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Next Generation Sequencing Improves Diagnostic 16S rRNA Amplicon-Based Microbiota Analyses of Clinical Samples Compared to Sanger Sequencing

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ABSTRACT

Sequencing of the 16S ribosomal RNA (rRNA) gene is an important tool in addition to conventional methods for the identification of bacterial pathogens in human infections. In polymicrobial samples, Sanger sequencing can produce uninterpretable chromatograms. This limitation can be overcome by Next Generation Sequencing (NGS) of the 16S rRNA gene. We investigated the applicability of Oxford Nanopore Technologies (ONT) sequencing of the partial 16S rRNA gene as a diagnostic routine method for pathogen detection in clinical samples. From June 2021 to August 2022, 101 clinical samples positive in PCR for partial 16S rRNA gene analysis were subjected to both Sanger and ONT sequencing. Sanger sequences were edited and compared with deposited sequences in the NCBI database using BLAST, while ONT data were processed using EPI2ME Fastq 16S. The positivity rate (clinically relevant pathogen) was higher for ONT vs. Sanger sequencing: 72% and 59%, respectively. Concordance between Sanger and ONT sequencing was 80%. Furthermore, ONT detected more samples with polymicrobial presence compared to Sanger (13 vs. 5) sequencing. Interestingly, in one joint fluid sample, *Borrelia bissettiiae* was identified by ONT but not by Sanger. The results show that the detection of both monobacterial and multiple bacterial species is improved using ONT.

1 | Introduction

Infectious diseases continue to pose an immense health burden. Globally in 2019, it was estimated that 704 million disability adjusted life years (DALYs) were associated with 85 pathogens, *Staphylococcus aureus* and several Gram-negative bacilli (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*) accounting for a substantial proportion of the DALYs [1]. Therefore, the need for fast and precise microbiological diagnostics is still called for. Traditionally, culture and subsequent identification of isolates by biochemical testing and Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) have been the mainstay of microbiological diagnostics. Detection of bacterial

DNA and sequencing of amplification products is an important diagnostic modality for identification of bacterial pathogens in samples from infectious foci [2–5], especially if antibiotic therapy has been administered or for nonculturable organisms like *Borrelia* sp. [6].

Amplification of the 16S ribosomal RNA (rRNA) gene and subsequent sequencing of the PCR product has become a widespread and inexpensive strategy for detecting pathogens. Sanger sequencing is considered reliable and accurate, with a positivity rate comparable to conventional culture methods [7]. However, Sanger sequencing can result in uninterpretable chromatograms for polymicrobial samples, limiting the sensitivity. Next Generation Sequencing (NGS) is believed to surpass Sanger

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sequencing by generating reads for individual bacteria in a sample, thereby identifying all pathogens present in samples with polymicrobial presence [8]. The clinical application of NGS in infectious disease is increasingly being investigated for different pathogens and localisations with varying outcomes [9–12]. This study investigated the value of 16S rRNA gene sequencing based on ONT compared to Sanger sequencing for routine microbiological diagnostics in a wide array of clinical samples.

2 | Material and Methods

Between June 2021 to August 2022, clinical samples were collected and analyzed prospectively at the Regional Department of Clinical Microbiology, Zealand University Hospital, Koege, which provides laboratory services to primary health care facilities and six hospitals with a catchment population of 821,000 citizens. Samples received in the study period for routine microbiological diagnostics at the department were analyzed following standard procedures, and 16S rRNA PCR was performed on samples meeting the following criteria: (1) negative culture, (2) antecedent antibiotic treatment, (3) samples from sterile sites, and (4) high suspicion of clinical infection. A senior clinical microbiologist assessed whether a sample was eligible for 16S rRNA gene analysis. Fulfilling all four criteria was not obligatory. Samples positive in the 16S rRNA PCR were subsequently sequenced by Sanger and ONT methodology. The clinical significance of the sequencing results was also evaluated by a senior clinical microbiologist. A total of 101 clinical samples positive by 16S rRNA gene PCR were included in the study. Concordance between the two sequencing methods was defined as 100% agreement in both the detected pathogen on genus level and number of detected pathogens, while negative samples were designated as such if no microorganisms or only contaminants were detected. If additional pathogen(s) were detected by one of the two sequencing methods, the result was categorized as partial concordance.

