Full Length Research Paper

Analysis of 16S ribosomal RNA gene segments for the diagnosis of Gram negative pathogenic bacteria isolated from urinary tract infections

Abdelaziz Elgaml[1]*, Ramadan Hassan[1], Rasha Barwa[1], Shadi Shokralla[1, 2] and Wael El-Naggar[1]

[1]Microbiology and Immunology Department, Faculty of Pharmacy, Mansoura University, Egypt.
[2]Biodiversity Institute of Ontario, University of Guelph, Canada.

Bacterial 16S ribosomal RNA (rRNA) genes contain nine "hyper-variable regions" (V1–V9) that demonstrate considerable sequence diversity among different bacteria. Species-specific sequences within a given hyper-variable region constitute useful targets for diagnostic assays and other scientific investigations. No single region can differentiate among all bacteria; therefore, systematic studies that compare the relative advantage of each region for specific diagnostic goals are needed. We characterized V6 and V1 to V6 regions of *16S-rRNA* gene in 20 *Pseudomonas*, 15 *Escherichia* and 15 *Klebsiella* species including common urinary tract pathogens using different primer sets specifically designed to amplify different regions of our marker. We determined that the 167 nucleotides-long V6 region is not suitable for distinguishing all bacterial strains to the genus and species level and could not distinguish among all bacterial species especially *Klebsiella* strains. The 1073 nucleotides-long V1 to V6 region is more accurate than identification using sequence analysis of V6 region only and could identify all strains of unknown species Id. We also focused on how much sequencing information is needed for blind identification of bacterial pathogens. In conclusion, the DNA sequencing based method provides a valuable tool for cheap and accurate diagnosis of Gram-negative bacteria in urinary tract infections which can be applicable in other infections and in the diagnosis of other pathogenic bacteria.

Key words: Gram negative bacteria, urinary tract infections, *16S-rRNA* gene, sequencing.

INTRODUCTION

Accurate identification of bacterial isolates is an essential task of the clinical microbiology laboratory (Tang et al., 1998). In clinical laboratories the present means of identification of bacteria rely on phenotypic tests. Traditional phenotypic identification is difficult and time-consuming. In addition, when phenotypic methods are used to identify bacteria, interpretation of test results involves substantial subjective judgment (Bosshard et al., 2004). Phenotypic tests are characterized by potential inherent problems; example, (i) not all strains within a given species may exhibit a common characteristic (Beighton et al., 1991), (ii) the same strain may give different results upon repeated testing (Tardif et al., 1989), (iii) the corresponding database does not enclose newly or not yet described species, and (iv) the test result relies on individual interpretation and expertise. Moreover, small alterations in the execution of an assay may give false test results. Consequently, identification based on phenotypic tests does not always allow an unequivocal identification (Poyart et al., 1998).

*Corresponding author. E-mail: elgamel3a@hotmail.com. Tel: +201006777315. Fax: +20502247496.

In the late of 1900s, genotypic identification is emerging as an alternative or complement to established phenotypic methods. Typically, genotypic identification of bacteria involves the use of conserved sequences within phylogenetically informative genetic targets, such as the small-subunit *16S-rRNA* gene (Wilson et al., 1990; Woese et al., 1990; Kirschner et al., 1993; Maurin et al., 1994).

Sequence analysis of the 16S ribosomal RNA (rRNA) gene has been widely used to identify bacterial species (Patel, 2001) and for diagnosing microbial infections (Trotha et al., 2001; Clarridge III, 2004). Ribosomal RNA molecules comprise several functionally different regions. Some of these are characterized by highly conserved sequences, that is, sequences that can be found among a wide range of bacteria. Other regions show highly variable sequences, that is, nucleic acid sequences that are specific for a species or a genus.

Thus, the *16S-rRNA* sequence of a species is a genotypic feature which allows the identification of microbes at the genus or the species level (Boettger, 1996). In addition, molecular identification offers the possibility of recognizing yet un-described taxa, because ribosomal DNA similarity reflects phylogenetic relationships (Woese and Fox, 1977) and allowing independence from growth conditions (Clarridge III, 2004).

