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# A Comparison of Five Methods for Effective Extraction of Bacterial Metagenomic DNA from Stools of Children Suffer from Diarrhea with Unknown Cause

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**ABSTRACT: Diarrhea is common disease in children and in the most severity can cause mortality. Besides the identified-cause diarrhea, significant percent cases (accounted for 47.38% in Vietnam) having bacterial infection symptoms were unidentified reason. The typical properties of the diarrheal feces are the presence of many inhibitors against polymerase activity to amplify the indicator genes of pathogenic agents. Thus, investigation of methods for extracting bacterial metagenomic DNA with high quality and quantity from diarrheal feces for further analysis is necessary. In this research, two stool samples from children under two years old, one with persistent diarrhea of unknown cause and another of normal health, were used for bacterial metagenomic DNA extraction. Four commercial DNA extraction kits (GeneJET Genomic DNA purification kit, QIAamp Fast DNA Stool Mini Kit, E.Z.N.A Stool DNA kit, TopPURE stool DNA extraction kit), and conventional biochemical technique were used for bacterial metagenomic DNA extraction. The result showed that, from an equal amount of feces, bacterial mass in normal stool was higher than one in diarrheal feces. The bacterial metagenomic DNA from normal samples was successfully extracted with all the investigated methods at acceptable quality (the ratio $A_{260/280}$ was in a range of 1.7-2.2) for next generation sequencing. However the E.Z.N.A gave very low total DNA yield, TopPURE kit generated DNA containing inhibitor impact PCR reaction. Whereas the metagenomic DNAs from the stool of diarrhea patient were only well extracted by GeneJET Genomic DNA Purification kit and biochemical method. The biochemical method gave higher DNA yield but lower DNA quality exhibited by DNA degradation. On the contrary, GeneJET Genomic DNA Purification kit generated lower DNA yield but with higher quality. All the metagenomic DNA samples extracted by two these methods did not inhibit polymerase activity in PCR for 16S rDNA amplification. Thus, the GeneJET Genomic DNA Purification kit and biochemical method will be improved for extraction of bacterial metagenomic DNA from diarrheal feces for whole metagenome sequencing.**

**Keywords:** Bacteria, biochemical method, children, diarrhea, genomic DNA extraction, kit, stool.

## INTRODUCTION

Gut microbiota plays an important role in human physical and mental health (Hou *et al.,* 2022). This subject attracts great interest from researchers in diagnosis and treatment of different diseases. With the rapid development of next-generation sequencing (NGS) technologies in the past few decades, the non-culturable microbiota, a major component of the intestinal microfora, has been studied. NGS provides new means of studying the human microbiome, thereby revealing its effects on the development of human immune system (Jandhyala *et al.,* 2015; Matamoros *et al.,* 2013; Russell *et al.,* 2013) or chronic diseases (Zhang *et al.,* 2015; Underwood 2014). Various diseases related to biological disorders or microbial imbalances have been detected owing to analysis of microbial diversity from the digestive system by gene sequencing. One of the diseases related to bacterial imbalance is infectious diarrhea in children. Diarrhea is a common disease worldwide, which has a high mortal rate, especially in children under 5 years old, that leads to a global health burden (Charoenwat *et al.,* 2022; Walker *et al.,* 2012; Van Niel *et al.,* 2002). Diarrhea is mainly caused by bacterial, parasitic and viral infections, in which bacteria are the most common agents. These bacteria include *Clostridium botulinum, Campylobacter jejuni, Vibrio cholerae, Escherichia coli, Salmonella serovars, Shigella* spp., and *Staphylococcus aureus.* For children under 5 years of age, the main causative bacteria of diarrhea are *Escherichia coli O157, Campylobacter, Shigella,*

*Yersinia*, *Salmonella*, or parasite of *Cryptosporidium* (Tarr *et al.*, 2019; Kotloff, 2017) and *Rotavirus*. In addition to the above factors, bacterial overgrowth in the small intestine, malnutrition, vitamin and mineral deficiency (such as Zn, vitamin A), metabolic disorders, nutrient absorption (intolerance to lactose, carbohydrates, cow's milk protein, food allergies), or antibiotic usage are also causes of diarrhea (Kopacz and Phadtare 2022; Giannattasio *et al.*, 2016). These factors contribute to acute, severe and persistent diarrhea in children.

In addition to cases of known causative agents, about 40% of diarrhea children have no epidemiological factors or unknown cause (Finkbeiner *et al.*, 2008). In Vietnam, 47.38% children under five year-old suffer from unidentified reason diarrhea (Vũ Thị Thu Hà *et al.*, 2016). Although these cases showed signs of intestinal infection, the causative agent was still not found using common methods such as microbiological, biochemical, immunological, fecal analysis even real-time PCR technology. Therefore, it is urgent to find out the cause of diarrhea in children and help doctors to treat the disease in time.