2.1 | Cultures

All clinical samples, irrespective of the location, received as part of routine patient care were processed according to the department's standard procedures. Tissue and fluid samples from sterile compartments were cultured on agar plates for both aerobic and anaerobic microorganisms; aerobic agar plates (Lactose agar with Bromothymol blue, 5% Horse Blood agar plate, and Chocolate agar plate), anaerobic agar plate, and for yeast on CHROMagar Candida. Microscopy of Gram-and methylene blue-stained samples was also performed. Plates and broths were incubated for 6 days. For identification of bacterial isolates, MALDI-TOF MS (Bruker, Bremen, Germany) was applied.

2.2 | 16S rRNA Gene PCR and Sanger Sequencing

Samples were processed using the Micro-Dx kit in combination with SelectNA plus (Molzylm GmbH & Co. KG, Bremen, Germany). The Micro-Dx kit contains primers targeting conserved regions of the 16S (comprising V3 and V4 regions) and the 18S (regions V8 and V9) rRNA genes. The 16S rRNA gene

analysis was performed according to the manufacturer's protocol. The PCR products were considered positive if the melting curve in real-time PCR was within the temperature range of 86°C to 92°C. PCR-positive samples were Sanger sequenced by Eurofins GATC Biotech NPL (Cologne, Germany). Sequencing data were downloaded from the Eurofins website and edited with CLC Main Workbench (Qiagen) and compared with deposited sequences in the NCBI database using the BLAST search engine, which includes sequences from type strains as well as nontype strains. Genus and species designations were based on the BLAST results, taking into consideration the number of identical bases, MaxScores, the number of identical taxon matches before the appearance of the next best taxon match, and E values. A minimal difference of 15 in MaxScore between the best and the next best taxon match was required for closely related taxons. In accordance with the literature, MaxScore differences varied according to the genera/species identified.

2.3 | Oxford Nanopore Technologies

In parallel, all the samples were sequenced using ONT methodology. The DNA libraries were prepared according to the SQK-SLK109 protocol from Oxford Nanopore Technologies with additional reagents from New England Biolabs (Cat. E7564, M0367, and E6056S). The ONT sequencing was run on a GridION (Oxford Nanopore Technologies) with FLO-MIN104/R9.4.1 flow cells. Sequencing run settings were Super-accurate basecalling, trim_barcodes="on", require_barcodes_both_ends="off", detect_mid_strand_barcodes="off", min_score=60, Read filtering min_qscore=10, min_bases=200, max_bases=500. MinKNOW version was kept up to date for the duration of the study. Settings not described were set to default. ONT data were processed using the EPI2ME platform's Fastq 16S workflow as well as an in-house pipeline using the k-mer alignment (KMA) tool [12], which maps reads to a database built from the NCBI RefSeq database and the SILVA 138.1 database.

Whether a microorganism was categorized as a contaminant or a significant pathogen depended on clinical data, recent positive microbiological history, and response to administered antibiotic therapy.

3 | Results

Between June 2021 and August 2022, 101 clinical culture-negative samples that were positive in 16S rRNA gene PCR were subjected to subsequent sequencing both by ONT and Sanger sequencing. These samples were predominantly from orthopedic surgery departments (58.4%), followed by the department of pulmonology (9.9%), the department of internal medicine (6.9%), and the department of infectious diseases (4.9%). The remaining 19.9% were from a wide array of departments (otorhinolaryngology, endocrinology, cardiology, gynecology, obstetrics, urology, nephrology, gastroenterology). Tissue samples comprised almost half of the total number of 16S rRNA PCR-positive samples, with hip, knee, and joint tissues accounting for most samples in this category (48.5%). Other common sample types were joint fluid (16.8%), pleural fluid (14.8%), pus from the abdomen, joint, liver, and pleural cavity (12.8%) while 4.0% were from various

localizations (cerebrospinal fluid, ascites and abdominal drainage fluid). The samples with bacterial identification of environmental species or microorganisms interpreted as contamination were designated as negative.

