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Metagenomic profiling of elephant dung using V3 – V4 amplicon sequencing of 16S rRNA

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Abstract

Metagenomic DNA extraction and subsequent bioinformatic analysis is an easy method to analyse the divergence and relative abundance of microflora present in environment samples. Extraction of DNA from faeces of elephant is challenging due the presence of inhibitory substances in dung. DNA was extracted from faeces using cTAB lysis method and commercial kits. The V3-V4 region of 16S rRNA was amplified and was further used for library preparation using NEBNext® Ultra™ DNA Library Prep Kit for Illumina®India. Taxonomic analysis was done using V search against SILVA data base. Metagenomic analysis revealed that Bacteriodata, Verrucomicrobiota, Firmicutes, Spirochaetota, Fibrobacterota, Synergistota, Proteobacteria were the major phyla seen in elephant dung samples.

Keywords: *Extraction of DNA, dung samples, elephant*

Hind gut fermenters are monogastric herbivores, the intestinal microflora of which have many beneficial effects. The gut microbiome of wild animals are a prominent source of many enzymes that have fibrolytic properties (Biddle *et al*., 2013). Metagenomic analysis of the DNA of microbes present in faeces provides information about the microbes present in the gut (Kouakou *et al.*, 2022). Information about the bacteria present in the fresh dung of elephants is scanty. Knowledge on this would help in the characterisation of bacteria based on their ability to degrade cellulose (Ilmberger *et al.*, 2014). Presently, metagenomic approaches help to identify the relative

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abundance of microorganisms present in the gut. Metagenomic approaches involve the direct extraction of metagenomic DNA from gut samples. Literature survey shows that there are a combination of different methodologies for DNA extraction with satisfactory concentration and purity. Luikart *et al*. (2008) extracted DNA from the faeces of sheep by using DNeasyTM Blood Kit (Qiagen, Valencia, CA). An extra step of washing the faecal pellets with lysis buffer (0.1 M Tris-HCl, 0.1 M ethylene-diamine-tetra-acetic acid, 0.01 M NaCl, 1% N-lauroyl sarcosine), pH 7.5 for 15 min was done before performing the kit protocol. Vaid *et al*. (2019) extracted DNA from mule dung samples using DNA commercial QIAamp® DNA stool mini kit (Qiagen Inc., Valencia, CA). They modified the procedure by doubling the quantity of InhibitEX tablet of the QIAamp® kit and removing the ethanol precipitation step with additional step of adding 15 µL of RNase before heat incubation and obtained good concentration and appreciable quality of DNA. Purity and concentration of metagenomic DNA is important for further PCR amplification and sequencing. The metagenomic DNA could be used as a template to amplify the 16S/18S rRNA coding sequences by PCR to study the microbial communities (Simon and Daniel, 2009). Hence, different protocols used for extraction of metagenomic DNA from the dung samples have been evaluated in this study and were used for taxonomic profiling of bacteria in elephant dung.

Materials and methods

The fresh dung samples of six elephants were collected from Punnathur kotta elephant sanctuary under the Guruvayoor Devaswom. Collected dung samples were stored in an ice box and transported to the Animal Nutrition laboratory within two hours.

Metagenomic DNA from the elephant dung samples were extracted using Qiagen QIAamp fast DNA tissue kit and commercial QIAamp® DNA stool mini kit as per the instructions of the manufacturer. In addition, DNA extraction and purification were also done by cTAB method (Tina *et.al.,* 2019). A gram of the faecal sample was placed in a centrifuge tube containing 6.6 ml of cTAB lysis buffer, 0.33 ml of 20% Sodium Dodecyl Sulfate (SDS), and 20 µl of proteinase K. The mixture was then incubated overnight at room temperature. Afterwards, the solution was mixed by vortexing for one minute, followed by an additional overnight incubation at room temperature. Three minutes of inversion mixing was performed, and the sample was further incubated for three hours at 65°C. Subsequently, the sample was centrifuged at 1300 rpm and 26°C for 30 minutes, and 1.5 ml of the supernatant was collected from the centrifuged sample. Purification was done using QiagenQIAamp fast DNA tissue kit as per manufacturer's instructions.

Quality of the isolated genomic DNA was checked by agarose gel electrophoresis and the concentration and purity were estimated by spectrophotometer (NanoDropTM 2000C). Metagenomic profiling was outsourced at MedGenome Labs, Narayana health city, Bengaluru. The V3-V4 region of 16S rRNA was amplified using specific V3 forward primer 5´-CCTACGGGNBGCASCAG-3´ and V4 reverse primer 5´-GACTACNVGGGTATCTAATCC-3´ and the amplified product was used for library preparation using NEBNext® Ultra™ DNA Library Prep Kit for Illumina®India. The library quantification and quality estimation were done using Agilent 2200 Tape Station. The prepared library was sequenced in Illumina HiSeq 2500 platform. Taxonomic analysis was done using V search against SILVA data base (Rognes *et al.* 2016).

