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# *Prevalence of opportunistic pathogenic Gram-negative bacteria from the Enterobacteriaceae family in the cloacal microflora of Testudo graeca and Testudo hermanni – a pilot study*

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**Abstract.** Reptiles can harbour a variety of zoonotic bacteria in their gut, often asymptomatically. In this study, we report our preliminary data on the occurrence of opportunistic pathogenic Gram-negative bacteria in the cloacal microbiota of tortoises housed in the Rescue, Rehabilitation and Breeding Center in Banya village, Burgas District. A total of 24 healthy adult individuals were sampled: 12 spur-thighed tortoises (*Testudo graeca*) and 12 Hermann's tortoises (*Testudo hermanni*), with equal numbers of each gender. Representatives of 7 genera from Enterobacteriaceae family, known as causative agents of rare but serious infections both in humans and animals, have been identified. The most common bacteria were *Klebsiella* spp., *Salmonella enterica, Citrobacter braakii* and *Enterobacter cloacae*, detected in over 75% of the individuals sampled. *Morganella morganii, Pantoaea agglomerans* and *Escherichia coli* were found less frequently, in 20% - 30% of individuals. No significant difference in bacterial load between tortoise species or between sexes was found. The high prevalence of opportunistic pathogens that tortoises host may pose a potential health risk, so strict hygiene practices are recommended when handling animals.

**Key words:** reptiles, chelonians, spur-thighed tortoise, Hermann's tortoise, cloacal microbiota, opportunistic pathogens, Enterobacteriaceae.

## Introduction

Chelonians are among the most imperiled vertebrates, with more than half of all extant species included in the IUCN Red List as threatened with extinction (i.e., in the categories Vulnerable and [Critically] Endangered) (IUCN, 2025). The main causes of population decline worldwide are habitat loss, over-consumption and harvesting for food, use of body parts for traditional medicine, poaching and pet trade (Galosi et al., 2021; McKnight et al., 2020). In addition, the emergence of certain infectious diseases can also seriously harm the populations of some species (Berish et al., 2010; Galosi et al., 2021; Lecis et al., 2011; McKnight et al., 2020). Potential pathogens, believed to be one of the threatening factors, are insufficiently studied. Various bacteria may be present in clinically healthy specimens but become pathogenic in susceptible animals living under stressful conditions. Environmental stress can trigger pathogen proliferation as a result of an affected host immune system or a change in microbial community interactions (Acevedo-Whitehouse & Duffus, 2009; Weitzman et al., 2018). The role of the micro-

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biome, as it determines the health and fitness of animals, remains poorly understood, ultimately for the survival of endangered species in conservation programs (West et al., 2019). Accordingly, pathogens can pose a challenge to wildlife conservation, particularly when acting in conjunction with anthropogenic stressors (Galosi et al., 2021). Therefore, assessing the composition and epidemiology of host-associated microbial communities, both in wild and in captivity, may be essential for positive conservation efforts for vulnerable species. Overall, in Europe, studies focused on different pathogens that affect tortoises are few (Casalino et al., 2021; Di Ianni et al., 2015; Laroucau et al., 2022; Lecis et al., 2011; Marenzoni et al., 2022). The composition and structure of the bacterial flora of tortoises are still unknown in detail (Golawska et al., 2019).

In this study, we report our preliminary data on the prevalence of opportunistic Gram-negative bacteria in the cloacal microbiota of the spur-thighed tortoise (*Testudo graeca ibera* Pallas 1814) and the Hermann's tortoise (*Testudo hermanii boettgeri* Mojsisovics 1889). Both are included in the Red Book of the Republic of Bulgaria (2015) and in the IUCN Red List (2022) with the status of endangered species. The examined population was reared in a semi-free environment close to their natural habitat, at the Tortoise Rescue, Rehabilitation and Breeding Center in Banya village, Burgas District. Some of the animals residing at the center were undergoing treatment after suffering road accidents, carapace traumas or other injuries, as well as those kept illegally as pets. They were raised for a period necessary for their recovery and, if possible, released back into the wild. The aim of the study was to investigate the epidemiological status of the tortoise group. Screening is important for the detection of opportunistic pathogens that, in certain cases, can be transmitted to humans or other animals.

