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IN SILICO CHARACTERIZATION OF PLASMID-MEDIATED ANTIMICROBIAL RESISTANCE IN *STREPTOCOCCUS*

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Abstract

*Antimicrobial resistance (AMR) is a significant issue of global health concern that causes significant deaths around the world. Overuse and misuse of antibiotics are the main causes of the rapid development of AMR. Global estimates suggest that about 4.95 million AMR-related deaths occurred in 2019 and that the number is expected to increase to 10 million deaths per year by 2050 unless effective mitigation measures are taken. The problem of multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogens is becoming a major concern in such countries as Pakistan. Earlier research has mainly been conducted on phenotypic expression of antimicrobial resistance and the mechanisms like horizontal gene transfer, but detailed data on the expression of individual resistance genes is still scarce. The species of Streptococcus cause a great variety of infections in humans and animals some of which may be life-threatening. The genes on plasmids can easily mediate resistance in these bacteria and hence, they can be easily spread. This paper had the purpose of analyzing the acquired antimicrobial resistance genes in the plasmids of the bacterium, *Streptococcus, using bioinformatics tools and predicting the resistance phenotypes based on functional analysis. Pathogenic species of streptococci were examined and a total of 34 plasmids were analyzed. The acquired AMR genes were identified by the ResFinder 3.0 database, after which the results were confirmed by the KmerResistance database using the query coverage scoring. To determine levels of resistance, the potential multiple antibiotic resistance (p-MAR) index was determined. In total, 16 AMR genes were found in 10 antibiotic classes, such as aac(6')-aph(2")-cat, catA1, cfr, erm, and tet variants. Such results offer valuable information on plasmid-mediated resistance and can be used to develop new treatment approaches to address the growing multidrug resistance.*

Introduction

Antimicrobial resistance (AMR) has become a key global health issue, whose emergence was mainly due to the capacity of bacteria to survive the impact of antibiotics 【1】. To come up with effective strategies to counter the menace of bacterial resistance, it is important to understand the underlying mechanisms of bacterial resistance. The main mechanism is the enzymatic breakdown

of antibiotics, e.g. hydrolysis, making drugs useless. Moreover, commensal bacteria have also been found to be important reservoirs of AMR genes, which makes them persist and spread 【2】. The abuse and over prescription of antibiotics such as improper prescriptions, wrong dosages and lack of the full course of treatment also increase resistance. Horizontal gene transfer (HGT) is a key factor in the spread of antimicrobial resistance between bacterial groups. Bacteria can obtain and share resistance determinants between species by means of conjugation (direct cell-to-cell contact), transformation (absorption of free DNA in the environment), and transduction (gene transfer through bacteriophages)【3】. Of particular value in this regard are plasmids, which are mobile genetic factors that can carry and transfer several resistance genes at a time. The plasmids are classified into conjugative, mobilizable and non-mobilizable based on their transfer abilities 【4】. The growing resistance of plasmids, particularly epidemic plasmids, is a serious threat since it rapidly diffuses in human and animal populations. The genus Streptococcus contains Gram-positive, facultatively anaerobic bacteria that are usually coccobacillus in pairs or chains 【5】. These are organisms that are common to the environment and are often found on the skin and mucosal surface of human beings and animals. Most of the species are commensals, although some are pathogenic and cause a wide range of diseases, such as pharyngitis, pneumonia, meningitis and systemic infections 【6】. Virulence factors including surface proteins, toxins, biofilm formation and host immune resistance evasion have been largely attributed to their pathogenicity 【7】. Over 100 species of Streptococcus have been described, among which clinically important ones are streptococcus pyogenes (Group A) and *streptococcus agalactiae* (Group B). A significant human pathogen, S. pyogenes, causes mild (such as strep throat) and severe (such as necrotizing fasciitis and streptococcal toxic shock syndrome) diseases 【8】. Conversely, the S. agalactiae is typically present in the gastrointestinal and genitourinary tracts as a commensal organism but has the potential to cause life-threatening infections, especially among neonates, pregnant women, the elderly, and those with compromised immune system 【9】. It also has zoonotic potential, with animals including fish, cattle, and camels, and may be spread to humans via contaminated food chains. Traditionally, streptococci are assigned to a hemolytic group (alpha, beta and gamma hemolysis) and Lancefield grouping, which characterizes the species by cell wall carbohydrates antigens 【10】. But the classification may be complicated by the overlapping antigenic properties of species. Such clinically relevant resistance patterns in the species of Streptococcus are: resistance to macrolides, lincosamides, streptogramins, tetracyclines, beta-lactams and fluoroquinolones 【11】. These resistance characteristics are commonly linked to mobile genetic factors like plasmids and integrative conjugative factors (ICEs). The mediation of plasmid-borne resistance in the species of Streptococcal is done in various ways such as alteration of the target site (e.g., methylation), active efflux of antibiotics and enzymatic inactivation 【12】. These processes enable bacteria to survive in the presence of antibiotics and they help in the dissemination of multidrug resistance worldwide. What is more, genetic research has also shown that resistance genes can be easily spread among bacterial populations via HGT pathways 【13】. The increased resistance of AMR in Stenotococcus species highlights the critical importance of thorough molecular studies to determine resistance factors and how they are transmitted. These findings can play a significant role in informing the creation of new therapeutic strategies, enhancing antibiotic stewardship, and adopting competent infection control strategies to curb the effects of antimicrobial resistance on