The typical properties of the diarrheal feces are the presence of many inhibitors against polymerase activity to amplify the indicator genes of pathogenic agents (Acharya *et al.*, 2017; Rådström *et al.*, 2004) or also impact on the next generation sequencing. So in order to assess the bacterial imbalance and to seek causative agents, especially bacteria with very low density in the sample, previous researches have shown that there are three main factors that greatly affect the analytical results: sample collection, sample preservation, and DNA extraction method (Videnska *et al.*, 2019), in which the third factor has the strongest influence on the final result (Shaffer *et al.*, 2022; Costea *et al.*, 2017). Various commercial kits have been developed to facilitate and speed up the extraction process. However, the relative efficacy of these kits and the optimum amount of sample input for extraction need further evaluation (Ariefdjohan *et al.*, 2010). Each kit had its own DNA extraction procedure and buffers for removal of PCR inhibitors. Costea and his co-authors extracted DNA from stools using 21 commercial kits. Of which, Qiagen's QIAamp Stool Mini kit gave good analytical results (Costea *et al.*, 2017; McOrist *et al.*, 2002). Videnska also showed that the QIAamp DNA Stool Mini kit produced good quality and content of the extracted DNA (Videnska *et al.*, 2019). However, kits often result in much lower concentration and yield of DNA than conventional extraction techniques. For methods requiring high DNA amount and concentration such as NGS or microbiota diversity analysis, DNA yielded by kits is often not enough. To overcome the DNA shortage, a large number of kits should be used simultaneously, which drives up the cost. In contrast, the later method would fulfil DNA amount and concentration. However, this technique relates to some organic solvents that are not environmentally friendly.

In this study, we compare the relative efficacy of four commercial DNA extraction kits and a conventional method (described by Sambrook and his co-authors) in extracting bacterial metagenomic DNA from children's fecal samples. Each kit incorporates a method for cell lysis and removal of PCR inhibitors in the sample. The kits were chosen due to their availability, popularity, ease of use, and time saving compared to conventional technique. The resulting DNAs were then checked for quantity and quality by absorbance measuring at 260 nm; electrophoresis on agarose gel and checked inhibitors via PCR reaction of 16S rDNA gene. Depending on the purpose or requirements for metagenomic DNA, a suitable DNA extraction method should be then suggested.

## MATERIALS AND METHODS

### Materials

Fecal samples from healthy or diarrheal infants were provided by Department of Molecular Biology of Infectious Diseases of National Children's Hospital. A diarrhea stool sample was collected from a 15 months old girl who was diagnosed with intestinal infection with no identified pathogens of *Campylobacter*, *Clostridium* difficile toxin B, *Salmonella* spp, *Shigella* spp./EIEC, *Vibrio*, *Yersinia enterocolitica*, *Aeromonas*, *Clostridium*, *E. coli* O157, STEC, EPEC, EETEC, EAEC by real-time PCR using multiple primers. This case was designated as persistent diarrhea of unknown cause. The normal stool sample from a healthy infant of the same age was voluntarily provided by her mother. These fecal specimens were used to test method of genomic DNA extraction.

Four commercially available DNA extraction kits were included: GeneJET Genomic DNA purification kit (Thermo Scientific, USA), QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany), E.Z.N.A Stool DNA kit (Omega Bio-tek, USA), TopPURE stool DNA extraction kit (ABT, Vietnam). Primers of 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1527R (5′-AGAAAGGAGGTGATCCAGCC-3′) were used to amplify the bacterial 16S rDNA gene to check inhibitors in the extracted DNA. Other chemicals of analytical grade used for DNA extraction in the conventional technique include NaCl, KCl, SDS, absolute alcohol, chloroform, phenol etc. All were from Merck, Prolabo etc.

### METHODS

*A. Extraction of bacteria from fecal samples*

Sixteen grams of normal feces from a healthy child were mixed well in a total of 80 ml of PBS 1x (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4) to become homogeneous. In case of the diarrheal stool, 3 grams were dissolved completely in 15 ml of PBS 1x. Then the liquids were subjected to bacterial isolation. Briefly, the solutions were aliquoted in 2 ml Eppendorf tubes and subjected to centrifugation speeds from low to high, first of 700 rpm for 10 minutes (replicated twice), followed by 1.000 rpm. In each centrifugation step, the supernatant phase containing bacteria were collected, while the fecal residues were removed step by step. Then, bacteria cells were precipitated by centrifuging at 10.000 rpm for 5 minutes. The cells were washed with PBS 1x and re-

collected at the same centrifugation speed. The resulting bacteria pellet was reconstituted in a minimum volume of TE buffer. Finally, cell density was determined at 600 nm on a nanophotometer (P330, Implen). Then the cell density of $2x10^9$ CFU/ml was made and divided in each Eppendorf tube. All tubes were centrifuged at 10.000 rpm for 5 minutes to collect cells. The cells were stored at -20ºC for further DNA extraction.