## Materials and methods

### *Sample collection*

A total of 24 adult individuals were sampled: 12 spur-thighed tortoises (*Testudo graeca ibera* Pallas 1814) and 12 Hermann's tortoises (*Testudo hermanni boettgeri* Mojsisovics 1889), with equal numbers of each gender (Fig. 1a,b). All animals were clinically healthy.

Cloacal samples were collected using sterile cotton swabs inserted carefully into the coprodeum and with a gentle rotating motion (Fig. 1c). The cotton swabs were placed immediately in Amies transport medium (Biolab Inc., Hungary) and stored at 4°C for 48 h until further processing in the laboratory.

Handling of animals was performed according to the necessary regulations and ethical requirements of the Ministry of Environment and Water, Republic of Bulgaria.

### *Isolation and identification of microorganisms*

Cotton swabs were transferred to tubes with 5 mL Nutrient Broth (HI Media, India) to enrich the cultures and incubated at 37°C for 24 – 48 h, depending on bacterial growth. For initial screening, the FECAL WELL D-ONE identification kit (Biolab, Italy) was used. Then the mixed cultures were inoculated in Petri dishes on the following selective and differentiation media: HiCrome UTI Agar (HI Media, India), Cetrimide agar (Merck Group, Germany), TCBS agar (Biolab Inc., Hungary), Brilliant green agar (after pre-enrichment in Rappaport-Vassiliadis Broth) (Biolab Inc., Hungary), and incubated for another 24 h (Fig. 1d). Isolated pure cultures obtained from single colonies were examined morphologically, microscopically (Gram staining) and biochemically by tests for catalase, glucose fermentation (OF test), cytochrome oxidase detection (OXI strip test), trypotphanase (INDOL test) and acetoin production (Voges–Proskauer reaction). The MICROLATEST® ID: ENTERO 24N identification kit Test (Erba Lachema, Czech Republic) and specialized software ErbaExpert Identification Program (www.erbalachema.com) were used for identification and interpretation of the results.

### *Statistical analysis*

Chi-square test was used to evaluate differences between tortoise species and sexes according to number of bacterial isolates. In all cases, $p < 0.05$ was considered a significance level (Preacher, 2001).

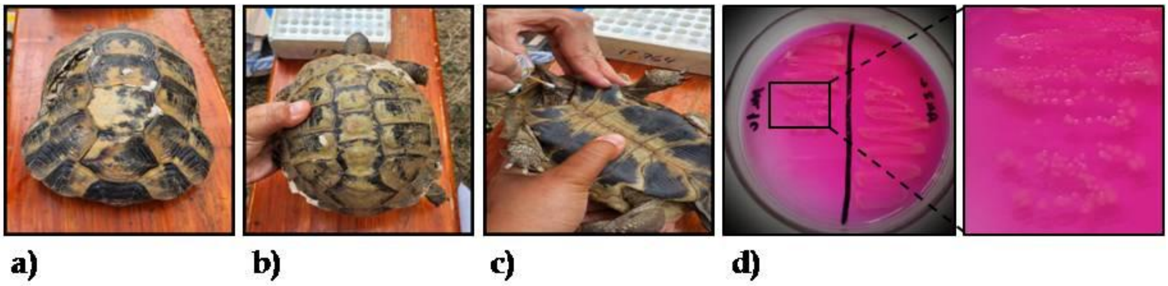

**Fig. 1.** Illustrative panel of photos from field and laboratory work: a) *T.graeca*; b) *T. hermanni*; c) sample collecting; d) a Petri dish with *Salmonella* spp. on the Brilliant green agar.

