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Research article

Bacterial and fungal flora in faecal samples from the Balkan snow vole (*Dinaromys bogdanovi*) at the Zagreb Zoo, Croatia

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Abstract

The Balkan snow vole (*Dinaromys bogdanovi*) is a poorly understood arvicoline rodent endemic to the western Balkan Peninsula. Little is known about its biology and there is no information regarding its microbiology. To increase knowledge of the normal microflora and potentially pathogenic and/or zoonotic microorganisms in this species, faecal samples were collected from animals of the F1 generation housed offshow at the Zagreb Zoo, Croatia, as a less invasive method able to provide insight into the bacteria and fungi colonising the gastrointestinal tract of these animals. Faecal samples of 20 animals were analysed using standard microbiological procedures for the detection of aerobic bacteria and fungi, and by real-time PCR for the detection of *Chlamydia* spp. Isolated faecal bacteria showed a statistically significant predominance of Gram-negative over Gram-positive species (P=0.026, Fisher's exact test). A total of 20 bacterial species were isolated, the most common of which was *Escherichia coli*, present in 55% of animals. All samples were negative for *Salmonella* spp., *Campylobacter* spp. and *Chlamydia* spp. Seven fungal species were isolated, most of which were *Mucor* sp. isolates. Most of the microorganisms identified belong to the soil bacteria and fungi species, and some are potentially harmful for both humans and animals. The present study provides the first report about the intestinal microflora of the Balkan snow vole that could be useful in protecting animals and persons involved in their handling.

Introduction

The Balkan snow vole (BSV) (*Dinaromys bogdanovi*) is an Arvicolinae rodent endemic to the western Balkan Peninsula including the Balkan states of Croatia, Bosnia and Herzegovina, Serbia, Montenegro and western Macedonia (Bužan et al. 2010). The species is dependent on rocky habitats, particularly cave entrances, accumulations of rocks, cracks in cliffs and crevices in rocky substrates (Kryštufek and Bužan 2008). The BSV is an elevation generalist, inhabiting areas at elevations of 10 to 2200 m, though most BSV suitable habitats are found at altitudes of over 1400 m (Bužan et al. 2010). Spatial genetic analysis has confirmed that BSV populations are highly fragmented, even in those areas with the highest population densities (Bužan et al. 2010). The BSV belongs to the family Cricetidae and subfamily Arvicolinae and is thought to be the only surviving member of the Pliomys lineage. The species' range has been larger in the past and has contracted over time (Kryštufek and Bužan 2008).

Special habitat requirements and small population sizes make this animal highly vulnerable. Due to its specific way of life in inaccessible karst habitats, the BSV is not directly threatened by human activities, though habitat degradation, such as that caused by climate change, could additionally reduce its biotope and further threaten this species (Bužan et al. 2010). Since these animals avoid forest habitats (Kryštufek and Bužan, 2008), any changes of the forest line toward higher elevations could further decrease their optimal habitats and increase population fragmentation (Bužan et al. 2010). Another factor that may threaten their survival is competition with the relatively recently expanded European snow vole (*Chionomys nivalis*), which has similar food and habitat preferences (Kryštufek and Bužan 2008).

The BSV is listed as vulnerable according to the IUCN criteria (Kryštufek 2008). In the Red Book of Mammals of Croatia, it has been listed as a data deficient species and is strictly protected (Tvrtković 2006; MCRC 2009).

There is little information on the biology and distribution of BSV, and a lack of data on its normal and potentially pathogenic microflora that could additionally endanger and reduce the population. To obtain insight into the normal intestinal microflora and potential pathogens which these animals could harbour, faecal samples of captive BSV were analysed using standard microbiological procedures and molecular methods.

Materials and methods

Subjects

Twenty BSV were housed offshow at the Zagreb Zoo, Croatia. They offshow to the F1 generation of animals captured on the northern slopes of Mt. Mosor in Croatia. Captive husbandry backstage at the Zagreb Zoo and research of these animals was approved by the Croatian Ministry of Environment and Nature Protection (Approval No. UP/I-612-07/12-33/0338).

Husbandry and sampling

Animals were kept individually in glass terrariums of dimensions 80×60×60 cm. Terrarium roofing was made of cord mesh for ventilation. A combination of gravel and dust-free sawdust was used as a substrate, and all the enclosures were enriched with limestone rocks and branches. Each terrarium had its own light, mimicking the natural photoperiod. Relative humidity in all enclosures was maintained at 50-60%, and the room was kept at temperatures of 10-20°C, depending on the season. Each terrarium was equipped with a water bowl, food storage, toilet and hiding place. The last three items were terracotta pots furnished with one or two entrance holes (Figure 1). Faeces were removed on a daily basis. Fresh food and water were provided daily. The food included meadow plants, such as dandelion leaves, clover, hollyhock and hay; occasionally, pieces of apple, chicory, rocket, lamb's lettuce and seed mix were also provided.