world health【14】.

Methodology

Genome sequences of the genus Streptococcus were compiled and examined in a systematic manner to examine antimicrobial resistance determinants. Firstly, the species diversity in the genus was measured based on the LPSN database which contained 159 species with 56 pathogenic, 2 opportunistic and 101 non-pathogenic species. Out of them, only species that have had plasmid data were further analyzed. The NCBI nucleotide database was used to extract plasmid sequences using certain filters to gain plasmid entries. Duplication was avoided by eliminating redundant sequences, such as sequences with the same base-pair length but with different accession numbers. A total of 540 distinct plasmids were completed to be analyzed but the preliminary search in the database suggested that there were more but probably because of updates or eliminations in the database over time. In order to supplement the collection of sequences, a literature survey was conducted to find out the already reported resistance genes to Streptococcus species with the help of Google Scholar. The keywords were antimicrobial resistance, antibiotic resistance and species-specific resistance terms. The resulting data consisted of plasmid sequences, resistance genes and included with specific prevalence in tetracycline, aminoglycoside, macrolide-lincosamide-streptogramin (MLS) and beta-lactam resistance genes. The acquired antimicrobial resistance genes were identified by means of the database ResFinder 3.0. The uploading of plasmid sequences was done one at a time and the analysis parameters were set to 90 percent identity and 60 percent minimum length to be sure of detection. Other options were given default settings. The outputs were received as phenotype tables and gene-specific outputs, which gave more specific information about resistance genes present in every plasmid. Also, the potential multiple antibiotic resistance (p-MAR) index was computed to assess the level of resistance in each antibiotic classes. The Kmer Resistance 2.2 database was used to confirm the identified resistance genes【15】. The bacterial plasmid host database and resistance gene database were analyzed with a 70 percent identity and 10 percent depth coverage parameter. The use of maximum query coverage scoring method was used to identify species and genes with accuracy. FASTA【16】-formatted plasmid sequences that did not have formatting inconsistencies were uploaded to ensure appropriate analysis. The results of the confirmed resistance genes obtained with Kmer Resistance were cross-tabulated with the results of ResFinder to make sure that the results are consistent and accurate. The additional functional characterization of antibiotic resistance determinants was conducted with the help of the ResFinderFG 2.0 database that made it possible to predict the resistance phenotypes basing on the functional metagenomics【17】. This step gave us an insight on the expression and the functional relevance of identified resistance genes. Lastly, statistical tests were performed to test the associations between plasmid characteristics. XLSTAT software was used to conduct principal component analysis (PCA) to test the relationships between GC content and plasmid distribution. NCBI BLAST software and MEGA X software were used to measure sequence similarity and homology by default maximum likelihood methods. This holistic methodology made it possible to identify, validate and functionally determine plasmid-mediated antimicrobial resistance genes in Streptococcus species【18】.

