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Original Article

Antibiotic Susceptibility Profiling, Molecular Characterization, and 16S rRNA-Based Phylogenetic Analysis of Pseudomonas Aeruginosa Isolates from Musculoskeletal Infections

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Abstract

Objective: The primary goal of this research was to describe antibiotic resistance and identification of virulence genes in Pseudomonas aeruginosa.

Methods: The study was cross-sectional, featuring a randomized selection of samples. Conducted from January to December 2023, the study encompassed a sample size of 320 including blood, pus, and wound swabs [Male:169(52.8%), Female 151(47.1%)]. Age-based categories were ascertained, G1:13-20 years, G2:21-40 years, G3:41-60 years, G4:61-80 years. Identification tests for Pseudomonas aeruginosa included API20NE (bioMérieux®, France). Antimicrobial susceptibility testing was following the CLSI,2020 for the Disc Diffusion Test.DNA extraction and purification were carried out through a Genome Jet DNA Purification kit and 16S rRNA primers for the identification of resistance genes exoA and oprL. Phylogenetic and taxonomic identification was conducted via ribosomal RNA sequencing of PCR-amplified products(BIO-RAD T100TM Thermocycler) and sequencing with an automated sequencer (Illumina MiniSeqTM). Phylogenetic tree was constructed by MEGA11.p-value<0.05 was statistically significant.

Results: Prevalence of Pseudomonas aeruginosa was 22%. Positivity was recorded from pus (152/320:47.5%),blood(124/320-38.75%) and wound swabs(44/320-1 3.75%).G4 showed maximum positive isolates(91.25%).Maximum resistance was exhibited against Meropenem(76%) and Imipenem(70%).PCR identified the presence of resistance genes,exoA gene(125 bp), and oprL(105 bp). The phylogenetic tree was constructed through ribosomal RNA.The gene bank accession number for 16S rRNA gene of P. aeruginosa is PQ269824.

Conclusion: Antimicrobial susceptibility testing helps improve the treatment alternatives for stubborn strains. The presence of resistance genes highlights how research at the molecular level enhances treatment approaches for managing multi-drug resistant pathogens.

Keywords: Pseudomonas aeruginosa, antibiotic susceptibility, musculoskeletal infections, phylogenetic tree

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Introduction

Musculoskeletal (MSK) infections are the infections of muscular tissues, bones and joints. These infections are usually underdiagnosed as most people coming to hospitals have atypical presentations and their diagnosis is definitely a time consuming and invasive procedure. Pseudomonas aeruginosa is a non- lactose fermenting Gram-negative organism relating to Kingdom Monera, Phylum Pseudomonadota, class gamma proteobacteria, order Pseudomonadales, genus Pseudomonas and species Pseudomonas aeruginosa.[1,2] Bacterial toxins play role in pathophysiology of MSK infections and their

evaluation needs effective antitoxic and antimicrobial agents which further will reduce the production of biofilm and the release of toxins.[3] Inflammatory involvement of joints is enhanced in persons with age > 80 years , recent joint surgery or prosthesis, diabetes mellitus, rheumatoid arthritis and immunocompromised individuals are potential target for P. aeruginosa infection. P. aeruginosa can develop in hospital reservoirs such as sinks, cleaning supplies, breathing equipment such as ventilators.[4,5] Overproduction of alginate, "glycocalyx" or "mucoid exopolysaccharide," causes the mucoid colony phenotype. The size of P. aeruginosa genome is 6.26 mega-base pairs (Mbp), encompassing 5567 genes. For cell growth, division, metabolism, and the stability of proteins, about 1500 genes are necessary.[6,7] The increased coding capacity of the P. aeruginosa genome enables more metabolic flexibility and environmental adaptation ability.[8]