Bacterial *16S-rRNA* genes generally contain nine "hyper-variable regions" (V1–V9) that demonstrates considerable sequence diversity among different bacterial species and can be used for species identification (Van de Peer et al., 1996). Species-specific sequences within a given hyper-variable region constitute useful targets for diagnostic assays and other scientific investigations. These nine hyper-variable regions are flanked by conserved stretches in most bacteria, enabling PCR amplification of target sequences using universal primers (Baker et al., 2003; Munson et al., 2004).

Identification of bacteria based on the sequences of their *16S-rRNA* gene process carried out by extracting genomic DNA from bacteria is amplified and then sequenced. Sequence analysis software compares genes analyzed from unknown bacteria to a proprietary *16S-rRNA* sequence library. The process has been done by using broad-range PCR primers (universal primers) to recognize conserved sequences in a variety of bacteria, while amplifying highly variable regions between the primer binding sites. The amplified segment is sequenced and compared with known databases to identify a close relative (Tang et al., 1998).

Unfortunately, *16S-rRNA* hyper-variable regions exhibit different degrees of sequence diversity, and no single hyper-variable region is able to distinguish among all bacteria. In the present study we conducted a comparative evaluation of the bacterial identification which is based on sequence analysis of 16S Ribosomal RNA gene segments using different Gram negative bacterial clinical isolates from urine specimens collected from Mansoura University Hospitals, Dakahlia governorate, Egypt.

MATERIALS AND METHODS

Clinical isolates

Twenty five isolates of *Pseudomonas*, twenty five isolates of *Escherichia* and twenty five isolates of *Klebsiella* were isolated and identified from Mansoura University Hospitals, Dakahlia governorate, Egypt. All the bacterial isolates were obtained from urine clinical specimens. The specimens were processed immediately using standard procedures and were identified according to the study of Barrow and Feltham (1993) and Collee et al. (1996).

Phenotypic characterization of tested isolates

Pseudomonas isolates

The samples were inoculated on cetrimide agar and nutrient agar plates. The overnight formed colonies at 37°C were examined and identified. The isolates were tested for their negative Gram-stain, colonial morphology, pigment production according to the study of Govan (1996), gelatin liquefaction, oxidase test and oxidation of sugars according to the study of Collee et al. (1996).

Escherichia isolates

The samples were inoculated on plates of nutrient agar and MacConkey's agar and incubated aerobically at 37°C for 24-48 h. The isolates were tested for their negative Gram stain, oxidase test, fermentation of lactose on Macconkey's agar, methyl red test, Voges Proskauer's reaction, indole production and citrate utilization. All tests were performed according to the study of Barrow and Feltham (1993).

Klebsiella isolates

The samples were inoculated on plates of nutrient agar, blood agar and MacConkey's agar and incubated at 37°C for 24-48 h. The isolated colonies were tested for their colonial morphology and negative Gram stain. Oxidase test, citrate utilization, methyl red test, Voges-Proskauer test, indole test, sugar fermentation and urease test were performed according to the study of Collee et al. (1996).

Molecular experimental design

Genomic DNA extraction

The genomic DNA of 50 isolates (20 *Pseudomonas*, 15 *Escherichia* and 15 *Klebsiella isolates*) were prepared using QIA amp® DNA mini Kit Cat. No. 51304 supplied by Qiagen Inc. according to the manufacturer instructions for bacteria 2003. DNA was eluted by adding 50 µl Qiagen EB buffer (10 mM Tris-Hcl, pH 8.5) and visualized by electrophoresis on horizontal gels containing 1% agarose and Invitrogen 1 kb ladder.

Primer design

The primers used in this study were specifically designed before in Biodiversity Institute of Ontario, University of Guelph, Canada. Bact. 16S V6 Fw and Bact. 16S V6 Rv primer set to amplify the V6 region of *16S-rRNA* gene of the bacterial isolates with a size of 167 bp and Bact. 16S V1 Fw and Bact. 16S V6 Rv primer set to amplify from V1

Table 1. Specific amplification primer sets for the V6 and V1 to V6 regions of *16S-rRNA* gene (Sundquist et al., 2007).

Gene name	Type	Sequence
V6 region of	Fw	5`…AAACTCAAATGAATTGACGG
16S-rRNA	Rv	5`… ACGAGCTGACGACATCCATG
V1 to V6 region of	Fw	5'… AGAGTTTGATCATGGCTCAG
16S-rRNA	Rv	5`… ACGAGCTGACGACAGCCATG

Fw: Forward primer, Rv: Reverse primer.

to V6 region of *16S-rRNA* gene of the bacterial isolates with a size of 1073 bp (Table 1) (Sundquist et al., 2007).

