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Deciphering the Fecal Microbiome of Indian Rhinoceros (*Rhinoceros unicornis*) by Metagenomic Approach

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ABSTRACT

With the advent of high throughput next generation sequencing (NGS) technology, it has become possible to study the diversity in microbial community of the gut with extraordinary resolution and accuracy. A number of sophisticated bioinformatics tools have also been developed to analyze the enormous data generated by such study. The greater one-horned rhinoceros (*Rhinoceros unicornis*) is native to the Indian subcontinent and majority of its population is found in Assam. The rhinoceros is one of the largest mammalian herbivores and has the ability to utilize fibrous plant matter through microbial fermentation in the hindgut. So far, there has been no report relating to study of the gut microbiota of the one-horned rhinoceros using metagenomic approach. In this study, we extracted genomic DNA from fecal sample of one-horned rhinoceros using commercially available kit as per the manufacturer's protocol. The QC passed DNA sample was used for amplicon generation, targeting V3 -V4 region of 16S rRNA genes. Library was constructed using Nextera XT index kit as per manufacturer's protocol. Further, to obtain an unbiased measure of bacterial diversity in the gut by metagenomic approach, we sequenced the nucleic acid by IllumiaMiseq 2x300 chemistry. Altogether 418,890 sequence reads were generated to characterize the gut microbiome of rhinoceros. The results showed that Firmicutes, Verrucomicrobia, Proteobacteria and Bacteroidetes were the predominant phyla occupying 74.87%, 14.83%, 6.86% and 2.29%, respectively of the microflora. A large proportion was found to comprise with unclassified bacteria. At the genus level, 4.71% *Ruminococcus* bacteria were detected which is an important member of the microbial community in the hindgut of non-ruminant herbivores, which enabled the host to gain nutrients from fibrous plant materials. The present work provides a framework for understanding the complex microbial community of the one-horned rhinoceros; however, further studies are required to link the distinctive microbiota with their digestive role in the hindgut of the one-horned rhinoceros.

Key words: Metagenomics; Microbiome; Next generation sequencing; *Rhinoceros unicornis*.

INTRODUCTION

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 (Rookmaaker *et al*., 2016). The wildlife sanctuaries of Assam are the home for the major portion of world's one-horned rhinoceros population and the population has increased by 27% in Assam since 2006 (Hance, 2014).

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 and Leptospirosis (Silberman and Fulton, 1979; Fowler and Miller, 2003). The major gastrointestinal problems are gastric ulcers and impactions, suspected to be caused by dietary factors rather than an infectious agent (Wyss *et al*., 2012). Among bacteria, *Salmonella* is the common cause of enteritis in young rhinoceros (Windsor and Ashford, 1972).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 (Garrett *et al*., 2010; Flint *et al*., 2012; Nicholson *et al*., 2012; Brown *et al*., 2013; Yoon *et al*., 2014). Hence, it has been

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Figure 1. Map showing the sampling site (Kaziranga National Park, Assam)

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 (Handelsman *et al*., 1998; Lopez-Garcia and Moreira, 2008). Almost 99% of bacteria present in the environment cannot be cultured (Amann *et al*., 1990); so high-throughput metagenomics approach could be the path-finder to discover the valuable bacterial diversity including those 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 present in the faeces of White Rhinoceros (*Ceratotherium simum*) and woolly rhinoceros has been documented (Mardanov *et al*., 2012; Bian *et al*., 2013) similar reports on *Rhinoceros unicornis* couldn't be traced out in the available literature. Faecal metagenome bears a signature of the gut microbiota, therefore, the present 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. This may be the first report on microbial diversity of the hindgut of Indian rhinoceros.

MATERIALS AND METHODS

Ethical approval

This article does not contain data from any study directly 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 Environmentand Forests, Govt. of India. For sampling, approval was taken (vide letter no. WTI/GM/18/02) fromField Director, Kaziranga Tiger Reserve and Project Leader, CWRC and Forest Department, Govt. of Assam, India.

