Identification of bacterial pathogens from clinical samples using 16S rRNA sequencing

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Abstract

Introduction: Bacterial infections have a substantial impact on global health and can become serious if misdiagnosed with several diseases related to the central nervous, cardiovascular, and respiratory systems. The prognosis in patients with infectious disease strongly depends on early diagnosis and appropriate antibiotic therapy. We aimed to compare the accuracy of genus and species-level identification bacteria using biochemical testing and 16S rRNA sequence analysis. **Material and methods:** 50 clinical samples were isolated and identified the pathogenic bacteria by routine laboratory methods. In parallel, DNA was extracted from isolate's colonies and amplified the 16S rRNA gene by using specific primers. The PCR products were evaluated by agarose gel electrophoresis and direct sequencing by the Sanger method. The sequence data were manipulated by Geneious Prime software. The sequence data matching the Prokaryotic 16S Ribosomal RNA database with a similarity score of ≥ 98% were selected. **Results:** Total of 50 clinical samples were isolated and identified the pathogenic bacteria with common biochemical test and API® Microbial Identification. The sequencing data showed that almost species identified by 16S rRNA sequencing matched the biochemical test method. There are 3 species (6%) were identified as different species with the routine methods. **Conclusions:** 16S rRNA gene sequencing is more sensitive, easier to manage, more accurate and especially for bacteria that are difficult to identify. 16S rRNA sequencing is considered an effective method to early identify pathogens in clinical samples, and this technique is increasingly being used in microbiology laboratories.

Keywords: *16S rRNA gene, Sanger sequencing, bacterial identification, misdiagnosed.*

1. INTRODUCTION

Bacterial infections have a substantial impact on global health and can become serious if misdiagnosed with several diseases related to the central nervous, cardiovascular, and respiratory systems. These contribute to increased morbidity and mortality rates, especially in immunodeficiency patients. The prognosis in patients with infectious disease strongly depends on early diagnosis and appropriate antibiotic therapy [1,2]. Therefore, rapid and sensitive identification of pathogenic bacteria is essential for initiating timely and effective antibiotic treatment and preventing disease spread [3]. Cultivation and phenotypic identification methods (culture-dependent methods) for determining antimicrobial resistance remain the gold standard approach in clinical microbiology. However, the sensitivity of culture methods is influenced by patient characteristics, laboratory practices, and the spectrum of bacterial pathogens. These are also time-consuming, taking at least 24 - 48h to complete which leads to delayed appropriate treatment in critically ill patients. Such delays may worsen the patients'conditions and increase mortality. In addition, it is challenging to identify by culture with fastidious, slowly growing microorganisms or antibiotic exposure prior to sample collection and generally fails to differentiate between species of the genus. The other methods for microbial identification in the laboratory is the genotypic identification - molecular diagnostic method [4].

Molecular approaches have been offered as an alternative or complement to phenotypic methods. Typically, conserved sequences within phylogenetically informative genetic targets, such as the 16S rRNA coding gene, are used for bacterial genotypic identification [5,6]. In this study, we report a comparison of two bacterial identification methods which rely on phenotypic/biochemical tests and 16S rRNA gene sequence analysis. The ability of these two methods to accurately identify 50 clinical isolates at levels of specificity: genus and species, was examined.

2. MATERIALS AND METHODS

2. 1. Materials

Forty clinical samples were collected from Dec 2020 to April 2021 at Hospital of Hue University

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DOI: 10.34071/jmp.2022.7.9
Recieved: 12/10/2022; Accepted: 15/11/2022; Published: 30/12/2022

of Medicine and Pharmacy. All samples were transported to the Microbiology Department of Hospital of Hue University of Medicine and Pharmacy within 2 hours after collection for microbiological analysis.

Ten QA-QC samples from OUCRU (Oxford University Clinical Research Unit - Vietnam) were performed the species identification as control group.

2.2. Methods

Research method: cross-sectional study.

Isolation and phenotypic identification

The samples were processed for bacterial isolation and identification by routine microbiological methods such as culture and biochemical tests following the guideline of the Ministry of Health - Vietnam. These strains were isolated and phenotypically identified by means of the API® 20 E for Gram-negative bacilli; API® 20 NE for Gram-negative non-*Enterobacteriaceae*; API 20 strep for Streptococci and API® Coryne for Corynebacteria. The isolates were stored in Brain Heart Infusion Broth (E&O Laboratories, Bonnybridge, Scotland) with 20% of sterile glycerol in a cryovial at -80°C for long-term storage.