PCR

The PCR reaction mixture was composed of 12.5 µl GEN-Master Mix (2x) (dNTPs, MgCl2 and polymerase), 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 5 µl of the isolated DNA template and 5.5 µl of nuclease free water were added for a total of 25 µl per reaction. We performed PCR in a Mastercycler epgradient S thermalcycler (Eppendorf, Mississauga, ON, Canada) including a negative control reaction with each primer set employing Platinum *Taq* polymerase supplied by Invitrogen (Burlington, ON, Canada). The amplification cycling was performed as follows, primary initial activation step at 95°C for 5 min, followed by 40 cycles of 94°C for 15 s, 46°C for 30 s, and 72°C for 30 s, followed by final extension of 72°C for 5 min. The generated amplicons were visualized on 1.5% agarose gel electrophoresis stained with ethidium bromide and illuminated under UV transilluminator. Sequences were obtained using ABI 3730XL Big dye terminator Sanger sequencer (Applied Biosystems, Foster City, CA) (Sanger et al., 1977) and edited by the Codon-code aligner software, version 3.0.3.

Sequence analysis and neighbor-joining tree construction

Sequence analysis software compares genes analyzed from unknown bacteria to a proprietary *16S-rRNA* sequence library. The amplified regions of *16S-rRNA* gene of tested bacterial isolates were compared with known databases to identify a close relative. The amplified segments' sequences were compared online with standard *16S-rRNA* sequences of bacteria in the gene bank at website (http://www.ncbi.nlm.nih.gov/) using nucleotide BLAST (Basic Local Alignment Search tool).

After the accurate identification of each tested isolate to the species level, alignment and comparison of each V1 to V6 region of *16S-rRNA* gene sequences among each group of tested bacterial isolates with each other were performed and genetic diversity trees were constructed using megablast aligner software version 4.1 at website (http://www.megasoftware.net).

RESULTS AND DISCUSSION

Phenotypic identification of bacterial isolates (Biochemical identification)

By using biochemical identification methods, all the 75 clinical isolates were identified to the genus level as, 25 isolates of *Pseudomonas*, 25 isolates of *Escherichia* and 25 isolates of *Klebsiella* species.

PCR amplification of V6 region and V1 to V6 region of *16S-rRNA* gene

The V6 and V1 to V6 regions of *16S-rRNA* gene were amplified from genomic DNA of 20 isolates of *Pseudomonas*, 15 isolates of *Escherichia* and 15 isolates of *Klebsiella*. The generated amplicons were visualized with a size of 167 and 1073 base pair respectively as illustrated in Figure 1.

Sanger sequences FASTA files and sequence analysis of tested isolates

Nucleotide sequencing and data analysis of V6 and V1 to V6 regions of *16S-rRNA* gene were carried out for the amplified PCR products. The obtained sequences were converted to Fasta file format and were compared online with standard *16S-rRNA* sequences of bacteria in the gene bank using nucleotide BLAST to identify close relative.

The identification using sequence analysis of V6 region of *16S-rRNA* gene could not identify all isolates to the species level. On the other hand, the identification using sequence analysis of V1 to V6 region of *16S-rRNA* gene was more accurate than identification using sequence analysis of V6 region of *16S-rRNA* gene and could identify all isolates of unknown species Id. This in accordance with the study of Kim et al. (2011), who showed that, by using longer reads spanning several hyper-variable regions gives accurate identification of bacteria and can produce results comparable with those obtained using full-length *16S-rRNA* reads.

Pseudomonas

Regarding the 20 *Pseudomonas* tested isolates; they could be classified into three groups, 15 isolates of *Ps. aeruginosa*, 2 isolates of *Ps. putida* and 3 isolates of *Pseudomonas* without species Id.