The positivity rate for identification of clinically relevant microorganisms was 72% (73/101) and 59% (60/101) by ONT and Sanger, respectively. Thus, 28 samples were negative by ONT while Sanger sequencing was not able to detect any microorganisms in 41 samples.

Concordance between ONT and Sanger sequencing analysis was 80% (81/101). Among these, 28 samples were negative, while the same pathogens were identified by both methods in 53 samples.

Seven (7.9%) samples showed partial concordance. In seven samples, additional pathogens were identified using ONT sequencing analysis, while in one sample, only Sanger sequencing analysis identified an additional pathogen compared to ONT sequencing analysis; in this sample from a lumbar joint abscess, only Sanger sequencing analysis detected *Pasteurella* sp., while *Cutibacterium acnes* was found by both sequencing methods.

Discordant results were observed in 13 (12.9%) samples; pathogens were detected by ONT sequencing but not Sanger sequencing analyses (Table 1). In one of these 13 samples, ONT sequencing analysis identified a rare pathogen in synovial fluid from a knee (41.667 mapped reads): *Borrelia bissettiae*. Furthermore, ONT sequencing analysis found *S. capitis* in this sample, while Sanger sequencing analysis missed both microorganisms. The mapped reads in this sample were distributed as follows: *S. epidermidis* 79% and *B. bissettiae* 17%. Pathogens most frequently detected by ONT but not by Sanger sequencing analyses were *Streptococcus pneumoniae* (22%) and *Fusobacterium* spp. (16%). In four samples, Sanger sequencing analyses did not detect *Fusobacterium* spp. detected by ONT sequencing.

The average number of mapped ONT reads per sample was 406.736. Three samples resulted in low read counts, with 350, 336, and 11 reads, respectively. In the sample with a read count of 350, ONT sequencing detected *Streptococcus intermedius* in pleural fluid from a patient with empyema. Thus, despite the low read count, *S. intermedius* was accepted as the plausible etiological pathogen. The two other samples were negative by both sequencing methods.

Tissue from joints, pleural fluids, and pus from abscesses was most likely to be polymicrobial (> 1 bacteria genus detected). For Sanger sequencing analyses, more than one bacterium was detected in 21 samples. However, only five (23.8%) of these samples were interpreted as clinically significant and categorized as polymicrobial infection. ONT sequencing analyses detected more than one bacterium in 21 samples, of which 13 (65%) were assessed to be relevant pathogens and categorized as polymicrobial infection. In the five polymicrobial samples, all pathogens detected by Sanger sequencing were also detected by ONT sequencing analysis.

A ZymoBIOMICS Microbial Community DNA standard (ZYMO research) was sequenced with ONT to investigate correct identification of bacteria in polymicrobial samples (Figure 1). The

TABLE 1 | Pathogens identified by ONT sequencing but not Sanger sequencing.

Sample type	Location	Pathogens (% reads) identified by 16S nanopore
Tissue	Shoulder	<i>Fingoldia magna</i> (36%)
Tissue	Hip	<i>Streptococcus pneumoniae</i> (76%)
Pus/aspirate	Hip	<i>Streptococcus pneumoniae</i> (52%)
Tissue	Hip	<i>Streptococcus pneumoniae</i> (68%)
Tissue	Hip	<i>Streptococcus pneumoniae</i> (25%)
Synovial fluid	Hip	<i>Staphylococcus epidermidis</i> (98%)
Tissue	Hip	<i>Cutibacterium acnes</i> (85%)
Tissue	Hip	<i>Cutibacterium acnes</i> (78%)
Synovial fluid	Knee	<i>Staphylococcus capitis</i> (79%), <i>Borrelia bissettii</i> (17%)
Pleural fluid	Pleural cavity	<i>Streptococcus intermedius</i> (92%)
Pleural fluid	Pleural cavity	<i>Fusobacterium nucleatum</i> (52%), <i>Parvimonas micra</i> (48%)
Pus/aspirate	Pleural cavity	<i>Fusobacterium canifelinum</i> (49%), <i>Treponema maltophilum</i> (29%), <i>Tannerella forsythia</i> (16%), <i>Porphyromonas gingivalis</i> (5%)
Pus	Liver	<i>Parvimonas micra</i> (32%), <i>Fusobacterium nucleatum</i> (25%), <i>Porphyromonas endodontalis</i> (6%)

standard underwent PCR in triplicate, and relative distributions of detected organisms were compared to the manufacturers' theoretical distributions. All organisms were reliably detected by ONT sequencing; distributions were similar to the theoretical distributions.