Results

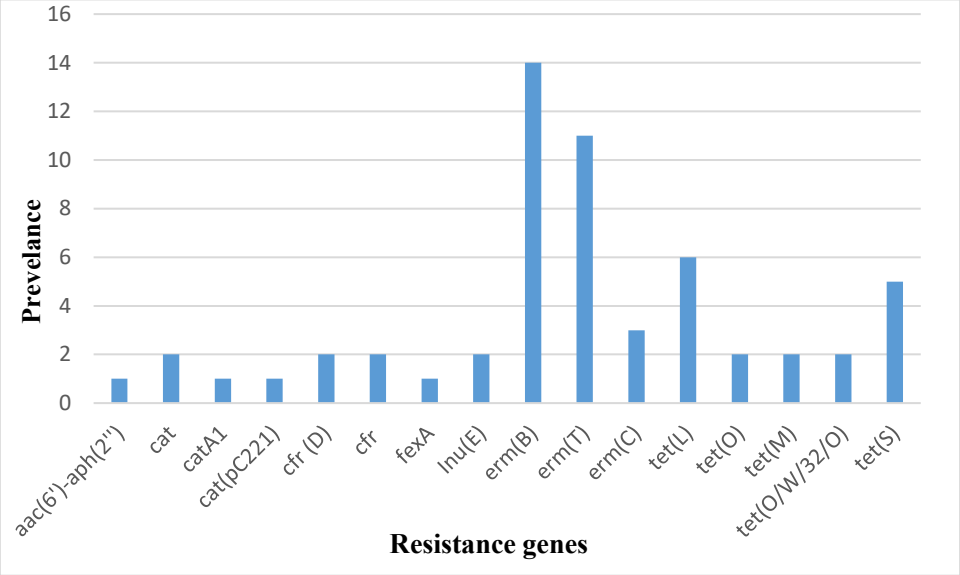
Thirty-four plasmids belonging to a variety of different species of Streptococcus were examined in order to identify the distribution of the antimicrobial resistance (AMR) genes within the key antibiotic classes. In general, the determinants

of resistance were not numerous but highly dispersed, and the majority of plasmids had resistance to one antibiotic group only, as the potential multiple antibiotic resistance index (p-MAR) was low (0.06). Most common resistance was found in macrolide– lincosamide streptogramin (MLS) antibiotics (21 occurrences) then tetracycline (11 occurrences) finally phenicol (8 occurrences). Aminoglycoside resistance was uncommon (1 case), and beta-lactam, fluoroquinolone, rifampicin, sulfonamides, and trimethoprim resistance occurred rarely or not at all. The pattern of the plasmids of the Styptococcus pyogenes was consistent, with most of the strains and plasmids encoding the single genes of MLS resistance. In the same way, plasmids of Streptococcus agalactiae were predominantly MLS-associated, although some plasmids, including pMV158, had several resistance determinants, which lead to an increased p-MAR index (0.13). More variation was found in the case of species like, *Streptococcus suis, Streptococcus mutans and Streptococcus pasteurianus. It is important to note that the plasmid of Streptococcus mutans (strain MD) and Streptococcus suis plasmid pStrcfr exhibited resistance to several classes of antibiotics, with the highest p-MAR index (0.2) suggesting the possibility of multidrug resistance. Conversely, single-class resistance, mostly tetracycline or MLS, was linked to plasmids of species like *Streptococcus infantis, Streptococcus parauberis and Streptococcus gallolyticus. To recap it all, the plasmid-mediated resistome of the Streptococcus prevails with MLS and tetracycline resistance genes. Most plasmids have a low resistance diversity, but a subgroup carries multiple resistance determinants, emphasizing their possible contribution to the spread of multidrug resistance.

Category	Description
Low resistance plasmids	Majority (single gene; p-MAR = 0.06)
Moderate resistance plasmids	Few (2–3 genes; p-MAR ≈ 0.13)
High resistance plasmids	Rare (≥3 genes; p-MAR = 0.20)
Major resistance drivers	MLS and Tetracycline genes
Epidemiological concern	Multidrug-resistant plasmids (e.g., *S. suis*, *S. mutans*)