## Results and Discussion

A total of 149 isolates were obtained from cloacal samples (n=24). Between 4 and 9 isolates were recovered from each cloacal swab. A median of 6 isolates per individual was indicated in 37.5% of animals. More than 8 isolates were observed in 12.5% of individuals. The identified members of 7 genera of Gram-negative bacteria belonging to the family Enterobacteriaceae are listed in Table 1, along with the frequency of occurrence in the overall data set.

**Table 1.** Gram-negative bacteria isolated from the cloacal microbiota of tortoises.

| Bacteria | Total number/Percent of individuals-carriers |
|---|---|
| *Klebsiella spp.* | 22 (91.7%) |
| *Citrobacter braakii* | 21 (87.5%) |
| *Enterobacter cloacae* | 19 (79.2%) |
| *Salmonella enterica subsp. enterica* | 18 (75 %) |
| *Morganella morganii* | 7 (29.1%) |
| *Pantoaea agglomerans* | 5 (20.8%) |
| *Escherichia coli* | 5 (20.8%) |

The results obtained demonstrated that the identified opportunistic pathogens are widespread in the examined group. *Klebsiella* spp., *C. braakii, E. cloacae* and *S. enterica* were detected in over 75% of samples. The *Klebsiella* spp. isolates were identified as *Klebsiella oxytoca, K. pneumoniae* and *K. aerogenes*. *Morganella morganii, P. agglomerans* and *E. coli* were more limitedly distributed (in 20% - 30% of individuals). It should be noted that the presence of *Providencia/Proteus* was also suspected in the initial bacterial suspension based on a positive biochemical test. However, we failed to isolate a pure culture from a single colony; therefore, these microorganisms were not included in further analysis.

Both tortoise species appeared to share a similar composition of cloacal microflora (Fig. 2a). Heavier colonization with *Klebsiella* spp., *S. enterica* and *P. agglomerans* was observed in *T. graeca*. One or more *Klebsiella* species were detected in all sampled *T. graeca*, while they were less common in *T. hermanni*, especially *K. aerogenes*. All but one *T. graeca* were positive for *Salmonella*, while hosts in *T. hermanni* were half the specimens. Interestingly, *P. agglomerans* was found in only one *T. hermanni*, in contrast to one-third of the *T. graeca* individuals. However, a greater number of *T. hermanni* were carriers of *E. cloacae, C. braakii* and *M. morganii*. Despite some bacteria being represented in variable proportions, overall, the differences between species were not found to be significant ($\chi^2 = 0.329$, $p = 0.566$).

Regarding the number of isolates, differences between sexes were also not significant ($\chi^2 = 0.051$, $p = 0.821$ in *T .graeca* and $\chi^2 = 0.014$, $p = 0.905$ in *T. hermanni*). However, some gender differences were observed in terms of bacterial load (Fig. 2b), more pronounced in *T. hermanni*, where *S. enterica* and *M. morganii* isolates were detected in a greater number of males than females.

