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Evaluation of DNA Extraction Methods of Mule Dung

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ABSTRACT

DNA isolation is a critical step in microbial community analysis of animal dung. DNA isolation from mule dung is challenging due to microbial diversity, composition and chemical nature of mule dung. Therefore, selection of an appropriate DNA isolation method is important to analyse the complete microbial diversity. In the current study, we evaluated the DNA isolation from mule dung samples (n=11) using QiAmp Mini stool kit as per manufacturer's procedure with modifications. The results suggest that modifications in proprietary column based method improved the DNA quality and quantity suitable for mule dung microbial community analyses.

Keywords: DNA extraction; Kit; Dung; Metagenomics; Mule.

1. INTRODUCTION

The last quinquennial has been very important in equine hindgut metagenome research as many studies have reported the equine hind gut and gastrointestinal metagenome[1-4]. These metagenomic studies employing next generation sequencing (NGS) reveal a more comprehensive picture of the diversity of microbes in the horse gut, majority of which cannot be successfully cultured in the laboratory.

These studies have employed many methods for DNA extraction from equine fecal samples, which has been predominantly used to analyse the equine hind gut microbial profile. Although most of the studies have used fecal DNA kits[5-6], many have also used soil microbe DNA kits[7-8] or total DNA isolation kits[9]. The DNA extraction method is a critical step in microbial community profiling as apart from its effect on sequencing output and microbial community profiling[10], it adversely affects the result comparison across studies, especially in physiological and pathological studies[11-12]. In addition to the differences in methods and protocols, the fecal samples also contain inhibitory compounds like humic acid, fulvic acid and proteins interfering with the PCR in amplicon generation before actual sequencing[13]. Therefore, after representative sample collection by aseptic techniques, DNA extraction is one of the most critical steps in metagenome data generation. However, little attention has been paid to standardisation of DNA extraction methods, which remain a major bottleneck in the process of analyzing different types of microbiome samples. Besides, the small laboratories initiating the metagenomic analysis usually find it difficult to choose appropriate method and to follow proprietary kits and protocol, which always do not function in a desired manner.

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Apart from improving the efficiency of DNA extraction protocols, another challenge is to isolate microbial community DNA accurately representing the diversity of microbes, as DNA extraction procedures also influences extraction of DNA from certain types of microbes[14-15]. The involved factors are crucial to consider before choosing a method, as both yield and purity will affect the applicability of the DNA for next generation sequencing (NGS) for microbial community analysis.

This study evaluates DNA extraction with respect to quality and quantity of DNA using the QIAamp® mini stool kit from mule dung and further evaluates these parameters by modifications in the protocol.

2. MATERIALS AND METHODS

2.1 Collection of Mule Dung Samples

Mule (*Equus asinus* × *Equus caballus*) dung samples (n=45) were collected aseptically by rectal palpation of mule by use of sterile gloves, or through catching of feed pellet during the process of defecation. Samples were immediately stored at 4 °C and during transportation and were subsequently stored frozen at −20 °C until used.

2.2 DNA Extraction

DNA extraction was performed using commercial QIAamp® DNA stool mini kit (Qiagen Inc., Valencia, CA). Briefly, four procedures were evaluated for DNA quality and quantity; procedure 1 consisted of DNA extraction as per manufacturer's instructions, whereas other three procedures (viz., 2, 3 and 4) incorporated modifications in the original protocol.

2.2.1 Procedure 1

In 1 g of mule dung sample, 10 mL of buffer was added

and vortexed for 1 min. for complete homogenisation. From the homogenate, 2 mL was transferred into an eppendorf tube, heated at 95 °C for 5 min, vortexed and centrifuged at 14,000 rpm for 1 min. to pellet the suspension. Supernatant (1.2 mL) was taken into 2 mL micro-centrifuge tube in which InhibitEX tablet (n=1) was added and immediately vortexed for complete dissolution. The suspension was incubated for 1 min. followed by centrifugation at 14,000 rpm for 3 min. to pellet the stool particles/inhibitors.

