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Clostridioides difficile in bat guano

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ABSTRACT

Bats are associated with the emergence of several mammalian diseases. Their sessional migration, and tendency to form large colonies in close proximity to human habitats enables effective intra- and inter-species transmission of pathogens. *Clostridioides difficile* is an important enteric pathogen in humans and animals; however, the source of its dissemination in the population is unknown. The purpose of this study was to determine the prevalence of *C. difficile* in bats, and to characterize *C. difficile* isolates.

Feces (n = 93) was sampled from bats during their migration across Europe. Eighteen samples (19.4%) were positive for *C. difficile*; ribotypes 078, 056, and a new ribotype CDB3 were identified.

Clostridioides difficile ribotypes 078 and 056 are associated with human and animal diseases. The *C. difficile* prevalence and ribotypes in this study do not necessarily identify bats as a significant source, but more likely as an indicator of *C. difficile* perpetuation in the environment.

1. Introduction

Bats are a diverse group of flying mammals, belonging to the order *Chiroptera*. They are the only mammal capable of true flight and are adapted to a variety of ecological niches. They are present on all continents except Antarctica and can form large multi species colonial populations [1]. Some species of bats are territorial, but many migrate over long distances. Bats in temperate zones of northern and central Europe migrate to warmer southern locations or use torpor and hibernation in colder months [1,2]. Bats are gaining significant attention as a potential host for pathogens that can affect humans, livestock and wildlife [2]. They have developed an interesting microbiota and have a benign phylogenetic relationship with some of the most virulent viruses and bacteria with zoonotic character [3–5].

Clostridioides (formerly *Clostridium*) *difficile* is an anaerobic bacteria capable of inducing severe disease in humans and some animal species. Because of its spore forming ability it is very resilient in the environment [6,7]. *Clostridioides difficile* infection (CDI) in humans can be hospital-associated or community–acquired with the possibility of transmission between animals and humans, as this bacterium can be found in a wide range of animal species and food [8,9]. Highly pathogenic and antibiotic resistant *C. difficile* strains are now frequently isolated among community-associated CDI cases [10,11]. Many species of farm and companion animals, and wild animals associated with human or/and farming environment, have been shown to carry *C. difficile* [12–18]. However, the recognition of a critical and/or unique source for *C. difficile* dissemination remains a pressing clinical and investigative quest [16]. Bats are present in a variety of human habitats and because of their migratory patterns, they are able to transfer *C. difficile* across vast geographical areas. Therefore, the aims of this study were to determine the prevalence of *C. difficile* in bats and to characterize *C. difficile* isolates.

2. Materials & methods

This study was carried out during the Slovene national bat monitoring program ”Monitoring of population of selected bats species in years, 2014/2015″ [19], commissioned by the Republic of Slovenia Ministry of the Environment and Spatial Planning. Bats were handled according to the European and Slovenian Nature conservation

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Table 1
Bat species, age and sex, and *C. difficile* ribotypes isolated from fecal samples.

Bat species	No. of samples positive for *C. difficile* / Total No. of Samples	Juvenile / Adult/ Undetermined	*C. difficile* ribotype
Barbastella barbastellus	2/4	0/4	078
Miniopterus schreibersii	3	0/3	
Myotis bechsteinii	3	0/3	
Myotis blythii	1/11	0/11	078
Myotis daubentonii	2/5	2/3	056, 078
Myotis emarginatus	3/19	1/18	078
Myotis myotis	3/11	0/7/4	078, CDB3
Myotis nattererii	1/3	0/3	078
Nyctalus leisleri	1	0/0/1	
Pipistrellus pipistrellus	2	0/2	
Plecotus auritus	1	0/1	
Rhinolophus ferumequinum	1	0/1	
Rhinolophus hipposideros	6/29	15/14	078
Σ	18/93 (19.4%)	18/70/5	

regulations with bat catching and sampling conducted under the Slovene Environment Agency license (document No.: 35601-35/2010-6).

