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Isolation, Identification and Comparative Analysis of 16S rRNA of Multidrug-resistant Bacteria Clinically Isolated from Al Qassim Region Hospitals in Saudi Arabia

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Abstract

Objective: This study concluded that the antibiotic resistance and gene transfer across bacterial strains in the hospital setting are two possible explanations for the observed sequence changes in the target microorganisms.

Methods: The 16S rDNA genes of all isolates were effectively amplified using PCR, and detailed identification results were derived from GenBank databases. The BLAST search resulted in the classification of 28 isolates into five strains. The GC content of bacterial sequences varies greatly between single species.

Results: 16S rDNA was utilized to identify bacterial species from isolates strains results demonstrated that identification of bacterial strains. Sequences varied between and within strains, also, variation are noticed in genomic nucleotide content of isolated and identified strains. Results of the present study demonstrated that the observed heterogeneity in the sequences of our target bacterial strains may be linked to antibiotic resistance and gene transfer between bacteria that evolved as a result of the hospital environment.

Conclusion: This study concluded that antibiotic-resistant bacteria are capable of transferring copies of their DNA encoding a resistance mechanism to other bacteria, even those that are distantly related to them. The observed heterogeneity in the sequences of our target bacterial strains may be linked to antibiotic resistance and gene transfer between bacteria that evolved as a result of the hospital environment.

Keywords: Multidrug-resistant, molecular identification, RNA, ribosomal, 16S, polymerase chain reaction

Introduction

Because of the high prevalence of infectious diseases, poor hygiene, and inadequate health systems, developing nations bear an outsized burden of antimicrobial resistance (AMR). In developing nations, a large proportion of hospital-acquired infections is a significant concern since they continue to have an impact on treatment outcomes and the evolution of antimicrobial resistance (AMR). If existing AMR conditions persist unchecked, more than 10 million fatalities will be caused by AMR by 2050. Pathogens such *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*.

Enterobacteriaceae are the most common ones that cause AMR because of their high risk of spreading among patients. The continual over- and imprudent use of antimicrobials has enriched for bacteria that are innately or acquired resistant to antibiotics. More and more clinical emphasis is being placed on bacteria like *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Acinetobacter baumannii* species because of their inherent resistance to numerous treatments as well as their capacity for developing high levels of MDR.[1] The creation of a multidrug-resistant (MDR) hospital environment microbial for particular applications is a necessary component of knowing how antibiotic resistance genes are transferred across various bacterial species. MDR microorganisms are difficult to isolate and identify because they display extremely specialised traits that enable them to exist in their natural settings and so cannot be cultivated using ordinary laboratory procedures.[2] Competition for space and resources in the hospital environment exerts a strong selection pressure on MDR bacteria, which may result in increased resistance to multiple medications.[3] The 16S rDNA sequence has characteristics that make it an excellent candidate for use as a universal phylogenetic marker. Additionally, 16S rDNA gene sequencing is a valuable approach for bacterial classification, since it involves determining the nucleotide sequences of this region and comparing them to sequences available in databases to find homology matches, allowing for bacterial identification of target samples.[4] MDR bacterial strains were identified and cultured on a variety of culture medium in this study from patient samples taken from different hospitals in Al Qassim region. Samples were pathogenic bacterial strains such as *Pseudomonas aeruginosa, Escherichia coli, Acinetobacter baumanni and Providencia Stuartii*. In this experiment, a total of 28 MDR bacterial strains were found using 16S rDNA sequence analysis and bioinformatics analysis. The evolutionary and phylogenetic relationships between isolated strains were also examined. The effectiveness of the strategy was shown by comparing it to a database of varied bacterial populations.