Then 200 µL of supernatant was pipetted into 1.5 mL micro-centrifuge tube to which 15 µL of proteinase K and 200 µL of AL buffer was added and vortexed. The mixture was heated at 70 °C for 10 min. and then 200 µL of ethanol was added and vortexed. This mixture was passed through the column which was washed with 500 µL of AW1 buffer, followed by washing of column with 500 µL of AW2 buffer. DNA was eluted in 200 µL of AE buffer by using the columns provided in the kit (Qiagen, Valencia, CA) and stored at -20 °C till use.

2.2.2 Procedure 2

This procedure was similar to that of procedure 1, except, that DNA was eluted for four times using 50 µL of elution buffer at each time instead of a single 200 µL of elution buffer.

2.2.3 Procedure 3

In this procedure, sample was incubated at 95 °C for 10 minutes. Sample treatment by proteinase K (>600 m AU/ml solution) was increased from 15 µL to 50 µL. Total DNA was eluted as mentioned in the procedure 2 and an ethanol precipitation step was introduced after final elution in 50 µL of TE buffer**.**

2.2.4 Procedure 4

This procedure consisted of doubling the quantity of InhibitEX tablet of the QIAamp® kit to two tablets. The ethanol precipitation step was not included; however additional 15 µL of RNase was used before heat incubation.

2.2.5 Samples Processed

Initially, 2 fecal samples *viz*., Mu1 and Mu34 were randomly chosen, on which all 4 procedures were applied. Additionally, 6 and 9 mule dung samples were used for extraction of DNA by procedure P3 and P4, respectively (Table 1, Table 2). All samples were used after thorough homogenisation.

2.3 DNA Quantity and Quality Evaluation

The quantity and quality of extracted DNA were determined using a NanoDrop spectrophotometer (Nanodrop 1000 Spectrophotometer, Thermo Fisher Scientific, Massachusetts). The purity was assessed via 260/280 and 260/230 absorbance ratios. The quality of the DNA extracts was also estimated by electrophoresis of 3 µL of purified DNA on a 0.8 per cent agarose gel. Samples were stored at -20 °C. The quality of metagenomic DNA is also important. As the procedure P1 and P2 gave low yield, the quality of DNA was evaluated in the DNA extracts from procedure P3 and P4 only.

3. RESULTS AND DISCUSSION

Many studies have been conducted using different methods/kits for DNA isolation from equine metagenomic samples, however studies on mule dung samples in Indian conditions are lacking. Moreover, data on DNA yield and quality is not generally available in studies. In addition, the DNA extraction method has bearing on true microbial diversity of the sample and inefficient DNA extraction may lead to erroneous interpretations on microbial richness[14,16].

In this study, the yield and quality of DNA extracted from mule dung samples using a column based QIAamp® DNA stool kit was performed as per manufacturer's instructions. In addition, 3 modified procedures with the same kit were as evaluated and compared in terms of improvement in DNA yield and quality. This kit, which uses silica spin filter technology, was chosen for its easy and fast extraction method. The method did not include bead beating step which is harsh as it shears the DNA and may reduce the quality of metagenomic DNA[16]. As the kit-based methods rely solely on retention of DNA in a column-based matrix, therefore the loss of DNA is minimum[17]. The kit was selected owing to its use for rapid DNA extraction

Table 1. Yield of DNA extracted after thermal and proteinaseK treatment (P3)

Sample	Yield of DNA (ng/µL)							
	E1 (50 µl)	E2 (50 µl)	E3 (50 µl)	E4 (50 µl)	After combining four elution (200 µL)	Total DNA/ gm (µg)	After ethanol precipitation (50 µL)	Total DNA/ gm (µg)
Mu1	22	25.5	16.5	19	25	5.0	30.5	1.5
Mu34	25.5	25	23.5	20.5	26.5	5.3	37	1.8
Mu12	19	21	26	90	40.5	8.1	35.5	2.2
Mu21	23	29.5	23	27	22	4.4	33	1.6
Mu22	25.5	24	18.5	13	29.5	5.9	44	2.2
Mu24	27	23.5	18.5	17	37	7.4	47	2.3
Mu26	38.5	31	37	18	45.5	9.1	52.5	2.6
Mu38	45	51.5	36	35.5	35	7.0	48	2.4