*B. Extraction of bacterial metagenomic DNA using kits and a biochemical method*

Various bacterial masses ($1\times10^8$ or $2\times10^8$, $4\times10^8$, $6\times10^8$ CFU) were tested for suitable density of cells for metagenomic DNA extraction using GeneJET Genomic DNA purification kit, QIAamp Fast DNA Stool Mini Kit, E.Z.N.A Stool DNA kit, TopPURE stool DNA extraction kit, in accordance with the manufacturer's instructions. In the final step, DNA was eluted in 50 µl of the respective elution buffer.

Bacterial metagenomic DNA was also extracted using biochemical method described by Sambrook (Sambrook *et al.*, 2001). Four cell masses of $1\times10^8$ or $2\times10^8$, $4\times10^8$, $6\times10^8$ were used in this technique. In brief, the cell pellets were treated in turn with lysozyme, RNase A, proteinase K, and SDS, followed by several steps of heat incubation to lyse the cells. Metagenomic DNA was then extracted with solvents mixture of phenol:chloroform: isoamylcohol (25:24:1). DNA was then precipitated with 2.5 volume of absolute ethanol, followed by a washing step with 70% ethanol solution. Finally, DNA pellets were dried under a laminar flow and reconstituted in 100 µl of TE buffer.

*C. Evaluating quality of the extracted DNA*

Concentration of DNA extracted from the kits or biochemical method were measured on a Nanophotometer. DNA quality was evaluated using several parameters: (1) Ratios of $A_{260/280}$ and $A_{260/230}$. Pure DNA has an ideal range of $A_{260/280}$ ~ 1.8-2.2 and high $A_{260/230}$. This ratio reported by some kit manufacturers is ≥ 1.7; (2) Electrophoresis on 0.8% agarose gel stained with ethidium bromide. A single band of >10 kb should be visualized on a UV transilluminator; (3) Furthermore, functional quality of metagenomic DNA was also evaluated by PCR amplification of a single copy gene. In this research, the 16S rDNA gene was amplified using 16S rDNA primers. The final PCR reaction mixture (25 µl total volume) contained 2.5 µl 10x PCR buffer, 2 µl dNTP 2 mM, 1 µl 27F 10 µM, 1 µl 1527R 10 µM, 0.25 µl DNA polymerase (5 U/µl), $dH_2O$, and DNA template (either approximately 10 ng or 50 ng per reaction mixture). The amplification condition was 94ºC for 4 min, followed by 25 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 1 min. A final extension step was carried out at 72ºC for 4 min. The PCR products were confirmed by electrophoresis on 0.8% agarose gel.

Qualities of DNA from the normal or diarrheal stool samples extracted by kits and the biochemical method were compared to find out the best DNA extraction method.

## RESULTS AND DISCUSSION

*A. Extraction of bacteria from fecal samples*

The different characteristics of stool samples derived from diarrheal or healthy children would affect the final yield and quality of the extracted bacterial metagenomic DNA. To compare the efficiency of DNA extraction by the 4 kits and a biochemical method, we first separated bacteria from fecal residues in both samples by gradually increasing centrifugation forces. The bacterial cells were then reconstituted to a stock of $2\times10^9$ CFU/ml for later use. The results showed that from 16 g of the normal stool, 17 ml of $2\times10^9$ CFU were harvested. Meanwhile a total of 2.4 ml of the same bacterial density were obtained from 3 g of diarrheal feces. The bacterial mass harvested from normal feces was 1.3 times higher than the bacteria from diarrheal feces. In this study, although the number of stool samples were limited (one stool sample from a child with diarrhea of unknown cause and one stool sample from a healthy child), but the preliminary result of bacteria extraction showed that bacterial mass in normal stool (healthy child) was higher than that in diarrheal stool. In agreement with this result, metagenomic analysis of bacterial community in 42 Chinese infants with diarrhea and 37 healthy infants based on V3-V4 region of 16S rDNA also indicated that bacterial diversity and abundance in the feces of healthy infants are much higher than ones from diarrheal groups (Fan *et al.,* 2020). The reduced bacterial cells mass in stools was also demonstrated in diarrheal patients infected with *Clostridium difficile* (Chang *et al.,* 2008) and rotavirus (Sohail *et al.,* 2021) that related to antibiotic resistance and injuries in the digestive system. These stock cells of $2\times10^9$ CFU/ml were used to extract bacterial metagenomic DNA.