The sequencing of V1 to V6 region of *16S-rRNA* gene could discover the species Id of the three *Pseudomonas* isolates identified without species Id using sequencing of V6 region of *16S-rRNA* gene and the net identification
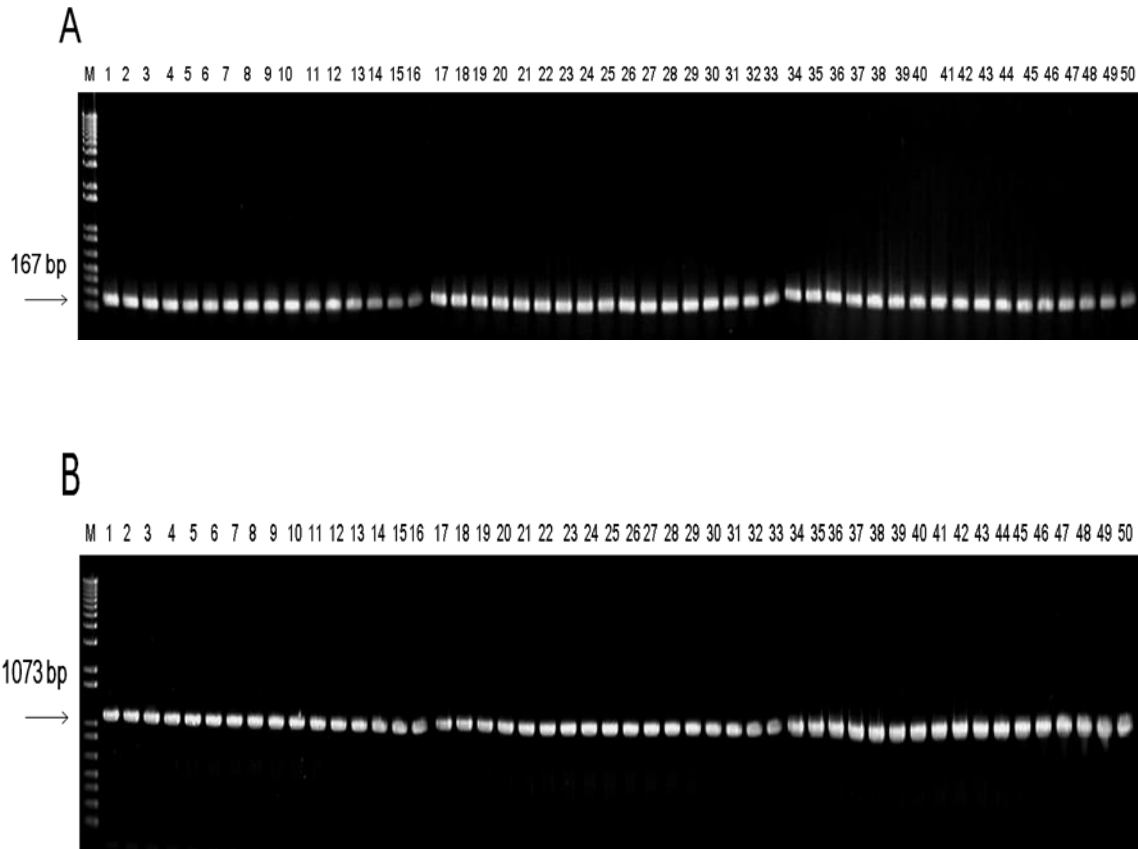
Figure 1. Agarose gel electrophoresis of V6 and V1 to V6 regions of *16S-rRNA* gene amplicons. A: universal primers amplifying V6 region; B: universal primers amplifying V1 to V6 region. Lane M is 1Kb DNA Ladder, lanes 1 to 20 are *Pseudomonas* isolates, lanes 21 to 35 are *Escherichia* isolates and lanes 36 to 50 are *Klebsiella* isolates.

results of the 20 tested *Pseudomonas* isolates; 15 isolates of *Ps. aeruginosa*, 2 isolates of *Ps. putida* and 3 isolates of *Ps. fluorescens*.

Escherichia

All *Escherichia* confirmed as *E. coli* by the molecular approach except *Escherichia* isolates No. 10 and 11, their sequencing results showed that these isolates were mixed samples with a majority of *E. coli* and a minority of *Klebsiella* without species Id.

All *E. coli* identified using sequencing of V6 region of *16S-rRNA* gene confirmed as *E. coli* by sequencing of V1 to V6 region of *16S-rRNA* gene, moreover sequencing of V1 to V6 region of *16S-rRNA* could discover the species Id of the two *Klebsiella* species which were mixed with *E. coli* in the isolates No.10 and 11 and identified as *K. pneumonia*.

Klebsiella

Regarding the *Klebsiella* isolates, out of the 15 tested isolates, 11 isolates were identified as Unknown Enterobacterial Bacterium and 4 *Klebsiella* isolates

without species Id.