Animals were sampled once for the identification of aerobic bacteria and fungi, and at three different occasions for the identification of *Salmonella* spp.

Detection of aerobic bacteria and fungi

Fresh faecal samples, immediately after defecation of each animal, were collected on a one-time basis in sterile test tubes and transported to the Faculty of Veterinary Medicine, University of Zagreb, Croatia. Samples were plated using a sterile microbiological loop, as described by Brown (2005), on nutrient agar (Difco Nutrient Agar, Becton, Dickinson and Company, France), brilliant green agar (Brilliant Green Agar, Oxoid Ltd., UK) and Sabouraud dextrose agar (Oxoid, UK) under aerobic conditions. Nutrient agar and brilliant green agar were incubated at 37°C and plates were checked after 24 and 48 hr. Sabouraud dextrose agar plates were incubated at room temperature and examined after five days. Bacterial colonies were examined microscopically to detect their morphology. Catalase (Catalase reagent, 3%, Hardy Diagnostics, USA), and oxidase tests (Oxidase strips, Oxoid Ltd., UK) were conducted, as described by Brown (2005), and Gram staining was carried out (BioGram 4 kit, BioGnost, Croatia). Further bacterial identification was carried out using the Analytical Profile Index (API; API 20 E, API 20 NE and API Staph System, BioMérieux S.A., France) and matrix-assisted laser desorption/ionization time of flight (Maldi – Tof) method. Identification of fungi was conducted based on morphological characteristics and microscopically, using lactophenol staining (Lactophenol blue solution, Sigma-Aldrich, France).

Salmonella spp. detection

Fresh faecal samples, taken on three different occasions to address intermittent shedding, were enriched in selenite cysteine broth (Becton-Dickinson and Company, France), incubated for 24 h at 37°C, then plated on BGA and xylose lysine deoxycholate agar (Merck, KgaA, Germany), incubated for 24 h at 37°C and then for 24 h at room temperature.

Campylobacter spp. detection

Faecal samples were aseptically transferred to the Campylobacter selective enrichment broth (Oxoid, Unipath, UK) and incubated for 48 h at 37°C in a microaerobic atmosphere (85% N^2, 10% CO^2 and 5% O^2). One loopful of the 48-h cultures was streaked onto modified charcoal cefoperazone desoxycholate agar plates (Modified CCDA; Oxoid, UK) and incubated in a microaerobic atmosphere for 48 h at 37°C (Bolton et al. 1992).

Chlamydia spp. detection

Fresh faecal samples of each animal were processed at the Laboratory for *Chlamydia* at the Department of Poultry Diseases Clinic, Faculty of Veterinary Medicine, University of Zagreb, Croatia. DNA was extracted using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, USA) according to the manufacturer's instructions and examined by a *Chlamydiaceae*-specific real-time PCR test, as previously described by Ehricht et al. (2006). Each sample was analysed in duplicate, with duplicates of both positive (*C. psittaci* strain, NPU 137/12) and negative (nuclease free water) controls. Aliquots of 5 µl DNA in a total reaction mixture volume of 20 µl were used for each faecal and/or control sample. Samples were analysed by a Mx3005P (Agilent Genomics, USA) instrument with TaqMan system for replicated segment identification under the following program: 95°C for 10 min, 50 replication cycles, denaturation 15 sec at 95°C, primer annealing at 60°C for 60 sec, when fluorescence was recorded.

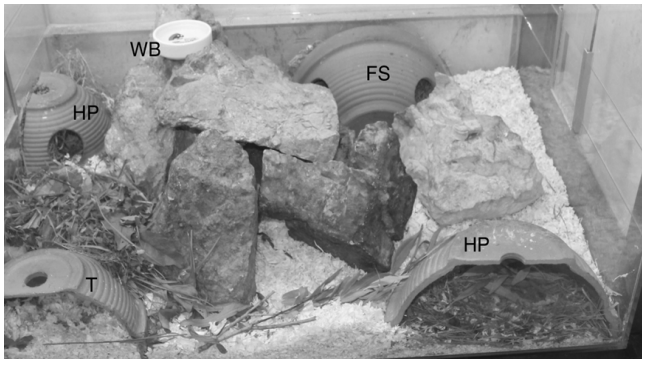

Figure 1. Glass terrarium for Balkan snow vole housing. Each terrarium is equipped with water bowl (WB), food storage (FS), toilet (T) and hiding place (HP).