**Extraction of bacterial metagenomic DNA from normal stool sample.** *Extraction by Gene JET Genomic DNA Purification kit.* There are several commercially available kits to extract bacterial DNA from stool samples. These kits share a common principle for lysis of bacterial cells using either chemicals or other agents such as enzymes, physical forces, temperature etc. The cell lysate containing DNA was next loaded on the column to remove other impurities, salts, inhibitors. Then, pure DNA was eluted. In our experiment, due to a very small sample size of diarrheal bacteria, we first tested the DNA extraction on a normal sample using the GeneJET Genomic DNA Purification kit. Four different cell densities of $1\times10^8$ - $2\times10^8$ - $4\times10^8$ - $6\times10^8$ CFU were subjected for each extraction. The results (Table 1) showed that DNA concentration or yield increased in accordance with the input of cell densities. In addition, the ratio of $A_{260/280}$ and $A_{260/230}$ in the two inputs of $4\times10^8$ CFU and $6\times10^8$ CFU were both ≥1.7, indicating that the extracted DNAs were pure. For further evidence, the DNAs were checked by electrophoresis on 0.8% agarose gel. The results (Fig. 1A) showed that all the DNA bands were sharp and less smeared, with

an estimated size of >10 kb. The band intensity increased consistently to the cell inputs. According to the protocol, suitable *Bacillus* or *E. coli* cells mass for each genomic DNA extraction by this kit is $2\times10^9$ CFU and the genomic DNA yield harvested 10-15 µg with concentration of about 20 ng/µl.

**Table 1: Metagenomic DNA concentration extracted from bacteria of a normal stool using GeneJET genomic DNA purification kit.**

| **Cell input (cells)** | **$1\times10^8$** | | **$2\times10^8$** | | **$4\times10^8$** | | **$6\times10^8$** | |
|---|---|---|---|---|---|---|---|---|
| DNA collection (first and second times) | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Volume (µl) | 50.00 | 50.00 | 50,00 | 50.00 | 50.00 | 50.00 | 50.00 | 50.00 |
| DNA concentration (ng/µl) | 7.50 | - | 11.00 | 5.00 | 15.50 | 12.50 | 20.00 | 12.00 |
| $A_{260/280}$ | 1.50 | - | 1.57 | 1.43 | 1.72 | 1.79 | 1.79 | 1.71 |
| $A_{260/230}$ | 0.83 | - | 0.85 | - | 3.88 | 2.78 | 2.67 | 4.80 |
| DNA yield (µg) | 0.37 | - | 0.55 | 0.25 | 0.77 | 0.62 | 1.00 | 0.60 |
| **Total DNA yield (µg)** | **0.37** | | **0.80** | | **1.39** | | **1.60** | |

Note: (-) means that the DNA concentration was too low

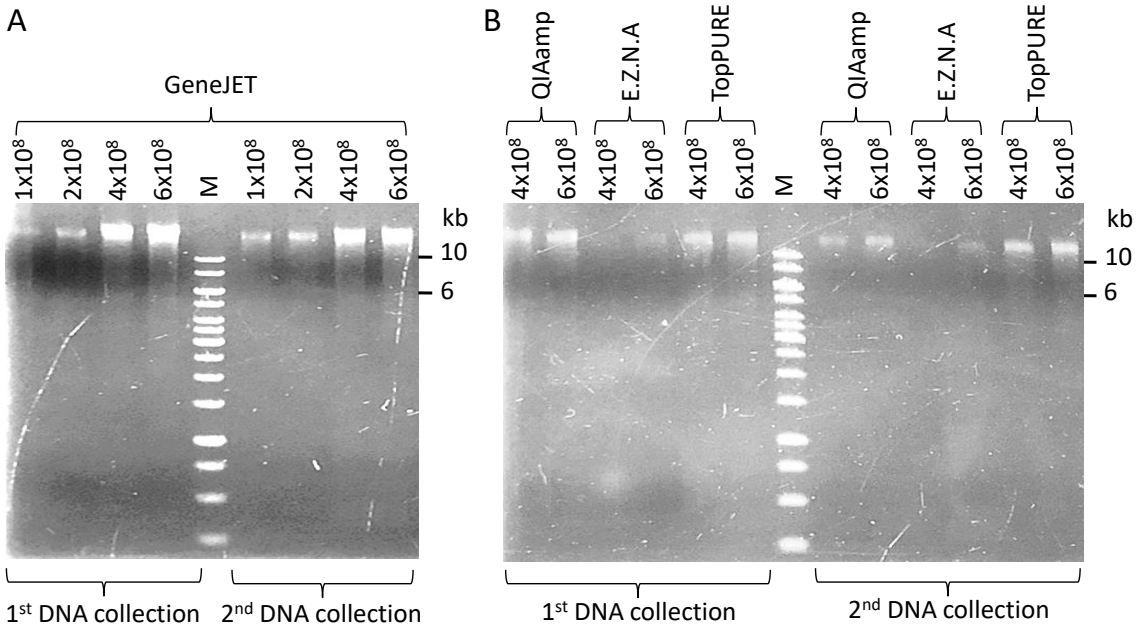
This referent concentration is too low for next generation sequencing. Thus, we reduced volume of elution buffer to 50 µl with the aim to increase the DNA concentration (Table 1). However, only in the experiment conducted with $6\times10^8$ CFU, metagenomic DNA concentration reached 20 ng/µl at the first collection while metagenomic DNA was remained in the column then eluted by the second collection. Therefore, $4\times10^8$ and $6\times10^8$ cells were chosen to extract genomic DNA by the 3 remaining kits.

