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RESEARCH ARTICLE

Molecular Analysis of Gram-Negative Bacterial Strains in Patients from Koya City: Implications for Diagnostic Approaches

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ABSTRACT

This study addresses the critical issue of Gram-negative bacteria (GNB) in patients from Koya City. A total of 14 samples were collected from different patient demographics and sources, including urine, wounds, vaginal swabs, seminal fluid, and throat swab. The samples were subjected to analysis through a multifaceted approach involving culturing and molecular identification. Following the isolation of bacterial samples, the 16SrRNA gene was targeted using polymerase chain reaction (PCR). Subsequently, the obtained PCR products underwent Sanger sequencing, providing a detailed analysis of the nucleotide sequences. The resulting sequences were then compared against existing prokaryotic DNA sequences in the BLAST database to determine the identity of the isolated bacteria. Out of the 14 samples, 92.9% yielded sequences of good quality, while 7.1% exhibited a noise sequence. The sequences were further aligned, and 11 samples demonstrated a 100% identity with previously identified bacteria such as *Escherichia coli* (100%), *Klebsiella pneumoniae* (100%), and *Shigella flexneri* (100%). Notably, two samples displayed a >99% identity with *E. coli* (99.5%), highlighting the diversity within the bacterial strains. This study sheds light on the prevalence and identity of GNB in Koya City patients the findings underscore the necessity of molecular techniques, such as PCR and sequencing, in clinical diagnostics. The diverse bacterial strains identified highlight the complexity of bacterial infections, emphasizing the importance of targeted approaches in addressing antibiotic resistance.

Keywords: Gram-negative bacteria, 16SrRNA gene, sequencing, molecular identification

INTRODUCTION

Gram-negative bacteria (GNB) represent a pervasive and formidable global health concern, characterized by their significant resistance to antibiotics. This resistance not only intensifies the risk of morbidity and mortality, particularly within the confines of intensive care units but also poses a persistent challenge in healthcare facilities worldwide.[1,2] The clinical implications of GNB infections are profound, as these bacteria exhibit a remarkable ability to infiltrate diverse organ systems, leading to a spectrum of human illnesses[3,4] The urgency to address the rising threat of GNB is underscored by their adeptness at causing infections in individuals with compromised immune systems.[5]

The encapsulation of GNB, coupled with an outer membrane acting as a protective layer, further complicates treatment strategies, as the destruction of this membrane releases a potent endotoxin.[6]

Well known for its high conservation, specificity, and substantial gene sequence length, the 16SrRNA gene serves as a robust tool for the identification of prokaryotic organisms. Understanding the internal structure, which comprises variable and conserved regions, allows for the design of universal primers targeting conservative areas and specific primers for variable regions. This innovative approach enhances detection specificity, providing valuable insights into the diversity of GNB at the genus or species level.[7,8]

Aims

This study aims to explore the genetic diversity of GNB in clinical samples using the 16SrRNA gene as a marker. Specifically, we hypothesize that the analysis of the 16SrRNA

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gene will reveal distinct genetic signatures, contributing to a deeper understanding of the clinical implications of Gram-negative bacterial infections.

MATERIALS AND METHODS

Sample Collection

In this study, a total of 14 samples were collected from various patients, each representing a diverse array of biological sources. These samples included urine, stool, wound sites, vaginal swabs, throat swabs, and seminal fluid, as detailed in Table 1.

Cultivation and Isolation of Bacterial Colonies on MacConkey Agar

To obtain pure bacterial colonies, the collected samples underwent a cultivation process on MacConkey agar, following inoculation, all MacConkey agar plates were incubated at 37°C for a standardized duration of 24 h. Subsequently, single colonies were isolated from each plate, forming the basis for subsequent Gram stain.