2.1. Study population and sampling

Ninety-three bats belonging to 13 species were sampled in Slovenia at eight different locations during their autumn migration across central Europe (Table 1). Bats were captured with specially designed mist nets. Fecal samples (guano) were collected, if excreted naturally, before bats were individually placed in clean, custom made bags for further identification of species, age and sex. The age of bats was determined as juvenile (born that breeding year), subadults (individuals that have passed through the juvenile period, but have not yet attained typical adult characteristics) and adults (one year old or older) [19,20]. Bats were released in the same location no more than 30 min after being caught. Fecal samples were collected with sterile gloves (Ansell Ltd., UK) and placed into 2 mL sterile tubes (Eppendorf Tubes, Germany). They were stored at −20 °C within 4 h after collection until processed.

2.2. Clostridioides difficile culture and ribotype determination

All samples collected were subjected to a selective *C. difficile* culture. Samples were inoculated in 9 mL of Tryptone Soy Agar (TSA) broth and incubated for 5–7 days anaerobically at 37 °C. After incubation, a 2 mL aliquot was alcohol-shocked with 2 mL of anhydrous alcohol for 1 h at room temperature. Samples were further centrifuged at 4000 rpm for 10 min to create a pellet, which was inoculated onto *C. difficile* moxalactam-norfloxacin (CDMN) agar (Oxoid, Nepean, Ontario, Canada). The anaerobic incubation at 37 °C lasted for 1–4 days. Colonies suspected of being *C. difficile* were further cultured onto Columbia blood agar (Oxoid) and incubated anaerobically at 37 °C for further 1–2 days. *Clostridioides difficile* was presumptively identified based on characteristic morphology of the colonies, odor and production of L-proline-aminopeptidase (Prodisk, Remel, Lenexa, KA, USA).

All isolates were further analysed for the presence of toxin (*tcd*A, *tcd*B, *cdt*A) genes using PCR [21–24]. Ribotyping of *C. difficile* was done with capillary ribotyping and analyses using the Webribo server (https://webribo.ages.at/) as previously reported [25]. Internationally identified ribotypes were assigned an appropriate numerical designation based on reference strains [26]. An internal laboratory number was assigned, when no matching reference strain was found.

2.3. Statistical analysis

The independent *t*-test was used to compare *C. difficile* positive samples between adult and juvenile bats using R language for statistical computing (R version 3.5.2) [27]. Significance level was set to 0.05.

3. Results

Eighteen samples (18/93; 19.4%) belonging to seven species of bats were positive for *C. difficile* (Table 1). The prevalence was 50% (2/4) in *Barbastella barbastellus*, followed by *Myotis daubentonii* (40%, 2/5), *Myotis nattererii* (33.3%, 1/3), *Myotis myotis* (27.3%, 3/11), *Rhinolophus hipposideros* (20.7%, 6/29), *Myotis emarginatus* (15.8%, 3/19) and *Myotis blythii* (9.1%, 1/11). In relation to age, 12 positive samples (12/70, 17.1%) were collected from adult bats (subadult/adult) and 4 (4/18, 22.2%) from juvenile bats ($P = 0.3$). Two positive samples were collected from bats where age could not be determined.

Three different *C. difficile* ribotypes were identified in this study. Ribotype 078 (A + B+CDT+) was the predominant strain (16/18; 88.8%). *Clostridioides difficile* ribotype 056 (A + B+CDT-) was isolated from one *Myotis daubentonii* (1/18, 5.6%). A previously unreported *C. difficile* ribotype (designated CDB3) was also isolated from one *Myotis myotis* guano, which was toxin A and toxin B positive and negative for binary toxin (A + B+CDT-).

4. Discussion

Bats are consistently considered as potential reservoirs for a variety of pathogens important in human and veterinary medicine. They can spread pathogens over large geographical area because of their seasonal migration and tendency to form large colonies with close contact between individual animals.