Collection of Fecal Samples

Samples were collected from one-horned rhinoceroses inhabiting the Kaziranga National Park, Assam, India (Figure 1). Fresh faecal samples (approximately 100 g each) were collected from 10 different animals in the morning hours in plastic containers with dry ice.The samples were immediately brought to the Department of Animal Biotechnology, CVSc, AAU, Khanapara for further downstream processing. Equal amount of faecal samples (50 g each) were prepared and pooled for DNA extraction as previously described (Wei *et al*., 2007).

Extraction of nucleic acids and Quantification

Total genomic DNA was extracted from the pooled samples. DNA extraction was done using the phenol-chloroform method with some modification. DNA was treated with DNase free RNase (Macherey-Nagel, Germany) to remove contaminating RNA. The quality and quantity of the DNA was measured by using Qubitflorometer 2.0 (Invitrogen, Thermo Fisher scientific) and gel electrophoresis. Extracted DNA was stored at −20 °C until further processing.

Amplification of 16S RNA gene and next generation sequencing

Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair, which targets the V3 and V4 region of the 16S rRNA gene sequence (Milani *et al*., 2013). For library preparation, 10 ng of PCR

amplicon were taken as starting material. The amplified region was cleaned up using Agencourt Ampure SPRI beads (Beckman Coulter). The equi-molar pooled amplified product was subjected to the next round of PCR using a Thermocycler (Applied Biosystems). 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 florometer and validated for quality by running an aliquot on high sensitivity Bioanalyser chip (Agilent Technologies, USA). The DNA library was outsourced for sequencing paired end 2 × 300 sequences on Illumina MiSeq platform.

Bioinformatics data analysis

The paired end reads were trimmed of their set primer sequences and the sequences were then *de novo* assembled into contigs using CLC genomics workbench software, with criteria of at least 95 % identity over 35-bp to merge two fragments and minimum contig length of 100 bases. The assembled contigs and singleton sequences greater than 100 bp were compared to the NCBI GenBank non-redundant nucleotide databases using BLAST. On the basis of the best BLAST result, sequences were classified into their likely taxonomic groups of origin based on the best hit (lowest E score) sequence match. An E value of $<10^{-5}$ was taken as cut-off value for significant hits.

RESULTS

Total 418,890 sequences were clustered to provide a complete set of 896 OTUs. Even though the primers were used to amplify each bacterium and archaea, most of the OTUs were found to be of bacterial origin, representing 326 bacterial families and 925 bacterial genera. A lesser wide variety of archaeal sequences were detected and the majority of these sequences belonged to the genus *Methanobrevibacter*. These sequences were found 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 2). 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 3). 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 were 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 *Rummelii bacillus* of Planococcaceae family. *Akkermansia* was found to be the major genus (14.81%), followed by *Lactobacillus* (2.33%) and *Clostridium* (0.81%). The microbial community was more diverse and consisted of *Enterococcus*, *Wautersiella*, *Clostridium*, *Streptococcus*, *Succinivibrio*, *Anaerofustis*, *Acinetobacter* and *Bacteroides*.

