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Original article

Effect of different DNA polymerases on 16S rDNA amplification for gut microbiota classification

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Abstract

Background: Gut microbiota plays vital role in enhancing the intestinal immune system and influencing nutrient metabolism. To study these microbial communities, 16S rDNA sequencing is commonly employed. However, the variability in reagents used for 16S rDNA amplification, especially in terms of DNA polymerases, can affect the quality and accuracy of the data.

Objective: This study aims to examine the effect of two commercially high-fidelity DNA polymerases on the gut microbiota profile based on full-length 16S rDNA amplification.

Methods: Fecal samples from lung cancer patients at King Chulalongkorn Memorial Hospital were collected for DNA extraction with the ZymoBIOMICS™ DNA Miniprep Kit. The extracted DNA was amplified using the Ultra HiFidelity PCR Kit and KOD One™ PCR Master Mix, then sequenced with Oxford Nanopore Technologies. The filtered sequences were clustered, polished, and classified using NanoCLUST, and visualized by MicrbiomeAnalyst.

Results: Gel electrophoresis showed that DNA polymerases from different PCR kits yielded identical PCR products. The KOD One™ PCR Master Mix (Toyobo) produced a more intense band, indicating possible overamplification, which may have reduced microbial richness and certain bacterial taxa abundance. No significant differences were noted in Shannon diversity, beta diversity, or the top 20 bacterial species' relative abundance between the PCR kits.

Conclusion: These results suggest that the choice of high-fidelity DNA polymerases can influence gut microbiota diversity and classification. This highlights the importance of selecting appropriate PCR kits and optimizing amplification conditions in future microbiome research.

Keywords: DNA polymerases, full-length 16S rDNA sequencing, gut microbiota, Oxford Nanopore Technologies.

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Gut microbiota potentially contributes to human health and disease processes.[1] Normally, gut microbiota promotes the maturation of the intestinal immune system as well as digestion by providing the extraction of calories and nutrients that would otherwise be excreted in feces.[2] The advancement of high-throughput DNA sequencing has enabled researchers to investigate the alteration of gut microbiota composition and functional processes associated with various diseases mechanisms, for example, cancer [3, 4], autoimmune diseases [5, 6], and others. Moreover, there are two approaches of DNA sequencing for microbiota identification including amplicon sequencing and shotgun metagenomics. [7]

The 16S rDNA amplicon sequencing has been applied in many research studies and routine diagnostic laboratories for bacterial identification since it detects the unculturable strain of bacteria and generates high data accuracy and quality. [8] The workflow of 16S rDNA amplicon sequencing has included DNA extraction, amplification of 16S rDNA gene, library preparation and sequencing, and data analysis. However, the different uses of reagents and bioinformatic tools in those processes have been addressed in many studies which mostly resulted in the discrimination of data quality and resolution. Increasingly, the different uses of reagents in DNA extraction processes have been shown to vastly impact the DNA yield, DNA quality, and microbiome results.[8] A previous study showed that the use of the bead-beating method coupled with the commercial kit in the DNA extraction process resulted in the better DNA quality and improved microbial diversity, although the taxonomic profile showed less abundant in some bacterial strains.[9-11] Furthermore, the primer design also relatively affected the microbiota results since the 16S rDNA gene has 9 hypervariable regions and some of them were more compatible for bacterial amplification.[12]) Previous evidences used specific hypervariable regions as targeted regions (V3-V4 or V4-V5) for amplification and discovered that those regions provided an accurate taxonomic profile, although the data shown only further to genus-level resolution which would not clearly explain the relationship between the gut microbiota and human diseases. [7, 13, 14] Regardless, different types of DNA polymerases might also be affected by the amplification since it essentially used to amplify DNA from the DNA templates.[15] The distinct properties of polymerases including the fidelity, thermostability, processivity, and specificity were varied, however the fidelity of the polymerases were crucial since it refers to the proofreading activity of the enzyme which can incorporates the accurate nucleotide during extension processes.[16] Yet there is no evidence of a comparison between DNA polymerases from different PCR commercial kits are reported.

To examine the effect of two DNA polymerases on 16S rDNA amplification for the gut microbiota classification, this study used the same extraction kit and primer sequences for the amplification. However, these DNA polymerases are high-fidelity with different processivity properties. Furthermore, Oxford Nanopore Technologies were used as DNA sequencing techniques since it improved the accuracy, read length and high-throughput.[17]

Materials and methods

Sample collection

The fecal samples were collected from the previous study. [18] In this study, fecal samples were selected from lung cancer patients who were treated at King Chulalongkorn Memorial Hospital. Patients who had taken antibiotic therapy four weeks prior to sample collection or missed follow-up appointments were excluded. Fecal samples were self-collected and stored in tubes containing DNA/RNA™ Shield-reagents (Zymo Research, California, USA). The collected sample was extracted using ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, California, USA) and stored at -80°C until further use.