Materials and Methods

Collection of Pathogenic Bacterial Strains

Within a two-month period, 38 clinical samples of blood, wounds, sputum, and urine were obtained from hospitalised patients at Al Qassim hospitals in Saudi Arabia. Clinical samples were collected under aseptic circumstances and promptly sent to the microbial genetics' laboratory at King Abdulaziz University's department of Biology, college of science. Isolates were Re identified depending on morphology and

biochemical test as compared with MicroScanWalkAway plus System Kit (Beckman Coulter) as confirmatory test. The liquid medium used for bacterial growth was Luria Bertani (LB) broth. The liquid cultures were incubated at 37°C was provided growing on a nutrient agar plate. An inoculating loop was sterilised with flame and was used to pick up a colony of each strain, to inoculate into 20 ml of LB broth. The inoculated LB broth was then incubated for 24 hours.

DNA Extraction, PCR Amplification and Sequencing of 16S rDNA16S Gene Sequencing and Analysis

Genomic DNA was isolated from the cell pellets of all Multidrug-resistant Bacteria isolates according to the manufacturer's instructions using a DNA extraction kit (Promega, USA). A single DNA fragment (about 1200 bp) encoding the 16S rDNA gene was amplified for each isolate using previously published procedures.[5] Electrophoresis in 1.5 percent agarose gel was used to identify the amplified PCR products of the 16S rRNA gene bacterial gene fragments. Purification and sequencing of the amplified fragments were performed at MACROGEN sequencing laboratory in Seoul, Korea, using an automated sequencer ABI 3100 (Applied Biosystems) equipped with the BigDye Terminator Kit v. 3.1. (Applied Biosystems). Sequencing was performed using primers 518 F (5′ C CA GCAG CC GC GG TAATACG - 3′) and 800 R (5′- TA CC AG GG TA TC TA AT CC -3′). The sequences were modified using the Vector NTI Suite 9 programme and compared to the NCBI database using BLAST searches. In this comparison, we looked for sequences of type strains that were most closely linked to the isolates' sequences. A similarity threshold of 97 percent was used to define operational taxonomic units (OTUs).[5]

Bioinformatics Analysis

Out of 38 samples, 28 samples were selected for the study. All the 28 sequences were subjected to ncbi BLAST search tool http://blast.ncbi.nlm.nih.gov to detect non-chance sequence similarity. BLAST search was restricted to 16S ribosomal RNA database, where models (XM/XP) as well as uncultured/environmental samples were also filtered out, such that more reliable results would be attained. Each individual sequence was solely blastd, where blast hit with the lowest expect-value (which indicate number of non-chance alignments) was picked. In order to ensure that Blast out puts were governed by expected-value (aka e-value), Blast algorithm parameter was decreased such the expected threshold was set to more stringent value of $1e^{-6}$. Alignment of the 28 sequence was carried out using version 2 of Clustalx.[6] Exploratory data and phylogenetic analyses were carried out under R Project for Statistical Computing.[7] Where exploratory data analysis was done using Seqinr R package.[6] Phylogenetic analysis was carried out by ape package.[8] Reconstruction of the phylogenetic tree was done using neighbour joint method.[8] DnaSP software was used to analyse the haplotype diversity (Hd), the average number of nucleotide differences, the average number of nucleotide differences,[9] the nucleotide diversity (π). The polymorphic site (S), the singleton variable sites (SP), and the parsimony informative sites (PIP) for each gene, and the average number of nucleotide substitutions per site between species (D_{xy}).[10]

Results

The isolated DNA of all 28 isolated strains is presented. The findings indicate that all isolates strains have effective DNA isolation procedures. As shown in Figure 1, 16S rDNA was utilised to identify bacterial species from 28 isolates strains and two ATCC strain No. (1, 2), on nutrient agar medium. The 16S DNA bands are 500 bp in length. The 16S rRNA was identified using a Macrogen universal primer as described in the materials and methods. As demonstrated in Figure 1, the findings of 16S gene separation are pure 16S rRNA gene isolation.

BLAST Analysis and Sequence Variation

The BLAST search resulted in categorizing 28 isolates into 5 strains namely 4 isolates of *Acinetobacter baumannii*, 15 isolates of *Escherichia coli*, 2 isolates for each of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and 5 isolates of *Providencia stuartii* (Table 1).