Results

Aerobic bacteria and fungi isolated from the faeces of 20 BSV kept in captivity are listed in Table 1. A total of 20 bacterial species were isolated, with the significant predominance of Gram-negative (14) over Gram-positive (6) species (P=0.026) and with a significant predominance of Gram-negative (37) over Gram-positive (15) isolates (P<0.001) (Table 1). The most represented species was *Escherichia coli*, which was present in 13 (65%) out of 20 samples, followed by *Bacillus cereus* (7.35%), *Proteus vulgaris* (6.30%), *Enterobacter cloacae* (4.20%) and *Staphylococcus lentis* (3.15%). *Staphylococcus xylosus, Acinetobacter johnsonii, Citrobacter braakii* and *Morganella morganii* were each detected in two (10%) samples, and *Hafnia alvei, Providencia rettgeri, Enterobacter cowanii, Serratia liquefaciens, Klebsiella oxytoca, Citrobacter sedlakii, Citrobacter gillenii, Bacillus pumilus, Micrococcus luteus, Micrococcus roseus* and *Serratia ureilytica* were each found in one (5%) sample (Table 1).

Table 1. Bacterial and fungal species isolated from the faeces of 20 Balkan snow voles.

Bacterial species	Number of solates (n)	%
Gram-negative*		
Escherichia coli	13	65
Proteus vulgaris	6	30
Enterobacter cloacae	4	20
Acinetobacter johnsonii	2	10
Citrobacter braakii	2	10
Morganella morganii	2	10
Citrobacter gillenii	1	5
Citrobacter sedlakii	1	5
Enterobacter cowanii	1	5
Hafnia alvei	1	5
Klebsiella oxytoca	1	5
Providencia rettgeri	1	5
Serratia liquefaciens	1	5
Serratia ureilytica	1	5
Gram-positive		
Bacillus cereus	7	35
Staphylococcus lentis	3	15
Staphylococcus xylosus	2	10
Bacillus pumilus	1	5
Micrococcus luteus	1	5
Micrococcus roseus	1	5
Fungal species		
Mucor sp.	11	55
Candida albicans	6	30
Aspergillus fumigatus	6	30
Rhodotorula rubra	5	25
Aspergillus flavus	4	20
Cladosporium sp.	1	5
Penicillium sp.	1	5

*Number of Gram-negative vs Gram-positive species, P=0.026; Number of Gram-negative vs Gram-positive isolates, P<0.001 (Fisher's exact test).

All faecal samples were negative for *Salmonella* spp., *Campylobacter* spp. and *Chlamydia* spp. Seven fungal species were isolated. *Mucor* spp. was present in 11 (55%) out of 20 samples, *Candida albicans* and *Aspergillus fumigatus* each in six (30%) samples, *Rhodotorula rubra* in five (25%) samples and *Aspergillus flavus* in four (20%) samples. *Cladosporium* sp. and *Penicillium* sp. were each detected in one (5%) sample (Table 1).

Discussion

BSV is an endemic mammal inhabiting the western Balkan Peninsula (Bužan et al. 2010). To obtain insight into the physiological microflora and potential pathogens, microbiological analysis of faecal samples of 20 captive animals housed offshow at the Zagreb Zoo, Croatia, was performed.

Twenty different bacterial species were identified, which is in line with the high diversity of the known bacterial population in the mammalian large intestine (Sorum and Sunde 2001). A significantly higher number of Gram-negative versus Gram-positive species was isolated. Also, a significantly higher total number of Gram-negative versus Gram-positive isolates was obtained. Generally, both findings indicate potential pathogenicity of isolated species, as Gram-negative bacteria are known to be more pathogenic and more resistant to antibiotics than Gram-positive bacteria (Wilson et al. 2002).