DNA extraction

The colonies were picked up from the primary plate of each isolate and resuspended in 200 uL 1× TE buffer. The samples were centrifuged at 15,000 × g for 15 min. The supernatant was eliminated, and the pellet was resuspended in molecular biology-grade water (Eppendorf, Hamburg, Germany), then centrifuged at 15,000 × g for 10 min. The supernatant was eliminated, and the pellet was resuspended in 40 µL of molecular biology-grade water, subjected to boiling at 100°C in a water bath for 10 mins, cooled on ice, and centrifuged at 15,000 × g for 10 s. The supernatant was transferred to a new tube before it was stored at -20°C [7 - 9].

PCR amplification of 16S rRNA gene

The 16S rRNA gene has been a mainstay of sequence-based bacterial analysis until today. The gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene. Conventional PCR was performed by using forward primers 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse primer 5'-TACGGYTACCTTGTTACGACTT -3 located at position 27 and 1492 respectively , which specifically targets approximately 1500 bp of the 16S rRNA gene [10]. A total of 50 ng genomic DNA, 0.5 µM for each primer, and 12.5 µL MyTaq Mix 2× Bioline (Meridian Bioscience International Limited) were combined in a 25 µL total volume reaction. The PCR amplification was profiled as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 90 seconds, then 72°C for 7 minutes in Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA). 4µL of PCR products were separated by electrophoresis on 1% agarose gel with 1× GelRed and digitized with GelDocs XR (Biorad, CA, USA).

16S ribosomal RNA gene profile analysis

10 ng of PCR products having 16S rRNA amplicon and 0.32 µM of each primer were used for direct sequencing. To sequence both strands, two primers were run for each isolate. Forward and reverse sequences were assembled into consensus sequences using Geneious Prime v2020.0.3 to get the consensus 16S rRNA sequences, primers were trimmed manually, and ambiguous bases were resolved based on visual inspection of the chromatograms. Consensus sequences were taxonomically classified via Geneious Prime BLAST Plugin. The sequence data matching the partial sequence of the Prokaryotic 16S Ribosomal RNA databases with a similarity score of ≥ 98% were selected.

3. RESULTS

Fifty clinical samples composed of 11 types of clinical samples were isolated the pathogenic bacterial by the biochemical tests-based method (Fig.1). There are 35 different strains with 21 samples identified as more than one strain, 6 samples were identified as *Stenotrophomonas maltophilia* (12%), 3 were identified as *Klebsiella pneumonia* (6%), 4 were identified as *Morganella morganii* (8%), 2 were identified as *Serratia odorifera* (4%), 2 were identified as coagulase-negative Staphylococci (CoNS) (4%), 4 were identified as *Klebsiella oxytoca* (4%), and 29 samples were identified in single species (Table 1).

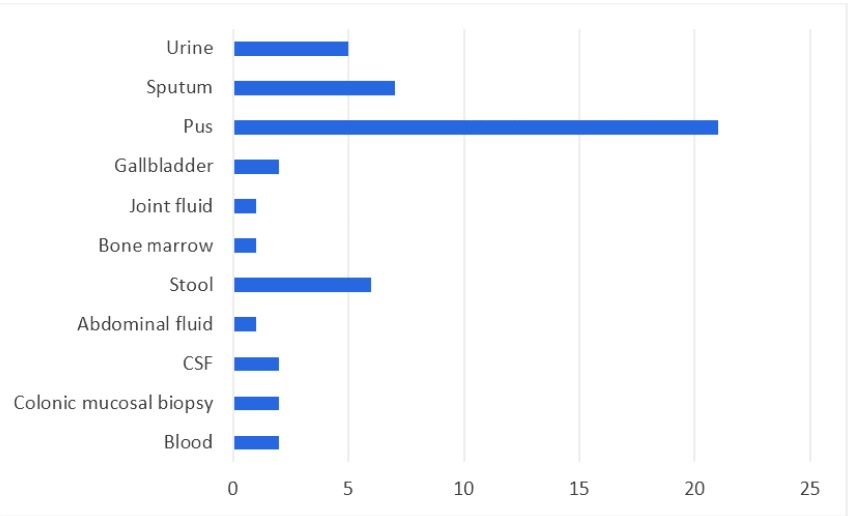

Fig.1. Type of clinical sample and the number of isolates of each type of clinical sample.