Validation of gram-negative bacterial presence

To provide robust confirmation of the presence of GNB within the isolates, a Gram stain analysis was conducted. MacConkey agar culturing and Gram stain analysis are used in a dual-stage approach to identify and understand bacterial composition in samples. The selection process focuses on 14 distinct colonies based on unique morphologies, providing a diverse representation of bacterial entities. Morphology-based selection can summarize variations in species, preparing for genomic DNA extraction procedures. This approach strengthens the identification process and provides a robust foundation for understanding bacterial composition.

DNA Extraction

A singular pure colony from each of the 14 isolates underwent cultivation in 10 mL Falcon tubes, containing 5 mL of Nutrient broth. This cultivation process unfolded over a regulated period of 24 h within a shaker incubator, at 37°C. DNA extraction from all isolates was then performed using the (QIAGEN) kit in strict adherence to the manufacturer's instructions. To confirm the integrity and purity of the extracted genetic material, Nanodrop measurement (Nanovue plus USA) and gel electrophoresis were employed. The extracted DNA samples were subjected to electrophoresis on a 0.7% agarose gel, infused with 0.07% ethidium bromide, and run in a 1X TBE buffer for 1 h at 80V.[9]

Table 1: Source of samples, across patient demographic data

Sample's code	Source of samples	Patient's gender	Patient's age
S1	Wound	Male	36 years
S2	Urine	Female	44 years
S3	Urine	Female	58 years
S4	Urine	Female	27 years
S5	Vaginal swab	Female	36 years
S6	Wound	Male	28 years
S7	Urine	Male	30 years
S8	Vaginal swab	Female	34 years
S9	Vaginal swab	Female	25 years
S10	Seminal fluid	Male	30 years
S11	Urine	Female	33 years
S12	Throat swab	Male	31 years
S13	Urine	Female	22 years
S14	Urine	Male	53 years

Specifically, this validated DNA served as the template for polymerase chain reaction (PCR), with the target set on amplifying the 16SrRNA gene for precise identification of bacterial strains.

PCR Amplification of the 16S rRNA Targeted Region

The amplification of the targeted region within the 16S rRNA gene was carried out through a PCR approach, ensuring precision and reliability in the analysis. The final reaction volume, set at 25 μL, including 12.5 μL of the ready-to-use Mastermix (Promega), 4 μL of template DNA, 1 μL (5 pmol/μL) each of the forward primers (16FWD-GTAATACGGAGGGTGCAAGC) and reverse primers (16REV-TCTAATCCTGTTTGCTCCCCA), and 6.5 μL of nuclease-free water. The primer sequences were taken from.[10]

The thermal cycler (BIO-RAD) initiated the procedure with an initial denaturation phase at 95°C, lasting 3 min. Subsequently, 30 cycles were used, involving a 30-s secondary denaturation step at 95°C, followed by an annealing phase lasting 45 s at 58.5°C. The primary extension was carried out at 72°C for an additional 45 s, with a final extension set at 72°C for 5 min.

To determine the efficacy of the PCR process, the accuracy of the PCR amplicon size was verified through gel electrophoresis. A 1% agarose gel, subjected to an electric field of 80 V for 45 min, served as a method for this crucial evaluation. The DNA bands were distinctly visualized post-electrophoresis, with ethidium bromide incorporated into the gel during its preparation, facilitating the staining of DNA bands. Subsequently, examination of the electrophoresis results allowed for the identification of samples displaying a correctly sized band, measured at 100 base pairs (bp). These chosen samples, with accurately sized bands, were then sent for the subsequent critical phase of the study, which was DNA sequencing.

Analysis of the Amplified 16SrRNA Gene Sequencing for in-Depth Insights

The crucial phase of our study involved the nucleotide sequencing of the PCR products obtained from our selected samples. This process was entrusted to Macrogene company situated in South Korea, leveraging the cutting-edge AB DNA sequencing system. Specifically, the sequencing was conducted in the reverse direction of the 16SrRNA gene using 16REV primer, ensuring a comprehensive exploration of the genetic information encoded within our bacterial samples.

