Mismatches in 16S rRNA Gene Primers: An Area Worth Further Exploring

Wenting Ren 1, Yingwen Zhong 1,2, Yi Ding 1,2, Yuehong Wu 1,2, XueWei Xu 1,2 and Peng Zhou * 1

1 Key Laboratory of Marine Ecosystem Dynamics, Ministry of Natural Resources and Second Institute of Oceanography, Ministry of Natural Resources, Hangzhou, China, 2 School of Oceanography, Shanghai Jiao Tong University, Shanghai, China

Keywords: metagenomic sequencing, 16S rRNA gene amplicon sequencing, universal primers, mismatch, microbiome

INTRODUCTION

The previous study has shown that ~10% of environmental microbial sequences might be missed from classical PCR-based SSU rRNA gene surveys, and primer mismatches would probably significantly reduce or prevent the recovery of taxonomic “blind spots” in PCR-based surveys (Eloe-Fadrosh et al., 2016). In spite of its deficiency, currently, 16S rRNA gene amplicon sequencing remains widely used in the studies on microbial communities (Liu et al., 2019; Zhang et al., 2019; Deng et al., 2020; Kitamoto et al., 2020; Gonzalez et al., 2021). The literature on 16S rRNA gene amplicon showed a significant upward trend based on the search against PubMed (https://pubmed.ncbi.nlm.nih.gov) (Supplementary Figure 1). However, in 2021, Palkova et al. reported that certain different primer sets toward 16S rRNA gene amplicon sequencing could provide rather opposite Bacteroidetes/Firmicutes ratio while investigating the outcome of sequencing analysis on intestinal microbiota from children with autism spectrum disorder (Palkova et al., 2021). Currently, the microbiome, as a potential diagnostic and predictive biomarker in severe alcoholic hepatitis, has been surveyed by 16S rRNA gene amplicon sequencing (Kim et al., 2021), to which coverage and accuracy are very important. It is necessary to draw attention to defects in 16S rRNA gene amplicon sequencing and call for further exploring mismatches in 16S rRNA gene primers, especially for diagnosis by surveying the microbiome.

PRIMER MISMATCHES

The 16S rRNA gene sequences mismatched with 18 frequently used bacterial universal primers (details shown in Supplementary Table 1) were screened by using BLASTN (e-value ≤ 0.001) against 592,605 bacteria rRNA gene sequences in the SILVA SSURef_NR99 database (release 132, https://www.arb-silva.de), which provides comprehensive, quality checked, and regularly updated datasets of aligned rRNA sequences. For each primer, the number (percentage) of mismatched sequences and the top three families with mismatched sequences are shown in Table 1. Among the surveyed forward and reverse primers, 515F and U529R had the lowest percentage of mismatch, 1.08 and 0.79%, respectively (shown in Table 1). There are 14 complimentary nucleotides overlapped between the forward primer 515F and reverse primer U529R (Supplementary Table 1), which explains similar taxa with mismatches to primers 515F and U529R, such as that the family Lachnospiraceae showed a high percentage of mismatch to primers 515F (0.06%) and U529R (0.04%), as shown in Table 1. Outside the 14 complimentary nucleotides, there are fewer mismatches to U529R (0.021%) than those to 515F (0.031%), which resulted in a lower percentage of mismatch to U529R in Lachnospiraceae (0.04%). Moreover, the family Lachnospiraceae had a high percentage of mismatches with primers U341F, 515F, 517F, 338R, U529R, 533R, and 907R (Table 1). The family Lachnospiraceae belongs to the core of gut microbiota, and its abundance...
was associated with aging (Odamaki et al., 2016), within which specific taxa were involved in different intra- and extra-intestinal diseases (Vacca et al., 2020). Other families closely related to human health and disease, Propionibacteriaceae, Bacillaceae, Burkholderiaceae, Staphylococcaceae, and Veillonellaceae, showed highly mismatched rates as well. For example, certain species of the family Propionibacteriaceae were considered potential pathogens in acne and other skin conditions (Berman, 2012).

**CASE STUDY ON PRIMER 515F**

Therefore, to emphasize that even the primer 515F, which showed the lowest percentage of mismatch (1.08%) against the SILVA bacteria database, may have significant effects on certain taxa due to primer mismatch in analyzing microbial community composition using 16S rRNA gene amplicons, six sequencing datasets on three stool samples from the human gastrointestinal tract, including both 16S rRNA gene amplicon sequencing and metagenomic sequencing (Peters et al., 2019), were chosen for further investigation. Three stool samples (S1, S2, and S3) were collected from patients with melanoma receiving different times for immunotherapy. The three immunotherapy times of S1, S2, and S3 were baseline, week 6, and week 12, respectively. The metagenomic datasets for S1, S2, and S3 were named 1-M, 2-M, and 3-M, respectively, whereas the amplicon datasets were named 1-A, 2-A, and 3-A. Details about datasets are available in Supplementary Table 2. Estimated by Nonpareil software, the coverage of the actual sequencing depth for the three metagenomic datasets 1-M, 2-M, and 3-M was 0.99, 0.98, and 0.95, respectively, indicating that sufficient sequencing depth was achieved for further analysis (C = 0.95, as a rule-of-thumb for nearly complete coverage) (Rodriguez and Konstantinidis, 2014; Rodriguez-R et al., 2018). The estimated coverage curves for these datasets are shown in Supplementary Figure 2.