Amplification of 16S rRNA gene

A sequence including the near full-length of the 16S rRNA gene was obtained from PCR reactions with 27F and 1492R universal primers in all of the samples. Amplification of the 16S rRNA gene was confirmed by gel electrophoresis. The expected size of approximately 1500 bp was amplified (Fig. 2). The specific primers worked correctly in all samples. The remaining PCR product was sequenced in a total of 50 samples.

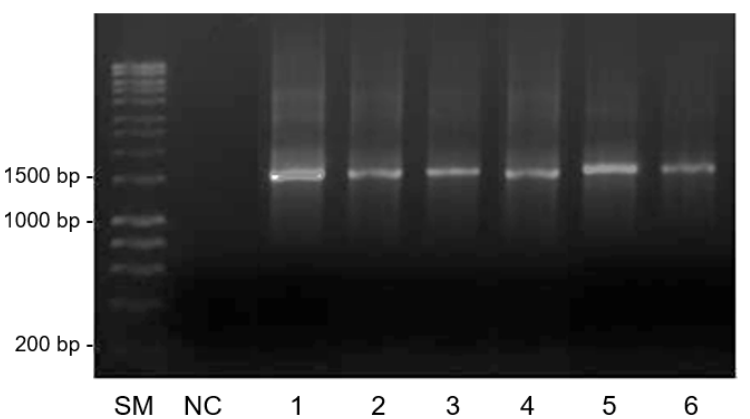

Fig.2. Amplicon of 16S rRNA gene on 1% agarose gel. SM: 1kb plus DNA ladder (ThermoFisher, USA); NC: non-template control; lane 1-6: amplicons of 16S rRNA gene

Sequencing and species classification

Fifty PCR products were sequenced and the data were manipulated with Geneious Prime software. The primers were trimmed manually, and ambiguous bases were resolved based on visual inspection of the chromatograms (Fig.3). The forward and reverse sequences were assembled into consensus of 16S rRNA gene for each isolate. Consensus sequences were taxonomically classified via Geneious Prime BLAST Plugin. All 50 sequence data were matching the partial sequence of the prokaryotic 16S ribosomal RNA databases with a similarity score of ≥ 98-99%. The species classification was shown in the right column of table 1. There are 3 samples in which the species taxonomy is different from the conventional microbiology method (grey row with underlined taxonomy name) (Table 1): *Burkholderia pseudomallei, Escherichia coli, Streptococcus constellatus* (Fig.4).

