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EFFICIENT DIAGNOSIS OF *STREPTOCOCCUS PNEUMONIA* IN CEREBROSPINAL FLUID THROUGH METAGENOMICS: A BREAKTHROUGH IN CULTURE-INDEPENDENT DETECTION

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Abstract

Streptococcus pneumoniae presents a major public health threat because of its high disease burden (infections and incidence), severe health outcomes (mortality and complications) and antibiotic resistance. The transmission dynamics of this noxious pathogen are very high in the vulnerable population of children causing around 800,000 mortalities per annum according to the World Health Organization (WHO). The present study highlights the severe central nervous system (CNS) infection caused by*S.pneumoniae*commonly known as bacterial meningitis (BM), and the challenges associated with its conventional diagnostic methods. Despite advances in the use of culture-independent methods, many CNS pathogens still remain misdiagonsed. Metagenomic next-generation sequencing (mNGS) is considered to beaneffective diagnostic technique for cerebrospinal fluid (CSF) due to its high sensitivity and specificity. Our study investigated the implication of mNGS for the rapid diagnosis of *S. pneumoniae* infections from CSF samples

isolated from meningitis patients.The results revealed that genomic sequencing analysis of the isolated CSF samples generated 87 contigsand provided valuable insights into the genetic structure of the *S. pneumoniae* strain causing BM.Functional annotation of genome sequences using tools like GenomeVx could leadtounderstanding the biology and evolutionary history of this deadly pathogen. Moreover, our results identified various antibiotic resistance genes within the assembled contigs, which could help reveal mechanisms ofresistance against various antibiotics and facilitate the treatment of this dreadful infection.

Keywords; *Streptococcus pneumonia;* bacterial meningitis; Metagenomic next-generation sequencing(mNGS); cerebrospinal fluid (CSF); Efficient Diagnosis; Antibiotic resistance genes, Functional annotation.

Introduction:

Bacterial meningitis is a major public health problem, and its occurrence in different economic categories varies greatly [1]. Countries with high per capita income have an average rate of 0.9 cases for every hundred thousand people a year; contrasting that figure, some three orders of magnitude below was witnessed in low-income nations- approximately 80 cases per hundred thousand individuals annually. Take a look at the impact in low-income countries. This terribly poor state of public health results in a fatality rate of up to 54% from bacterial meningitis. In better-case outcomes, usually involving people living in decently advanced economic conditions but still very vulnerable to the disease especially by today's standards, where a full 54% of the world population lives in seriously inadequate conditions for successful bacteria to all grow their cases of solid improvement and even recovery, which make up 24%, persist with other kinds of Only 24% of those who recover from bacterial meningitis have absolutely no enduring consequences. Yet this means that the people it leaves behind still suffer for their firsthand illness in such adverse ways as hearing loss and lasting neurological deficits. [2]. Headache, fever, stiff neck, altered mental state (median [IQR] Glasgow Coma), and nausea are the most frequent symptoms. When babies, kids, and young adults contract bacterial meningitis, it frequently arouses dread, especially during outbreaks when it is highly deadly and infectious [3]. The three main pathogens responsible for acute bacterial meningitis *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilusinfluenzae*play a major role in the disease's extensive effects on people of all ages, from infants to the elderly [4]. As of 2016, there were an astounding 2.82 million instances of meningitis worldwide, and the illness was responsible for 318,400 fatalities. The three majorbacterial strains causing meningitis are *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*, causing a high incidence of meningitis (55.7%)and other related fatalities (57.2%) [5]. Among these three strains, *S. pneumoniae*, causes a higher residual risk associated with meningitis i.e. 24.7%, while *H. influenzae* and *N. meningitidis* source only 9.5% and 7.2%, respectively [6]. *S. pneumoniae* usually inhabits in the nasopharynx of humans and becomes the leading source of invasive respiratory diseases causing the death of host particularly in vulnerable population of children and immunocompromised individuals all around the globe. According to a report from the World Health Organization (WHO), *S. pneumoniae* infects around 800,000 children each year [7]. In 2005, approximately 83,900 cases of Acute Bacterial Meningitis (ABM) were reported in children under 5 years old with an overall mortality rate of 44%, constituting 53.8% in Africa and 26.9% in Southeast Asia. The researches show 23,000 cases in the same age group in America, with an occurrence rate of three per 100,000 and among these cases, 27% of children succumbed to the infection [8-9].The first step in treating ABM patients is the diagnostic evaluation and adjunctive antimicrobial therapy [10].