Full-length 16S rDNA gene sequencing based on Oxford Nanopore Sequencing (ONT)

Full-length 16S rDNA gene amplification of fecal DNA was performed using V1-V9 hypervariable region primers (forward primer: 27F 5' – T T T C T G T T G G T G C T G A T A T T G C AGRGTTYGATYMTGGCTCAG – 3' and reverse primer: 1492R 5' – ACTTGCCTGTCGCTCTATC TTCCGGYTACCTTGTTACGACTT– 3'). [19] The two commercial kits were applied for the polymerase chain reaction (PCR) amplification, Ultra HiFidelity PCR Kit (TIANGEN BIOTECH, Beijing, China) and KOD One™ PCR Master Mix (Toyobo, Japan). The PCR products were subsequently purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions. The concentration of purified DNA was

measured with the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). A DNA library pool was prepared using the Ligation Sequencing Kit V14 (SQK-LSK114, Oxford Nanopore Technologies, Oxford, UK). The R10.4.1 flow cell (FLO-MIN114) was utilized and subsequently loaded into the MinION Mk1C for sequencing.

Data and statistical analysis

The raw FAST5 files were further pre-processed by Guppy base-caller (v6.5.7) for base calling to convert the raw electrical signal into a nucleotide sequence with quality scores (>15) in the FASTQ format. This FASTQ format was subsequently demultiplexed and adapter trimmed with Poreshop (v0.2.4). The filtered sequences were clustered, polished, and assigned taxonomy using the Ribosomal Database Project (RDP) database with the NanoCLUST. [20] MicrobiomeAnalyst (v2.0) was utilized for data visualization and statistical analysis. Chao1 and Shannon's diversity indexes were applied to observe changes in alpha diversity while the Bray-Curtis dissimilarity index was executed for the beta diversity, with principal coordinate analysis (PCoA). Relative abundance. The Mann-Whitney *U* test and permutational multivariate analysis of variance test (PERMANOVA) were employed to statistically analyze alpha and beta diversity, respectively. A *P*-value of less than 0.05 in all analyses indicated a significant difference.

Results

Effect of different DNA polymerases on the 16S rDNA amplification

To examine the effect of different DNA polymerases for 16S rDNA amplification, three representative samples were comparatively validated by 1% agarose gel electrophoresis. The bands of PCR product obtained from different DNA polymerases were similar in fragment length (1,500 bp.). However, the PCR products obtained from KOD One™ PCR Master Mix (Toyobo) yielded significant higher band intensity than those from the Ultra HiFidelity PCR Kit (**Figure 1**), indicating that this kit yielded more amplification efficiency with the same amount of DNA template and amplification cycles.

Effect of different DNA polymerases on the microbial diversity

To compare the gut microbiota diversity from 16S rDNA amplification using two different PCR commercial kits, the alpha and beta diversity analyses were performed. In **Figure 2A**, Chao1 diversity index of amplicons obtained from KOD One™ PCR Master Mix (Toyobo) was significantly lower ($P = 0.00003$) than those found in Ultra HiFidelity PCR Kit (Tiangen). There was no significant differences in Shannon's diversity index between PCR commercial kits ($P = 0.39$) (**Figure 2B**). Moreover, beta diversity also demonstrated no significant differences between two different PCR commercial kits ($P = 0.114$) (**Figure 2C**).

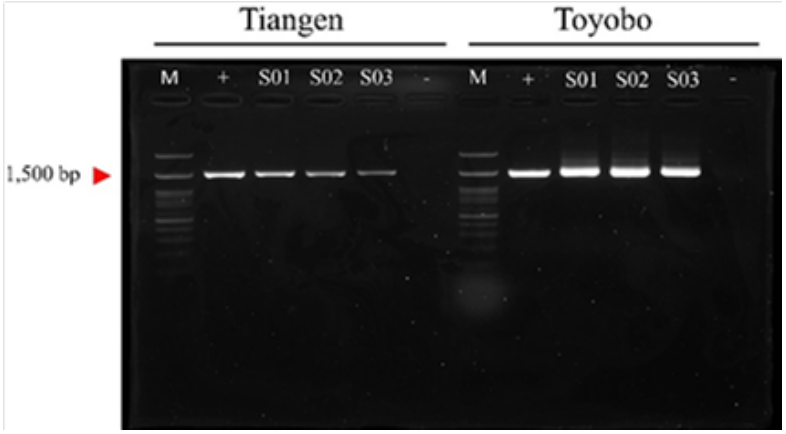

Figure 1. Representative of agarose gel electrophoresis showing bands intensity of PCR products (approximately 1.5 kb.) obtained from different DNA polymerases by using the same amount of DNA templates and amplification cycles to compare the amplification efficiency between Ultra HiFidelity PCR Kit (Tiangen) and KOD One™ PCR Master Mix (Toyobo).