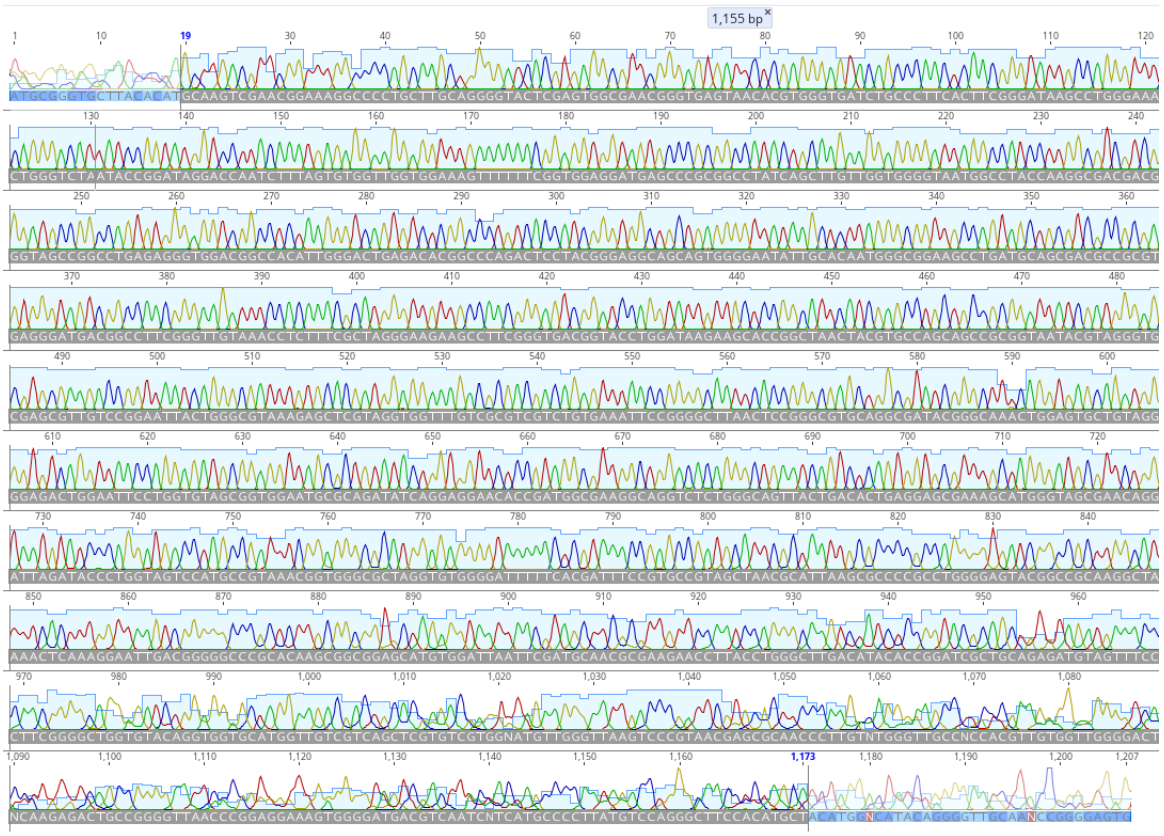

Fig 3. Raw data of forward sequence by the Sanger sequencing method (chain-termination). The good quality sequenced bases are more than 1100bp for each strain.

Table 1. Result of bacterial identification by biochemical tests-based method versus 16S rRNA gene sequencing method

Sample ID	Biochemical test methods	Specimens	16S rRNA sequencing
1	*Morganella morganii*	Pus	*Morganella morganii*
2	*Stenotrophomonas maltophilia*	Pus	*Stenotrophomonas maltophilia*
3	*Providencia stuartii*	Urine	*Providencia stuartii*
4	*Stenotrophomonas maltophilia*	Pus	*Stenotrophomonas maltophilia*
5	*Providencia rettgeri*	Urine	*Providencia rettgeri*
6	*Morganella morganii*	Sputum	*Morganella morganii*
7	*Chryseobacterium eningosepticum*	Urine	*Chryseobacteriumm eningosepticum*
8	*Morganella morganii*	Pus	*Morganella morganii*
9	*Stenotrophomonas maltophilia*	Pus	*Stenotrophomonas maltophilia*
10	*Klebsiella oxytoca*	Colonic mucosal biopsy	*Klebsiella oxytoca*
11	*Stenotrophomonas maltophilia*	Blood	*Stenotrophomonas maltophilia*
12	*Stenotrophomonas maltophilia*	Stool	*Stenotrophomonas maltophilia*
13	*Alcaligenes spp.*	Pus	*Alcaligenes spp.*
14	*Plesiomonas shigelloides*	Gallbladder	*Plesiomonas shigelloides*