Bacterial colonization of host tissues is dependent upon different virulent factors including lipopolysaccharide capsule, flagella and pili. Certain bacterial species have a cell signaling mechanism called the quorum-sensing phenomenon that allows the bacterial cell to respond to external signals.[9,10] The genes las and rhl control its activity. For the rapid molecular identification of P. aeruginosa, specific primers are designed especially for gene sequencing of oprL proteins. The toxA gene is the most dangerous gene in its severity, encoding the exotoxin A and inhibiting protein synthesis. Elastase B (las B) is a significant metalloenzyme that promotes tissue adhesion, colonization, and invasion during host infection, leading to chronic pulmonary inflammation.[11-13] Antibiotic resistance in P. aeruginosa is significantly influenced by the outer membrane proteins (opr L). The most prevalent ESBLs genes associated with P. aeruginosa are blaCTX-M and blaTEM.[14,15]

Methods

It was a cross- sectional study conducted in age groups from 13 years to 80 years, distributed into 4 groups G- 1 to G- 4 as described in Table 1. Samples were collected through blood, pus, tissue and wound swabs. The study was conducted from January to December 2023 at Institute of Molecular Biology and Biotechnology, University of Lahore, Lahore. A total of 328 samples were taken of which 320 samples were of diagnosed patients and 8 samples (n=8) will serve as control group

Table 1: *Samples collection designed to isolate P. aeruginosa from blood, pus and wound swabs.*

Groups	Age	Blood	Pus	Wound swab
Group 1	13-20 yrs	33	40	7
Group 2	21-40 yrs	24	44	12
Group 3	41-60 yrs	29	41	10
Group 4	61-80 yrs	38	27	15
Total		124	152	44

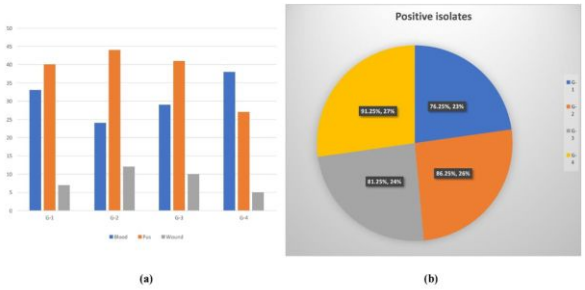

Figure I: *Graphical representation of sample distribution and positive isolates according to age groups*

(a) Graphical representation of sample distribution according to age groups

(b) Illustrated number of positive isolates in different age groups

Figure 1 (a) shows the distribution of blood, pus and wound swabs in group 1 to group 4 with more percentagein pus samples (152), followed by blood samples (124) and wound swab (44) Antimicrobial susceptibility testing was carried out by disc diffusion method on the Muller Hinton agar following the guidelines of Clinical and Laboratory Standards Institute (CLSI 2020).[16] DNA was isolated and purified from *Pseudomonas aeruginosa* using the Genome Jet DNA Purification Kit (Themo-Scientific). The phylogeny and taxonomy of the bacterium was evaluated by the use of 16S ribosomal RNA gene sequencing.[17] Using nucleotide BLAST, the isolated sequences were compared to the readily available sequences in GenBank at NCBI, by generating the phylogenetic tress. Detection of resistant genes exoA and oprL were done on isolated samples.[18] For the amplification of full length 16S ribosomal RNA, set of universal primers were designed.

27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and

1492 R (5′-TACGGYTACCTTGTTACGACTT-3′)

Table 2: *The percentage growth of different organisms in different age groups.*

	Group I 13-20yrs Total =80 NG = 20	Group II 21-40yrs Total= 80 NG = 11	Group III 41-60yrs Total=80 NG = 15	Group IV 61-80yrs Total= 80 NG = 7	Percentage %
S. aureus	24	29	21	32	39.55%
P. aeruginosa	10	12	16	21	22.01%
S. epidermidis	8	6	1	2	6.34%
Acinetobacter	3	3	9	8	8.58%
K. pneumoniae	8	13	12	2	13.05%
E. coli	2	4	5	9	7.46%
Enterococcus	3	0	0	0	1.11%
Proteus mirabilis	2	1	1	0	1.49%
Streptococcus	0	1	0	0	0.37%
Total	60	69	65	74	

