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Research Article
A Novel Mutation in the Conserved Region of 16S rRNA Genes of *Escherichia coli* Clinical Isolates

[1,2]Ahmed S. Kabbashi, [1]Arwa M. Hassan, [2]Mohammed I. Garbi, [3]Hisham N. Altayb, [4]Salah Eldin G Elzaki, [5,6]Ashraf Siddig Yousif and [1]Ehssan H. Moglad

[1]Department of Microbiology, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), P.O. Box 2404, National Center for Research, Khartoum, Sudan
[2]Department of Parasitology, Faculty of Medical Laboratory Sciences, International University of Africa, Khartoum, Sudan
[3]Faculty of Medical Laboratory Science, Sudan University of Science and Technology, Sudan
[4]Department of Epidemiology, Molecular Epidemiology Laboratory Biology, Tropical Medicine Research Institute, National Centre for Research, Khartoum, Sudan
[5]Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology and Harvard University, 400 Technology Square, Cambridge 02139, USA
[6]Department of Immunology and Biotechnology, Tropical Medicine Research Institute, National Centre for Research, Khartoum, Sudan

Abstract

Background and Objective: New pathogens in clinical samples that are suspected to carry bacterial infection can be effectively characterized by sequence analysis of the rrs gene (16S rRNA). This study aimed to identify and characterize *Escherichia coli* isolated from clinical samples (wound, urine and stool) by sequencing analysis of 16S rRNA. **Materials and Methods:** *Escherichia coli* isolated from clinical samples identified using enrichment selective media and biochemical tests. The DNA was isolated from *E. coli* by Chelex® method and subsequently, specific primers were used to amplify 16S rRNA genes through a conventional PCR technique. The amplified PCR product was sequenced by Macrogen Campany, Korea. The chromatogram sequences visually analyzed using Finch TV program version 1.4. The similarity and identity of the nucleotide sequence from the isolated strains compared with sequences published in the NCBI database applying the local alignment search tool BLASTn. A Phylogenetic tree generated via the Phylogeny.fr software. **Results:** Sequencing revealed that isolates 76 and 77 contain a novel inserted G at position 884 of reference from France (FJ544921), China (KU156692), Portugal (JQ781608), Argentina (FJ997269), Korea (FJ4638197), China (FJ803886), USA (KF574802), Korea (FJ405334), Pakistan (KR822241) and Belgium (KJ016265). **Conclusion:** This is one of the very few documents that shows the sequencing data of *E. coli* isolated from Sudanese patients and revealed a novel insertion mutation in the conserved region of 16S rRNA genes. Also, this study raises the important issue of whether conserved regions are totally conserved or not, which might have implications for the use of 16S rRNA as a biomarker, therefore, more studies are needed to confirm this result.

Key words: 16S rRNA, *E. coli*, alignment, phylogenetic tree, sequencing

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Corresponding Author: Ehssan H. Moglad, Department of Microbiology, Medicinal and Aromatic Plants and Traditional Medicine Research Institute, P.O. Box 2404, National Center for Research, Khartoum, Sudan Tel: 00249-922283316

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Escherichia coli is a Gram-negative, facultative anaerobic, non-sporulating bacterium, belongings to the (Enterobacteriaceae family). A Wide distribution of *E. coli* has been documented in the intestinal microbiota of humans and other homoeothermic animals as well as their environment when it is polluted with feces[1]. The *E. coli* strains are commonly restricted to the intestinal lumen but can lead to infectious diseases in immunosuppressed hosts or in case of the violation of the gastrointestinal walls. Furthermore, clones that pathogenically adapted can create *E. coli* strains that lead to disease[2]. The pathogenicity of *E. coli* strains also extends to potential extraintestinal infection for instance, respiratory diseases in birds and pyometra and urinary tract infection in canines[3].

E. coli is presently considered the most important example of Gram-negative bacteria linked to various diseases due to several mechanisms of pathogenicity[2]. The conventional process of identifying the pathogenic bacteria was generally achieved by bacteria isolation, Gram staining and culturing in addition to biochemical approaches; these methods have been the most commonly used criteria for bacteria identification[4]. However, as these conventional methods are not rapid, reliable and efficient enough to detect and characterize the pathogenic strains, the application of the molecular biology-based approaches for more effective detection and characterization became more popular[5,6].