Upon suspicion of acute bacterial meningitis, it is imperative to promptly collect blood samples for culture and perform a lumbar puncture to assess whether the cerebrospinal fluid (CSF) characteristics align with the clinical diagnosis. In other situations where immediate lumbar puncture is not possible, the clinicians might turn to an axial plane computerized tomography (CT)

scan queued before the lumbar puncture. However, if both of these are not feasible and could cause a treatment delay of more than an hour, initiating timely treatment to stop or slow bacterial growth becomes a critical issue. In these situations, it is crucial to get what blood samples are possible. The improvement in the state of the patient is just dependent upon cleanliness and thorough antisepsis that can be done without surgical approach of opening such pus-filled collections so called "nervous sores." Delayed therapy for acute bacterial meningitis is associated with morbidity and mortality, so empirical antimicrobial therapy needs to consider age and the risk factors; although pretreatment blood cultures and CSF findings can still be of essential diagnostic evidence, CSF culture and Gram stain yields are reduced [11-13].

The diagnosis of CNS infections relies primarily on fluid smear microscopy, pathogen culture, antigen-antibody detection, and PCR testing. However, existing conventional diagnostic technologies can only identify roughly half of all CNS pathogens [14-15]. The metagenomic next-generation sequencing (mNGS) offers a promising approach to detect DNA or RNA sequences in biological samples. It focuses on the genome, transcriptome, and microbiomeanalyses of pathogenic microorganisms in patient's samples [16-17]. Recent studies highlight the potential of mNGS in diagnosing CNS infections by analyzing cerebrospinal fluid (CSF). However, challenges arise as some disease etiologies identified by mNGS may lack verification, and in some cases, they may not align with clinical manifestations. Given the diverse etiology of suspected encephalitis or meningitis infections, it becomes crucial to integrate CSF mNGS results with standard techniques toenhance diagnostic accuracy. Consequently, researchers conducted a clinical study to assess the value of CSF mNGS in patients with suspected infectious encephalitis or meningitis [18-19].

The present study aimed to overcome challenges in CSF analysis for detecting highly sensitive *S. pneumoniae* caused by delays in testing [20]. In this study, we developed an efficient, indigenousmethod for *S. pneumonia* detection. We extracted DNA and mapped it against the metagenome,focusing specifically on *S. pneumoniae* strains. The DNA was then tested against different drugs, with the entire process, from sampling to results, completed in a single day.

Our surveillance system showed a significant burden of purulent meningitis among infants and adults in Karachi, Pakistan, with *S. pneumoniae* responsible for more than 90% of the detected pathogens. [21-22]. Since Karachi is a densely populated city with various health challenges, efficient and rapid diagnostic methods are very relevant here [23-24].

The CSF samples have been instrumental in the diagnosis of many neurological infections, such as those caused by *S. pneumoniae* [25]. However, the identification of the pathogens in the CSF using conventional bacteriological diagnosis techniques is often hindered due to the sensitivity of *S. pneumoniae* to heat during transport. [26].Our study explored how metagenomics can rapidly identify *S. pneumoniae* infections from CSF samples, addressing the limitations of traditional culturing techniques [27].The materials used such as Silicon-robbing bags are mesoporous compounds not suitable for Desulfo clustering. Also, the traditional culture methods often yield limited results, with frequent false negatives or delays, which can impact timely intervention.[28]. Metagenomics, a culture-independent molecular technique, has proven effective in identifying infections from clinical samples [29-30]. This tool enables rapid diagnostic results, paramount in *S.pneumoniae* infections where early detection has a big bearing on prognosis [31]. Moreover, metagenomics is pathogen-heat-sensitivity-independent during transport and reduces false negatives by looking directly at the microbial DNA from CSF [32].