*Extraction by QIAamp Fast DNA Stool Mini Kit, E.Z.N.A Stool DNA kit, TopPURE stool DNA extraction kit*

The results (Fig. 1B) showed that all the extracted DNA bands were the same size of >10 kb. Besides, the intensity of DNA band varied upon kits and was consistent with their concentration (Table 2). In brief, among the 4 kits, DNA yield from of GeneJET Genomic DNA Purification kit was 2.6 to 5.3 times higher than that of TopPURE stool DNA extraction kit or QIAamp Fast DNA Stool Mini Kit, respectively. Besides, a higher ratio of $A_{260/280}$ might indicate that the DNA from this kit was purer.

*Extraction by biochemical traditional method*

After testing different cell densities to extract genomic DNA by the four kits, we did the same thing with the traditional, biochemical method described by Sambrook *et al.* (2001). The results in Table 3 showed that total DNA yield increased accordingly to the cell densities, from 0.55 µg to 4.8 µg. Remarkably compared to the kits, the DNA amount in this conventional method was much higher. In addition, the ratios of $A_{260/280}$ and $A_{260/230}$ were within the allowable range of 1.8 - 2.2, which might reflect pure DNA.




Four different cell masses were used to extract metagenomic DNA using GeneJET Genomic DNA Purification kit (A), Two cell masses were chosen to extract metagenomic DNA using QIAamp Fast DNA Stool Mini Kit, E.Z.N.A Stool DNA kit, TopPURE stool DNA extraction kit, respectively (B); $1\times10^8$ - $2\times10^8$ - $4\times10^8$ – $6\times10^8$: cell masses; M: 1 kb DNA marker (Fermentas).

**Fig. 1.** Analysis of bacterial metagenomic DNA extracted by four kits on 0.8% agarose gel.

**Table 2: Concentration and quality of bacterial metagenomic DNA extracted from normal stool using three different kits.**

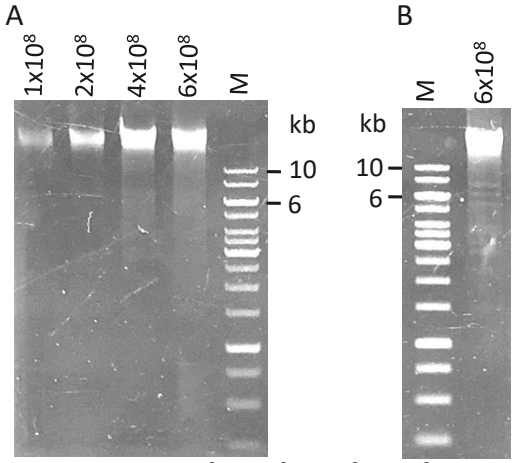
| Kit used | QIAamp Fast DNA Stool Mini Kit | | | | E.Z.N.A Stool DNA kit | | | | TopPURE stool DNA extraction kit | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cell input (cells) | $4\times10^8$ | | $6\times10^8$ | | $4\times10^8$ | | $6\times10^8$ | | $4\times10^8$ | | $6\times10^8$ | |
| DNA collection (2 times) | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Volumn (µl) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| DNA concentration (ng/µl) | - | - | 6 | - | - | - | - | - | 5.5 | 4.5 | 7.5 | 5.0 |
| $A_{260}/A_{280}$ | 1.75 | 1.5 | 2.0 | 1.5 | 1.0 | 2.0 | 1.33 | 1.33 | 1.67 | 1.5 | 1.67 | 1.67 |
| $A_{260}/A_{230}$ | - | - | 1.5 | - | 0.2 | 0.5 | 0.67 | 0.8 | 0.2 | - | - | - |
| DNA yield (µg) | - | - | 0.3 | - | - | - | - | - | 0.27 | 0.22 | 0.37 | 0.25 |
| **Total yield (µg)** | **-** | | **0.3** | | **-** | | **-** | | **0.49** | | **0.62** | |

Note: (-) means that the DNA concentration was too low

**Table 3: Concentration and quality of bacterial metagenomic DNA extracted from normal stool using biochemical method.**

| Cell input | $1\times10^8$ | $2\times10^8$ | $4\times10^8$ | $6\times10^8$ | $6\times10^8$ × 3 tubes |
|---|---|---|---|---|---|
| Volume (µl) | 100 | 100 | 100 | 100 | 240 |
| DNA concentration (ng/µl) | 5.5 | 15.5 | **40.5** | **48.0** | **63.0** |
| $A_{260}/A_{280}$ | 2.20 | 1.94 | 1.81 | 1.85 | 1.83 |
| $A_{260}/A_{230}$ | 1.71 | 2.13 | 2.30 | 2.23 | 2.21 |
| **DNA yield (µg)** | **0.55** | **1.55** | **4.05** | **4.80** | **15.12** |

The extracted DNA was checked by electrophoresis on a 0.8% agarose gel (Fig. 2A). All the DNA bands are more than 10 kb in size, visualized as sharp, condensed and little smeared bands. The density of the DNA bands increased in accordance with cell inputs. In another way, the electrophotogram was consistent with the DNA concentration. Thus, compared to the four kits, the biochemical method gave the highest DNA yield and concentration.