Sequence length percentage of GC content for the 28 isolates are shown numerically in Table 1 and graphically in Figures 2 and 3. Sequence length varied greatly between and within strains. In general one of the *A. baumannii* strains has the shortest length of (366 base), where one of *E. coli* has the longest sequence length (1263 base). When it comes to the length range *K. pneumoniae* had the narrowest length range (801–1017) base followed by *E. coli* (914–1263 base), where *A. baumannii* has the widest length range (366–1189 base).

In the present study the percentage of GC content ranged from 50 to 56% among the 28 isolates (Table 1). Considering percentage of GC content within species *K. pneumoniae* has the narrowest GC% range (55–56%) where *P. stuartii* has the widest range (Figure 3).

Figure 4 shows the maximum likelihood phylogenetic tree of the 28 isolates along with heat map. The phylogenetic tree consists of two large clades. The clade comprised *A.baumannii*, and only one of the tow *P.aeruginosa* isolates. The second clade consisted of two huge clusters. The first cluster composed of *K. pneumoniae* and *E. coli*, where the second cluster comprised *P. stuartii* and the second *P. aeruginosa* strain.

Principle component analysis (PCA) was carried out for a better understanding of the diversity between and within the five species. The general idea underlying PCA similar species should cluster, there for PCA outlines factors basically responsible for differences between strains. As such, the more similar strains the more the closer they located. Results of PCA are presented graphically in Figure 5. That is *A. baumannii* and *P. aeruginosa* clustered together and a bit further *P. stuartii* where

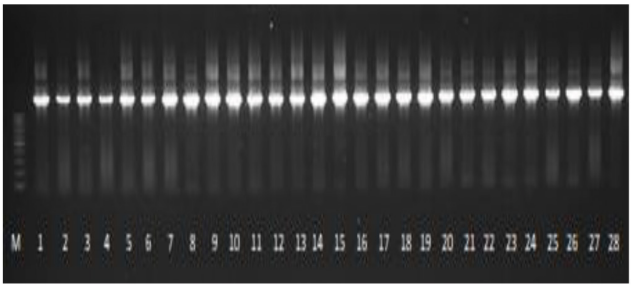

Fig. 1 **Agarose gel electrophoresis 16S PCR products out of 38 samples, 28 samples were selected for the study.**

Table 1. **16S rDNA sequence lengths and %GC values of 28 visolates from Al Qassim hospitals in Saudi Arabia**

Strains	Sequence length	GC%
Acinetobacter baumannii	1189	52
Acinetobacter baumannii	774	52
Acinetobacter baumannii	366	50
Pseudomonas aeruginosa	544	53
Acinetobacter baumannii	669	50
Providencia stuartii	814	53
Providencia stuartii	595	52
Providencia stuartii	922	54
Providencia stuartii	722	53
Providencia stuartii	836	51
Escherichia coli	1158	55
Escherichia coli	1224	54
Escherichia coli	1226	55
Escherichia coli	914	55
Escherichia coli	964	55
Escherichia coli	937	55
Escherichia coli	1036	55
Escherichia coli	1191	55
Escherichia coli	1227	54
Escherichia coli	1234	55
Escherichia coli	1259	54
Escherichia coli	1236	55
Escherichia coli	1202	55
Escherichia coli	1202	56
Klebsiella pneumoniae	801	56
Klebsiella pneumonia	1017	55
Pseudomonas aeruginosa	1172	53
Escherichia coli	1263	54