Now a days, a combination of tools from molecular biology and bioinformatics have advanced bacterial identification and characterization. Sequence analysis of the 16S rRNA gene for instance is applied to recognize different species of bacteria[6]. In general, 16S-rRNA genes consist of nine hypervariable regions (V1-V9) that display significant sequence diversity between diverse species of bacteria[7-9]. Different regions in the genome with different functions form 16S rRNA molecules; while sequences in some of these regions are highly conserved, sequences are substantially variable in others, with nucleic acid sequences particular to one genus or species. Hence, microbes can be discovered on the level of genus or species through the genotypic quality of the sequence[10].

There are many studies that applied 16S rRNA for bacterial characterization and phylogenetic tree relationships[5,6,11-14]. By employing 16S rDNA sequencing, 29 out of 215 novel bacteria species from human specimens between 2001-2007 have been discovered to belong to novel genera[14]. The method was also used for the diagnosis of microbial infections[5,15]. This shows that 16S rRNA sequencing is an important tool in clinical microbiology for identifying bacterial isolates and discovering novel mutations in bacteria[14].

Molecular identification using 16S rRNA gives furthermore the opportunity to recognize taxa that were not described yet. Since similarity indicates phylogenetic relationships and permits independence from growth conditions, the 16S rRNA gene serves as a housekeeping genetic marker that facilitates the study of bacterial taxonomy and phylogeny[5,12,15].

In Sudan, 16S rRNA sequencing is generally known and applied[16,17] but there is still a scarcity of bacterial sequencing data. At the same time, bacterial infections, for instance from *E. coli* are a significant public health issue. Therefore, this study used a clinical specimen from Sudanese patients to characterize *E. coli* isolates by sequencing of the 16S rRNA *E. coli* strain.

MATERIALS AND METHODS

Study area and clinical isolates: This study was carried out in Khartoum state, Sudan, during the period from January till June, 2016; specimens were collected from Soba Hospital University and Al Ribat Hospital. Study subjects were patients suffering from bacterial infections. Bacteria were isolated and identified following standard biochemical tests[18]. Forty *E. coli* isolates were collected from different sites of infections (29 from urine culture, 2 from stool culture and 9 from wound infection). Control strain in all procedures was *E. coli* (ATCC 25922). All the procedures were carried out at Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), Department of Microbiology.

DNA extraction: Bacterial DNA was extracted by the Chelex® method. The extraction process involved boiling suspension bacteria in a 6% suspension of deionized water and Chelex® 100. The suspension was then vortexed and centrifuged, separating the resin and cellular debris from the supernatant that contained the DNA and afterward stored at -20°C. It was then used for conventional PCR[19,20].

Conventional Polymerase Chain Reaction (PCR): Bacterial genomic DNA served as templates for PCR amplification of the 16S rRNA gene using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') as forwarding primer and 1495R (5'-CTACGGCTACCTTGTTAC GA-3') as reverse primer in a total reaction volume of 25 µL, in

detail 5 µL Master mix (iNtRON Biotechnology, Seongnam, Korea), 1.0 µL forward primer, 1.0 µL reverse primer, 5 µL DNA and 13.0 µL nuclease-free water. The PCR amplifying procedure was as follows: Initial denaturation 5 min at 94°C, 30 cycles of 1 min denaturation at 94°C, annealing for 1 min at 58°C, an extension for 2 min at 72°C and final extension for 10 min at 72°C performed on a Bio-Rad automatic thermal cycler. The PCR was duplicated for every sample to confirm. The products of amplification were checked through running on 1.5% dissolved in 1X TBE Agarose gel electrophoresis[21]. To identify the specific amplified products, they were photographed under an ultraviolet light machine (Transilluminator; Uvite, UK) and compared with the 100 bp standard DNA ladder (Fig. 1). The remnants of PCR products were stored at -20°C until sequencing.

Sequencing of the 16S rRNA genes: Sequencing of the coding sequence of 16S rRNA genes was performed by Macrogen Company (Seoul, South Korea). The ABI Genetic Analyzer (Applied Biosystems) conducted the purification and standard forward and reversed sequencing of 16S rRNA. Only 15 isolates were selected randomly for DNA sequencing due to financial constraints.

Bioinformatics analysis: The relationship of the 16S rRNA gene sequences to other 16S rRNA gene sequences available in the NCBI GenBank database analyzed with the BLASTn algorithm[22]; highly similar sequences were found to be accession numbers KX108935.1, KT943978.1, JN609194.1, KX214108.1, KU672378.1, KU764451.1 and FJ648815. BioEdit software version[23] 7.0.9.1 utilized for ClustalW multiple alignment to compare the study isolates sequence with other highly similar published sequences from different selected countries (France (FJ544921), China (KU156692), Portugal (JQ781608), Argentina (FJ997269), Korea (FJ4638197), China (FJ803886), USA (KF574802), Korea (FJ405334), Pakistan (KR822241) and Belgium (KJ016265)). The phylogenetic and molecular evolutionary analyses were done with the online software Phylogeny.fr as well as MEGA6 software (version 0.06) to confirm [24].