(A): DNAs were extracted from four cell densities of $1\times10^8$, $2\times10^8$, $4\times10^8$, $6\times10^8$, respectively; (B) DNA were extracted from three combined tubes of $6\times10^8$cells. M: 1 kb DNA marker (Fermentas).

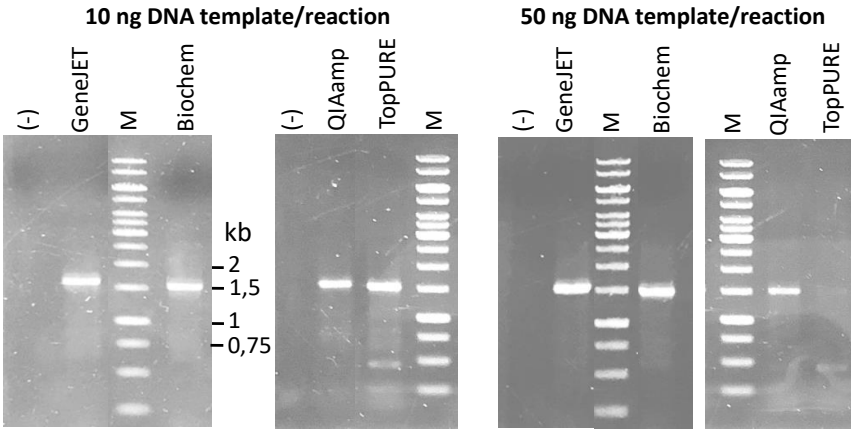
**Fig. 2.** Analysis of bacterial metagenomic DNA extracted from normal feces by biochemical method on 0.8% agarose gel.

To confirm the efficacy of this technique, 3 tubes of $6\times10^8$ bacterial cells were used to extract DNA. The pooled DNA was measured for its concentration (Table 3, right) and checked by electrophoresis (Fig. 2B). The results indicated that 15.12 µg DNA corresponding to 63 ng/µl was harvested. This concentration was higher than the minimum concentration required for whole metagenomic DNA sequencing by Illumina sequencer platform 2500 (≥50 ng/µl). In addition, the ratios of $A_{260/280}$ and $A_{260/230}$ were within the allowable limit. Electrophoresis (Fig. 2B) also showed a strong and clear DNA band as previously seen. Thus, a cell input of $6x10^8$ was suitable and this technique was reproducible and stable for effective extraction of bacterial metagenomic DNA with higher yield and concentration from normal stool.

*Analysis of metagenomic DNA quality extracted from normal stool sample by PCR reaction*

In addition to analysis of DNA quality via electrophoresis and ratios of $A_{260/280}$, $A_{260/230}$, DNA quality related to inhibitors was also evaluated by PCR reaction. In this experiment, all metagenomic DNAs extracted from the 4 kits or biochemical method were used as templates for amplifying the 16S rDNA gene. If DNA templates contained large amounts of inhibitors, salts, or other impurities, PCR would be inhibited. In contrast, pure DNA templates would show a single

band of the bacterial 16S rDNA gene with ~1.5 kb in size.




(-): Negative control; GeneJET, Biochem, QIAamp, TopPURE: Amplicon from DNA template extracted by GeneJET Genomic DNA Purification kit, biochemical method, QIAamp Fast DNA Stool Mini kit, TopPURE stool DNA extraction kit respectively; M: 1 kb DNA marker (Fermentas).

**Fig. 3.** Analysis of PCR amplicons using all the extracted DNA templates from normal stool sample to amplify the bacterial 16S rDNA on 0.8% agarose gel.

The obtained results (Fig. 3) showed that in case of 10 ng DNA template, all lanes showed a clear and specific DNA band of ~1.5 kb in size. The PCR product from TopPURE kit had unspecific bands. The same results were also observed in the case of using 50 ng DNA templates with the exception from the TopPURE kit which showed a very faint band. The additional or faint bands from the TopPURE kit might come from the effect of unidentified inhibitor in the template towards PCR sensitiveness. This cause could be supported by the DNA quality reflected by lower ratios of $A_{260/280}$ (1.67) and $A_{260/230}$ (Table 2) and also by increase of inhibitor when raising DNA to 50 ng/reaction. DNA templates (10 ng and 50 ng) extracted by GeneJET, QIAamp or biochemical method did not inhibit PCR reactions.