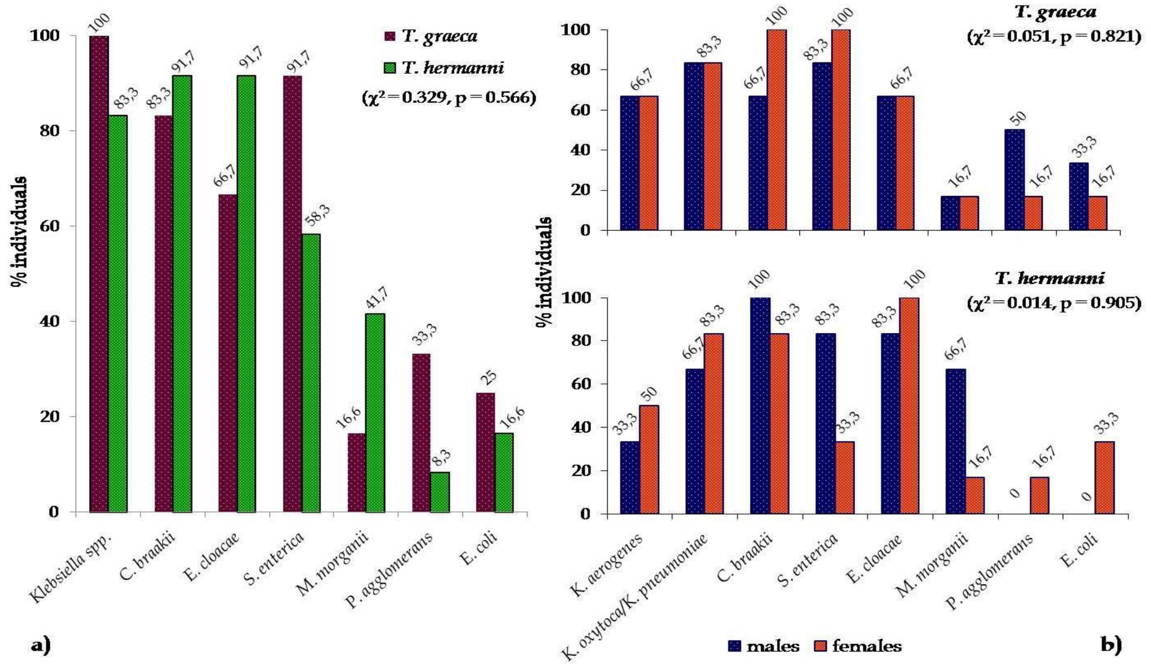

**Fig. 2.** Distribution of the identified Gram-negative bacteria according to: a) tortoise species; b) sex (with $\chi^2$ and *p*-value indicated).

## Discussion

It is well known that chelonians can be carriers of many zoonotic bacteria, often asymptomatically, and contribute to their spread in the environment (Di Janni et al., 2015; Ebani, 2017; Marenzoni et al., 2022; Laroucau et al., 2022). Various Gram-negative bacteria are commonly reported as pathogens of reptiles, although they are often considered part of the normal gut microbiota. Commensals are not thought to pose an immediate threat in the absence of specific stressors. "Commensalism" is defined as a condition that results in no or clinically invisible harm to the host, although it may elicit an immune response. Evolutionary co-existence has provided both the microorganisms and the host immune system with similar mechanisms of defense and selection, but opportunistic infections may occur (Galosi et al., 2021). Both pathogenic and opportunistic bacteria can affect other poikilothermic and homoeothermic animals as well as humans (Golawska et al., 2021). Captive animals may be prone to infections due to stress, weakened immunity, being raised in inappropriate conditions or overcrowding. In this study, all individuals sampled were clinically healthy. However, it should be noted that the identified Gram-negative bacteria are known as opportunistic pathogens and causative agents of rare but serious diseases. *Escherichia coli, Klebsiella, Proteus, Citrobacter, Morganella morganii, Enterobacter* and *Salmonella* are commonly implycated in various infections and abscesses in reptiles, sometimes persisting in combinations (Divers, 2022).

*Salmonella* deserves particular attention as it is an important zoonotic agent of public concern, and reptiles are a common reservoir of a wide variety of serovars (Briones et al., 2004; Hidalgo-Vila et al., 2008a; Pasmans et al., 2000; Zajac et al., 2021). It is often isolated from healthy, asymptomatic reptiles and is generally considered a normal component of their microbiota (Gowalska et al., 2019; Pees et al., 2023). Despite frequent detection, in many cases these bacteria can be implicated in diseases with non-specific clinical signs, such as anorexia, lethargy and weight loss, as well as severe symptoms such as skin inflammation, pneumonia, abscesses, sepsis, osteomyelitis, splenitis, hepatitis, etc. (Pees et al., 2023). Clinical salmonellosis in tortoises appears to be relatively rare, compared to other reptile groups (Pees et al., 2013). However, a case report provided evidence of pathological lesions in the liver of a spur-thighed tortoise (*T. graeca*) caused by *S. typhimu-*