Data availability: The obtained 16S rRNA gene nucleotide sequences were submitted to the GenBank database; registered under the accession numbers KX650757, KX650758, KX650759, KX650760 and KX650761.

RESULTS

Sequencing of the 16s rRNA genes: In the 40 clinical isolates, *Escherichia coli* were confirmed in 72.5% from urine samples, 22.5% from wound infection samples and 5% from the stool samples. All samples were 16S rRNA positive; 15 representative isolates were selected for 16S rRNA sequencing, 5 out of these went further for sequence analysis because they demonstrated clear chromatogram results on Fitch TV. These five samples were from community origin, including isolate 73 from stool culture, isolates 76 and 77 from urine culture and isolates 78 and 80 from wound culture.

16S rRNA genes' sequencing analysis: Their sequence analysis by BLASTn revealed that isolates 73, 76 and 77 were 100% matched with samples from France FJ544921, China KU156692, Portugal JQ781608, Argentina FJ997269 and Korea FJ4638197, while isolate 78 was 100% matched with samples from China FJ803886, USA KF574802 and Korea FJ405334 (Fig. 2). Isolate 80 was 100% identical with samples from Pakistan KR822241 and Belgium KJ016265 (Fig. 3). However, isolates 76 and 77 were revealed to contain a new

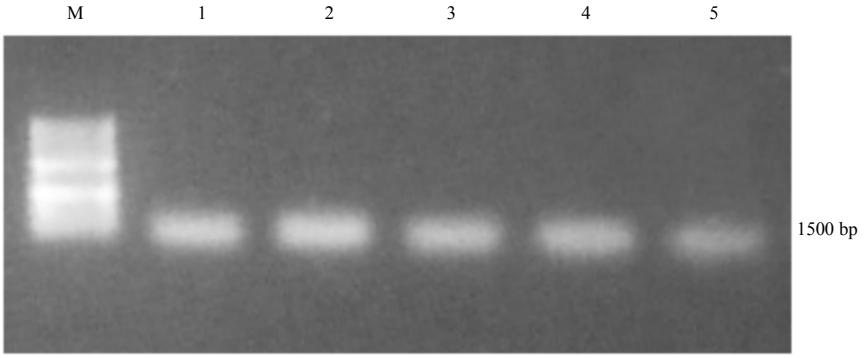

Fig. 1: A representative agarose gel electrophoresis of PCR products after amplification of the16S rRNA gene
Lanes: 1-5 positives 16S rRNA PCR products and (M) a molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas)

Fig. 2: BioEdit multiple sequence alignment of the 5 isolates and other selected strains from the database. Isolate 78 is 100% matching with references from China, USA and Korea

Fig. 3: BioEdit multiple sequence alignment of the 5 isolates and other selected strains from the database. Isolate 80 was 100% identity with samples from Pakistan KR822241 and Belgium KJ016265

insertion of G in position 884 (Fig. 4). A phylogenetic tree of the 16S rRNA gene was constructed which showed the relationship between strains from Sudan and other countries as shown in Fig. 5.

DISCUSSION

The result revealed that *E. coli* was dominant in the urinary cultures affecting all age groups. This is in agreement with previous studies which reported that urinary tract infections are among the most common infections worldwide[25].

The present study has also demonstrated the suitability of partial 16S rRNA gene sequencing for the identification of bacterial strains; 2 isolates showed a novel insertion mutation in the 16S rRNA gene at position 884. The mutation position is in the Domain II at the conservation region between the hypervariable region V and VI. According to the previous

Fig. 4: BioEdit multiple sequence alignment of the 5 isolates and other selected strains from the database
There is new sequencing for isolate 76 and 77 which revealed new insertion of G in position 884