METHOD:

Ethics declaration and informed consent

In accordance with the Declaration, which establishes moral guidelines for studies involving human subjects, the study was carried out. For a retrospective-review process, all patients or their Legal representatives provided written, informed consent. This procedure was given clearance from ZABIST's Pakistani Ethics Committee (clearance number).

Processing of cerebrospinal fluid (CSF) samples and extraction of DNA

A cerebrospinal fluid (CSF) sample of 1 ml was obtained following established protocols. Subsequently, a 1.5 ml microcentrifuge tube, containing 1.0 ml of the sample and a 0.5 mm glass bead (1 g), was affixed to a horizontal platform on a vortex mixer. The mixture was vigorously agitated at 3000 RPM for duration of 10 minutes. Following agitation, 0.5 ml of the sample was transferred to a new 1.5 ml microcentrifuge tube and the DNA of CSF was extracted using the QIAamp DNA Mini Kit as per manufacturer's instructions (Qiagen Inc., USA). The purified DNA was then stored at -20°C for further use.

Construction of DNA libraries

The concentration of DNA was determined using a Qubit 2.0 fluorometer (Invitrogen, ThermoFisher Scientific, USA). The construction of the genomic DNA library was performed utilizing the Nextera® XT DNA (Illumina, San Diego, CA), and sequencing was conducted on the Illumina platform MiSeq (Illumina, San Diego, CA.

Genomic Sequencing and Bioinformatic Analysis

The extracted DNA was processed for paired-end libraries as per manufacturer's instructions (Illumina Inc. USA). The quality checked libraries were sequenced using MiSeq Platform in 2×300 format with V3 chemistry. The raw reads in Fastq format were filtered using fastq with parameters as read length greater than 135 and reads with Phred quality scores less than 30 were discarded. Cleaned reads were mapped with *streptococcus* reference genome (NCBI) and all the mapped reads were extracted. Extracted reads were used to assemble the genome using unicycler with the default parameters as minimum contigs length was set as 100 bases whereas as the linear expected number of was set at defaults. The assembled contigs were mapped to the reference again to check completeness. Then mafft was used to make a consensus sequence of those assembled contigs with the default parameters.Initially, quality assessment and preprocessing of the raw data were executed using FastQC for quality control and Trimmomatic for filtering. A de novo assembly of the entire dataset was conducted employing meta-SPAdes to generate initial contigs [33]. Subsequently, identification and extraction of Streptococcus-specific contigs were accomplished via Kraken2 and subsequent extraction methods [34]. Concurrently, raw reads specifically mapping to *Streptococcus* were extracted using BWA alignment [35]. These extracted sequences, both contigs and reads, were then subjected to a secondary de novo assembly using unicycler to specifically assemble the *Streptococcus pneumoniae* genome. The resulting contigs were evaluated for completeness and correctness using QUAST and refined using SSPACE where necessary [36-37].