Results and discussion

Concentration and purity of extracted DNA from dung samples of elephant were estimated using nanodrop TM spectrophotometer 2000C (Table 1). The CTAB DNA extraction method yielded DNA concentrations ranging from 6.2 to 1716.9 ng/µL, and was highly variable. Further trials are needed for revising the protocol to yield good quality DNA. It was also found that metagenomic DNA extracted using QIAamp® DNA stool mini kit (Qiagen Inc., Valencia, CA) yielded better quality (Table 1). The DNA samples obtained from elephant dung by commercial QIAamp® DNA stool mini kit were used for 16SrRNA sequencing and bioinformatic analysis. Vaid *et al.* (2019) extracted DNA from mule dung

Table 1. Concentration and purity of DNA samples obtained from elephant dung by 3 different methods.

Sample	Qiagen QIAamp fast DNA tissue kit		cTAB method		QIAamp® DNA stool mini kit	
	Concentration ng/µl	260/280	Concentration ng/µl	260/280	Concentration ng/µl	260/280
Sample1	2.7	2.17	54.4	1.9	54.4	1.9
Sample2	2.4	2.34	52.2	1.9	52.2	1.9
Sample3	2.8	2.17	439.9	1.76	39.9	1.86
Sample4	1.7	4.33	499.6	1.7	49.6	1.8
Sample5	2.7	4.15	863.7	1.71	83.7	1.81
Sample6	3	2.37	83.4	1.9	83.4	1.9

sample by using QIAamp® DNA stool mini kit (Qiagen Inc., Valencia, CA) and obtained DNA concentration in the range of 25-65 ng/µl. Tang *et al.* (2022) conducted comparative metagenomic analysis of microbiome in wild horses and resident Asiatic wild asses. The microbial DNA present in the dung sample was isolated using the E.Z.N.A Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) yield high quality DNA with good concentration for further metagenomic analysis. Ilmberger *et al.* (2014) conducted a study on metagenomic analysis of the faecal microbiota of a breast-fed and a plant-fed Asian elephant. The DNA isolation was done with the QIAamp DNA Stool kit from Qiagen (Hilden, Germany) which resulted in good quality metagenomic DNA. Kouakou *et al.* (2022) isolated DNA from dung sample of an elephant using a method similar to Qiagen QIAamp fast DNA tissue kit method in this study.

Metagenomic profiling of elephant dung was using V3 – V4 amplicon sequencing of 16 S rRNA. The taxonomic analysis of the metagenomic DNA revealed that the elephant dung contained bacterial phyla such as *Bacteriodata*, *Verrucomicrobiota*, *Firmicutes*, *Spirochaetota*, *Fibrobacterota*, *Synergistot*a, and *Proteobacteria*. Relative abundance of these species at phylum level in elephants were 37.4 per cent, 28.7 per cent, 22.7 per cent 3.85 per cent, 0.97 per cent, 2.40 per cent and 1.77 per cent, respectively (Fig. 1).

Ilmberger *et al.* (2014) analysed the gut bacterial population of elephants by 16S rRNA gene analysis and concluded that their

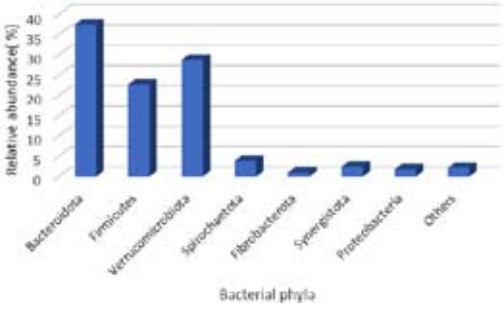

Fig.1 Relative abundance of bacterial phyla present in elephant dung

population was dominated by *Bacteroidetes* (47%) and *Firmicutes* (36%) other bacterial phyla including *Spirochaetes* (4%), *Fibrobacteres* (2%) and *Lentisphaeria* (5%). They also did a comparative study of the faecal microbiota of adult and baby elephants which revealed that the majority of bacteria belonged to Bacteroidetes. More than 50 per cent of the microbes were *Flavobacteriales* (22%) or *Bacteroidales* (34%). Further, the sample of the baby elephant comprised of a high number of *Proteobacteria* (32%) and *Firmicutes* (11%). The bacterial profiling of different species is very important in many aspects. Studies have shown that gastrointestinal difficulties in African elephants resulted in lower bacterial amplicon sequence variant (ASV) richness, and the gut microbiome makeup was connected to certain reproductive and metabolic hormones. Bacterial ASV abundances in African elephants had substantial linear correlations with prolactin, luteinizing hormone, and follicle stimulating hormone, which in turn affected the reproductive functions (Keady *et al.*, 2021).

Conclusion

The DNA extracted using QIAamp® DNA stool mini kit from elephant dung was of consistently good quantity as well as high quality. The relative abundance of bacterial phyla in the faeces of elephant were *Bacteroidota* (37.5 %), *Verrucomicrobiota* (28.7 %), *Firmicutes* (22.7 %), *Spirochaetota* (3.85%), *Synergistota* (2.40 %), *Proteobacteria* (1.76%) and *Fibrobacterota* (0.96%). Further studies can be directed to isolate the celluloytic bacteria for production of probiotics and fibrolytic enzymes.

Conflict of interest

The authors declare that they have no conflict of interest

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