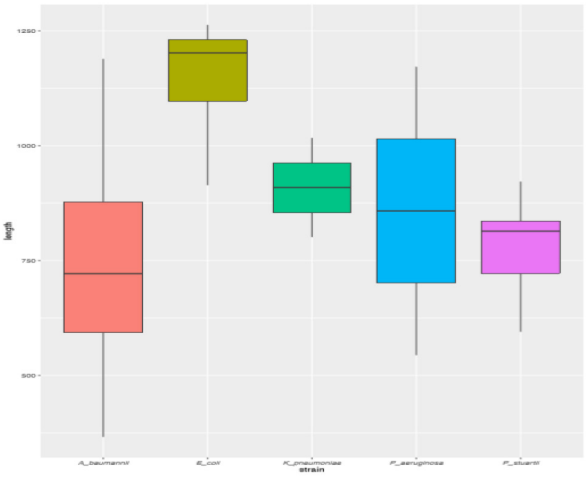

Fig. 2 **Boxplot displaying the distribution of sequence length for the strains, where top and bottom lines represent the maximum and minimum values, the top and bottom of each box represent the first quartile (Q1) and third quartile (Q3) where the line inside each box is the median (second quartile Q2).**

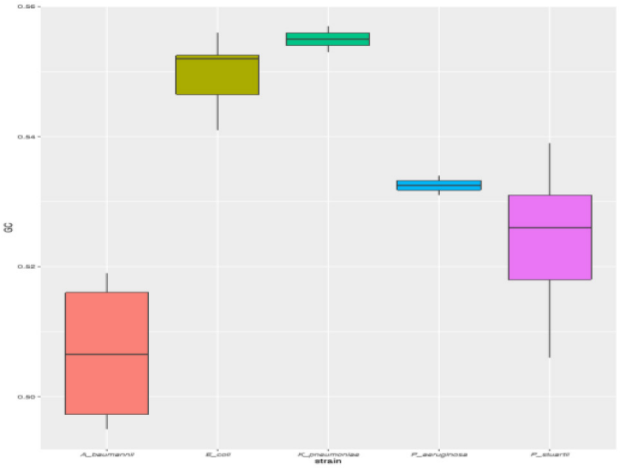

Fig. 3 **Boxplot displaying the distribution of percentage of GC content of the strains where top and bottom lines represent the maximum and minimum values, the top and bottom of each box represent the first quartile (Q1) and third quartile (Q3) where the line inside each box is the median (second quartile Q2).**

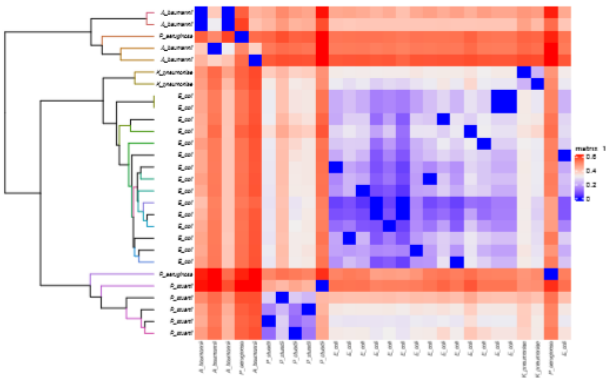

Fig. 4 **Maximum likelihood phylogenetic tree of the 28 isolates along with heat map.**

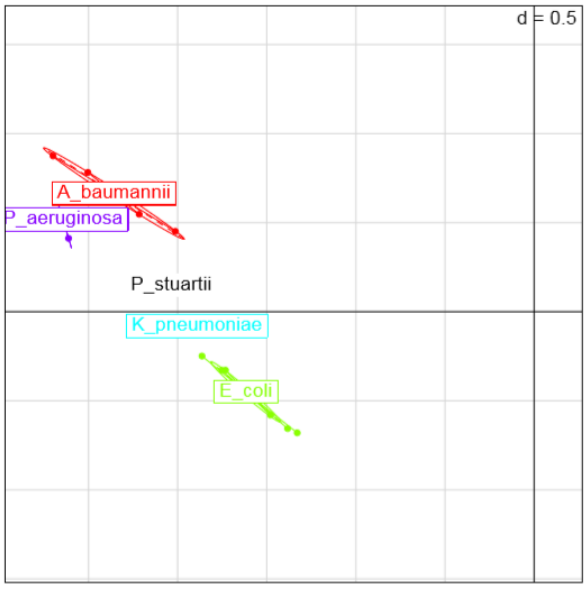

Fig. 5 **Principal component analysis of the five strains, X-axis is the first principal component and Y-axis is the second.**

P. aeruginosa and 2 *E. coli* constituted a different component. This pattern is somehow similar to phylogenetic tree Figure 4.