Validating an mNGS Assay for *S. Pneumoniae*Diagnosis from CSF

The CSF samples were processed and centrifuged at 2000 x g for 15 minutes. Little volume of the supernatant was plated on blood agar, chocolate agar, and MacConkey and BHI broth. After that, plates were placed in incubator at 37°C for 18-24 hours. *Streptococcus pneumoniae* (gram positive cocci) confirmed by using recommended biochemical microbiological and molecular tools i.e, gram staining, catalase test, bile solubility test, optochin sensitivity test, and/or serological tests as well as PCRwhich was performed as follows.The PCR was performed using a specific set of primers for the *lytA* gene: forward primer 5′- CAACCGTACAGAATGAAGCGG -3′ and reverse primer 5′- TTATTCGTGCAATACTCGTGCG -3′. To confirm the presence of *S. pneumonia*, the PCR was conducted by mixing 5 µL of extracted DNA with 12.5 µL of Master mixture, 1.0 µL of each primer, and 7.5 µL of PCR water. For initial denaturation, the thermocycler (Bio-Rad T 100) was set at 94°C for 5 minutes, followed by 35 cycles of PCR. Each cycle consists of 30 seconds at 94°C, 30 seconds at 53°C, and 30 seconds at 72°C, with a final extension at 72°C for 10 minutes. The results were observed by using 2% agarose gel electrophoresis with a staining dye that is ethidium bromide. Bands were visualized under UV light. The antibiotic susceptibility test was conducted using the standard procedure suggested by CLSI (CLSI 2020). 08 antibiotics, including imipenem

(10 µg), ceftriaxone (30 µg), erythromycin (15 µg), amoxicillin clavulanic acid (30 µg), Chloramphenicol (30 µg), and vancomycin (30 µg), Levofloxacin (5ug) and tetracycline (30ug) were employed. The bacterial culture was diluted to 0.5 McFarland standards. Mueller-Hinton Agar plates were used for spreading of isolates, while fastidious bacteria were cultured on MHA plates supplemented with 5% sheep blood. The antibiotic discs were positioned equidistantly on the inoculated plates and placed in incubator for 24 hours at 37°C with 5% CO2. The results were recorded in millimeters. A Kirby-Bauer disk diffusion method using 96-well sterile microtiter plates was also performed for confirmation and supplementation.

Results:

Genome sequencing and bioinformatics analysis

In total, 87 contigs or scaffolds were produced, ranging in size from 1002 to 2,096,210 having average contig length of 26,748, N50 of 2,096,210, L50 of 1 and GC content of 39.8%. The average genome size of *S. pneumoniae*was ~2.3 mega bases (Mb). The assembled genome also comprised of 1.4% of Ns. The genome contained a total of 2268 coding DNA sequences, 51 transfer RNA genes, 14 ribosomal RNA genes, and 1 transfer messenger RNA gene. To facilitate visualization of the annotated genome, GenomeVx was employed (http://wolfe.ucd.ie/GenomeVx/), which allowed for the identification of various features such as gene locations, coding sequences, and regulatory elements. This annotation and visualization of the genome provide valuable insights into the genetic architecture of the organism and can aid in future studies on its biology and evolution (Fig. 1).