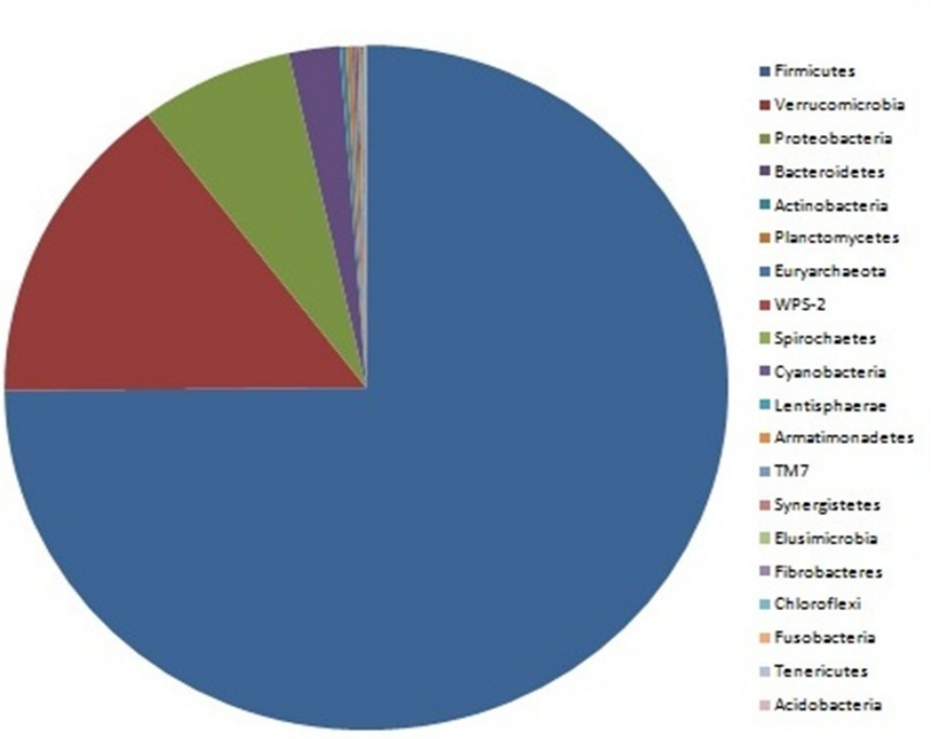

Figure 2. Fecal bacterial community at the phylum level. Relative abundance of bacterial groups (phylum level) in the feces of one-horned rhinoceroses.

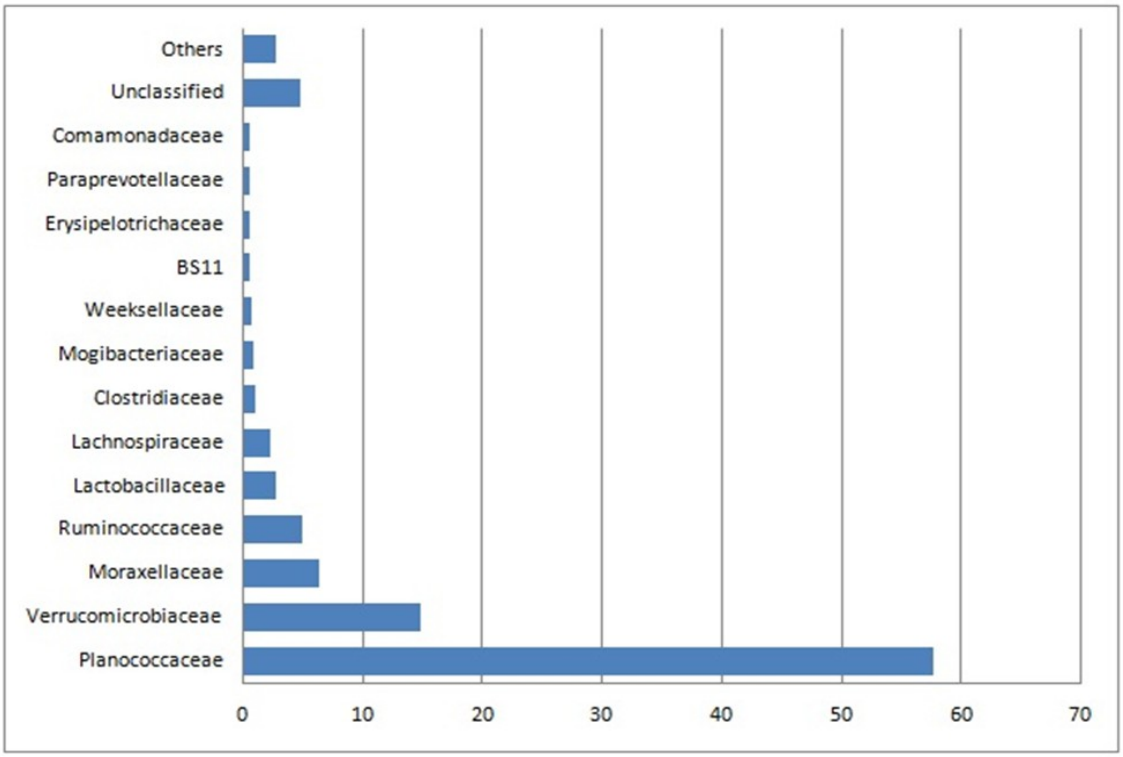

Figure 3. Fecal bacterial community at the family level. Relative abundance of bacterial groups (family level) in the feces of one- horned rhinoceroses.