Table 2. Yield of DNA extracted with procedure 4

Sample	Yield of DNA (ng/µL)				After combining	Total DNA/gm (µg)
	E1	E2	E3	E4		
Mu 1	29.5	23	22	17.5	28	5.6
Mu 34	43.5	48	27.5	23	35	7.0
Mu 12	49	28	18	6	39	7.8
Mu 21	45.5	43.5	54	27.5	49	9.8
Mu22	77.5	88.5	21	15	65	13
Mu 23	51	43	30	28.5	43	8.6
Mu 24	57.5	70.5	32	28.5	53	10.6
Mu 26	52	42	26	7.5	47	9.4
Mu 31	56	43	28.5	17.5	39	7.8
Mu 35	28.5	33.5	27.5	21.5	28	5.6
Mu 40	46.5	33	20	5	35	7.0

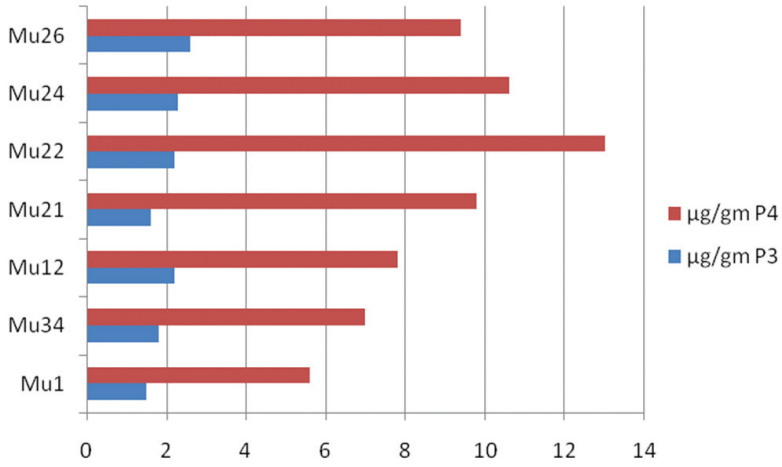

Figure 1. Appreciable increased in total DNA yield/gram of mule dung sample by procedure 4 (P4) over procedure 3 (P4).

from equine and other animal feces without requirement of special equipments[1,3,6,18].

There was a significant effect of modifications in the standard DNA extraction protocol on the amount and quality of DNA obtained from mule dung samples, as observed among four procedures. Procedure P1 and P2 provided a lower yield than P3 and P4 (Table 1, Table 2, Fig. 1).

The Mu1 and Mu34 fecal samples processed by P1 resulted in 2 and 1 ng DNA/µL respectively, which amounted to a yield of 0.4 and 0.2 µg total DNA from 1 gm of sample. Similarly, the yield improvement with P2 was approximately 1 µg/g of sample. The poor yield from P1 and P2 may be due to the incomplete lysis of bacterial cells, as the QIAamp® DNA stool kit is based on enzymatic lysis method, as compared to the bead-beating or mechanical disruption methods, which reportedly improve the DNA yield[19]. The microbiome analysis of animal fecal samples demands efficient and pure DNA extraction, however such samples pose challenges due to their composition and chemical nature, apart from their microbial complexity[20]. The lysis may be improved by increasing the incubation time; however a higher incubation time may compromise DNA quality owing to DNase activity[21].

Apart from lysis, DNA elution method from the columns has a bearing on the total DNA yield. The comparative higher yield from P2 than P1 may be due to inclusion of 2 or 4 successive elution steps in P2. Desneux and Pourcher (2014)[22] also reported an increase in DNA yield by a factor of 2 with the use of 4 successive elution steps with lagoon effluent. However, they reported that same level of improvement in yield was not obtained from high organic matter containing raw manure. In mule dung sample, presence of large amount of organic matter and undigested fibre may affect DNA yield.