The sequencing of V1 to V6 region of *16S-rRNA* gene could discover the species Id of *Klebsiella* isolates. Out of the 15 tested isolates, the 11 isolates that were identified as Unknown Enterobacterial Bacterium using sequencing of V6 region of *16S-rRNA* gene was confirmed as *K. pneumonia* using sequencing of V1 to V6 region of *16S-rRNA* gene. Interestingly, the 4 *Klebsiella* isolates without species Id were classified as 2 isolates of *K. oxytoca* and 2 *Klebsiella* isolates gave a close hit of 96.2% to *Klebsiella* species in the gene bank without species Id.

Comparison between *16S-rRNA* V6 region sequencing and *16S-rRNA* V1 to V6 region

sequencing methods of identification

The comparison is shown in Tables 2 and 3. Our results showed that, sequence analysis of conserved "house keeping" genes such as the bacterial *16S-rRNA* gene are increasingly being used to identify bacterial species in clinical practice and scientific investigations. In the case of *16S-rRNA* analysis, species identification is easiest when

Table 2. V6 region versus V1 to V6 region sequencing identification of *Pseudomonas* isolates.

Strain ID	16S-rRNA V6 region sequence Id	*16S-rRNA* V1 to V6 region sequence Id	Geographical Source	Strain ID	16S-rRNA V6 region sequence Id	*16S-rRNA* V1 to V6 region sequence Id	Geographical Source
1	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(UNC)	11	*Pseudomonas Spp.*	*Pseudomonas fluorescens*	(MUH)
2	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(MUH)	12	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(UNC)
3	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(UNC)	13	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(UNC)
4	*Pseudomonas putida*	*Pseudomonas putida*	(MUH)	14	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(MUH)
5	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(MUH)	15	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(PUH)
6	*Pseudomonas Spp.*	*Pseudomonas fluorescens*	(MUH)	16	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(PUH)
7	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(PUH)	17	*Pseudomonas putida*	*Pseudomonas putida*	(PUH)
8	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(PUH)	18	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(PUH)
9	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(PUH)	19	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(PUH)
10	*Pseudomonas Spp.*	*Pseudomonas fluorescens*	(MUH)	20	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	(UNC)

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most or the entire gene can be sequenced. However, DNA sequencing is impractical in medical diagnostics where speed is often of the essence. Species-specific sequences can be identified very rapidly in assays that combine nucleic acid amplification and a sequence-specific probe of the amplified product. These approaches are usually only able to query short DNA sequences; therefore, it is important to identify the regions within the target gene that supply the most taxonomic information in the smallest stretch of nucleotides. Additional benefits of small amplicon size may include increased assay sensitivity and applicability to archival specimens.

Since The hyper-variable 167 nucleotides-long V6 region (907–1073) is the shortest hyper-variable region with the maximum degree of sequence heterogeneity (Chakravorty et al., 2007), so the sequences obtained in this study may be added to the existing sequences in the gene bank to facilitate the identification process especially in the case of *Klebsiella* isolates and *Pseudomonas fluorescens* isolates.

Interestingly our results put the light on the power of *16S-rRNA* sequence analysis to identify novel pathogens and indicate the criteria for identification using *16S-rRNA* sequence analysis where, identification to the species level was defined as a *16S-rRNA* sequence similarity of more than or equal to 99% with that of the prototype strain sequence in the gene bank; identification to the genus level was defined as a *16S-rRNA* sequence similarity of more than or equal 97% with that of the prototype strain

sequence in the gene bank. A failure to identify was defined as a *16S-rRNA* sequence similarity score of lower than 97% with those deposited in the gene bank at the time of analysis (Drancourt et al., 2000). Therefore it's suggested that the two *Klebsiella* strains No. 9 and 12 lacking species Id are two novel *Klebsiella* species as they are 96.2% similar to *Klebsiella* species in the gene bank and this is in accordance with the study of Petti (2007), who stated that *16S-rRNA* gene sequence data on an individual strain with a nearest neighbor exhibiting a similarity score of lower than 97% represents a new species.