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 (Barnes *et al*., 1972) and early molecular fingerprinting methods (Amit-Romach *et al*., 2004; Gong *et al*., 2007). Despite the extensive use of NGS in unraveling the function and importance of human gut microbiome (Arumugam *et al*., 2011; Lagier *et al*., 2012; Yatsunenko *et al*., 2012), there is currently 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 (Flint *et al*., 2008). Moreover, presence of effective fibrolytic bacteria in the hindgut aids in conversion of fibrous feeds into volatile fatty acids and imparts significant energy requirements of the host (Daly *et al*., 2012). Studies regarding the intestinal microbial flora of the one-horned rhinoceros have not been reported so far. The faecal bacterial community of the one-horned rhinoceros was reported for the first time in the present study using high throughput sequencing technology. Considerable numbers of bacteria present in the faeces of the one- rhinoceros were of unclassified genera according to 16S RNA gene sequence database which was not surprising as limited work has been reported pertaining to gut metagenomics of wild fauna. 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 diversity of Rhino gut microbiota (Facklam and Elliot, 1995; Hildebrandt *et al*., 2009). 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 (Bian *et al*., 2013; MohdShaufi *et al*., 2015). The composition of the resident gut microbes is significantly determined by diet of a host (Turnbaugh *et al*., 2009a). The human gut microbiome is shared among family members and possesses similar microbiota even if they live at different locations (Turnbaugh *et al*., 2009b). From human faecal samples, 66 dominant and prevalent operational taxonomic units were reportedwhich included the genera of *Faecalibacterium*, *Dorea*, *Eubacterium*, *Ruminococcus*, *Alistipes, Bacteroides* and *Bifidobacterium* (Tap *et al*., 2009).Diversity in the faecal bacterial and fungal communities was also reflected in studies on canine and feline gut samples (Handl *et al*., 2011). In canine,the most copious microbial phyla present in gut were *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, whereas the most customary orders were *Clostridiales*, *Erysipelotrichales*, *Lactobacillales*(*Firmicutes*) and *Coriobacteriales*(*Actinobacteria*). The common microbes harbouring rumen of ruminants were revealed to be *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus, flavefaciens*, *Butyrivibrio fibrisolvens*, and *Prevotella* (Flint *et al*., 2008).The existence of a core stable gut microbiota is dynamic and remains to be a prominent debatable factor among microbiologists. Almost 5000 unique bacterial OTUs have been estimated in the gut of human under different spatial and temporal

conditions but approximately 300 OTUs were considered as core stable microbiome present in healthy individuals (Frank *et al*., 2007; Manichanh *et al*., 2008). In the present study, 896 OTUs were detected in the faecal microflora of one-horned rhinoceros representing more than 75% of abundance within the total microbiota. In addition, we found that the gut microbiota of one-horned rhinoceroses was 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 GaoruiBianfor mammals (Ley *et al*., 2008). On the other hand, the faeces of healthy horse were found to be dominated by Lachnospiraceae(Costa *et al*., 2012). In the rumen of cows, the predominant core bacteria belonged to the genus *Prevotella* and *Butyrivibrio* and family Lachnospiraceae(Jami and Mizrahi, 2012).

Akkermansia, a widely studied microorganism that is inversely associated with obesity (Santacruz *et al*., 2010; Karlsson *et al*., 2012), was found to be abundant in one-horned Rhino gut inthe present study. *Akkermansia* has been reported to be a mucin degradation-specialized bacterium that utilizes mucus as a sole carbon and nitrogen source (Derrien *et al*., 2004). An increase in *Akkermansia* has been shown to protect the niche from obesity (Santacruz *et al*., 2010; Zhang *et al*., 2009), and type I and type II diabetes mellitus (Hansen *et al*., 2012). 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 almost 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.

CONCLUSION

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 and cattle. 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 in the same habitat.

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