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Research Article

Fecal Microbiota of *Rhinoceros Unicornis* as a Reservoir of Multi-Drug-Resistant Bacteria

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Abstract

The presence of Antimicrobial Resistance (AMR) bacteria in wildlife indicates the possible role of wild animals as efficient AMR reservoirs and dispersers of resistant bacteria to the human, livestock and natural environments. The presence of AMR bacteria not only has serious public health consequences, but also threatens native wildlife populations. In this study, we investigate the occurrence and antibiotic resistance patterns of fecal microbiota of *Rhinoceros unicornis* in Assam. Sixty two freshly voided dung samples of rhinoceros were collected from Kaziranga National Park of Assam in 2018. Fecal samples were tested for the presence of bacterial species and submitted to National Centre for Veterinary Type Cultures, National Research Centre on Equines, (NC-VTCC, NRCE) Hisar for identification. Antimicrobial Susceptibility Testing (AST) was determined using the disk diffusion method and antibiotic resistance patterns were assessed according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. Overall, 24 isolates were identified that belonged to 19 different bacterial genera including *Klebsiella* spp., *Achromobacter* spp., *Pseudomonas* spp., *Alishewanella* spp., *Wautersiella* spp., *Moraxella* spp., *Inquilinus* spp., *Weeksella* spp., *Oligella* spp., *Myroides* spp., *Paracoccus* spp., *Ochrobactrum* spp., *Psychrobacter* spp., *Pannonibacter* spp., *Shewanella* spp., *Sphingobacterium* spp., *Sphingomonas* spp. (4% each), *Escherichia coli* (8%) and *Acinetobacter* spp. (21%). Of the 24 isolates found in rhinoceros fecal samples tested for antimicrobial susceptibility, 13 isolates showed resistant to three or more than three substance classes. Results indicate that overall, most of the bacterial species from *R. unicornis* were multi-drug-resistant, which may reflect not only several risk factors leading to the origin of AMR in wild animals but also wildlife as natural reservoir of resistant bacteria. Therefore, efforts must be initiated to monitor the occurrence of such AMR bacteria in wildlife and understand their potential effect on wildlife conservation and public health.

Introduction

Antibiotic resistance is an emerging worldwide concern to both humans and animals. The key factors leading to antibiotic resistance are the overuse and misuse of antibiotics [1]. It is high time to lessen the growth of antibiotic resistance and most importantly to understand the cause and underlying mechanism of substantial increase of antibiotic resistance bacteria in the environment. The intensive usage of antibiotics in human medicine, veterinary, aquaculture and agricultural settings are among its contributing sources that results in their continuous release into the environment. Consequently, there is widespread presence of antibiotic resistant microorganisms of human or animal origin in the natural environment. A wide range of Antimicrobial Resistant (AMR) microorganisms including the ESKAPE pathogens of public health concern were detected. In recent years, the antibiotic resistance has led to a growing number of health care problems as AMR pathogens are not responding to existing antibiotics, therefore, poor prognosis, heightened severity of infections etc [2].

Since the first description of Chloramphenicol resistant *Escherichia coli* in wild birds, the occurrence of AMR bacteria in wild fauna has been reported in diverse wild species [3,4]. Insects, wildlife, including rodents and birds, as well as companion animals or pets were reported to harbor AMR pathogens and/ or genes [5-7]. Although wild animals are unlikely of being treated with antimicrobials, many studies affirm that wildlife is reservoir and disperser of AMR pathogens [8]. There may be several unique risk factors, including expansion of urban populations that result in fragmentation, encroachment and loss of natural habitats, poorly enforced animal and human regulations etc [9-12]. When wild creatures lose their forest homes due to deforestation, they are often unable to subsist in the smaller fragments of forested land resulting in straying into human habitats. As a result, the possibility of spillover of pathogens at the human-animal interface increases. The epidemiology of AMR at the human-animal interface involves transmission routes of resistant bacteria and/ or genes, antimicrobial selective pressures in several reservoirs (animals, humans, and the environment) and other ecological drivers [12]. Additionally, often there is overlap between habitats resulting in transmission of AMR bacteria between the different niches [9]. There is evidence of spread of bacteria as well as antibiotic residues from food producing animals and inadequately treated human wastes in the environment as well as in wild fauna [13,14]. Both the environment and wild fauna can become reservoirs of AMR bacteria and at the same time act as a source of reintroduction of AMR bacteria into domestic animals and humans. Studies regarding the presence of AMR bacterial and/ or genes in wildlife are scarce. Therefore, it becomes important to study the presence of AMR bacteria and genes in wildlife and consider their role in the dynamics of AMR. The Indian rhinoceros (*Rhinoceros unicornis*) also called the Greater One-horned rhino is a vulnerable wildlife species found in the Indian subcontinent and 72% of the global Indian rhinoceros population is concentrated in Assam (15; https://en.wikipedia.org/wiki/Indian_rhinoceros). Kakati et al. [16] reported that 1% of the gut bacteria of wild greater one-horned rhinoceros are predicted to be harmful and are associated with different diseases. In this study, we investigate the occurrence and antibiotic resistance patterns of fecal microbiota of *R. unicornis* in Assam.