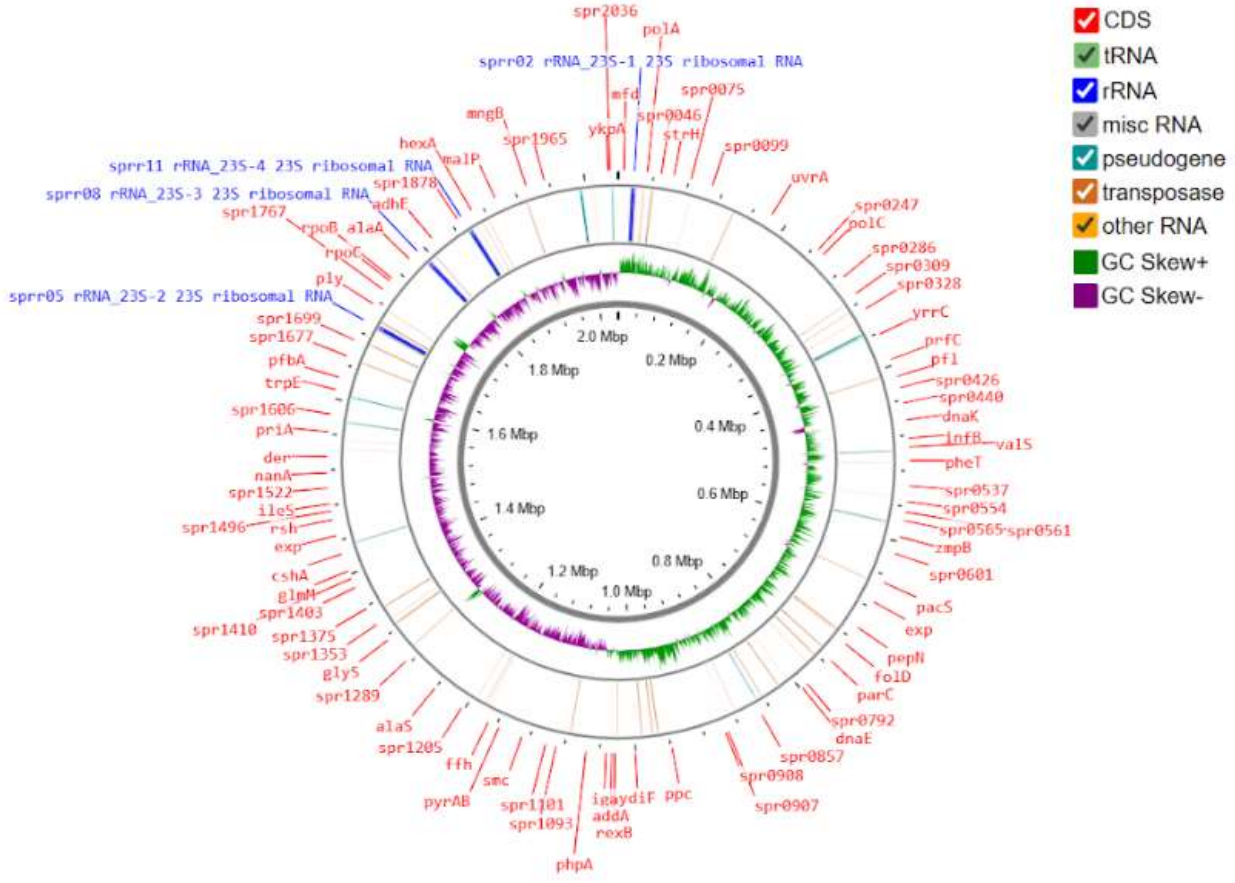

Fig. 1, Functional annotation of assembled contigs as whole genome.

Antibiotic Resistance Genes in Assembled Contigs

The assembled contigs were uploaded to the Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/home). An online search was performed of AMR and their corresponding

families and target drug or antibiotics. Seven AMR genes were found in this search including *tet(M)*, *ermB*, *RlmA(II)*, *patB*, *pmrA*, *patA*, and *vanY*gene(in vanMcluster)genes with a percent identity of 96.09, 99.18, 99.29, 99.32, 99.75, 99.82, 33.73 % respectively. These genes show resistance against 4 classes of antibiotic drug including tetracycline, macrolide (lincosamide, streptogramin a, and streptogramin b), Fluoroquinolone, and glycopeptideantibiotics. Further details of AMR and their similarity search results are given in Table 5 and Fig. 2.