Effect of different DNA polymerases on the microbial composition

Furthermore, the relative abundance of the microbial composition were also visualized at species levels (**Figure 3**). The result showed that there were similar trends of top 20 bacterial species obtained from two different PCR commercial kits. However, *Faecalibacterium prausnitzii* seem to be lower relative abundance in the 16S rDNA amplicons obtained from KOD One™ PCR Master Mix (Toyobo). These results may indicate that the use of different PCR commercial kit is likely to have some effect on microbiome analysis.

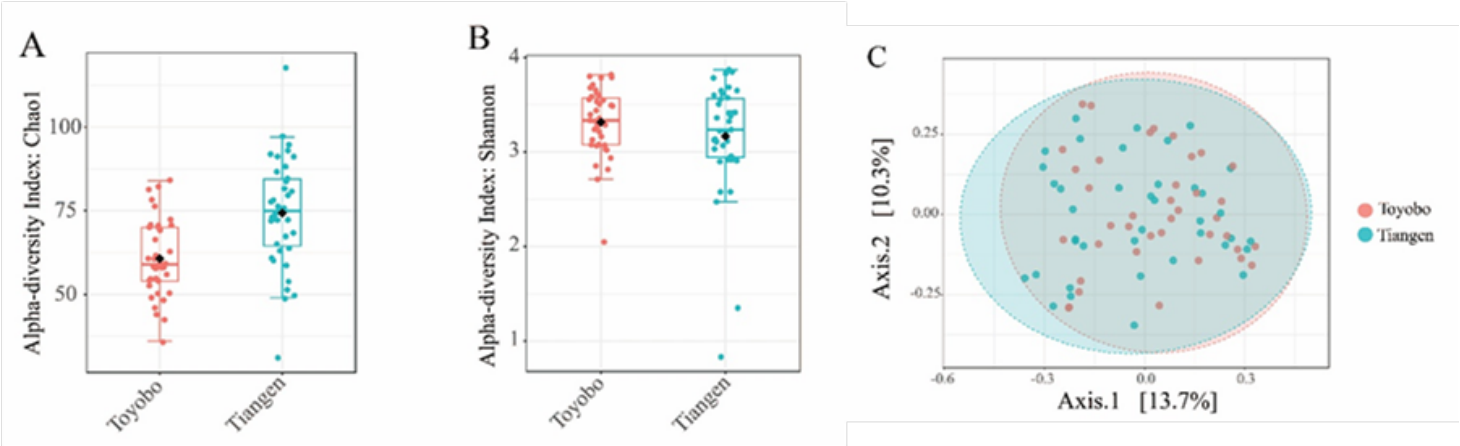

Figure 2. (**A**) Chao1 diversity index (**B**) Shannon's diversity index (**C**) Beta diversity of microbiota obtained from different PCR commercial kits: Ultra HiFidelity PCR Kit (Tiangen) and KOD One™ PCR Master Mix (Toyobo).