Materials and Methods

Sampling

In the present study, 62 freshly voided dung samples, specifically from the inner masses of the dung heap of rhinoceros were collected during the month of July 2018 from Kaziranga National Park of Assam. The selection of the freshly voided samples was based on the physical and visual parameters of the dung such as the shine, moisture content, presence of mucous, presence of dung beetles and surface growth of toadstool and mushrooms etc. Sample size was not specified under this study, as the investigation was performed depending on the availability of suitable samples. This work is a part of the research initiated on rhino health by the Assam Forest Department in partnership with College of Veterinary Science (CVSc), Assam Agricultural University (AAU), Khanapara, Guwahati-781022 and World Wide Fund for Nature-India. This study was approved by the Institutional Ethics Committee of College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781022.

Bacterial isolation and antimicrobial susceptibility analysis

Fecal samples were tested for the presence of bacterial species. Primary isolation was carried out using enriched media (Brain heart infusion agar). In the present study, identification of the bacterial isolates was accomplished with support from National Centre for Veterinary Type Cultures, National Research Centre on Equines, (NC-VTCC, NRCE) Hisar. Antimicrobial Susceptibility Testing (AST) was determined using the disk diffusion method (Kirby Bauer method) on Mueller-Hinton agar (Cat. M173-100G, HiMedia) according to Clinical and Laboratory Standards Institute (CLSI) recommendations. CLSI breakpoints were used to characterize the antibiotic resistance patterns [17]. The isolates were subjected to the examination spectrum of seven antimicrobial substance classes. The following nine clinical antibiotic disks (HiMedia) with concentration of the drug per disk as stated in parentheses were used in the test: beta-lactams [penicillin G (10 U)], aminoglycosides [kanamycin (30 mcg) and tobramycin (10 mcg)], tetracyclines [doxycycline (30 mcg), carbapenems [imipenem (10 mcg)], fluoroquinolones [ciprofloxacin (5 mcg)], including sulpha/ trimethoprim [co-trimoxazole (25 mcg)] and colistin (10 mcg). The inhibition zone diameters were interpreted from CLSI guidelines. A bacterial isolate is defined as multi-drug-resistant if it is resistant to three or more antimicrobial classes tested [18].

Results

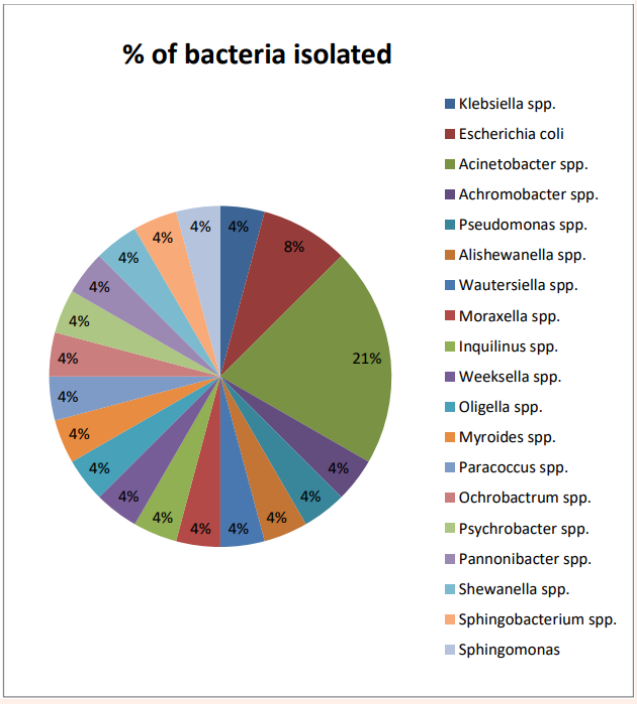

Figure 1: Isolation of bacteria (n=24) from rhinoceros fecal samples.