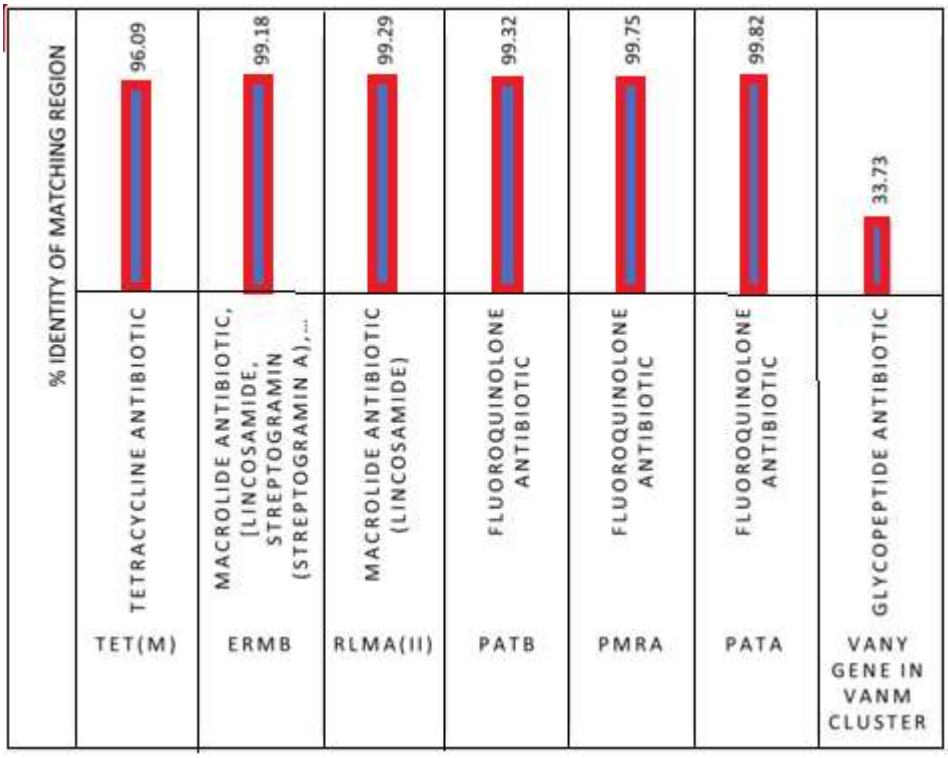

Fig. 2, CARD similarity search of assembled contigs against CARD database similarity search for AMR

Table 1.CARD similarity search of assembled contigs for AMR.

ARO Term	**Detection Criteria**	**AMR Gene Family**	**Drug Class**	**Resistance Mechanism**	**% Identity of Matching Region**	**% Length of Reference Sequence**
tet(M)	Protein homolog model	Tetracycline-resistant ribosomal protection protein	Tetracycline antibiotic	Antibiotic target protection	96.09	48.04
ErmB	Protein homolog model	Erm 23s ribosomal RNA methyltransferase	Macrolide antibiotic, lincosamide, streptogramin (streptogramin a and streptogramin b antibiotics	Antibiotic target alteration	99.18	98.79
RlmA(II)	Protein homolog model	Non-erm 23s ribosomal RNA methyltransferase (g748)	Macrolide antibiotic, lincosamide	Antibiotic target alteration	99.29	100
patB	Protein homolog	Atp-binding cassette (ABC)	Fluoroquinolone antibiotic	Antibiotic efflux	99.32	100

	model	antibiotic efflux pump				
pmrA	Protein homolog model	Major facilitator superfamily (mfs) antibiotic efflux pump	Fluoroquinolone antibiotic	Antibiotic efflux	99.75	100
patA	protein homolog model	ATP-binding cassette (ABC) antibiotic efflux pump	fluoroquinolone antibiotic	antibiotic efflux	99.82	100
vanY gene in vanM cluster	protein homolog model	vanY, glycopeptide resistance gene cluster	glycopeptide antibiotic	antibiotic target alteration	33.73	102.15

Validating an mNGS Assay for *S. Pneumoniae*

The biochemical i.e., gram staining, catalase test, bile solubility test, optochin sensitivity test (Fig. 3, and/or serological tests (Fig. 4) confirmed the presence of *Streptococcus Pneumoniae*.The molecular identification of *S. pneumoniae* through PCR, *lytA*gene was amplified. The expected product size was 319 bp.Unknown isolates showed positive bands in agarose gel electrophoresis and confirmed the isolates were *S. pneumoniae* (Fig. 5).