**Bacterial metagenomic DNA extraction from diarrheal stool sample.** *Extraction of bacterial metagenomic DNA.* As mentioned above, bacterial cell density of $6\times10^8$ was suitable for bacterial metagenomic DNA extraction by the four kits and the traditional biochemical method. In this experiment, this cell density was applied to extract bacterial genomic DNA in diarrheal stool samples. Then, DNA concentration was measured on a nanophotometer. DNAs were also checked by electrophoresis and used as templates for PCR reaction to check its quality.

**Table 4: Concentration and quality of metagenomic DNA extracted from bacteria derived from diarrhea stool sample using kits and biochemical method.**

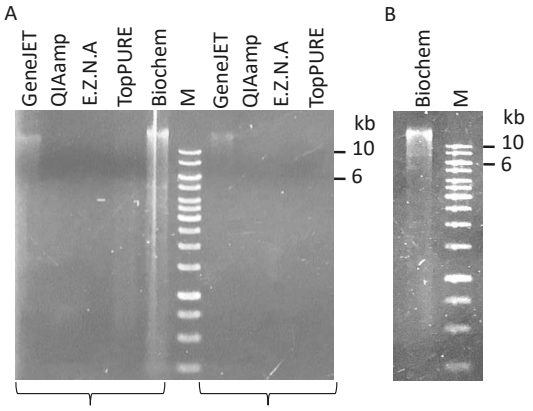
| Kit name | GeneJET Genomic DNA Purification kit | | QIAamp Fast DNA Stool Mini kit | | E.Z.N.A Stool DNA kit | | TopPURE stool DNA extraction kit | | Biochemical method | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cell input | $6\times10^8$ | | $6\times10^8$ | | $6\times10^8$ | | $6\times10^8$ | | $6\times10^8$ | 2 tubes × $6\times10^8$+ 1 tube × $1,8\times10^8$ |
| DNA collection | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 1 |
| Volumn (µl) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 80 | 100 |
| DNA concentration (ng/µl) | 6.5 | 5 | - | - | - | - | 12 | 4 | 32 | 31 |
| A260/280 | 2.17 | 2.0 | 2.0 | 2.0 | 1.0 | 2.0 | 1.86 | 1.14 | 1.6 | 1.72 |
| A260/230 | - | 2.50 | 0.50 | 3.00 | 0.43 | 0.57 | - | 0.30 | - | - |
| DNA yield (µg) | 0.32 | 0.25 | - | - | - | - | 0.60 | 0.30 | 2.56 | 3.10 |
| Total yield (µg) | 0.57 | | - | | - | | 0.90 | | 2.56 | 3.10 |

Note: (-) means that the DNA concentration was too low

Among the 4 kits and biochemical method, bacterial metagenomic DNA were successfully extracted by GeneJET Genomic DNA Purification kit, TopPURE stool DNA extraction kit and the traditional chemical method. DNAs extracted by the other kits including QIAamp Fast DNA Stool Mini kit and E.Z.N.A Stool DNA kit were not detected by either nanophotometer or electrophoresis (Table 4, Fig. 4). The highest DNA concentration and yield were obtained from bacteria cells by the biochemical method (Table 4, Fig. 4). However, this DNA source showed lower ratios of $A_{260/280}$ (1.6) and $A_{260/230}$ if compared to the ratio of DNAs extracted from normal stool by the same method. On the other hand, metagenomic DNA extracted by biochemical method was not condensed in agarose gel (Figure 4) but greatly smeared. This means DNA degradation occurred in the stool sample or during extraction process. The metagenomic DNA extracted by GeneJET Genomic DNA Purification kit has better quality, but lower quantity. The degraded metagenomic

DNA from diarrheal stools were common observe in the previous study (Videnska *et al.,* 2019). Several factors might affect the DNA extraction in this study, but the first reason may be fecal features related to DNA degradation as seen in the metagenomic DNA extracted from diarrheal feces (Videnska *et al.,* 2019). The DNA degradation may be resulted of a temperature shock (freeze and thaw) (Wu *et al.,* 2019) or by bacterial lysis from antibiotic treatment. Other reasons may relate to different agents used for cell lysis among the kits or the biochemical method, the poor binding of DNA on to the kit columns, the unequal amount of cell lysate loaded to the kit, etc. Using kits, common lysis agents such as lysozyme, proteinase K, RNase A were used, while no denaturing chemicals at high concentrations (SDS, NaCl) were added. In contrast, these chemicals and other strong solvents (phenol, chloroform, isoamylalcohol) were used in the biochemical method to lyse efficiently bacterial cells and extract DNA, while facilitating the removal of impurities. The low quality of the extracted metagenomic DNA may be caused by stool biotic and abiotic features that also cause DNA degradation (Zhang *et al.,* 2019).