*rium* (Candela et al., 2005). Reptile-associated salmonellosis (RAS) can pose a serious health threat to humans, especially to children, pregnant women, or immunocompromised patients, often presenting with acute clinical manifestations and requiring hospitalization (Bertrand et al., 2008; Hernandez et al., 2021; Pawlak et al., 2020; Zajac et al., 2021). *Salmonella* transmission to humans can occur through direct contact or indirectly through feces, contaminated surfaces, terrariums, water containers, soil substrate, etc. (Lockhart et al., 2008). In fact, turtles are most often reported to be responsible for salmonellosis in humans, due to their frequent use as pets (Bertrand et al., 2008; Hidalgo-Vila et al., 2008b; Zajac et al., 2021). Although *Salmonella* has been recorded among a variety of chelonian groups: terrestrial and aquatic, wild and pet (Hernandez et al., 2021; Nowakiewicz et al., 2015; Zając et al., 2021), relatively few reports have addressed its occurrence in free-living tortoise populations (Briones et al., 2004; Dickinson et al., 2001; Galosi et al., 2021; Hidalgo-Vila et al., 2007, 2008a; Lockhart et al, 2008). In general, in Europe, most epidemiological studies on tortoises have been conducted on animals, kept in various rescue centers (Casalino et al., 2021; Dipineto et al., 2011; Marenzoni et al., 2022; Pasmans et al., 2001; Percipalle et al.,2011; Strohl et al., 2004). Both *T. graeca* and *T. hermanni* were thought to be more predisposed to *Salmonella* spp. than other tortoise species when kept together (Barbour et al., 2013; Strohl et al., 2004). Our findings of high prevalence of this pathogen are consistent with the data reported by Pasmans et al. (2001) and Hidalgo-Vila et al. (2008a) (80-100%), exceeding the carriage rates between 37% and 60% in other populations (Casalino et al., 2021; Dipineto et al., 2011; Marenzoni et al., 2022; Percipalle et al., 2011; Strohl et al., 2004). Variations can be attributed to the sampling and isolation methodology, chelonian species, or location (Hernandez et al., 2021; Pasman et al., 2000). We also found a greater *Salmonella* load in *T. graeca* (91% of the individuals), compared to *T. hermanni* (58.3%). Uneven distribution of *Salmonella* spp. has been reported in different mixed populations of the two species: 36.8% *T. graeca* vs. 25.4% *T. hermanni* (Percipale et al., 2011), in contrast to 80% *T. hermanni* vs. 72% *T. graeca* (Pasman et al., 2001).