On the completion of the sequencing process, the resulting chromatogram, a graphical representation of the sequencing output, underwent careful analysis. This detailed examination aimed to validate the quality and integrity of the obtained sequences. Employing the MEGA7 software, we systematically assessed the chromatogram, ensuring that the sequenced DNA fragments met the stringent criteria for reliability and accuracy.

After this quality assurance step, the generated sequences were subjected to a comparative analysis by aligning our sequences with the extensive genetic information within the Basic Local Alignment Search Tool (BLAST) database, we were able to identify matching sequences from various species. This comparative approach served as a reference point, corroborating, and solidifying the accuracy of our study's results.

RESULTS AND DISCUSSION

Sample Collection

In our study cohort comprising 14 patients, a gender distribution revealed 8 females (57%) and 6 males (43%). The mean age of the participants was calculated at (34.78 ± 10.33) years, providing a snapshot of the age diversity within our sampled population in [Figure 1]. In addition, this diversity in sample types adds a layer of complexity to our study, allowing for a comprehensive exploration of Gram-negative bacterial presence across different physiological environments. The observed gender distribution and age range in our study cohort align with previous studies highlighting the susceptibility of individuals to Gram-negative bacterial infections across diverse demographics.[11,12] This balanced representation enhances the external validity of our findings and facilitates a more comprehensive understanding of bacterial prevalence.

The age-related variations in Gram-negative bacterial infections have been noted in the literature, with distinct patterns observed in different age groups.[13] Our study, covering participants aged 22–58 years, contributes valuable insights into the age-dependent dynamics of GNB in clinical settings.

The diversity in sample types collected in our study, as depicted in [Figure 1] Panel C, is essential for exploring the heterogeneity of bacterial communities in different anatomical sites. Previous research emphasizes the importance of considering varied sample sources in bacterial studies to capture the complexity of microbial ecosystems within the human body.[14,15] The inclusion of samples from urine, wounds, vaginal swabs, seminal fluid, and throat swabs broadens the scope of our investigation, shedding light on the potential variations in Gram-negative bacterial prevalence across different physiological niches.

As we progress to the subsequent sections, the identification of specific bacterial strains within these diverse samples will further elucidate the nuanced interactions between patient demographics, sample types, and the presence of GNB. This integrative approach, bolstered by existing literature, will contribute to a better understanding of Gram-negative bacterial infections and inform future diagnostic and therapeutic strategies.

Culture and Stain Identification

The cultured samples revealed diverse putative gram-negative bacterial species, including notable representatives such as *Escherichia coli* and *Klebsiella* spp., characterized by their ability to ferment lactose. In addition, the isolation identified slow lactose fermenter *Serratia*, non-lactose fermenter *Shigella* spp., and *Pseudomonas* spp. These findings align with the broader understanding of GNB, showing their varied metabolic capabilities and morphological distinctions.

These findings are in agreement with previous studies, highlighting the importance of Gram staining in initial bacterial characterization.[16,17] The ability to differentiate between lactose fermenters and non-fermenters is crucial for understanding the metabolic profile of isolated strains, further emphasizing the utility of culture and stain techniques in microbial identification.

DNA Extraction and Quality Assessment

The quality of extracted DNA is assessed for the reliability of downstream molecular analyses using 0.7% gel electrophoresis, a standard method for evaluating purity and integrity.[18,19] Notably, all extracted DNA samples exhibited a distinct, clear band on the gel [Figure 2], showing the purity of the DNA without signs of degradation or contamination.