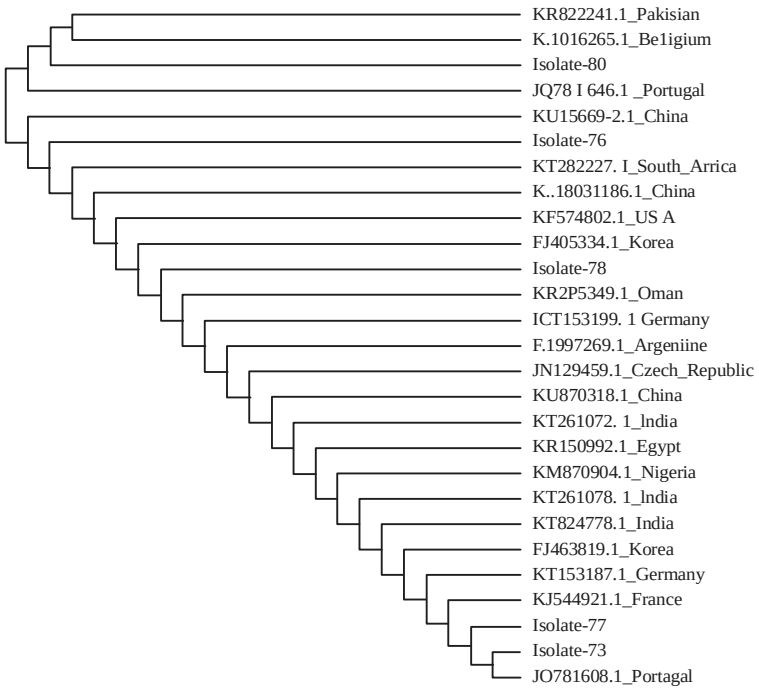

Fig. 5: Phylogenetic tree of the 16S rRNA gene and other 16S rRNA genes obtained from the database
The tree was divided into two branches the isolate 80 at the upper branch and isolate 76 and 76, 77 and 73 at the lower branch

study, the 9 hypervariable regions spanned nucleotides 69-99, 137-242, 433-497, 576-682, 822-879, 986-1043, 1117-1173, 1243-1294 and 1435-1465 for V1 through V9, respectively[9]. This point mutation within rRNA is a novel, it does not match any references in the NCBI database or other isolates sequences. These isolates; 76 and 77 were from urine culture from patients who suffered from recurrent UTI and resistant to Norfloxacin, Ceftazidime and Ceftrizone according to the hospital's lab manager. The mechanism seems to be restricted to organisms and might be due to prolonged exposure to the antibiotics in individual patients. It will be interesting to determine whether similar rRNA mutations are present in other bacterial multi-drug resistant pathogens.

Most importantly, this result suggested that conserved regions of the gene may not be as conserved as expected,

which agreed with a previous finding that conserved regions of the 16S rRNA gene reveal significant variation that has to be considered when using this gene for identification. Moreover, nucleotide frequency analysis of consensuses exhibited that small segments or single nucleotide positions were far from being constant within conserved regions[26]. While the result supported the use of 16S rRNA for the identification of pathogenic bacteria in the clinical laboratory and discovery of novel mutations, such mutations in the conserved region raises a serious concern about how long this gene will serve as universal gene for all bacteria; more and more mutation in the consensuses regions may lead to stopping using of 16S rRNA as biomarker in the near future.

The finding that sequencing of 16S rRNA is a successful tool for characterization of *E. coli* corresponds to the previous development of the 16S rRNA as a PCR target for detection of *E. coli* in Rainbow Trout[27], the finding of a novel mutation agreed with studies reporting 16S sequencing to be usable for reclassifying bacteria into new species or genera[28,29] or to characterize new species which were not successfully cultured before[30].

The authors supported, accordingly, Drancourt's procedures and proposed criteria for complete 16S rRNA gene sequencing as a reference method for bacterial identification whenever feasible[31]. The study benefitted from characteristics of the 16S rRNA genes making their sequencing a highly adept tool for bacterial phylogeny and taxonomy, among them high conservation within and among the species of the same genus; the stability of function over time connected to its existence as a multigene family or operons, suggesting random sequence changes as a more accurate measure of time; and the gene's size of 1500 bp rendering it accessible to bioinformatics analysis[6].

The study was limited in sample size, which started with forty and ended with only five samples, mainly due to constraints in financial sources and in the quality of available chromatographs.

CONCLUSION

This study discovered the novel insertion mutation of G in the 16S rRNA gene of *E. coli* bacteria in the conserved region at position 884 that can be beneficial for discovering new bacterial strains. This study will help the researchers to uncover the critical areas of limited bacterial sequencing data from Sudan that many researchers were not able to explore. Thus a new theory on the high prevalence of bacterial infections may be arrived at.

COMPLIANCE WITH ETHICAL STANDARDS

The Ethical and Scientific Committee of the Medicinal and Aromatic Plants and Traditional Medicine Research Institute, National Center of Research, Khartoum, Sudan, approved this study (approval number 02-16). It certified that acceptable ethical standards for the conduct of research with patients were followed in a way that protects their confidentiality and privacy. The hospital microbiological laboratories (laboratory manager) obtained the patients' informed consent to collect samples during routine procedures. All samples were anonymized; personal details were not relevant for the current study and thus not retained.

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