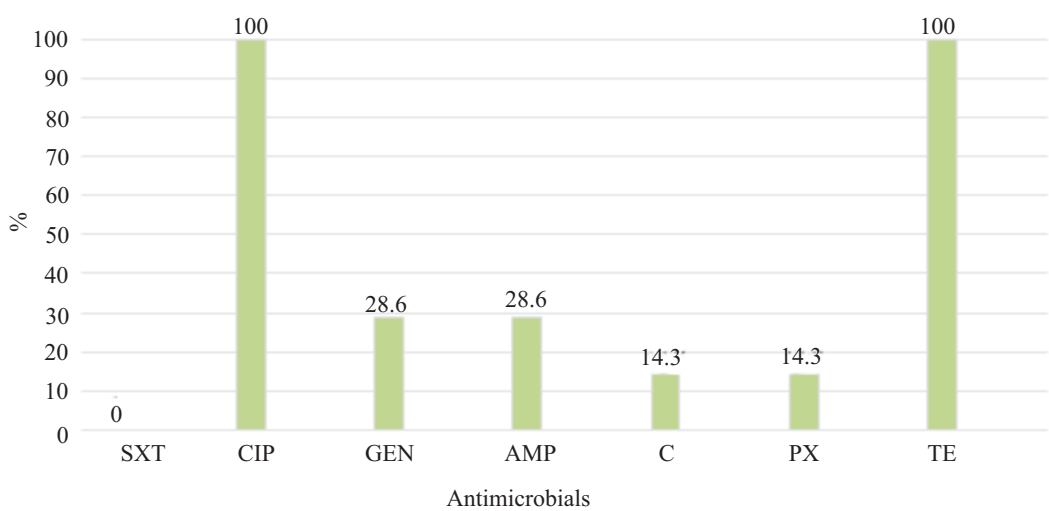
Fig. 3. Number of resistant isolates family *Enterobacteriaceae* isolated from European pond turtles (%).

Chryseobacterium (21.2%) and *Citrobacter* (19.2%). The rest of the genera included *Acinetobacter*, *Bacillus*, *Morganella*, *Sphingobacteria* and *Delftia*. Ten isolates were obtained from group I (old individuals), and the rest of the samples were group II (young individuals). Enterobacteriaceae and *Aeromonas* spp. were found in both old and young individuals while *Chryseobacterium* and *Acinetobacter* were found only in young turtles (Table 1). The antimicrobial susceptibility data of each isolate is presented in Table 1.



SXT – sulfamethoxazole/trimethoprim, CIP – ciprofloxacin, GEN – gentamicin, AMP – ampicillin, C – Chloramphenicol, PX – cefpodoxime, TE – tetracycline

Fig. 4. Number of resistant *Aeromonas* spp. isolates from European pond turtles (%).



SXT – sulfamethoxazole/trimethoprim, CIP – ciprofloxacin, GEN – gentamicin, AMP – ampicillin, C – Chloramphenicol, PX – cefpodoxime, TE – tetracycline

Fig. 5. Number of resistant *Acinetobacter*spp. isolates from European pond turtles (%). Bacteria of this genus were isolated only from juvenile turtles.

Figure 2 shows that the most common resistance rates of the isolates from both groups of turtles were toward ampicillin (86.6%), ciprofloxacin (61.5%) and gentamicin (40.4%). The least common resistance was detected to sulfamethoxazole-trimethoprim (26.9%). Statistically significant results ($p \leq 0.05$) between I and II groups were detected toward ciprofloxacin and tetracycline where the more frequent resistance was detected in the I group as well as toward cefpodoxime where the most frequent resistance was detected in the II group of the turtles. All of the Enterobacteriaceae isolates were resistant to ampicillin, tetracycline and sulfamethoxazole/trimethoprim. Less ciprofloxacin-resistant isolates were detected in the group of young turtles (71.4%) rather than in isolates obtained from old animals where all of the Enterobacteriaceae isolates were resistant to this antimicrobial (Fig. 3). Bacteria genus *Aeromonas* demonstrated high susceptibility rates to sulfamethoxazole-trimethoprim, gentamicin, ciprofloxacin and chloramphenicol in both groups of the turtles. The highest resistance rate was detected toward ampicillin

(100% in both groups) and tetracycline (100%) in the isolates obtained from old animals (Fig. 4). Bacteria of genus *Acinetobacter* spp. were isolated only from young individuals and had highest resistance rates to ciprofloxacin (100%) and tetracycline (100%) (Fig. 5).