The obtained identification results of *Escherichia* samples No. 10 and 11 show the ability of *16S-rRNA* sequence data analysis for diagnosis of mixed samples with major and minor or fastidious non-cultivable microorganisms as the sequencing chromatograms will show mixed signals and this is in accordance with study conducted by Rantakokko et al. (2000), who reported that, although conventional culture methods of identification detected *Staphylococcus* only in pus clinical sample, further *16S-rRNA* analysis suggested the presence of other bacterial species in addition to *Staphylococci*. On the other hand, other studies showed that mixed cultures led to 1% of *16S-rRNA* based identification failures, either because the wrong colony was selected on subculture or because more than one bacterial species was inadvertently included in the amplification, resulting in ambiguous *16S-rRNA* data. These studies recommend

Table 3. V6 region versus V1 to V6 region sequencing identification of *Escherichia* and *Klebsiella* isolates.

Strain ID	*16S-rRNA* V6 region sequence Id	*16S-rRNA* V1 to V6 region sequence Id	Geographical Source	Strain ID	*16S-rRNA* V6 region sequence Id	*16S-rRNA* V1 to V6 region sequence Id	Geographical Source
4	*Escherichia coli*	*Escherichia coli*	(MUH)	1	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)
6	*Escherichia coli*	*Escherichia coli*	(MUH)	3	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)
8	*Escherichia coli*	*Escherichia coli*	(MUH)	4	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)
9	*Escherichia coli*	*Escherichia coli*	(MUH)	5	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)
10	*E.coli Klebsiella* spp.	*E.coli Klebsiella pneumoniae*	(MUH)	6	*Klebsiella* spp.	*Klebsiella oxytoca*	(UNC)
11	*E.coli Klebsiella* spp.	*E.coli Klebsiella pneumoniae*	(MUH)	7	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)
12	*Escherichia coli*	*Escherichia coli*	(MUH)	8	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)
14	*Escherichia coli*	*Escherichia coli*	(PUH)	9	*Klebsiella* spp.	*Klebsiella spp.*	(UNC)
15	*Escherichia coli*	*Escherichia coli*	(MUH)	10	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)
16	*Escherichia coli*	*Escherichia coli*	(MUH)	11	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(PUH)
17	*Escherichia coli*	*Escherichia coli*	(MUH)	12	*Klebsiella* spp.	*Klebsiella spp.*	(PUH)
21	*Escherichia coli*	*Escherichia coli*	(MUH)	14	*Klebsiella* spp.	*Klebsiella oxytoca*	(PUH)
22	*Escherichia coli*	*Escherichia coli*	(PUH)	15	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)
23	*Escherichia coli*	*Escherichia coli*	(PUH)	16	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)
24	*Escherichia coli*	*Escherichia coli*	(PUH)	17	Unknown Enterobacterial Bacterium	*Klebsiella pneumoniae*	(UNC)

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that more attention should therefore be paid to achieving a pure culture prior to *16S-rRNA*-based identification (Drancourt et al., 2000). Alternatively, mixed *16S-rRNA* sequences could be separated by, for example, denaturing gradient gel electrophoresis prior to sequencing for more accurate identification (Teske et al., 1996).

Moving forward with the sequencing results, in this study, we could use the phylogeny to determine the relatedness between different isolates and the source of isolation Figure 2.

From the phylogenetic trees, it is clear that both *Pseudomonas* and *Escherichia* species were shared the

intra-species genetic distance between individual isolates of 0.005. On the other hand, the intra-species genetic distance between individual isolates of the *Klebsiella* species was higher up to 0.01. These findings can show the high rate of evolution and the genetic variation in *Klebsiella* species compared to *Pseudomonas* and *Escherichia* species.