The homologues of the antimicrobial resistance (AMR) genes in plasmid sequences of the species of Streptococci were identified using ResFinder database, which identifies the resistance genes only through acquired resistance genes. This method fails to identify chromosomal mutations, thus, phenotypic confirmation might be needed to fully confirm resistance profiles. The reliability of sequence-based methods in AMR surveillance has been proven through previous studies that have shown a high concordance (approximately 99.74) between phenotypic and whole-genome sequence-based predictions of antimicrobial susceptibility. Proper sequence alignment is essential to accurate determination and understanding of resistance determinants, especially when comparing many variants of genes in different plasmids. The analysis of the ResFinder in this study provided evidence of 16 obtained AMR genes spread among various antibiotic classes. The identified genes were aminoglycoside resistance gene aac(6 0)-aph(2 0)-; phenicol resistance genes (cat, catA1, cat(C221) -cfr); macrolide -lincosamide streptogramin (MLS) resistance genes (ermB, ermC, ermT, lnuE); and tetracycline resistance These results suggest that the plasmid-mediated

resistance in *Streptococcus* is mainly linked to the MLS, tetracycline, and phenicol classes of antibiotics with minimal representation of other classes of drugs. Figure 1 shows the entire distribution of identified AMR genes.



Discussion

(AMR) mediated by plasmids. In the first instance, 65 pathogenic species were determined, 28 of which species had plasmids in the NCBI database. Even though a total of 540 plasmids have been reported on the genus, about 190 plasmids were related specifically to pathogenic species, and included in this study. Some species, including, but not limited to, , *Streptococcus alactolyticus* and *Streptococcus pluranimaliu*m initially displayed plasmid records, but were subsequently no longer available in the database, probably as a result of updates or data maintenance 【19】. The ResFinder 3.0 database was used to screen all the selected plasmid sequences to determine acquired AMR genes (Table 1). The findings were also confirmed with the help of the KmerResistance database in order to be accurate and consistent 【20】. Each plasmid was given a potential multiple antibiotic resistance (p-MAR) index, with a range of 0.06 to 0.15, which implied that the general resistance was not high or low. The p-MAR index was calculated as the number of the antibiotic classes to which a plasmid was resistant to divided by the total number of antibiotics tested 【21】. The number of AMR genes was also found to be 16 in the analyzed plasmids, which are four large antibiotic classes. The aminoglycoside resistance gene aac(6)-aph(2)-aac(6): aph(2)-aac(6) changes antibiotics and prevents protein synthesis, which makes it resistant. Enzymatic inactivation was linked to phenicol resistance genes (*cat(pC221)*, *catA1*, cfr, cfr(D) and fexA) and was mainly via acetyltransferase activity. Macrolide-lincosamide-streptogramin (MLS) resistance genes (>lnu(E)*, erm(B)*, erm(C)*, erm(T)*) are effective by altering the ribosomal targets to prevent the elongation of peptide chains. The tetracycline resistance genes (tet(L) (tet(M)) (tet(O) (tet(S)) (tet(O/W/32/O)) (tet(O/W/32/O)) confer resistance either by the 30S subunit by ribosomal protection or efflux mechanisms 【22】. Of all the reported genes, the most common was the gene named erm(B) which suggested extensive resistance to the MLS antibiotics in the plasmids of the genus Streptococcus 【23】. On the whole, these results demonstrate that MLS and tetracycline resistance

mechanisms prevail and stress the importance of plasmids in the spread of AMR in the pathogenic species of the genus Streptococcus.

Conclusion

Pathogenic species of Streptococcus were systematically examined in this study on the basis of computational methods to determine plasmid-mediated antimicrobial resistance (AMR) genes. A total of 34 plasmids in 11 pathogenic species were identified to contain acquired resistance genes out of 194 plasmids investigated. A total of 16 AMR genes were identified, including **aac(6')-aph(2'')**, **cat**, **catA1**, **cat(pC221)**, **cfr**, **cfr(D)**, **fexA**, **lnu(E)**, **erm(B)**, **erm(C)**, **erm(T)**, **tet(L)**, **tet(O)**, **tet(M)**, **tet(O/W/32/O)**, and **tet(S)**. These genes were also linked to resistance to important antibiotic classes like aminoglycosides, phenicols, macrolide - lincosamide streptogramin (MLS), and tetracyclines. The presence of MLS- and tetracycline-related genes, especially, the most common, that is, the **erm(B)* gene, indicates their general distribution within the plasmids of the genus Streptococcus. The occurrence of these resistance determinants implies the possibility of AMR spreading plasmid-mediated, which leads to the development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains. Comprehensively, this work highlights the essentiality of plasmids as a reservoir and vectors of AMR genes in pathogenic species of Streptococci. The results offer useful information towards surveillance, risk evaluation, and subsequent formulation of specific measures to curb the transmission of antibiotic resistance.

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