15	*Serratia marcescens*	Pus	*Serratia marcescens*
16	*Aeromonas salmonicida*	Sputum	*Burkholderia pseudomallei** Accession: OP890627
17	*Streptococcus anginosus*	Pus	*Streptococcus anginosus*
18	*Klebsiella pneumoniae*	Urine	*Klebsiella pneumoniae*
19	*Burkholderia pseudomallei*	Blood	*Burkholderia pseudomallei* Accession: OQ076306
20	*Staphylococcus aureus*	Sputum	*Staphylococcus aureus*
21	*Streptococcus constellatus*	Pus	*Streptococcus constellatus*
22	Coagulase-negative Staphylococci	Pus	*Staphylococcus intermedius*
23	*Aeromonas hydrophila*	Abdominal fluid	*Aeromonas hydrophila*
24	*Serratia odorifera*	Bone narrow	***Escherichia coli****
25	*Serratia odorifera*	Pus	*Serratia odorifera*
26	Coagulase-negative Staphylococci	Pus	*Staphylococcus haemolyticus*
27	*Stenotrophomonas maltophilia*	Pus	*Stenotrophomonas maltophilia*
28	*Elizabethkingia meningoseptica*	Sputum	*Elizabethkingia meningoseptica*
29	*Chromobacterium violaceum*	Joint fluid	*Chromobacterium violaceum*
30	*Klebsiella pneumoniae*	Pus	*Klebsiella pneumoniae*
31	*Klebsiella oxytoca*	Sputum	*Klebsiella oxytoca*
32	*Klebsiella pneumoniae*	CSF	*Klebsiella pneumoniae*
33	*Gemella morbillorum*	Pus	*Gemella morbillorum*
34	*Kluyvera intermedia*	Gallbladder	*Kluyvera intermedia*
35	*Chryseobacterium indologenes*	Pus	*Chryseobacterium indologenes*
36	*Streptococcus agalactiae*	Pus	*Streptococcus agalactiae*
37	*Aerococcus urinae*	Pus	*Aerococcus urinae*
38	*Leuconostoc spp.*	Pus	***Streptococcus constellatus****
39	*Aerococcus viridan*	Pus	*Aerococcus viridan*
40	*Achromobacter xylosoxidans*	Pus	*Achromobacter xylosoxidans*
41	*Morganella morganii*	Sputum	*Morganella morganii*
42	*Klebsiella oxytoca*	Sputum	*Klebsiella oxytoca*
43	*Stenotrophomonas maltophilia*	Pus	*Stenotrophomonas maltophilia*
44	*Citrobacter koseri*	CSF	*Citrobacter koseri*
45	*Enterococcus spp.*	Stool	*Enterococcus spp.*
46	*Enterococcus durans*	Stool	*Enterococcus durans*
47	*Salmonella spp.*	Stool	*Salmonella spp.*
48	*Enterococcus faecium*	Urine	*Enterococcus faecium*
49	*Enterococcus faecalis*	Stool	*Enterococcus faecalis*
50	*Enterococcus group D*	Stool	*Enterococcus group D*

** Identification by 16S rRNA sequencing are different from biochemical tests-based method.*

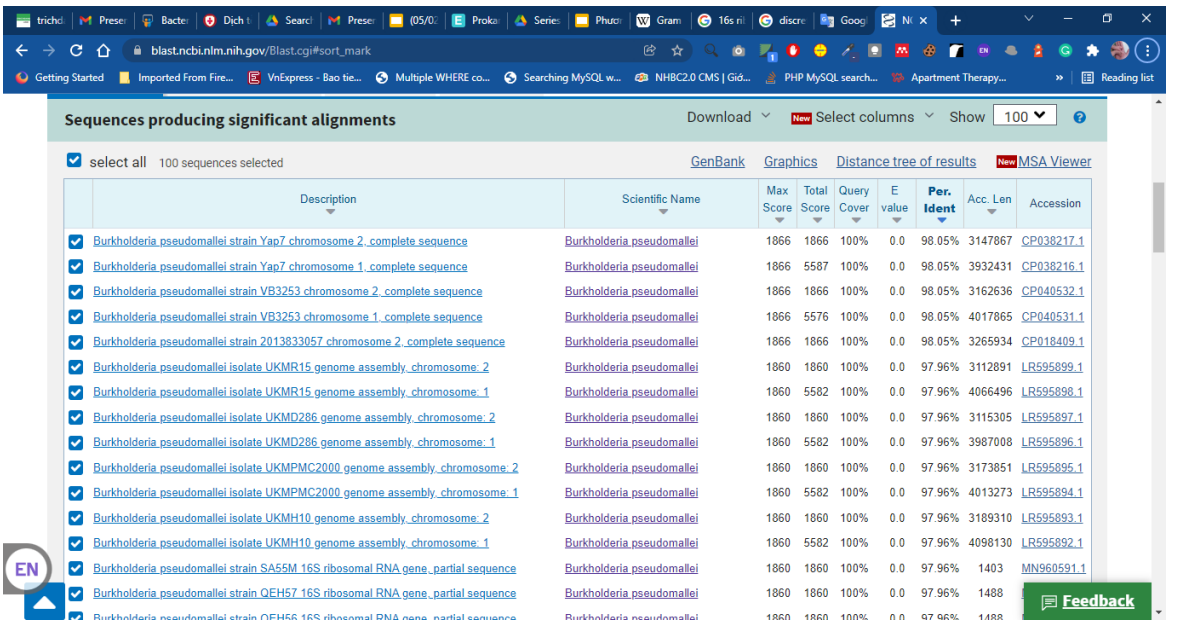

Fig.4. BLAST's result of analyzing raw data of *Burkholderia pseudomallei*'s 16S rRNA sequence on MegaBLAST NCBI

4. DISCUSSION

Accurate and early identification of infectious pathogens is crucial for clinical diagnosis, appropriate antibiotic therapies and transmission control. In microbiology laboratories, a variety of methods are employed to identify bacterial agents and chosen methods rely mainly on the financial capacity and human resources of the laboratory [11,12].