Discussion

Analysis of the predominant cultivable bacteria within the gut of turtles showed that *Aeromonas* spp. were the most common bacteria among the others. This genus is well-known because of its frequent prevalence in different aquatic organisms, especially fish (Isonhood and Drake 2002, Pridgeon and Klesius 2011). The isolation of *Aeromonas* species from diseased fish, reptiles and frogs was the first implication of *Aeromonas* as animal pathogens. *A. salmonicida*, *A. hydrophila* and *A. veronii* cause different pathologies in fish (Rahman et al. 2002, Igbinosa et al. 2012). Bacteria of this genus have long been recognized as pathogens of amphibians and reptiles (Vivas et al. 2004). An outbreak of *Aeromonas hydrophila* infection with a high rate of mortality (95%) in turtles in Italy was reported (Pasquale et al. 2004). The *Aeromonas* species and particularly *A. hydrophila* are well-known bacteria that cause different infections in humans (Igbinosa et al. 2012). *A. caviae* has been associated with human septicemia, soft tissue infection and necrotizing fasciitis (Kumar et al. 2012). The relationship between the presence of aeromonads in drinking water and their presence in the stools of patients with gastroenteritis has been reported as well (von Graevenitz 2007). The high prevalence of different *Aeromonas* species (*A. hydrophila, A. caviae and A. veronii*) in healthy European pond turtles revealed that this genus could be treated as a normal microbiota in the gut of captive turtles. On the other hand, it is well known that *Aeromonas* strains can differ in the presence of virulence factors such as exotoxins (Tomhs 2012). Therefore, the pathogenicity of the aeromonads should be determined in case of turtle diseases.

The second most prevalent genus within this study was *Chryseobacterium*. No identification even up to the species level within this genus was possible using 16S rRNA sequencing. The isolated colonies produced a yellow pigment although this property is characteristic for different species of *Chryseobacterium* (Bernardet et al. 2005). It is known that *Chryseobacterium* is most commonly found in environments including water sources (Chen et al. 2012). More recently, a number of *Chryseobacterium* spp. were recovered from fish – some of these were diseased (Loch and Faisal 2012). The bacteria of this genus were recovered from diseased turtles (Hernandez-Divers et al. 2009, Sarmiento-Ramtrez et al. 2014), frogs (Mauel et al. 2002), and humans (Calderón et al. 2011, McKew 2014).

Two genera from the family Enterobacteriaceae were isolated from the turtles in this study. The predominant genus was *Citrobacter*, and all of the isolates from this genus belonged to a single species – *C. freundii*. This species is found in different animals including domestic animals and humans. It is also known as a causative agent of septicemic cutaneous ulcerative disease (SCUD) in immunocompromised turtles (Lescano et al. 2013). This bacterium was isolated from 10 healthy turtles out of 32, and it might be treated as normal microbiota in turtles. Moreover, this species mostly cause health problems in case of low immunity status. The other species of *Enterobacteriaceae* isolated in this study was *Morganella morganii*. This species is commonly found in the environment and in the intestinal tract of humans, mammals, and reptiles as normal microbiota. *M. morganii* also has been regarded as a harmless opportunistic pathogen, but some strains carry plasmids encoding antimicrobial resistance and have been associated with nosocomial human outbreaks (Senior and Voros 1990). Recently, this microorganism has been isolated from the turtle with conjunctivitis (Di Ianni et al. 2015).

Acinetobacter is another genus that is wide-spread in the environment and identified in our study. There are data about the prevalence of *Acinetobacter* in sick turtles (Soslau et al. 2011). It is also known that this bacterium causes infections in immunologically compromised humans. Infections caused by *Acinetobacter* are difficult to treat because this genus is usually resistant to many antimicrobials (Manchanda et al. 2010).

The scientific data are scarce regarding the variety of microbiota in European pond turtles. A recent study performed in Poland by Nowakiewicz at al. in 2015 demonstrated that the predominant bacterial species in captive turtle cloaca were *Cellulomonas flavigena, Enterococcus faecalis*, *Escherichia coli* and *Proteus mirabilis*. Interestingly, we did not find any of those species in the cloaca of our turtle subjects. This implies that the microbiota of water reptiles is location-specific. It also may depend on feed, sanitary conditions, and direct or indirect contact with other animals.

Although no antimicrobials were used for the treatment of turtles, a high number of multi-resistant isolates was detected in this study. All of the isolates of *Chryseobacterium* were resistant to critically and highly important antimicrobials for human and animal treatment including fluoroquinolones, aminoglycosides, cephalosporins as well as to other beta-lactams and amphenicols. Bacteria of this genera is known of their wide resistance to different antimicrobial classes (Maravić et al. 2013). The *Enterobacteriaceae* also demonstrated multi-resistance however, all of the isolates

were susceptible to cefpodoxim which means that no isolates with production of extended spectrum beta-lactamases were detected. Resistance profile of aeromonads and *Acinetobacter* depended on the isolates which had different resistance profiles. Overall, the microbiota isolated from European pond turtles demonstrated a wide resistance to the main antimicrobial classes used for the treatment of animals and humans. Further studies on the European pond turtle microbiota should be performed both in a wild conditions as well as are those in captivity. This can facilitate a comparison of the prevalent bacterial species and resistance in the microbiomes i.e. to obtain knowledge on the origin (captivity vs natural aquisition) of gut microbiota within the turtles.

Acknowledgements

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