4 | Discussion

For the last two decades, microbiological routine diagnostics in clinical settings have been revolutionized by molecular techniques. A transition from clinical application of a metagenomic approach to amplicon-based NGS using the ONT platform with lower cost and fast bioinformatic outputs is gaining increasing interest. Despite the limitations associated with NGS, in line with previous studies, we found a higher positivity rate for 16S

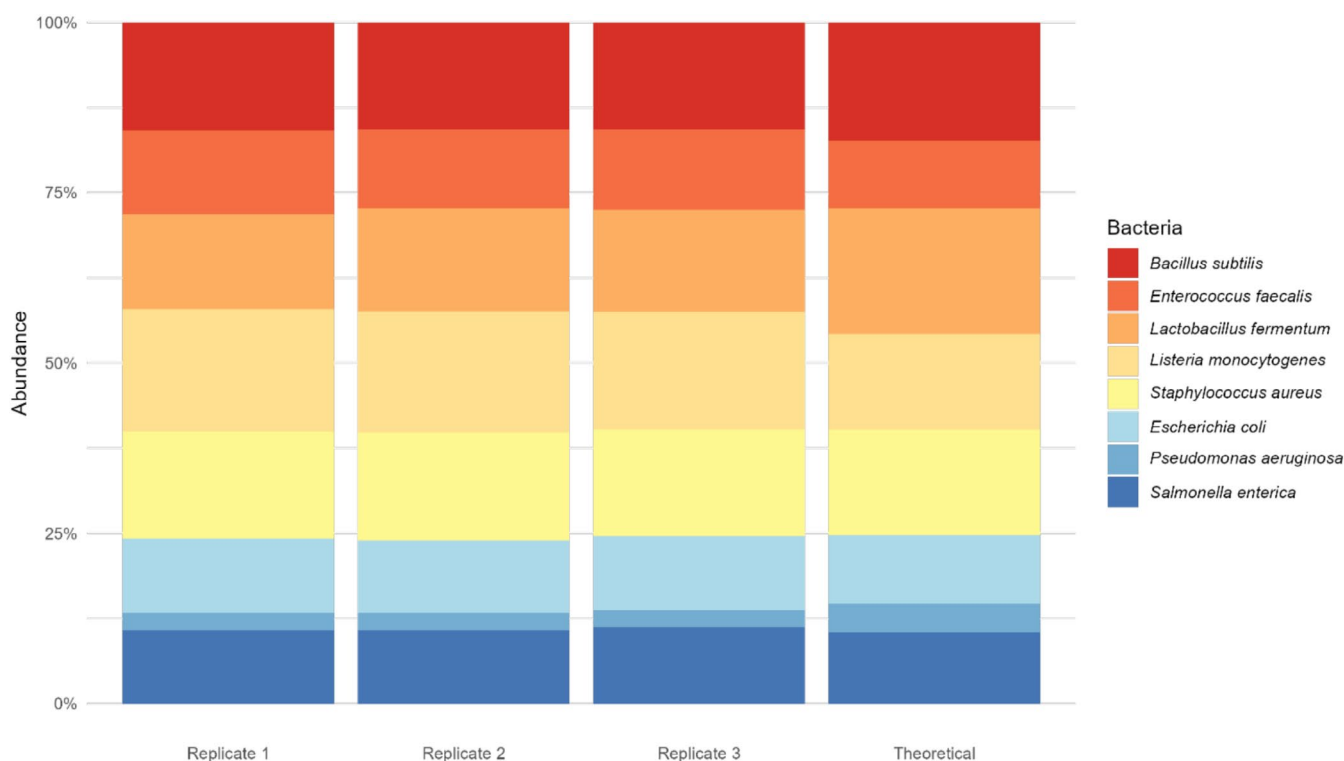


FIGURE 1 | ZymoBIOMICS Microbial Community DNA standard underwent PCR in triplicate and was sequenced with Oxford Nanopore Technologies. The figure shows relative species distributions of the triplicates compared to the theoretical composition stated by ZYMO Research.