Among the 14 Gram-negative species identified in this study, the most frequent was *E. coli*, followed by *P. vulgaris, E. cloacae, A. johnsonii, C. braakii, M. morganii, C. gillenii, C. sedlakii, E. cowanii, H. alvei, K. oxytoca, P. rettgeri, S. liquefaciens* and *S. ureilytica. E. coli* is one of the most prevalent bacteria in the large intestine of mice and rabbits (Sorum and Sunde 2001) and some serotypes have harmful effects on the gastrointestinal system. The enteropathogenic *E. coli* caused potentially fatal haemorrhagic typhlocolitis in common marmosets (Hayashimoto et al. 2016). In addition, wild animals can transmit enteropathogenic *E. coli* to humans, thus spreading infections in urban areas (Jay-Russell et al. 2014). Serotyping was not performed in this study, though none of the captive animals showed any clinical signs that would indicate gastrointestinal problems. Therefore *E. coli* can be assumed to be part of the normal intestinal flora of the BSV studied in this work, although it could be potentially harmful in the presence of concurrent disease(s), or in immunocompromised animals (Aisenberg and Aroch 2003; David et al. 2010). All bacterial species from the genera *Enterobacter, Proteus, Morganella* and *Providencia* belong to the Enterobacteriaceae family, and have been described as opportunistic bacteria able to cause primary or secondary infections in humans and animals (Papadogiannakis et al. 2007; Liu et al. 2016; Abe et al. 2017). *E. cloacae* is a common facultative anaerobic organism, but it was also described as an important opportunistic and multiresistant human pathogen (Davin-Regli and Pages 2015; Patel et al. 2016). *E. cowanii* was first described in 2000 by Inoue and colleagues as a new species in the Enterobacteriaceae family and it is not known whether this bacterium is part of the normal flora of the BSV. *M. morganii* has been associated with bronchointerstitial pneumonia in guinea pigs (Vandenberge et al. 2013). *Citrobacter spp., Klebsiella spp.* and *Acinetobacter spp.* are soil bacteria, especially present in wet areas (Lightfoot 2003). In this study *K. oxytoca* was detected in one animal, without any clinical signs. This organism was reported to cause suppurative endometritis, salpingitis, perioophoritis and peritonitis in sentinel mice (Davis et al. 1987; Rao et al. 1987). In humans, *K. oxytoca* is considered an opportunistic pathogen involved especially in clinical cases of nosocomial infections in hospitals (Savino et al. 2009). Bacteria of the genus *Serratia* are opportunistic pathogens capable of colonising different types of water and soil surfaces, as well as the digestive tract of rodents, insects, fish and humans (Grimont and Grimont 1978). In this study, both *S. liquefaciens* and *S. ureilytica* were isolated from two animals, with no clinical signs of disease.

Six Gram-positive bacterial species were identified in this study, belonging to the genera *Micrococcus, Staphylococcus* and *Bacillus.* All these bacteria have also been isolated from human bite wounds caused by mammals (Abrahamian and Goldstein 2011). *Staphylococcus xylosus* was associated with nasal dermatitis in breeding colonies of Mongolian gerbils, indicating its possible role as an opportunistic pathogen (Solomon et al. 1990). Since no clinical signs and/or disease related to Gram-positive bacterial flora were observed in this study, it can be assumed that these bacteria belong to the normal intestinal flora of this species.

All faecal samples had also negative results for *Salmonella spp., Campylobacter spp*. and *Chlamydia spp*., which are widely known

as zoonotic bacterial pathogens that can be transmitted from animals to humans via bite wounds or through direct or indirect contact with animals and husbandry equipment, respectively (Rubini et al. 2016). One possible explanation of the negative findings could be the fact that the BSV lives in cracks and crevices of rocky habitats in small fragmented populations, surrounded by a low number of other animal species able to transmit those bacterial infections.

Six fungal species were isolated from the faecal samples studied. *Mucor spp.* was the most abundant fungi, present in 55% of the tested animals. Fungi from the Mucorales order are ubiquitous and could cause mucormycosis. Mucormycoses are very well described in humans, particularly in immunocompromised patients (Kyriopoulos et al. 2015; Guimaraes et al. 2016; Seo et al. 2016). Mucormycosis in animals has been reported in different organs and systems (Abdo et al. 2012; Hurley-Sanders et al. 2015). *C. albicans* and *R. rubra*, which were each present in 30% of the analysed faecal samples, are widely considered ubiquitous organisms commonly found in the environment and able to cause fungal infections in immunocompromised humans and animals (Naglik et al. 2008; Wirth and Goldani 2012). Although most *Aspergillus* species are saprophytic fungi commonly found in soil (Sejdemojtaba et al. 2015), *A. fumigatus* and *A. flavus* have been described as pathogens in different animal species (Peeters and Clercx 2007; Adamama-Moraitou et al. 2011; Barrs and Talbot 2014). Infections associated with *Cladosporium spp.* and *Penicillium spp.* are not frequent in mammals (Poutahidis et al. 2009; Chong et al. 2012).

In conclusion, 27 bacterial and fungal species were isolated from faecal samples of the BSV in this study. Most of these are ubiquitous soil microorganisms able to cause various diseases in animals and humans. The primary zoonotic pathogens *Chlamydia spp., Campylobacter spp.* and *Salmonella spp.* were not detected, which could be due to the elusive lifestyle of the study species. To date, no health problems have been detected in this group of animals kept in captivity or in the persons involved in their care at the Zagreb Zoo, Croatia. To our knowledge, this is the first report describing the intestinal microflora of the BSV. Further research is needed to increase the knowledge on gastrointestinal microflora of this endemic species.

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