Bacterial identification using enzymatic profile-based were performed by traditional biochemical test tube, API® strips, or automated system (most commonly used VITEK™, BD Phoenix™ instruments). Despite being inexpensive and allowing both quantitative and qualitative information about the diversity of microorganisms present in a sample, these methods are laborious and time-consuming, and results are only observed after several days. In some cases, false positives are obtained, especially when considering similar microbial species. Some properties change due to mutations or biochemical properties that are difficult to determine the results. This leads to incorrect bacterial identification [6]. Matrix-Assisted Laser Desorption Ionization time-of-flight mass spectrometry (MALDI-TOF/MS) is coming a reliable instrument for the identification of microorganisms and clinical diagnosis [11,13]. Species identification using MALDI-TOF/MS focuses on the spectrum between 2000 and 17,000 Daltons, thereby primarily analyzing ribosomal proteins. Ribosomal proteins demonstrate high species diversity and this perfectly serves the requirements for species identification and typing [14].

In our study, all samples were identified by using traditional biochemical tests and API® strips. 50 isolates were successfully identified to genus and species level. Biochemical tests remain critical to bacterial identification. Biochemical tests, however, have some disadvantages. Despite being inexpensive and allowing both quantitative and qualitative information about the diversity of microorganisms present in a sample, these methods are laborious and time-consuming, and results are only observed after several days. In some cases, false positives are obtained, especially when considering similar microbial species [15].

The 16S rRNA gene has been a mainstay of sequence-based bacterial analysis until today. The gene is large enough, with sufficient interspecific polymorphisms of the 16S rRNA gene, to provide distinguishing and statistically valid measurements. The small subunit ribosomal RNA (16S rRNA in prokaryotes) was the phylogenetic marker of choice from an early stage and has been used extensively to date[16]. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene (the initial 500-bp sequence) or at the end of the whole sequence (about the 1500-bp region). Our study shows that there are 3

specimens whose identification results from using 16S rRNA gene sequencing are different from those using conventional microbial culture in the microbiology laboratory. *Burkholderia pseudomallei* is the causative agent of melioidosis. This pathogen is listed in category B bio-threat agents by the Centers for Disease Control and Prevention's Strategic Planning Workgroup because of their availability and potential to cause illnesses with high morbidity and mortality. Therefore, the correct identification of this species is extremely important in clinical treatment [17]. Using the API® 20 NE may lead to confusion in the identification results with *Burkholderia pseudomallei* and affect to treatment process [18]. The sequencing data of *Burkholderia pseudomallei* strains were submitted to Prokaryotic ribosomal RNA (rRNA) database on Genbank NCBI with two accession number: OP890627, OQ076306.

The API® 10S system is designed uniquely for the identification of *Enterobacteriaceae* and non-fastidious, Gram-negative rods which may be encountered in clinical samples [19]. Therefore, obtained result when using the API® 10S system to identify in sample ID 24 is *Serratia odorifera*. The 16S rRNA gene sequencing was matched to *Escherichia coli*. Supplementary tests are sometimes necessary to differentiate between two species. Additionally, some identifications may be extended by the use of the API® 20E which provides 10 extra tests compared to the API® 10S strip.

The bacterial DNA extracted by using boiling method was used. This method is very simple and saves time. This method has been used on most Gram-negative and Gram-positive bacteria and gives results roughly equivalent to those used in commercial kits [7,20–22]. We use pure colonies for DNA extraction, which minimizes the components present in the samples due to being washed prior to boiling. The 27F and 1492R primers have been shown to be the universal primer used in most commercial kits for bacterial identification. The binding sites of the primers at both ends of the 16S rRNA gene are found in most bacteria [12,16,23–26].

5. CONCLUSION

16S rRNA gene sequencing is more sensitive, easier to manage, more accurate and especially for bacteria that are difficult to identify. 16S rRNA sequencing is considered an effective method to early identify pathogens in clinical samples, and this technique is increasingly being used in microbiology laboratories.

ACKNOWLEDGEMENTS

The 16S rRNA gene sequencing in this study was supported by project No. DHH 2020-04-122 of Hue University, Hue, Viet Nam

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