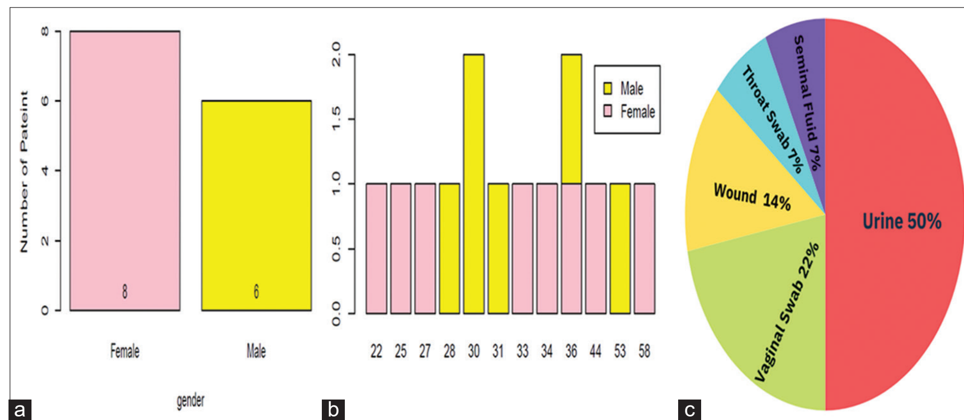

Figure 1: Illustrates the genders, ages, and diversity of sample types. (a) Visually represents the gender distribution, illustrating the numerical contrast between male and female participants. (b) The age demographics, showing a spectrum ranging from 22 to 58 years, thus summarizing a broad age range within our cohort. (c) The collection of various sample types from these participants

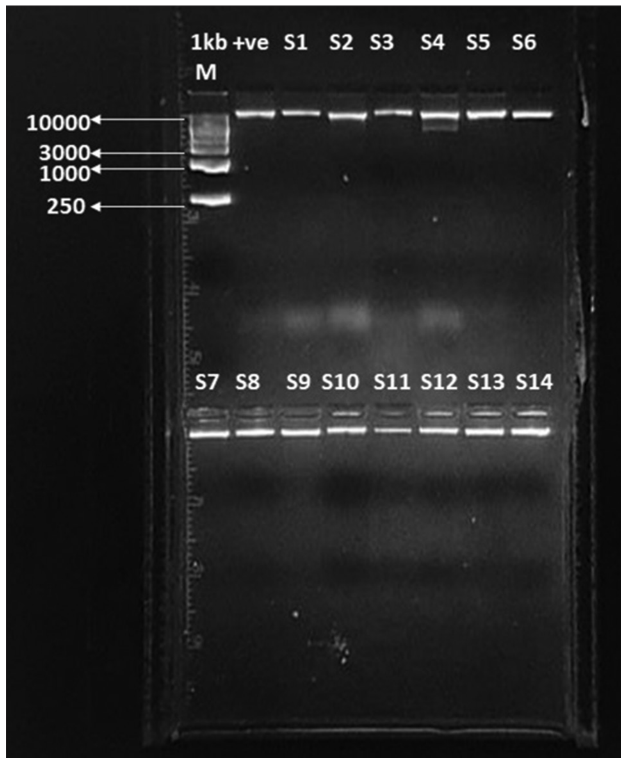

Figure 2: Agarose gel electrophoresis analysis of 14 DNA samples extracted from bacteria genera. Lane M is the 1 kb marker (Genedirex), +ve is a positive control DNA that has been extracted from *E. coli* strain ATCC 25218, S1 – S14 is *K. pneumoniae*, *E. coli*, *K. pneumoniae*, *E. coli*, *K. pneumoniae*, *K. pneumoniae*, Unidentified strain, *E. coli*, *E. coli*, *S. flexneri*, *S. flexneri*, *E. hermanii*, *E. coli* and *E. coli,* respectively. *E. coli*: *Escherichia coli*, *S. flexneri*: *Shigella flexneri*, *K. pneumoniae: Klebsiella pneumoniae*

This unequivocal visualization of a single, well-defined band is indicative of intact and high-quality DNA molecules. The absence of smearing or additional bands reinforces the reliability of the DNA extraction process, crucial for accurate downstream analyses such as PCR amplification and sequencing.[20]

These results confirm the success of the DNA extraction protocol employed, ensuring the production of pure and intact genetic material suitable for subsequent molecular investigations. The observed clarity in the gel electrophoresis reinforces the fidelity of the genetic material obtained, laying a robust foundation for the ensuing steps in the experimental workflow.