rRNA PCR using ONT sequencing compared to Sanger sequencing [13]; ONT sequencing analyses increased 16S rRNA gene positivity by 17% (73–60/73) compared with Sanger sequencing analyses. For polymicrobial samples, the additional yield by ONT sequencing analyses was even more prominent, resulting in a 61% increase (13–5/13). In a study evaluating the clinical performance of NGS (ONT) in infectious disease diagnostics on various sample types, comparing NGS with PCR followed by Sanger sequencing, very similar to our findings, reported a discrepancy rate of app. 13% (8).

Furthermore, ONT sequencing analyses showed a clear advantage over both Sanger sequencing analyses and culture for the detection of relatively fastidious pathogens such as *Fusobacterium* spp. and *S. pneumoniae*. This is important since infections with fastidious bacteria may delay diagnosis and de-escalation of broad-spectrum empirical antibiotic therapy. *Fusobacterium* spp. were predominantly detected in polymicrobial samples, which could explain why Sanger sequencing analyses failed to detect this pathogen. Pleural fluids were most likely to be polymicrobial, underlining the potential of ONT sequencing analysis in the diagnosis of infections from this localisation [14].

In samples with discordant results, *B. bissetiae* identified by ONT sequencing analysis only was considered a significant clinical finding, since the patient had relevant exposures to ticks and positive *Borrelia* serology.

In one of the polymicrobial samples, Sanger sequencing identified *Pasteurella*, while ONT sequencing analysis did not. This sample was an abscess aspiration from a patient who developed postoperative infection in relation to spinal stenosis surgery.

One week after aspiration from the abscess, the patient underwent repeated surgery in another hospital. Ten biopsies collected from the second operation were subjected to culture and Sanger sequencing analysis of the 16S rRNA gene in another microbiological laboratory than ours. *C. acnes* was cultured from one of the ten biopsies. *Pasteurella* sp. was not detected in any of the biopsies by either culture or 16S rRNA sequencing. It was concluded that the *Pasteurella* sp. DNA from the first abscess aspiration was contamination.

Results from 16S rRNA sequencing, both based on Sanger sequencing and ONT sequencing, are highly dependent on both the wet lab and the dry lab procedures. In the wet lab, the DNA extraction method used is pivotal. In our lab, we used the Micro-Dx kit from Molzym, which includes depletion of human DNA. However, there is a risk that the depletion procedure may potentially result in a biased recovery among the microbial species due to their unequal sensitivities to the lysing conditions [15]. Therefore, optimization of the DNA extraction method is desirable to achieve more sensitive results for the 16S analyses.

To minimize methodology bias, the same 16s V3-V4 PCR product was sequenced in parallel for comparison of ONT and Sanger sequencing.

In the dry lab, it is important that the bioinformatic pipeline used for mapping of sequences has access to an updated and validated database. In this study, we used the EPI2ME Fastq 16S pipeline from Oxford Nanopore Technologies and the SILVA database, which is a quality-controlled database for rRNA gene sequences. However, the number of sequences included in SILVA is limited [16]. Sequences from the manually curated NCBI

RefSeq database were added to remedy this. EPI2ME performs analysis of ONT data in real time, allowing rapid results.

A limitation of this study is the lack of data on the duration or timing of antibiotic therapy prior to sampling and any therapeutic consequences of the sequencing results. Although ONT sequencing analysis clearly performed better than Sanger sequencing analysis, the study cannot address whether this is particularly valid for samples from patients treated with antibiotics.