The primers were designed following forward primer at 27 bp position and reverse primer at 1492 bp position. The Thermo Scientific Master Mix was used in the PCR reaction using the Bio-Rad T100 thermocycler. The data was analyzed using (SPSS) version 25. The difference between percentages was interpreted by Chi-square test. The validity of all data was assumed with P value< 0.05.[19,20]

Results

Figure 1 (b) shows the number of positive isolates in different age groups with more 91.25% in group 4 (61-80 years) and lowest 76.25 % of positive isolates in group 1 (13- 20 years).

The lowest age groups were those between the ages of 13years to 20 years (76.25%) and 41years to 60years (81.25%), while those of between the ages of 61 years and 80 years (91.25%) had the highest prevalence. There were total 320 patients, 169 (52.8%) were male and 151 (47%) were females.

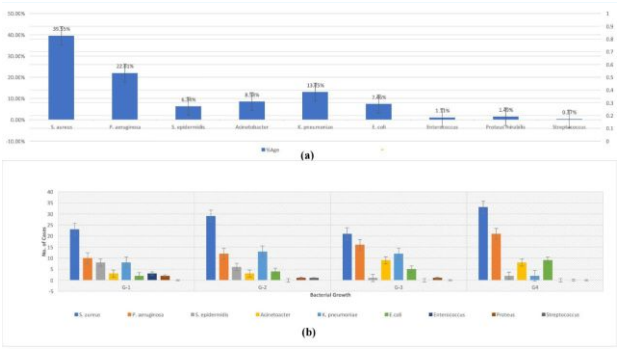

Figure 2: *Graphical representation of percentage growth of different organisms according to age groups.*

(a) Graphical representation of growth of different organisms

(b) Graphical representation of growth of different organisms in different age groups

Figure 2 (a) shows the percentage growth of different microorganisms with highest percentage 39.55% of S. aureus followed by 22.01% of P. aeruginosa. Figure 2 (b) shows the growth of different organisms according to age groups with highest percentage in group 4 (61 years to 80 years) and lowest percentage in group 1 (13 years to 20 years).

Table 3: *The positive isolates were collected from different specimens.*

Sr No.	Samples	Quantity	Percentage
1	Tissue swab	53	19%
2	Wound swab	35	13.05%
3	Pus swab	110	41.02%
4	Blood	70	26.11%

Identification up to species level of the P. aeruginosa strain was carried out via bidirectional sequencing (16S ribosomal RNA sequencing). Chromatograms of DNA sequencing files displayed the results of peaks between 110 and 170 nucleotides in various hues, each of which indicated a different pattern

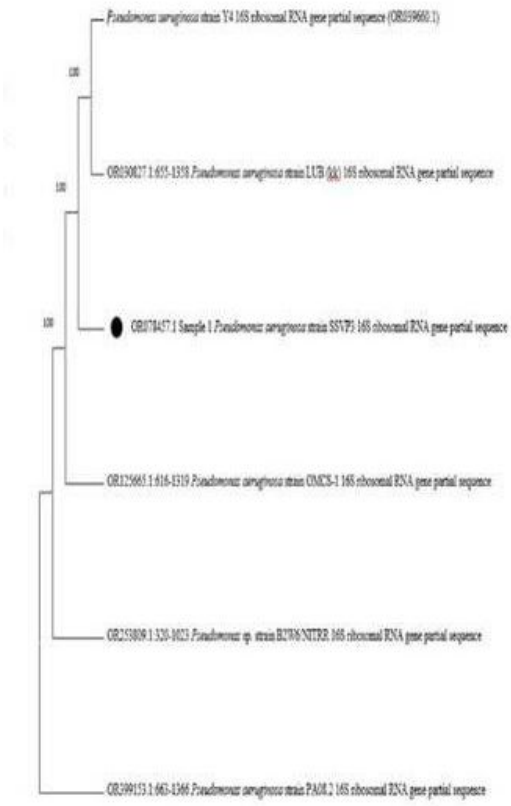

Figure 3: *Molecular characterization of Pseudomonas aeruginosa- Phylogenetic tree of query sequence showing neighbor alignment of SSVP3 Strain.*

