
The greater one-horned rhinoceros review

The greater one-horned rhinoceros (*Rhinoceros unicornis*), traditionally known as Indian rhinoceros, is a native of Indian subcontinent predominant in northern India and Nepal. Currently, the species is listed to be vulnerable in the IUCN Red List. In 2015, according to World Wide Fund for Nature (WWF), the total population of Indian rhinoceros in the globe was assessed to be around 3500 (1). The wildlife sanctuaries of Assam is the home for the major portion of world's one-horned rhinoceros population and the population has increased by 27% in Assam since 2006 (2).

Poaching and habitat destruction are the severest threat to the Indian rhinoceros population rather than the lethal diseases. Out of all bacterial diseases, most commonly encountered in Indian rhinoceros are *Salmonellosis*, Tetanus, Tuberculosis, Leptospirosis etc (3, 4). The customary spotted gastrointestinal problems are gastric ulcers and impactions, suspected to be caused by dietary factors rather than an infectious agent (5). Among bacteria, *Salmonella* is the common cause of enteritis in young rhinoceros (6). It is a well-established fact that the gut microbiota contains both pathogenic and non-pathogenic bacteria, and the gut microbiome plays a vital role in host immune system, enteric disease resistance, inhibition of pathogens and metabolism including synthesis of essential compounds like secondary bile acids, short chain fatty acids, vitamin B, and vitamin K (7-11). Thus, it has been hypothesized that the enteric disease resistance in Indian rhinoceros might be contributed by some novel bacteria present in the gut.

The advancement of post genomic technologies like metagenomics during the last decade and its anticipated appreciation in varied areas of biological science is going to aid the present era scientist in culture-independent genomic analysis and discovery of microbial diversity in a particular environmental niche such as soil, water and the gastrointestinal tracts (12-13). Almost 99% of bacteria present in the environment cannot be cultured (14); so high-throughput metagenomics approach could be the path-finder to discover the valuable bacterial diversity present in the gut of Indian rhinoceros. Extensive documentation has been done- in relation to human gut microbiome but gut meta-genomics in wild animals is still in its infancy. Although the microbial community in the faeces of White Rhinoceros (*Ceratotherium simum*) has been reported (15), similar reports on *Rhinoceros unicornis* are scanty. The intestinal microbiome community of woolly rhinoceros is also well documented (16). Faecal metagenome bears a signature of the gut microbiota, therefore, this study aimed to identify the potential, valuable and unique microbiota present in the faeces of Indian rhinoceros and to reveal its probable impact in the health of this non-ruminant herbivore and perhaps this is the first report on microbial diversity of the hindgut of Indian rhinoceros.

Material and Methods

Collection of Fecal Samples

Samples were collected from one-horned rhinoceroses housed in the Kaziranga National Park, Assam, India. Fresh faecal samples (approximately 100 g each) were collected from 10 different animals in the morning in plastic containers with dry ice. The samples were sent to the

Department of Animal Biotechnology, CVSc, AAU, Khanapara immediately after collection and stored at -20°C until processing. The samples were pretreated as follows: 50 g of faeces was suspended in a sterile plastic beaker containing 250 ml of sterile phosphate-buffered saline (PBS) (0.05 mol/L, pH 7.4) (17). The sample was stirred with a sterile plastic rod for about 30 min to remove the bacteria from the plant residue. The suspension was then divided into 60 ml aliquots and transferred to sterile centrifuge tubes and vortexed vigorously for 15 min. The samples were centrifuged at 2000 g for 5 min three times (each time the supernatant was transferred to a new tube) to remove coarse particles. The cells in the supernatant were collected and washed three times by centrifuging at 9000 g for 3 min with 30 ml fresh PBS. Finally, the washed cell pellets were re-suspended in one- tube in 10 ml of sterile PBS, divided into 1ml aliquots and stored at ?20°C for DNA extraction within one- week.

Extraction of nucleic acids and next generation sequencing

The samples were subjected to DNA extraction using the phenol-chloroform method. The quality and quantity of the DNA was measured by using picodrop. Partial 16S rRNA gene sequences were amplified by polymerase chain reaction using a Thermocycler (Applied Biosystems) and a primer pair, which targeted the V3 and V4 regions of the 16S rRNA gene sequence (Milani et al., 2013). An additional PCR was performed to amplify the larger V3 region before proceeding with the nested PCR. For library preparation, 10 ng of PCR amplicon were taken as the starting material. The amplified region was cleaned up using Agencourt Ampure SPRI beads (Beckman Coulter). The equi-molar pooled amplified product was used for next round of PCR. Index barcodes were added using modified primers which had adapter sequences. 10 cycles of PCR was performed and the product was cleaned up using Agencourt Ampure SPRI beads (Beckman Coulter). The prepared library was quantified using Qubit fluorometer and validated by running an aliquot on high sensitivity Bioanalyser chip (Agilent Technologies, USA). The DNA library was sequenced for paired end 2 × 300 sequences on Illumina MiSeq platform.