In line with previous findings [13] we conclude that 16S rRNA amplicon-based ONT sequencing analyses were superior to Sanger sequencing analyses for the identification of clinically relevant organisms in polymicrobial infections, making it a valuable addition to future routine microbiological diagnostics.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

References

1. M. Naghavi, T. Mestrovic, A. Gray, et al., “Global Burden Associated With 85 Pathogens in 2019: A Systematic Analysis for the Global Burden of Disease Study 2019,” *Lancet Infectious Diseases* 24, no. 8 (2024): 868–895.
2. R. W. Jordan, N. A. Smith, A. Saithna, A. P. Sprowson, and P. Foguet, “Sensitivities, Specificities, and Predictive Values of Microbiological Culture Techniques for the Diagnosis of Prosthetic Joint Infection,” *BioMed Research International* 2014 (2014): 180416.
3. R. Dyrhovden, R. M. Nygaard, R. Patel, E. Ulvestad, and Ø. Kommedal, “The Bacterial Aetiology of Pleural Empyema. A Descriptive and Comparative Metagenomic Study,” *Clinical Microbiology and Infection* 25, no. 8 (2019): 981–986.
4. J. Parvizi, E. Ghanem, S. Menashe, R. L. Barrack, and T. W. Bauer, “Periprosthetic Infection: What Are the Diagnostic Challenges?,” *Journal of Bone and Joint Surgery* 88 (2006): 138–147.
5. L. M. Abayasekara, J. Perera, V. Chandrasekharan, et al., “Detection of Bacterial Pathogens From Clinical Specimens Using Conventional Microbial Culture and 16S Metagenomics: A Comparative Study,” *BMC Infectious Diseases* 17, no. 1 (2017): 631.
6. M. J. G. Sigakis, E. Jewell, M. D. Maile, S. K. Cinti, B. T. Bateman, and M. Engoren, “Culture-Negative and Culture-Positive Sepsis: A Comparison of Characteristics and Outcomes,” *Anesthesia and Analgesia* 129, no. 5 (2019): 1300–1309.
7. M. Fida, S. Khalil, O. A. Saleh, et al., “Diagnostic Value of 16S Ribosomal RNA Gene Polymerase Chain Reaction/Sanger Sequencing in Clinical Practice,” *Clinical Infectious Diseases* 73, no. 6 (2021): 961–968.
8. Y. Fu, Q. Chen, M. Xiong, et al., “Clinical Performance of Nanopore Targeted Sequencing for Diagnosing Infectious Diseases,” *Microbiology Spectrum* 10 (2022): e0027022.
9. Y. Guo, Z. Li, L. Li, et al., “A Dual-Process of Targeted and Unbiased Nanopore Sequencing Enables Accurate and Rapid Diagnosis of Lower Respiratory Infections,” *eBioMedicine* 98 (2023): 104858.

10. D. Han, F. Yu, D. Zhang, et al., “Molecular Rapid Diagnostic Testing for Bloodstream Infections: Nanopore Targeted Sequencing With Pathogen-Specific Primers,” *Journal of Infection* 88, no. 6 (2024): 106166.
11. Y. H. Sung, Y. K. Ju, H. J. Lee, et al., “Clinical Performance of Real-Time Nanopore Metagenomic Sequencing for Rapid Identification of Bacterial Pathogens in Cerebrospinal Fluid: A Pilot Study,” *Scientific Reports* 15, no. 1 (2025): 3493.
12. P. F. Indelli, S. Ghirardelli, B. Violante, and D. F. Amanatullah, “Next Generation Sequencing for Pathogen Detection in Periprosthetic Joint Infections,” *EFORT Open Reviews (British Editorial Society of Bone and Joint Surgery)* 64 (2021): 236–244.
13. L. Flurin, M. J. Wolf, M. M. Mutchler, M. L. Daniels, N. L. Wengenack, and R. Patel, “Targeted Metagenomic Sequencing-Based Approach Applied to 2146 Tissue and Body Fluid Samples in Routine Clinical Practice,” *Clinical Infectious Diseases* 75, no. 10 (2022): 1800–1808.
14. Y. Shiraishi, K. Kryukov, K. Tomomatsu, et al., “Diagnosis of Pleural Empyema/Parapneumonic Effusion by Next-Generation Sequencing,” *Infectious Diseases* 53, no. 6 (2021): 450–459.
15. Y. Shi, G. Wang, H. C. H. Lau, and J. Yu, “Metagenomic Sequencing for Microbial DNA in Human Samples: Emerging Technological Advances,” *International Journal of Molecular Sciences* 23 (2022): 2181.
16. C. Quast, E. Pruesse, P. Yilmaz, et al., “The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools,” *Nucleic Acids Research* 41, no. D1 (2013): D590–D596.