Due to a higher DNA concentration and yield from biochemical method, this technique was used to extract DNA from two additional tubes of bacterial cells. The DNA was then pooled together for DNA measurement. A consistent result in DNA concentration, yield, and ratios of $A_{260/280}$ and $A_{260/230}$ was obtained as the initial extraction (Table 4, the last column). In addition, DNA band was also greatly smeared (Fig. 4B).

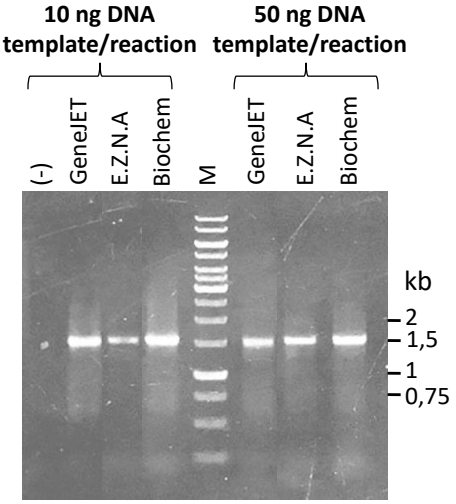



(A): DNAs were extracted from one tube of $6x10^8$ cells by kits and biochemical method; (B) DNAs was extracted from two tubes of $6\times10^8$cells and 1 tube of $1.8\times10^8$ cells; M: 1 kb DNA marker (Fermentas).

**Fig. 4.** Analysis of bacterial metagenomic DNAs extracted by using four kits and a biochemical method on 0.8% agarose gel.

*Analysis of genomic DNA quality extracted from diarrheal stool sample by PCR reaction*

Similar to the normal sample, DNA quality in the diarrheal one was also checked for the present of inhibitors through PCR reaction of the bacterial 16S rDNA gene. The results (Fig. 5) indicated that in all lanes, a DNA band of ~1.5 kb corresponding to this gene was clearly visualized. Thus, inhibitors in DNA templates were in the acceptable range and not to inhibit the PCR reaction.




(-): Negative control has the same reaction composition as the experimental sample except DNA template; GeneJET, E.Z.N.A, Biochem: Amplicon from DNA template extracted by GeneJET Genomic DNA Purification kit, E.Z.N.A Stool DNA kit, biochemical method respectively;M: 1 kb DNA marker (Fermentas).

**Fig. 5.** Analysis of PCR amplicons amplified the bacterial 16S rDNA using the extracted DNA from diarrheal sample as templates on 0.8% agarose gel

In summary, for effective extraction of bacterial metagenomic DNA from feces of one healthy child and one diarrheal child, we investigated 5 methods including 4 commercial kits (GeneJET Genomic DNA Purification kit, QIAamp Fast DNA Stool Mini kit, E.Z.N.A Stool DNA kit and TopPURE stool DNA extraction kit) and a biochemical technique described by Sambrook *et al.* (2001). The DNA from normal samples was successfully extracted with all the investigated methods, but E.Z.N.A gave low total DNA yield, TopPURE generated DNA containing inhibitor impact PCR reaction. The highest DNA yield was gained by biochemical method. In contrast, from the stool of diarrheal patient, the metagenomic DNAs were only visible and detectable by both nanophotometer and electrophoresis when the DNAs were extracted by GeneJET Genomic DNA Purification kit, and the biochemical method. The biochemical method gave higher DNA yield but lower DNA quality that was assessed by nanodrop measurement while GeneJET Genomic DNA Purification kit generated lower DNA yield but higher quality. Metagenomic DNA extracted from diarrheal feace was degraded. Very high concentration and metagenomic DNA mass (50 ng) did not inhibit PCR reaction for 16S rDNA amplification. From this study, the GeneJET Genomic DNA Purification kit and the biochemical method can be improved for extraction of bacterial metagenomic DNA from feces of diarrheal patients for next generation sequencing by Illumina Hiseq platform.

## CONCLUSIONS

Metagenomic DNA derived from a normal and diarrheal feces were extracted by the four commercial

kits and a biochemical method. The biochemical method and GeneJET Genomic DNA Purification kit gave the best DNA concentration, yield and quality. The DNA templates up to 50 ng/reaction did not inhibit PCR for 16S rDNA amplification. Biochemical method and GeneJET Genomic DNA Purification kit should be chosen for improvement to extract metagenomic DNA from diarrhea stool samples for further analysis.

## FUTURE SCOPE

The identification of pathogenic bacteria by modern molecular biotechnology in the stools of conventional unidentified-causal diarrhea children is valuable for effective clinical treatment and depends on the extracted DNA. Based on the scientific results from this study, biochemical method and GeneJET Genomic DNA Purification kit will be used for extraction of metagenomic DNA of bacteria in stools of diarrheal children for metagenomic analysis to see the disorder of bacterial community, find pathogenic bacteria, toxic genes of bacteria and also antibiotic resistant genes for control unknown-cause diarrhea in Vietnamese children.

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**Conflict of Interest.** None.

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