Bioinformatics analysis

The metagenomics pipeline performs quality control, protein prediction, clustering and similarity-based annotation on nucleic acid sequence datasets for annotations. We performed a protein similarity search between predicted proteins in the metagenome and database proteins. QIIMP is comprehensive software comprising of tools and algorithms such as FastTree for heuristic based maximum-likelihood phylogeny inference, the RDP classifier for the assignment of taxonomic data using naive Bayesian classifier and others. We performed stitching the PE data into single end reads and picked the Operational taxonomic units (OTU) based on sequence similarities within reads, and picked a representative sequence from each OTU. The OTUs were assigned a taxonomic identity from reference databases. Diversity matrices from each were calculated and the types of communities were compared using the taxonomic assignments.

OTU picking identifies highly similar sequences across the samples and provide a platform for comparison of community structure. Sequences from all the samples were clustered into operational taxonomic unit (OTUs) based on their sequence similarity. OTUs are the clusters of sequences, intend to represent the degree of taxonomic relatedness, may be made up of many sequences, we picked representative sequences for each OTU for downstream analysis. These representative sequences were used for taxonomic identification of OTU. It was done- by setting

assignment method to the RDP classification system and a confidence of 0.8. This is done by using uclust at 97% sequence similarity and each resulting clusters is typically represents a species.

Results

A total of 418,890 tremendous sequences have been clustered to provide a complete of 896 OTUs. Even though the primers were used to amplify each bacteria and archaea, most of the OTUs were found bacterial origin, deriving from a total of 326 bacterial families and 925 bacterial genera. A smaller wide variety of archaeal sequences had been detected and the majority of these sequences belonging to the genus *Methanobrevibacter*. The compositional distribution pattern underneath one-of-a-kind taxonomic type consisting of phylum, class, order, family and genus levels were in comparison. MiSeq sequencing of the V3-V5 region of 16S rRNA gene amplicons from one- horned rhinoceros samples generated a total of 418,890 sequences. These sequences had been clustered into 896 bacterial OTUs belonging to 20 phyla, 78 families and 105 genera. Firmicutes was the most abundant phylum (74.86%), followed by Verrucomicrobia (14.82%), Proteobacteria (6.86%) and Bacteroidetes (2.28%) (Figure.1). Sixteen out of 20 phyla constituted less than 1% of the microflora. At the family level, Planococcaceae (57.65%) was found to be the most abundant in the gut of rhino (Figure. 2). Verrucomicrobiaceae was found to be the next (14.81%) followed by Moraxellaceae (6.22%), Ruminococcaceae (4.93%), Lactobacillaceae (2.72%) and Lachnospiraceae (2.17%). However 71 families out of 78 found to occupy below 1% of the microflora. In all, 5.1% microbes were found to be of unclassified bacterial family, which belonged to phyla Firmicutes, Bacteroidetes, WPS-2, Cyanobacteria, TM7, Elusimicrobia, Armatimonadetes, Elusimicrobia, Proteobacteria, Actinobacteria and Chloroflexi.

Bacillales was the most abundant order from the class Bacilli (57.68%) and Lactobacillales, a representative order from the class Bacilli, was detected to be lower (2.87%) in the samples. At the genus level, one- horned Rhino gut microflora was dominated by *Rummeliibacillus* of Planococcaceae family. *Akkermansia* was found to be the major genera (14.81%), followed by *Lactobacillus* (2.33%) and *Clostridium* (0.81%). The microbial diversity was more diverse and consisted of *Enterococcus*, *Wautersiella*, *Clostridium*, *Streptococcus*, *Succinivibrio*, *Anaerofustis*, *Acinetobacter* and *Bacteroides*.