Overall, few studies have documented the presence of pathogens other than *Salmonella* in chelonians (Dickinson et al.,2001; Di Ianni et al., 2015; Galosi et al., 2021; Golawska et al., 2021). Their zoonotic potential seems underestimated. We found a wide distribution of *Klebsiella* and *Citrobacter* among the examined tortoises. In wildlife, *Klebsiella* has been reported in snakes, tuatara, turtles and lizards (Jackson, 2016; Lazarkevich et al., 2024; Singh et al., 2013). Despite its ubiquitous occurrence in the environment and in the gastrointestinal tract of animals and humans, it can cause various infections in cattle, poultry and pets, as well as the outbreak of nosocomial epidemics (Jackson, 2016; Quintelas et al., 2024; Vetere et al., 2021). Pet reptiles can suffer from *Klebsiella*-induced stomatitis, cloacitis, enteritis, ear infections, subcutaneous and visceral abscesses, upper respiratory tract infections, pneumonia, hypopyon, osteomyelitis, spondylitis (Divers, 2022; Hossain et al., 2020; Vetere et al., 2021). *Citrobacter* is among the frequently isolated bacteria from the reptile cloacal microbiota (Hossain et al., 2017; Lazarkevich et al., 2024; Nowakiewicz et al., 2015; Pawlak et al., 2020; Romero et al., 2015; Singh et al., 2013). We observed a large presence of *C. braakii* in the entire group of *T. graeca* and *T. hermanni* (87.5% of individuals). This result exceeded findings of other studies (Dickinson et al., 2002; Galosi et al., 2021; Golawska et al., 2019). In clinical cases, *Citrobacter* spp. may be involved in the etiology of Septicemic Cutaneous Ulcerative Disease (SCUD) (Divers, 2022) or necrotic lesions (Innuria et al., 2024; Nowakiewicz et al., 2015). *Morganella morganii* - closely related to *Providencia/Proteus* genera, is recognized as an increasingly important pathogen in recent years, implicated mainly in post-operative wound and urinary tract infections (Liu et al., 2016), skin ulceration, fin bleeding, scale shedding, pneumonia, hepatorenal enlargement, empyema, pericarditis, arthropathy, endophthalmitis, etc., in different species of animals, including chelonians (Joyner, 2006; Wimalasena et al., 2017; Zhai et al., 2024). Also, it is considered a dangerous vector for the dissemination of antibiotic resistance genes through the aquatic environment (Wimalasena et al., 2017). Severe clinical infections and mortalities caused by enteropathogenic *E. coli* strains in tortoises held in a controlled environment have been reported (Avsever & Tinaligil, 2016; Omuavanam et al., 2012). The suspected transfer was possible contamination from an external source - human visitors, other animals, soil manure, flies, feed supplies (Omuavanam et al., 2012). Anthropogenic *E. coli* strains might be found in chelonians due to intensive breeding and, if virulent, contribute to diarrhoea or animal death (Golawska et al., 2021). The isolation of multidrug-resistant *E. coli* isolates originating from a tortoise farm raises the question of the likelihood of the transmission of resistance genes located on mobile genetic elements (Golawska et al., 2021). Laroucau et al. (2020) hypothesized that *E. coli* found together with *Chlamydia* could favor their proliferation and spread in a population of captive spur-thighed tortoises (*T. graeca*), and also that co-infections could weaken the immune system and modulate the expression of virulence factors.

Wildlife rehabilitation centers provide an important infrastructure for care and conservation of many vulnerable species, but they could also represent potential reservoirs for pathogens, as often animals from different habitats are housed together in crowded conditions, sometimes for long periods of recovery (Lecis et al., 2010). The individuals that we sampled came to the shelter from various locations and spent different periods of time there. Likely, the observed similarity in the cloacal microbiota of *T. graeca* and *T. hermanni* is due to shared commensals during coexistence and the habits of feeding and sleeping collectively in the enclosures. Differences may occur in tortoises living in natural and controlled environments. As this is a pilot study, it does not claim to be comprehensive. A drawback includes the small sample size of individuals, as well as the fact that *Salmonella* spp. serotyping was not performed. Further research envisages repeated sampling and a larger number of screened specimens, more in-depth studies in wild populations, not simply limited to the detection of pathogenic bacteria, but also determining the antibiotic resistance and expression of virulence factors of the isolated strains, serotyping of *Salmonella serovars*, etc. This will contribute to clarifying the real epidemiological status of tortoises in their natural environment.

## Conclusions

Although conducted on a limited group of animals, this study provides baseline data on the variety of opportunistic Gram-negative bacteria of the family Enterobacteriaceae occurring in the cloacal microbiota of testudines. The most common bacteria that tortoises host are also human pathogens that may pose a potential health risk. Reptiles could represent a possible source for the dissemination of these pathogens if good hygiene is not maintained in their care and handling. This should be kept in mind by people who have closer contact with these animals, such as zookeepers, pet owners, and environmentalists.

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