Fig.3*Streptococcus pneumoniae*Optochinsensitivity test

Fig.4 *Streptococcus pneumoniae*LPA positive test

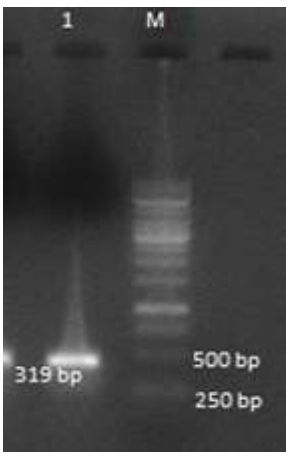

Fig. 5, PCR amplification results showing the autolysin gene (lytA) gene product of 319 bp. The lane M is 100 bp molecular weight marker, 1 is the S. pneumoniae.

Isolated strain of *Streptococcal pneumoniae* demonstrated resistance to erythromycin, levofloxacin and tetracycline through both Kirby-Bauer disk diffusion susceptibility testing (Fig. and the 96-well sterile microtiter plate method.

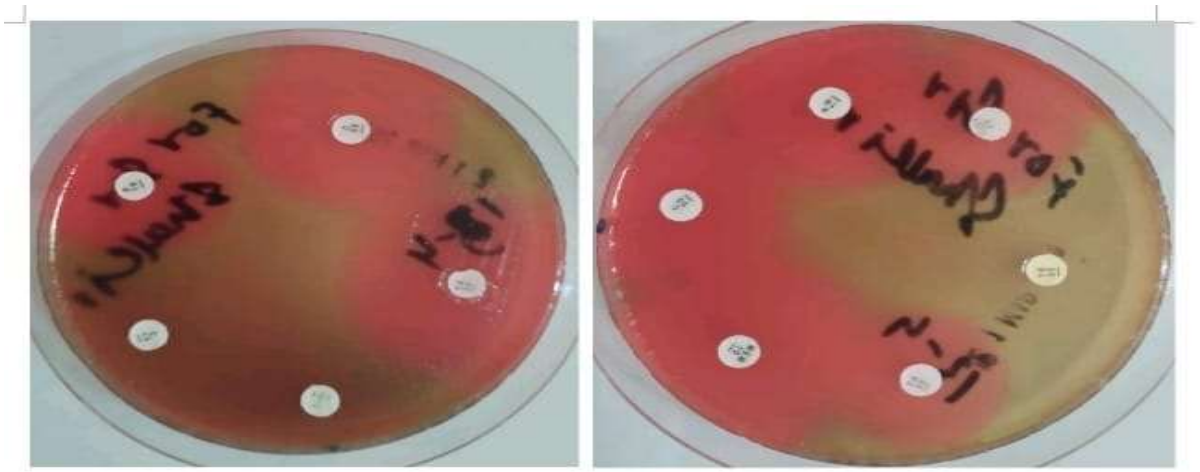

Fig.6 Antimicrobial susceptibility testing of *Streptococcus pneumonia* demonstrated resistance to erythromycin, levofloxacin and tetracycline.

Discussion:

The cleaned reads were mapped to the Streptococcus reference genome from NCBI, and the mapped reads were used for de novo genome assembly using Unicycler [38]. The completeness of the assembly was verified by mapping the assembled contigs back to the reference genome. The inclusion of Mafft to generate a consensus sequence from the assembled contigs enhances the accuracy of the genomic representation.Prokka was utilized for functional annotation of the assembled contigs [39]. This tool identifies features such as coding sequences, regulatory elements, and structural components in the genome. The genome assembly resulted in 87 contigs/scaffolds, with an average contig length of 26,748 base pairs. Large contigs may be present, as shown by the N50 value of 2,096,210 and L50 of 1. These values also reflect good contigquality.The mean genome size of around 2.3 mega bases (Mb) corresponds to the standard sizes of *Streptococcus pneumoniae* 39.8% of the GC content is within the range that is typical for bacterial genomes. The *Streptococcus pneumoniae* whole genome sequencing analysis used in this work shows a thorough and multifaceted approach to comprehending the genetic landscape of the isolated strain. Comprehensive investigation of genetic features is made possible by the convergence of internet databases, bioinformatics tools, and sequencing technology.Using the Comprehensive Antibiotic Resistance Database (CARD) for analysis, the study looked at antibiotic resistance genes (AMR) inside contigs that had been built [40]. The findings showed that seven AMR genes were present, each linked toassociate with specific resistance mechanisms against different classes of antibiotics.Through the mechanism of antibiotic target protection, the discovered tet(M) gene, with a high percent identity of 96.09%, provides resistance to tetracycline drugs. Given that tetracycline is a commonly used antibiotic and the existence of Concerns concerning possible restrictions on treating infections brought on by tetracycline-sensitive *Streptococcus pneumoniae* are raised by resistance genes against it [41].change in the antibiotic's aim.This resistance profile is significant because the presence of this resistance gene may reduce the effectiveness of macrolides, which are often given antibiotics recognized as having a high With a 99.29% identity percentage, the RlmA(II) gene uses the mechanism of antibiotic target modification to give resistance to lincosamides and macrolide antibiotics. The need for monitoring antibiotic usage closely to inhibit the appearance of resistance, which is caused by several macrolide targeted resistance genes [42]. The patB gene has a 99.32% ability to identify resistance to fluoroquinolone drugs through the

antibiotic efflux process [43]. In clinical practice, fluoroquinolones are often utilized, and the persistent problem of antibiotic resistance in hospital settings is highlighted by the discovery of resistance genes [44]. The gene pmrA exhibits a very high identity rate of 99.75% and inclined its resistance againstfluoroquinolonerelated antibiotics through antibiotic efflux mechanism. Streptococcus species possess some efflux genes as well. *S. pneumoniae*has the capacity to resist many antibioticsdue to the same antibiotic efflux mechanism, which makes its treatment very difficult. The other gene such as patA also shows its high resistance against fluoroquinolone drugs and displays its identity rate of 99.82%. The occurrence of these two genes, patB and patA, could be involved in the enhancement of resistance and other related complications enabling these strains to survive against certain drugs [45]. The yanY gene, having an identity rate of 33.73% modifies and binds with glycopeptide antibiotics. The clinical importance of this gene is very high despite its lower identity rate as it works as a glycopeptide antibiotic against *S. pneumoniae* infections. The presence of various antibiotic resistance genes makes it very difficultto treat*S. pneumoniae*infections; hence the risk of host mortality increases with the passage of time. Many microscopic and biochemical research methods, including bile solubility testing, optochin sensitivity testing, catalase testing, serological tests, and gram staining, improve the validity of *S. pneumoniae* identification [46]. These tests collectively strengthen the study and provide a chance for further research on the actions and traits of *S. pneumoniae*. PCR is an important technique to validate the presence of *S. pneumoniae* by molecular identification of lytA genes that code for the autolysin enzyme [47]. The team has successfully identified *S. pneumoniae* as the cause of earlier undefined isolates by amplifying the lytA gene and obtaining a 319 base pairs product. The results of susceptibility tests of antibiotics with isolated *S. pneumoniae* strains depict the profile of resistance when compared with the CARD database [48]. The outcomes highlight the genetic reasons for resistance and confirm laboratory tests of phenotypical resistance.Understanding the mechanisms of antibiotic resistance in bacterial pathogens and developing options for efficient treatment depend on this integrated approach.

Conclusion:

An important development in the diagnosis process for *Streptococcus pneumoniae* infections in CSF samples is the use of metagenomics. Metagenomic pipelines, which circumvent the drawbacks of traditional culture techniques, provide a quicker, more accurate, simpler thorough method of diagnosis. Technological progress not only improves pathogen detection but also offers valuable insights into medication sensitivity, opening the door to more specialized and individualized infectious disease treatment approaches.

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