PCR Amplification of 16s rRNA Gene

The PCR targeting the 16S rRNA gene was carried out with precision, resulting in the expected amplicon size of approximately 230 base pairs across all extracted bacterial DNA samples [Figure 3]. This consistent amplification signifies the reliability and efficiency of the PCR assay, ensuring the targeted genomic region's successful replication.

Interestingly, all 14 samples displayed a single clear band, the uniformity in band sizes across all the samples, maintaining a consistent 230 base pairs, reinforces the

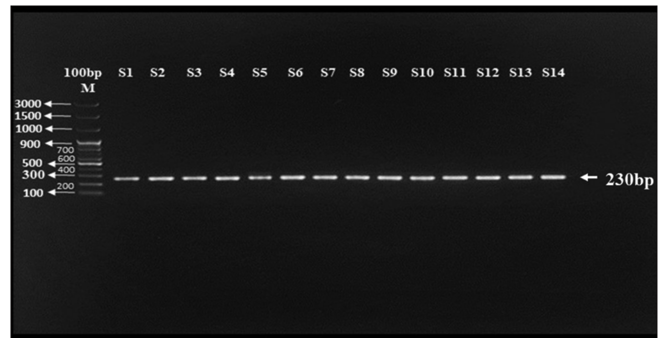

Figure 3: V4 amplification of 16s rDNA. Lane M is a 100bp marker (Genedirex), and Lanes S1 through S14 show ~230bp of PCR amplicons generated from *K. pneumoniae*, *E. coli*, *K. pneumoniae*, *E. coli*, K. *pneumoniae*, K. *pneumoniae*, Unidentified strain, *E. coli*, *E. coli*, *S. flexneri*, *S. flexneri*, *E. hermanii*, *E. coli,* and *E. coli,* respectively, using a DNA template from section 3.3. *E. coli*: *Escherichia coli*, *S. flexneri*: *Shigella flexneri*, *K. pneumoniae: Klebsiella pneumoniae*

reliability of the PCR technique in selectively amplifying the target region. The absence of aberrant banding in the samples further underscores the protocol's robustness.

The successful amplification of the 16S rRNA gene, evident in the distinct bands observed, aligns with the established efficacy of PCR in molecular biology.[21]

16S rRNA Sequencing: Profiling Bacterial Diversity in Isolated Samples

Following the genomic DNA PCR, sequences from 14 bacterial isolates underwent examination using MEGA7 software. To ensure high-quality data, sequences were trimmed at both ends, resulting in 13 samples (S1, S2, S3, S4, S6, S8, S9, S10, S11, S12, S13, S14, and S15) suitable for further investigation [Figure 4]. Notably, S7 exhibited a poor-quality, noisy sequence and was excluded from subsequent analyses [Figure 5].

Employing individual automated alignments with the BLAST database against prokaryotic 16S rRNA gene sequences facilitated the identification of bacterial species within the samples. The pairwise-similarity analysis revealed compelling insights into the diversity of bacterial strains present [Table 2].

The identification of bacterial species through 16S rRNA sequencing serves as a powerful tool in microbial taxonomy and diversity studies.[8] The high-quality sequences obtained from the majority of samples underscore the reliability of the applied methodology.[8] However, the detection of a bad and noisy sequence in S7 emphasizes the inherent challenges associated with sequencing, necessitating stringent quality control measures during data analysis.[22]

The identification results unveiled a spectrum of bacterial strains, with 100% identity matches including *E. coli*, *Klebsiella pneumoniae*, *Shigella flexneri*, and *Escherichia hermannii*. This alignment supports the prevalence of these bacterial species within the sampled population. In addition, the 99.5% identity match with *Escherichia* spp. in two samples further underscores the genetic diversity within the *Escherichia* genus.