The prevalence of *C. difficile* noted here (19.4%) was reasonably high for a healthy, non-antibiotic-exposed population. It is higher compared to other flying migrating wild animals (0–4%) [16,28] but more closely resembles the prevalence found in wild animals on farms (5.4–24.3%) [18,29]. Bats, as several species of migrating birds, are insectivorous animals [20]. The high *C. difficile* prevalence found in insects on farms (56–100%) [30] suggests potential exposure of insectivorous bats on and around farm environments. This trend was also found in barn swallows, which are closely related to farming habitats [16].

Identification of *C. difficile* in bats was not surprising since a range of other enteric pathogens, including *Salmonella* spp. [4,5,31,32], *Shigella* spp. [5,32], *Yersinia* spp. [5,33], *Listeria* spp. [32], *Campylobacter* spp. [4,32,34], *Clostridium perfringens* [4,5,35] and *Clostridium sordellii* [4,5,32] were previously identified in bats. This bacterial pathogen carriage, particularly within bats' excrements, have not proven to be of critical concern for human health and likely poses the greatest potential risk for people with occupational or recreational exposure to bat caves. However, increased urbanization and climate changes can alter the dynamics of direct and indirect exposure between animals, and between animals and humans, and increase the likelihood for pathogen transmission [5,36,37].

The sampled population in this study was predominantly adult bats on migration, which is in concordance with the typical colony age ratio [20]. Both juvenile and adult bats were found to carry *C. difficile* in their feces.

Three *C. difficile* ribotypes were isolated. Of particular importance is the predominant finding of ribotype 078, which is commonly found in production animals and has been increasingly associated with the community-acquired CDI in humans [11,38–40]. Farm environment, including pastures and water sources, and farm related products are considered to be the likely sources for *C. difficile* ribotype 078 [41]. *Clostridioides difficile* ribotype 078 isolated from humans and animals are genetically highly related; therefore, this ribotype can easily be transmitted between species [42]. The prevalence of *C. difficile* ribotype 078 in this study was similar to the prevalence reported in barn swallows (*Hirundo rustica*) [16], and in farm [11,15]. These data suggest that bats might transmit *C. difficile* ribotype 078; however, they more likely serve as an environmental indicator of *C. difficile* ribotype 078 rather than the source of contamination/infection in humans and other animal species.

Two other *C. difficile* ribotypes recovered in this study were the ribotype 056 and a previously unidentified toxigenic ribotype. Ribotype 056 was previously recovered from human samples in USA and Netherlands [15,43], from Australian calves [44] and from Dutch poultry [43]. *Clostridioides difficile* ribotype 056 is not often isolated from CDI cases. However, in the European survey of nosocomial CDI it was determined to be more likely associated with a complicated disease outcome compared to other *C. difficile* ribotypes associated with CDI [40]. It is also significantly more likely to be found in community acquired CDI cases than in hospital acquired CDI cases [45]. Only one bat was positive for *C. difficile* ribotype 056 and little is known about this strain in domestic animals in Europe and wildlife overall [46]. The previously uncharacterized *C. difficile* ribotype CDB3 was toxigenic and possesses genes for the production of toxin A and B. While potentially pathogenic, it has not been previously identified in this laboratory or documented in the WebRibo database, nor is the isolate available from the Cardiff ECDC collection. Therefore, it is unlikely to be of a major human or animal health concern.

5. Conclusions

Bats insectivore nature, their daily and seasonal migratory pattern and large colony congregations at roosting sites enable efficient intra- and inter- species transmission of pathogens. Three *C. difficile* ribotypes were identified in this study, two of which have been associated with CDI in humans. It is unlikely that bats are a significant source of *C. difficile* exposure for humans and might more likely be an indicator of environmental exposure. However, a potential for dissemination of previously identified and new *C. difficile* ribotypes from bats cannot be dismissed.

Competing interests

The authors declare that they have no conflicts of interest.

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