Discussion:

Investigation of one-horned Rhino gut microbiota is essential to understand the role of the resident microbes in host function. However, available reports were mainly focusing on data obtained through the culture-dependent techniques (18) and early molecular fingerprinting methods (19-20). Despite the extensive use of NGS in unraveling the function and importance of human gut microbiome (21-23), there is currently a lack of sufficient information relating to biodiversity assessment using HT-NGS to understand the topological differences and development of gut microbiota in one-horned Rhino intestines. The microbial population in the gut plays a key role in the health and welfare of the herbivores (24). An active and functional fibrolytic bacterial population in the hindgut converts fibrous feeds into volatile fatty acids which make a significant contribution to the energy requirements of the host (25).

So far, studies regarding the intestinal microbial flora of the one-horned rhinoceros are not

available. The faecal bacterial community of the one-horned rhinoceros was reported for the first time in the present study using high throughput sequencing technology. It was not unexpected to find that a large number of bacteria in the faeces of the one- rhinoceros belonged to the unclassified genera based on the current database of 16S RNA gene sequences, since little work on this kind of wild herbivorous animals has been reported so far. Due to differences in approaches and concepts of study, direct comparison of OTUs and taxonomic composition between the reported studies may not be accurate. Additional factors, such as environment, diet, horizontal gene transfer, geography and climate might also play role in the Rhino gut microbiota (26-27). Based on the present study, Firmicutes was found to be the most predominant phylum which was consistent with previous reports both in white Rhinoceros and chicken (28-29).

Diet plays a vital role in determining the composition of the resident gut microbes (30a). The human gut microbiome is shared among family members, who have similar microbiota even if they live at different locations (31b). From human faecal samples, 66 dominant and prevalent operational taxonomic units were reported which included the genera of *Faecalibacterium*, *Dorea*, *Eubacterium*, *Ruminococcus*, *Alistipes*, *Bacteroides* and *Bifidobacterium* (32). Diversity in the faecal bacterial and fungal communities was also reflected in studies on canine and feline gut samples (33). The most abundant phyla in canine gut microbiota were found to be Firmicutes, followed by Actinobacteria and Bacteroidetes, whereas the most common orders were Clostridiales, Erysipelotrichales, Lactobacillales (Firmicutes) and Coriobacteriales (Actinobacteria). In ruminants, the common rumen microbes were found to be *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens*, and *Prevotella* (24).

In the wider area of gut microbiology, there is active debate concerning the existence of a core stable microbiota. It is estimated that there are perhaps 5000 unique bacterial OTUs in the human gut when considered over a range of individuals under different spatial and temporal conditions (34). However, it is speculated that there are perhaps 300 OTUs that make up the core stable microbial population in a healthy individual (35). In the present study, 896 OTUs were detected representing more than 75% of abundance within the total microbiota. In addition, we found that one- horned rhinoceroses were dominated by phyla Firmicutes and Verrucomicrobia including Proteobacteria, Bacteroidetes, Actinobacteria and Planctomycetes, which was different from those reported by Ley and his co-workers and Gaorui Bian for mammals (36). On the other hand, the faeces of healthy horse were found to be dominated by Lachnospiraceae (37). In the rumen of cows, the predominant core bacteria belonged to the genus *Prevotella* and *Butyrivibrio* and family Lachnospiraceae (38).

Akkermansia spp., a widely studied microorganism that is inversely associated with obesity (39-40), was found to be abundant in one-horned Rhino gut of the present study. *Akkermansia* has been reported to be a mucin degradation-specialized bacterium that utilizes mucus as a sole carbon and nitrogen source (41). An increase in *Akkermansia* has been shown to protect the niche from obesity (39, 42), and type I and type II diabetes mellitus (43).

Possible reasons for the high percentage of core bacteria in the rhinoceros might be that only a few animals were screened and these animals had the same diet in the same habitat. A higher diversity of bacterial population in rhinoceroses compared to horses and cows might be responsible for its strong ability to adapt to the diet.

The work presented here describes the composition of the overall bacterial communities in the faeces of one-horned rhinoceros living in the Kaziranga National park, Assam. Our data reveals the presence of a complex bacterial community in the faeces of the one--horned rhinoceros. The rhinoceros possesses distinctive microbiota and core bacteria in the faeces compared to horses. These observations increased our understanding of the bacterial ecosystem of this endangered animal; however, further study is still needed to know whether rhinoceroses in the wild have specific gut microbiota compared to other non-ruminant herbivores.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies conducted on human or animal subjects. However, the study performed under the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Govt. of India. For sampling approval was taken (vide letter no. WTI/GM/18/02) from Field Director, Kaziranga Tiger Reserve and Project Leader, CWRC and Forest Department, govt. of Assam, India.