In conclusion, our results showed that identifying bacteria isolated in the clinical laboratory by DNA sequencing techniques can improve clinical microbiology by better identifying poorly described, rarely isolated, or biochemically aberrant strains. *16S-rRNA* gene sequences allow bacterial
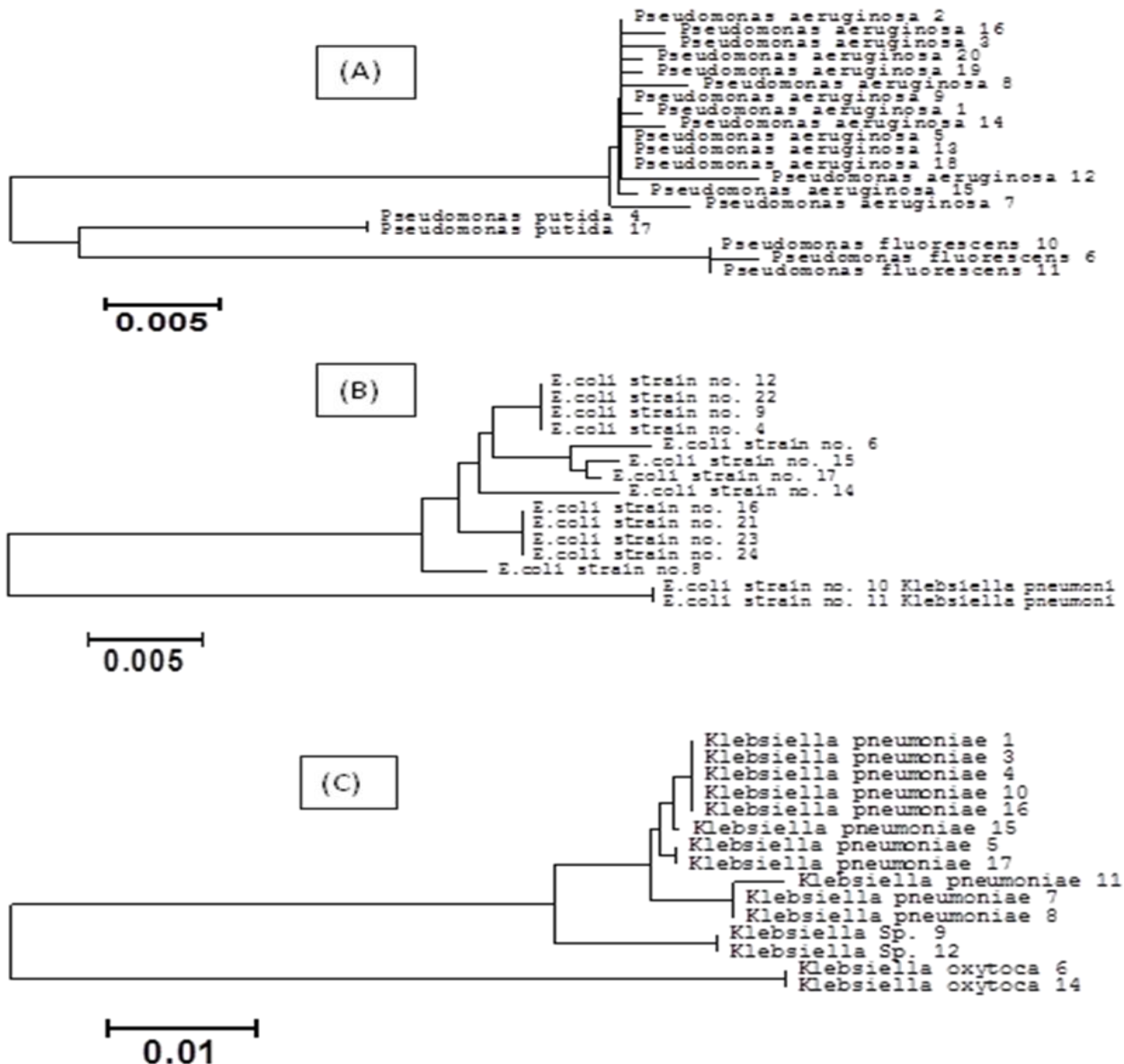
Figure 2. Phylogenetic neighbor joining trees A, B and C of *16S rRNA* amplified from tested isolates of *Pseudomonas*, *Escherichia* and *Klebsiella* species respectively.

identification that is more robust, reproducible, and accurate than that obtained by phenotypic testing. The test results are less subjective. Sequence analysis of *16S-rRNA* gene can lead to the discovery of novel pathogens, the identification of non-cultured bacteria and allowing independence from growth conditions. In addition our results can enrich the gene bank by the newly obtained V6 region sequences in the present study to facilitate the identification process.

Moreover, the DNA sequencing technologies have been evolved in the last 20 years from the dye termination to Pyrosequencing ending by single molecule sequencing. The race of different sequencing technologies will grantee high accuracy and low cost to be efficient and suitable for

medium and small microbiological laboratories.

Authors' contributions

EA, HR, SS and EW participated in the study design. EA, BR participated in sample collection and did the microbiological work. EA and SS did the molecular work. EA and SS participated in data analysis and wrote the manuscript. All authors read and approved the final manuscript.

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