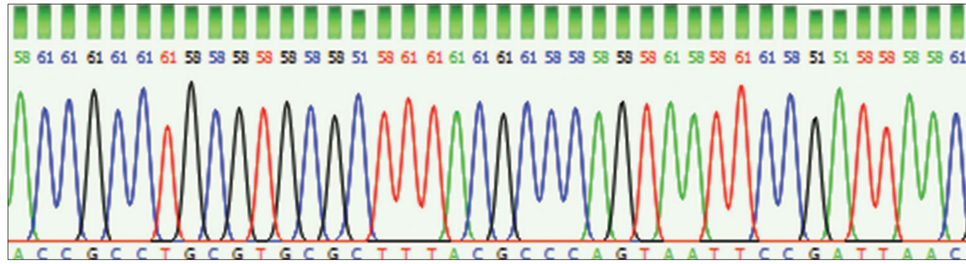

Figure 4: Represents the V4 of the 16s rDNA sequence. The top green bars show a single base's quality value, which is displayed as a numerical value below it, generally more than 40 QV. There is no noise, and the peaks are neatly and evenly distributed. The baseline remains steady and uniform over the whole sequence chromatogram. The base is named exactly beneath each peak (DNA Sequence Assembler v4 (2013), Heracle BioSoft, www.DnaBaser.com)

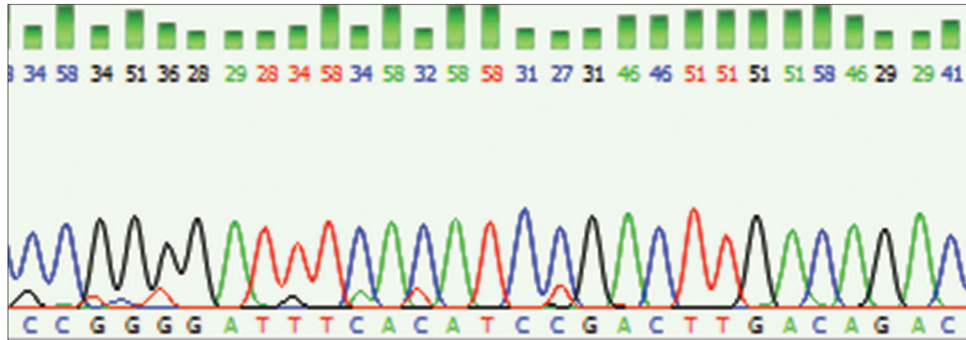

Figure 5: The noisy DNA sequence from sample 7 shows <40 QV at the top green bar, the peaks were not distributed evenly, and the baseline is not smooth and flat (DNA Sequence Assembler v4 (2013), Heracle BioSoft, www.DnaBaser.com)

Table 2: Bacterial identities according to automated alignments with the BLAST database against prokaryotic 16S rRNA gene sequences

Sample's code	Type of bacteria	Identities (%)
S1	*Klebsiella pneumoniae*	100
S2	*Escherichia coli*	99.5
S3	*Klebsiella pneumoniae*	100
S4	*Escherichia coli*	99.5
S5	*Klebsiella pneumoniae*	100
S6	*Klebsiella pneumoniae*	100
S8	*Escherichia coli*	100
S9	*Escherichia coli*	100
S10	*Shigella flexneri*	100
S11	*Shigella flexneri*	100
S12	*Escherichia hermannii*	100
S13	*Escherichia coli*	100
S14	*Escherichia coli*	100

CONCLUSION

Our study successfully identified some GNB through PCR amplification of the 16SrRNA gene, revealing key pathogens such as *E. coli*, *K. pneumoniae*, *S. flexneri*, and *E. hermannii*. The consistent 230bp DNA fragments highlighted the robustness of our approach. Despite one sample exhibiting noise, our findings emphasize the clinical relevance of GNB. This research underscores the importance of molecular techniques in diagnostics, contributing valuable insights into antibiotic resistance. Further studies can explore resistance profiles for enhanced treatment strategies.

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