Afterward, we compared the composition of the microbial community via the two sequencing methods and the histograms of relative abundance at the family level for six datasets shown in Supplementary Figure 3. The results showed that Bacteroidaceae was dominant in all six datasets. The relative abundance of this family in the datasets 1-M, 2-M, and 3-M was significantly higher than those in the datasets 1-A, 2-A, and 3-A (Supplementary Figure 4 and Supplementary Table 2). Also, the relative abundance of Lachnospiraceae, Ruminococcaceae, Enterobacteriaceae, and Fusobacteriaceae was obviously higher in datasets 1-M, 2-M, and 3-M, when compared to datasets 1-A, 2-A, and 3-A, respectively (Supplementary Figure 4 and Supplementary Table 2). Consistently, certain species of Bacteroidaceae and Lachnospiraceae were not detected, either at all or with sufficient abundance in the 16S rRNA gene amplicon sequencing datasets denoted with Greengenes Database (Peters et al., 2019).
Since the universal primer 515F was used for 16S rRNA gene amplicons, bacterial 16S rRNA gene reads covering the primer 515F region were screened in the metagenomic datasets, 37,028 in 1-M, 41,178 in 2-M, and 36,999 in 3-M, respectively (refer to Supplementary Methods for details). Notably, the numbers of reads mismatched to primer 515F in the datasets 1-M, 2-M, and 3-M were 4,619, 6,627, and 6,343, respectively, and the percentage of mismatched reads to primer 515F (PMR-515F) was 12.47 (4,619/37,028), 16.09 (6,627/41,178), and 17.14% (6,343/36,999) (Supplementary Table 4). Furthermore, PMR-515F for taxa (relative abundance > 0.04%) in the datasets 1-M, 2-M, and 3-M was analyzed. The family Bacteroidaceae showed the highest PMR-515F in the datasets 1-M, 2-M, and 3-M (7.51, 8.34, and 5.40%, respectively) (Supplementary Figure 4), which could be one of the possible explanations for its higher relative abundance in the metagenomic datasets than those in the corresponding amplicon datasets. If reads with mismatch to 515F were excluded from the metagenomic datasets, the relative abundance was closer to that in amplicon datasets (Supplementary Figure 4). Similarly, PMR-515F of the family Lachnospiraceae in the datasets 1-M, 2-M, and 3-M were 0.90, 2.78, and 3.48%, respectively (Supplementary Figure 4), which may result in the higher relative abundance in the metagenomic datasets compared with the amplicon datasets. It was consistent with the above result of Lachnospiraceae as one of the taxa with the most mismatched sequences by aligning primer 515F against the SILVA bacteria database. Besides Lachnospiraceae, some other families also showed relatively high PMR-515F in the metagenomic datasets, such as 3.34% for Ruminococcaceae in 3-M, 1.87% for Fusobacteriaceae in 1-M, 1.83% for Enterobacteriaceae in 2-M, and 1.95% for Tannerellaceae in 3-M (Supplementary Figure 4).

Furthermore, the reads mismatched to 515F in the top three families in the metagenomic datasets were analyzed at genus and species levels, and the genus Bacteroides showed high PMR-515F (details in Supplementary Table 5). For instance, in all three metagenomic datasets, some reads with mismatched sequences to primer 515F were found as segments of the 16S rRNA gene of Bacteroides vulgatus, (2 reads in 1-M, 13 reads in 2-M, and 7 reads in 3-M), whereas the downstream sequence of the primer 515F in those reads was not detected in the corresponding amplicon datasets using BLASTN. Similarly, some reads with mismatched sequences to primer 515F were annotated as segments of the 16S rRNA gene of B. thetaiotaomicron in the metagenomic datasets (3, 18, and 4), none of which were detected in the amplicon datasets. B. vulgatus and B. thetaiotaomicron were opportunistic pathogens, which could induce severe colitis. The results suggested that primer mismatches have an effect on the accuracy of detecting pathogenic bacteria in the 16S rRNA gene amplicon sequencing. Furthermore, to evaluate underestimation in amplicon sequencing at a higher level, the intra-family PMR-515F in the metagenomic datasets was investigated (shown in Supplementary Figure 5). The results showed significant intra-family PMR-515F for Bacteroidaceae (11.85% in 1-M, 15.74% in 2-M, and 16.89% in 3-M), Tannerellaceae (13.24% in 1-M, 47.06% in 2-M, and 17.58% in 3-M), Lachnospiraceae (12.16% in 1-M, 15.81% in 2-M, and 15.95% in 3-M), and Ruminococcaceae (14.19% in 1-M, 16.10% in 2-M, and 16.26% in 3-M). Consistent with the previous study, metagenomic sequencing could uncover a more comprehensive composition of microorganisms in the environment, including the microbial groups that were underestimated or ignored in the analysis of amplicon sequencing (Eloe-Fadrosh et al., 2016).