Twenty eight Gram negative rods or cocco-bacillary rods and one Gram positive bacilli were isolated from 62 tested rhinoceros fecal samples and were submitted to NC-VTCC, NRCE, Hisar. Identification of these isolates resulted in one *Klebsiella* spp. and two *Escherichia coli* belonging to the family Enterobactericeae; whereas other 21 isolates were identified as those belonging to glucose non-fermentor group (GNG). The biochemical identification of GNG group has classified seven isolates as five *Acinetobacter* spp., one *Achromobacter* spp., and one *Pseudomonas* spp. Rest fourteen isolates were classified into a large group of GNG which are indole negative, oxidase positive and further could be divided into sub-groups by presence or absence of benzyl arginine aminopeptidase (trypsin) activity. Based on this, the isolates belonged to genera viz., *Alishewanella, Wautersiella, Moraxella, Inquilinus, Weeksella, Oligella, Myroides, Paracoccus, Ochrobactrum, Psychrobacter, Pannonibacter, Shewanella, Sphingobacterium and Sphingomonas* spp. (Figure 1). Five of the isolates could not be re-cultivated.

From the bacterial isolates found in fecal samples from rhinoceros, *Oligella* spp. showed 100% sensitivity whereas *Escherichia coli* and *Inquilinus* spp. showed 100% resistance to all the antimicrobial substance classes tested in this study. One isolate of *Psychrobacters* spp. was susceptible to all antibiotics tested except beta-lactam group of antibiotic, penicillin G. *Paracoccuss* spp., isolate was resistant to penicillin G and co-trimoxazole. *Alishewanella* spp., isolate was resistant to aminoglycosides and colistin. *Moraxella* spp. isolate was susceptible to Kanamycin, Tobramycin, Doxycycline and Co-trimoxazole. One isolate of *Pseudomonas* spp., *Ochrobactrums* spp., *Shewanella* spp., and *Sphingomonas* spp. were susceptible to three classes of antibiotics tested. Likewise, one isolate of *Weeksella* spp., *Myroides* spp., *Klebsiella* spp., and *Achromobacter* spp., were susceptible to two antibiotic classes. Out of 5 isolates, *Acinetobacter* spp. showed only 20% sensitivity to kanamycin, ciprofloxacin and colistin and 40% sensitivity to penicillin G, co-trimoxazole and imipenem, and showed 100% resistance to tobramycin and doxycycline. Just two isolates, namely *Wautersiella* spp. and *Pannonibacters* spp. were susceptible to only co-trimoxazole and imipenem each respectively. Of the twenty four isolates found in rhinoceros fecal samples tested for antimicrobial susceptibility, 13 isolates showed resistant to three or more than three substance classes (Figure 2 & Table 1). It can be concluded from the results that most of the bacterial isolates were resistant to at least three classes of antibiotics and were thus classified as multi-drug-resistant.

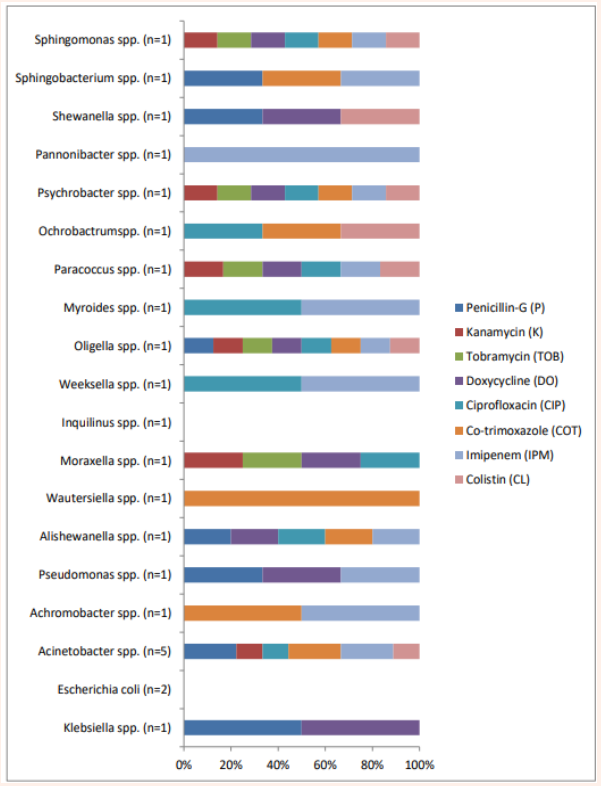

Figure 2: Antimicrobial susceptibility pattern of bacterial isolates (n=24) found in fecal samples from rhinoceros.

Table 1: Antimicrobial Susceptibility Testing (AST) of bacterial isolates from rhinoceros fecal.