DNA Sequences Analysis

Polymorphic Sites

Analyses of polymorphic sites for the 28 isolates are shown in Table 2. The total number of aligned sites was 1301 sites, the number of sites without alignment gaps or missing data was 238 of which 109 invariable (monomorphic) sites. The number of variable (polymorphic) site was 129, of which 42 singleton variable sites and 87 parsimony informative sites.

Haplotype & Nucleotide Analyses

Number of haplotypes, haplotypes diversity and Standard deviation (SD) of haplotypes diversity are shown in Table 3. Number of haplotypes was 14. Hapotype (gene) diversity was 0.8. Nucleotide diversity (π) was 0.13. 1 ± 0.8, where average number of nucleotide differences was 30.

Table 2. **Estimated parameters of the polymorphic sites for 28 isolates from Al Qassim Hospitals in Saudi Arabia**

Number of sites	Monomorphic sites	Polymorphic sites	
		Singleton sites	Parsimony informative
238	109	42	87

Table 3. **Estimated parameters of DNA polymorphism 28 isolates from Al Qassim Hospitals in Saudi Arabia**

No. haplotype	Haplotype diversity +sd	Nucleotide diversity π	Average number of nucleotide differences
14	0.8+0.1	0.13	30

Table 4. **Length of conserved regions, conservation, homzigosity and *P*-values of the 28 isolates sequence from Al Qassim Hospitals in Saudi Arabia**

Region Start-End	Conservation	Homozigosity	*P*-value
256–420	0.56	0.92	0.001
464–572	0.56	0.92	0.01
829–1079	0.62	0.93	<0.0001

Table 4 shows the conserved regions along the 28 antibiotic sequences and measurements of conservation (C), homosigoisty and *P*-value. Conservation (C) is calculated as the proportion of conserved sites in the alignment region, where homosigosity is measured as 1-heterzygosity. Only 3 conserved regions were observed. the length of the 3 conserved regions was not the constant, ranged from 109 bases to 251 bases. The *P*-values of the three conserved regions were less than 0.01.

Discussion

A considerable variation are noticed in genomic nucleotide content of bacteria, with GC content (number of the same strand guanine +cytosine sites divided by DNA sequence length) ranging from less than 13% to more than 75% between sole species.[11] Albeit the particular reasons for these GC varieties, wither within and between species, are still not known, it is largely thought that a number of variables in connection with evolutionary history as well as the environment are responsible.[12]

DNA sequence analysis introduces an efficient tool for understanding the evolutionary forces that shaped nucleotide variations as well as bring insight into the significance of specific genomic regions.[13]

Haplotype diversity (aka gene diversity) is the probability that two arbitrary sampled alleles are different.[14]

This study builds on the published evidence that, antibiotic-resistant bacteria are capable of transferring copies of their DNA encoding a resistance mechanism to other bacteria, even those that are distantly related to them. These bacteria are then capable of passing on the resistance genes, resulting in the production of generations antibiotic-resistant bacteria. Vertical gene transfer, which happens by cell-to-cell conjugation, is referred to as "horizontal gene transfer".[15]

Conclusion

Results of the present study demonstrated that the observed heterogeneity in the sequences of our target bacterial strains may be linked to antibiotic resistance and gene transfer between bacteria that evolved as a result of the hospital environment. Consequently, hospital environments are considered "hot spots" for antibiotic resistant bacteria, and this suggests that significant precaution should be taken at all stages of the health-care system to minimize.

Conflict of Interest

None.

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