Figure 3 shows phylogenetic tree of SSVP3 strain showing alignment with positive strain on 16S ribosomal RNA gene sequencing.

Phylogenetic tree of Pseudomonas aeruginosa: Phylogenetic examination reveals various connections between different species. It is the investigation of transformative relatedness among different gatherings of life forms (for instance, species, and populaces (Figure 3).

Discussion

MSK have imposed a major threat to public health. They result in major compromises in quality of life with structural and physical changes leaving long term effects in life. Pseudomonas aeruginosa is the third most prevalent pathogen in intensive care units.[21] Prevalence of P. aeruginosa in this study was 22 %, which also corelates with study reported by Chand et a. in which the organism causes about 18 to 63 % infections world-wide. These results were also parallel to data reported by Sultan et al. with prevalence rate of 32.5 %.[22,23] Out of the total 320 samples, the high level of infection rate was in group 4, 61-80 years with 91.25 %, 41-60 years in a 81.25 % and low level of infection was in group 1, 13- 20 years with (76.25%) respectively.

A variety of pathogenic elements, such as the lipopolysaccharide capsule, flagella, and pili, are necessary for the colonization of host tissues by bacteria. The quorum-sensing phenomenon is a cell signaling mechanism found in some bacterial species that enables the bacterial cell to react to outside stimuli. Its activity is regulated by the genes las and rhl. Particular primers are made specifically for oprL protein gene sequencing in order to facilitate the quick molecular identification of P. aeruginosa. The toxA gene, which codes for the exotoxin A and prevents the production of proteins, is the most harmful gene in terms of severity. Elastase B, also known as las B, is an important metalloenzyme that causes chronic pulmonary inflammation by encouraging tissue adhesion, colonization, and invasion during host infection. The outer membrane proteins (opr L) have a major impact on P. aeruginosa antibiotic resistance.

The similar results were also correlated with Hafiz *et al*. which also showed highest incidence of infection between 65-84 years.[24] Most positive samples were obtained from pus followed by blood and wound swabs, further confirmed by Farooq *et al*. which showed 33% from pus samples. To mitigate this computational cost, BLAST can efficiently identify places of local resemblance between the query sequence and the sequences within the database. BLAST typically takes input data in FASTA format showed a query sequence of 1301 nucleotides. The entire evolutionary analysis was carried out using MEGA11, which is a software tool used for conducting phylogenetic analyses.[25]

Conclusion

Pseudomonas aeruginosa has proved to be highly resistant, opportunistic and nosocomial pathogen affecting more males as compare to females with high percentage in age groups 60-80 year. Molecular characterization of Pseudomonas aeruginosa interpreted the presence of oprL and toxA resistance genes which emphasized that the research studies at molecular levels has improved the treatment strategies to control multidrug-resistant pathogens.

Ethical Approval: The study was conducted following ethical approval from the Institute of Molecular Biology and Biotechnology University of Lahore, Lahore viz Ref # IMBB/BBBC/23/910 dated 15-03-2023.

Ethical Approval: The IRB/EC approved this study via letter no. IMBB/BBBC/23/910 dated March 15,2023.

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Authors' Contribution

WB, SZHN: Conception

MM, MJI : Design of the work

AG, MQA: Data acquisition, analysis, or interpretation

MM, MJI, AG, MQA: Draft the work

WB, SZHN: Review critically for important intellectual content

All authors approve the version to be published

All authors agree to be accountable for all aspects of the work

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