	Penicillin-G (P)	Kanamycin (K)	Tobramycin (TOB)	Doxycycline (DO)	Ciprofloxacin (CIP)	Co-trimoxazole (COT)	Imipenem (IPM)	Colistin (CL)
Klebsiella spp. (n=1)	1(100%)	0%	0%	1(100%)	0%	0%	0%	0%
Escherichia coli (n=2)	0%	0%	0%	0%	0%	0%	0%	0%
Acinetobacter spp. (n=5)	2(40%)	1(20%)	0%	0%	1(20%)	2(40%)	2(40%)	1(20%)
Achromobacter spp. (n=1)	0%	0%	0%	0%	0%	1(100%)	1(100%)	0%
Pseudomonas spp. (n=1)	1(100%)	0%	0%	1(100%)	0%	0%	1(100%)	0%
*Alishewanella*spp. (n=1)	1(100%)	0%	0%	1(100%)	1(100%)	1(100%)	1(100%)	0%
Wautersiella spp. (n=1)	0%	0%	0%	0%	0%	100%	0%	0%
Moraxella spp. (n=1)	0%	1(100%)	1(100%)	1(100%)	1(100%)	0%	0%	0%
Inquilinus spp. (n=1)	0%	0%	0%	0%	0%	0%	0%	0%
Weeksella spp. (n=1)	0%	0%	0%	0%	100%	0%	100%	0%
Oligella spp. (n=1)	100%	100%	100%	100%	100%	100%	100%	100%
Myroides spp. (n=1)	0%	0%	0%	0%	100%	0%	100%	0%
Paracoccus spp. (n=1)	0%	100%	100%	100%	100%	0%	100%	100%
*Ochrobactrum*spp. (n=1)	0%	0%	0%	0%	100%	100%	0%	100%
Psychrobacter spp. (n=1)	0%	100%	100%	100%	100%	100%	100%	100%
*Pannonibacter*spp. (n=1)	0%	0%	0%	0%	0%	0%	100%	0%
Shewanella spp. (n=1)	100%	0%	0%	100%	0%	0%	0%	100%
Sphingobacterium spp. (n=1)	100%	0%	0%	0%	0%	100%	100%	0%
*Sphingomonas*spp. (n=1)	0%	100%	100%	100%	100%	100%	100%	100%

Discussion

The present study documents that the fecal micobiota of *R. unicornis* is dominated by Gram negative bacterial species. The results of our study are in agreement with [16] who reported Gram negative Proteobacteria being the most abundant phyla in the gut of Indian rhinoceros followed by Firmicutes and Bacteriodets. Limited data are available on the contribution of rhinoceros to the spread of AMR. In the present study, the antibiotic resistance patterns of the fecal micro biota from rhinoceros in Assam revealed the occurrence of multi-drug-resistant bacteria. This might indicate not only the high level of exposure of the rhinoceros to antimicrobials but also as carriers of resistant bacteria. However, to establish this, it would need to be further assessed by adequate epidemiological analysis. Several studies report that the wild animals may uptake resistant bacteria via feed and drinking water or through direct contact with garbage and sewages [19]. It has been also demonstrated that wildlife living with little direct human or livestock contact or due to anthropogenic activity in wildlife areas can harbor resistant bacteria [20]. Saha et al. [21] reported abundance of multiple-antibiotic-resistant *Salmonella* strains in the fecal samples of *R. unicornis* of the Kaziranga National Park, Assam. Available studies have shown wild boars, roe deer and wild ducks and geese as the carriers of bacteria with specific resistance traits including colstin, fluoroquinolones and cephalosporins (8). Resistance to colistin in *E. coli* isolates from wild birds has been previously described [22,23]. Guyomard-Rabenirina et al. [24] reported the prevalence of resistance to antibiotics in *E. coli* isolates collected from wild animals (iguanas, birds, anoles and rodents).

In the present study, most of the bacterial isolates were resistant to at least three classes of antibiotics and were thus classified as multi-drug-resistant. The presence of AMR bacteria in wildlife indicates the possible role of wild animals as efficient AMR reservoirs and dispersers of resistant bacteria to the human, livestock and natural environments. Moreover, several risk factors leading to greater proximity of the wild animals to urban and peri-urban areas play a relevant role in the origin of AMR in wild animals. Therefore, efforts must be initiated to monitor the occurrence of such AMR bacteria in wildlife and understand the dissemination of resistant gene among bacteria harbored by wild animals. The presence of resistant bacteria to critically important antimicrobials poses a serious public health concern as well. Therefore, "One Health" approach would be essential to study the possible role of wildlife as reservoir and the epidemiological links between human, livestock and environment [25].

Conclusion

The antibiotic resistance patterns of the fecal micro biota from rhinoceros in Assam revealed the occurrence of multi-drug-resistant bacteria. However, the finding of bacteria with AMR in rhinoceros does not, in itself, indicate that this is a source of AMR bacteria for domestic animals or humans. Thus, to establish the potential role of *Rhinoceros unicornis* in AMR, in depth study utilizing the landscape used for domestic animals and humans is encouraged. More to the point, the Indian rhinoceros are already listed as Vulnerable to IUCN Red List; therefore multi-drug-resistant infections might be a new and insidious threat for them as well. In view of that, it is the need of the hour to understand the potential effect of bacterial pathogens with AMR on wildlife conservation and public health.

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