SUMMARY
This study analyzed the primer mismatches from the SILVA database to the experimental datasets. The case study showed the effects of amplicon on the composition of a microbial community. Here, the importance of an approach with less bias is emphasized for the studies on a microbial community. Since the microbiome could be considered the potential diagnostic biomarker (Kim et al., 2021), the accuracy of inferred microbial community composition is essential for diagnosis. With the development in sequencing technology, the methods, which do not require sequence-dependent primer annealing, should be applied more extensively.

AUTHOR CONTRIBUTIONS
PZ: study concept and design. WR: acquisition of data and statistical analysis. WR, YZ, and PZ: analysis and interpretation of data. WR, YW, PZ, and XX: drafting and editing of the manuscript. WR and YD: manuscript revision. XX: study supervision. All authors contributed to the article and approved the submitted version.

FUNDING
This work was supported by grants from the National Science and Technology Fundamental Resources Investigation Program of China (2021FY100900), the Oceanic Interdisciplinary Program of Shanghai Jiao Tong University (No. SL2020MS027), Scientific Research Fund of the Second Institute of Oceanography, MNR (No. JZ1901), and Fund for International Cooperation, State Oceanic Administration of China (Nos. 17070393 and 18070323).

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.888803/full#supplementary-material
REFERENCES

Berman, J. J. (2012). "Actinobacteria," in Taxonomic Guide to Infectious Diseases, ed J. J. Berman (Boston, MA: Academic Press), 77–84. doi: 10.1016/B978-0-12-415895-5.00014-3

Deng, X., Tian, H., Yang, R., Han, Y., Wei, K., Zheng, C., et al. (2020). Oral probiotics alleviate intestinal dysbacteriosis for people receiving bowel preparation. Front. Med. 7, 73. doi: 10.3389/fmed.2020.00073

Eloe-Fadrosh, E. A., Ivanova, N. N., Woyke, T., and Kyrpides, N. C. (2016). Metagenomics uncovers gaps in amplicon-based detection of microbial diversity. Nat. Microbiol. 1, 15032. doi: 10.1038/nmicrobiol.2015.32

Gonzalez, E., Brereton, N. J. B., Li, C., Lopez Leyva, L., Solomons, N. W., Agellon, L. B., et al. (2021). Distinct changes occur in the human breast milk microbiome between early and established lactation in breastfeeding guatemalan mothers. Front. Microbiol. 12, 557180. doi: 10.3389/fmicb.2021.557180

Kim, S. S., Eun, J. W., Cho, H. J., Song, D. S., Kim, C. W., Kim, Y. S., et al. (2021). Microbiome as a potential diagnostic and predictive biomarker in severe alcoholic hepatitis. Aliment. Pharmacol. Ther. 53, 540–551. doi: 10.1111/apt.16200

Kitamoto, S., Nagao-Kitamoto, H., Hein, R., Schmidt, T. M., and Kamada, N. (2020). The bacterial connection between the oral cavity and the gut diseases. J. Dent. Res. 99, 1021–1029. doi: 10.1177/0022034520924633

Liu, J., Zheng, Y., Lin, H., Wang, X., Li, M., Liu, Y., et al. (2019). Proliferation of hydrocarbon-degrading microbes at the bottom of the Mariana Trench. Microbiome 7, 47. doi: 10.1186/s40168-019-0652-3

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J. Z., et al. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol. 16, 90. doi: 10.1186/s12866-016-0708-5

Palkova, L., Tomova, A., Repiska, G., Babinska, K., Bokor, B., Mikula, I., et al. (2021). Evaluation of 16S rRNA primer sets for characterisation of microbiota in paediatric patients with autism spectrum disorder. Sci. Rep. 11, 6781. doi: 10.1038/s41598-021-86378-w

Peters, B. A., Wilson, M., Moran, U., Pavlick, A., Izsak, A., Wechter, T., et al. (2019). Relating the gut metagenome and metatranscriptome to immunotherapy responses in melanoma patients. Genome Med. 11, 61. doi: 10.1186/s13073-019-0672-4

Rodriguez, R. L., and Konstantinidis, K. T. (2014). Nonpareil: a redundancy-based approach to assess the level of coverage in metagenomic datasets. Bioinformatics 30, 629–635. doi: 10.1093/bioinformatics/btu584

Rodriguez-R, L. M., Gunturu, S., Tiedje, J. M., Cole, J. R., and Konstantinidis, K. T. (2018). Nonpareil 3: fast estimation of metagenomic coverage and sequence diversity. mSystems 3, e00039–e00018. doi: 10.1128/mSystems.0039-18

Vaccaro, I., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., and De Angelis, M. (2020). The controversial role of human gut Lachnospiraceae. Microorganisms 8, 573. doi: 10.3390/microorganisms8040573

Zhang, L., Liu, Y., Zheng, H. J., and Zhang, C. P. (2019). The oral microbiota may have influence on oral cancer. Front. Cell. Infect. Microbiol. 9, 476. doi: 10.3389/fcimb.2019.00476

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Ren, Zhong, Ding, Wu, Xu and Zhou. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.