In the P3, apart from collection of successive elution steps, ethanol precipitation was performed to obtain a higher yield by concentrating the DNA. However, ethanol precipitation step was time consuming and it led to a loss of DNA (Table 1). The purity and integrity of the DNA is important parameter for gene amplification by PCR and hence metagenomics analysis. The purity and integrity of DNA from P3 and P4 were better than that of preceding procedures; however, the P4, i.e., doubling the InhibitEX tablets resulted in the best quality of DNA. Increasing the quantity of InhibitEX was done to adsorb impurities, which can degrade DNA and may result in inhibition of PCR components[23].

Procedure 3 showed multi-fold increase in DNA yield of 22 and 25.4 ng/µL in elute 1 (E1) for Mu1 and Mu34 samples, respectively. The higher yield may be due to increase in the duration of incubation at 95°C, which leads to better lysis of cells. The considerable increase in yield of DNA led us to use this procedure on more samples (Table 1). Both P3 and P4 showed dense bands of DNA, however, the DNA samples obtained from P3 began to degrade after a period of 1 week of storage at -20°C. On electrophoresis, smearing was observed indicating towards shearing (Fig. 2). As compared to P3, the P4 DNA was of better quality, as observed after 1 week of storage at -20°C storage (Fig. 3).

The P4 DNA may have showed less shearing because of reduced contaminants. The buffer provided should also protect the released DNA from degradation by DNases, which are very active in fecal samples[21]. The first 3 procedures may not have been able to satisfactorily remove the contaminants such as humic acid, fulvic acid polysaccharides, protein and RNA[24, 25]. Multiple elution steps might also help in the removal of contaminants from DNA. RNase and proteinase K treatment in P4 also added to DNA purity.

4. CONCLUSIONS

Due to complex nature of carbohydrates, and presence of chemical inhibitors in mule dung, the doubling of proprietary InhibitX tablet and increase in incubation temperature lead to good quantity and quality of DNA extraction obtained from

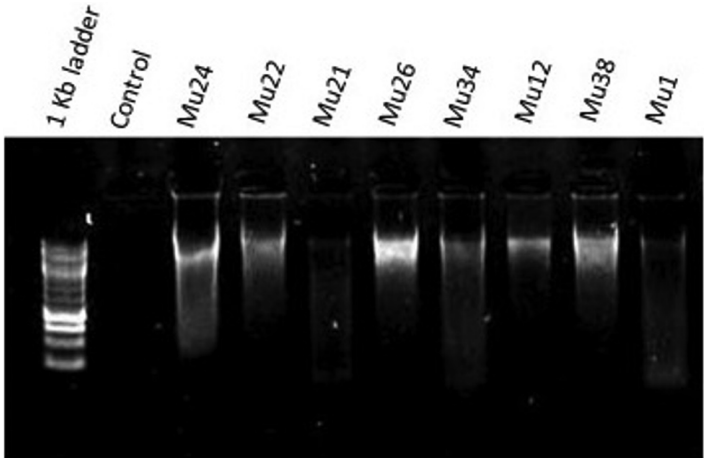

Figure 2. Smearing of DNA observed from DNA obtained by procedure 3.

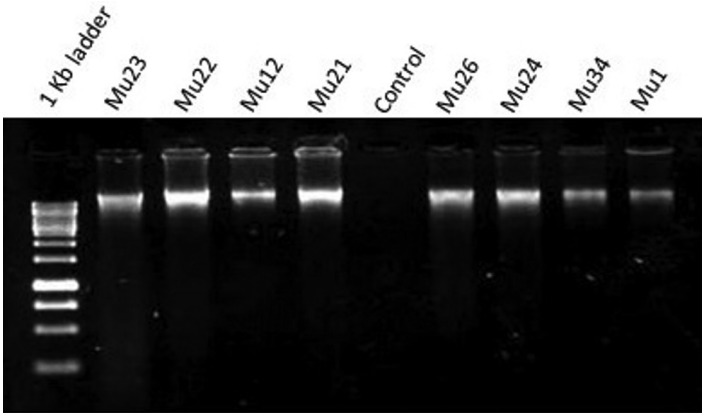

Figure 3. Better quality of DNA obtained by procedure 4.

the P4, therefore this procedure is recommended for DNA extraction from mule dung for microbial metagenomic analysis. This procedure can be adopted in the small laboratories without any specialised equipments and is easy to perform with high reproducibility.

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