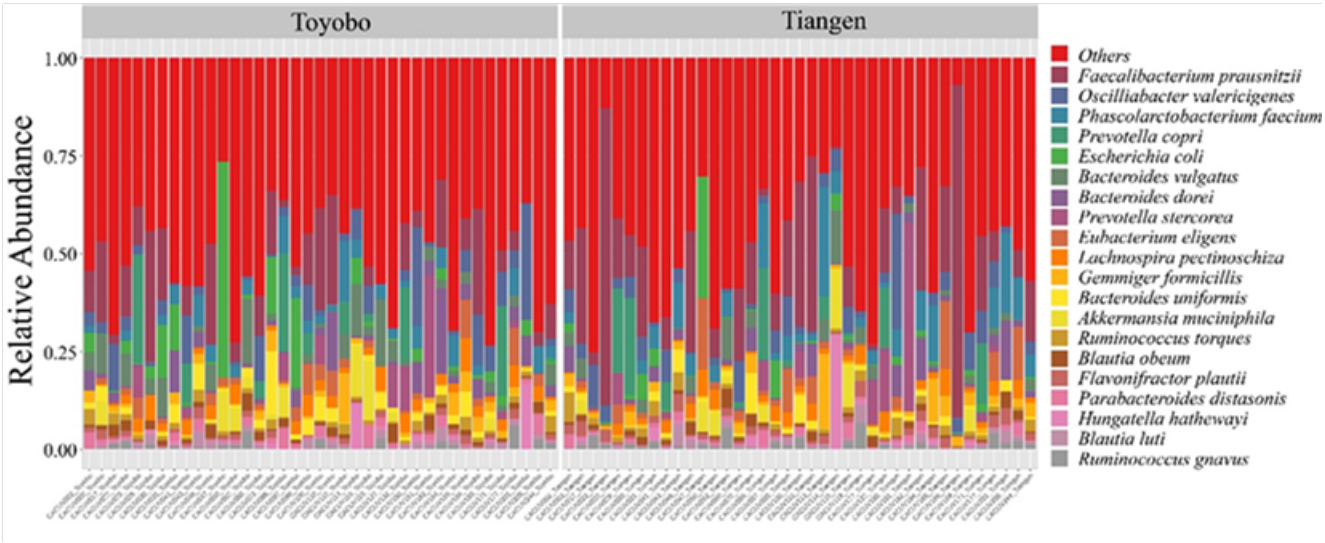

Figure 3. The relative abundance of gut microbiota composition at species levels obtained from different PCR commercial kits: Ultra HiFidelity PCR Kit (Tiangen) and KOD One™ PCR Master Mix (Toyobo).

Discussion

This investigation concentrated on examining the influence of DNA polymerase sourced from commercial PCR kits on microbiota profiles. A comparative analysis involving two commercial PCR kits, characterized by high-fidelity DNA polymerase with different processivity properties, was conducted for the amplification of full-length 16S rDNA to address this matter.

Nevertheless, the microbiota profiles from two different commercial PCR kits exhibited a high degree of similarity. DNA polymerases derived from hyperthermophilic archaea have been incorporated into numerous commercial PCR kits due to high-fidelity to withstand the high temperature and had proofreading activity. [15] The Ultra HiFi DNA polymerase, developed by Tiangen company, offers a fidelity rate 50 times greater than that of *Taq* DNA polymerase. Additionally, it enhances resistance and adaptability to PCR inhibitors that may interfere with the amplification process as manufacturer's description (https://www.tiangenbioem.com/ultra-hifidelity-pcr-kit-product/). KOD DNA polymerase was utilized in the PCR reagents manufactured by Toyobo company, offering an 80-folds increase in fidelity compared to *Taq* DNA polymerase as manufacturer's description (https://www.toyobo-global.com/products/). This polymerase is categorized as a family B DNA polymerase, demonstrating higher processivity and extension rates than other high-fidelity DNA polymerases. [21] A previous study demonstrated that KOD DNA polymerase is appropriate for serological studies; however, it may potentially lead to non-specific binding of primers and genomic DNA. [22]

According to our finding, the KOD One™ PCR Master Mix (Toyobo) yielded higher intensity of PCR products, indicating that more effective amplification occurred to reach the stationary phase of PCR (overamplification of specific templates) leading to reduced microbial richness (Chao1 diversity index) and lower relative abundance of some bacteria. Previous study found that high concentration of magnesium ions (Mg^{2+}) and different PCR condition were affected to the locus-specific amplification in DNA of rhesus monkey, additionally high concentration of Mg^{2+} with short annealing time and long extension time lead to increased PCR product yield with reduced non-locus specific amplification.[23] Recent study showed that PCR enhancer promoted the DNA structure stability and resistance to DNA inhibitors which affect to the catalytic activity of DNA polymerase.[24] However, these factors only suggested the possibility of the overamplification of specific template that may lead to reduced microbial richness and relative abundance. Due to these evidences, this study represents the inaugural investigation into the effects of different DNA polymerases from PCR kits on the gut microbiota profile, thereby lacking prior evidence to substantiate our findings. Notwithstanding these limitations, the present study has contributed valuable information pertinent to the selection of an appropriate PCR kit and optimization of the amplification protocol to avoid overamplification are important for microbiome studies. Moreover, this study utilized Oxford Nanopore Technologies for DNA sequencing which were more capable to generating a longer reads and provided better resolution for microbial community classification.[25] However, this study did not compare the effect of different sequencing platform for gut microbiota classification which was one of the crucial parameter in producing different microbial diversity and relative abundance. Despite the single use of sequencing platform, Oxford Nanopore Sequencing has provided sufficient microbiota profile for further downstream analysis and future studies.

Conclusion

In summary, the present study demonstrates that employing two different high-fidelity DNA polymerases might has some effect on the diversity and the relative abundance of microbiota profiles. These findings may raise a concern for selection of appropriate PCR kits with suitable amplification cycles for future microbiome research endeavors.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data sharing statement

The data produced or examined throughout the current study are encompassed within this published article. Additional information may be obtained for noncommercial purposes by contacting the corresponding author, pursuant to a reasonable request.

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