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UNCOVERING THE GENETIC MAKEUP OF A HOSPITAL-ASSOCIATED MULTIDRUG RESISTANT PATHOGEN *E. CLOACAE* MB649 FROM INFECTED ORTHOPAEDIC IMPLANTS

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Abstract

Hospital environment may serve as important reservoirs of multidrug resistant pathogenic bacteria, or they can also emerge in the healthcare facilities as a result of mutation or horizontal gene transfer. In the present study, we sequenced and phenotypically characterized, a Gram-negative, rod-shaped isolate recovered from infected orthopaedic implant samples collected from a hospital in Islamabad, Pakistan. The optimum growth temperature and pH was 37°C, pH 7, respectively under laboratory conditions. Colonies on BHI and LB agar were round, smooth, raised and off-white in colour. The genome of the isolated strain denoted as MB649 was sequenced, the results showed 94% similarity to *Enterobacteriaceae* family, 85% to genus *Enterobacter* and 82% with specie *cloacae*. The genome analysis revealed presence of several secretion systems such as type II, IV, V, VI, VIII along with several heavy metal resistant genes such as systems for copper, nickel, tellurium, and tellurite resistance and multidrug resistance genes as well. The strain also encoded two plasmids IncHI1A(NDM-CIT) and IncHI1B(pNDM-CIT). Assembly resulted in 5297873 base pairs and 239462 reads distributed in 104 contigs, with a G + C content of 54.46 %. Sequence and annotation data of the *E. cloacae* MB649 genome have been deposited at GenBank under accession JABWTL000000000.

BACKGROUND INFORMATION

Members of the genus *Enterobacter* belong to the class *Gammaproteobacteria* and are rod-shaped, Gram-negative, motile, facultative anaerobes. They are commonly found in soil, water, sewage, and intestines of animals and humans (Davin-Regli and Pagès*.* 2015*)*. *Enterobacter* species are important human opportunistic pathogens, responsible for nosocomial infections such as osteomyelitis, cholecystitis, urinary tract infections, and neonatal meningitis (**Sanders and Sanders,** 1997; Habib *et al*., 2022). *Enterobacter cloacae* is a prevalent nosocomial pathogen exhibiting high-level resistance to disinfectants and antimicrobial agents (Mishra *et al*., 2017; Senchyna *et al*., 2019; Muhammad *et al*., 2022). Here, we report the morphological, phenotypic characteristics and the genome sequence of *Enterobacter cloacae subsp. cloacae strain* MB649, isolated from infected orthopaedic implant samples collected from a hospital in Islamabad.

MATERIAL AND METHODS

Sample Collection and Isolation

The sample for this study was collected from a 65-year-old diabetic female undergoing removal of an infected surgical knee implant in the orthopaedic department of a hospital in Islamabad, Pakistan. The samples were collected aseptically, inoculated into Luria-

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Bertani (LB) broth, and cultured aerobically for 24 hours at 37°C. Serial dilutions of the bacterial culture were made, and single bacterial colonies were obtained following serial dilution and streaking.

Crystal Violet (CV) Adherence Assay In Microtiter Plates

Biofilm forming ability was observed using the crystal violet assay. Briefly, an aliquot (100 µL volume) of culture suspension diluted to an OD_{600} of 0.05 was added to each well in a 96-well microtiter plate. The plate, sealed with a sterile gas-permeable film was incubated under static condition at 37°C for 24 hours. The biomass attached to the walls of the plates was quantified using crystal violet stain. The amount of crystal violet stain absorbed by the biomass was extracted with 200 µL acetic acid (30% v/v) in each well. After 10 min, the OD_{595} was measured spectrophotometrically. A well containing sterile LB served as a negative control. All assays were conducted in triplicate.

Siderophore (Pyoverdine and Pyochelin Measurement)

The siderophore secretion was measured using the method purposed by Schwyn and Neilands (1987). Briefly, 2 mL of the overnight culture of MB649 was spotted into the centre of the chrome azurol S (CAS) plate's agar plate. Formation of an orange halo around the colonies after 24 hours incubation at 37°C indicated presence of siderophore-producing bacteria.

16S Rdna Sequencing

The identity of the isolate was established via 16S rDNA sequencing and subsequent comparison of the output sequence data against the NCBI database (http://www.ncbi.nlm.nih.gov/). The 16S rRNA-encoding genes typically contain 9 hypervariable regions (V1 – V9) (Kameoka *et al*., 2021). Initial identification of MB653 was confirmed through 16S rDNA analysis using the V3 and V4 hypervariable regions. Genomic DNA was extracted from the bacteria following the protocol described by Vingataramin and Frost (2015). Colony PCR was carried out to amplify the 16S rRNA-encoding gene. Briefly, 250 ng of bacterial DNA was used as template with 0.5 µM (each) of the degenerate primers 341F [5-CCTAYGGGRBGCASCAG-3] and 806R [5-GGACTACNNGGGTATCTAAT-3], which anneal to the sequences flanking the V3 and V4 regions, 200 µM deoxynucleoside triphosphate, and 2 U of *Taq* DNA polymerase (Boehringer Mannheim) in a 1× amplification buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$). The PCR comprised an initial denaturation (94°C for 2 min), followed by 40 cycles of denaturation (94°C, 1 min), annealing (56°C, 1 min), and elongation (72°C, 1 min). The final extension step was at 72°C for 10 min. PCR products were resolved by electrophoresis on a 1% agarose gel before excision of the gel fragments and sequencing. The sequences obtained were confirmed as *Enterobacter cloacae* by comparison with sequences deposited in the NCBI gene bank, http://www.ncbi.nlm.nih.gov/. MEGA X software was used to generate a phylogenetic tree (Kumar *et al*., 2018).

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Whole Genome Sequencing, Assembly and Annotation

The whole genome sequence of MB649 was generated by Illumina sequencing (250 bp paired end protocol) performed by a sub-contractor (MicrobesNG). The sequence reads (mean depth: 65×) were assembled using SPAdes version 3.7, and the resulting contigs were annotated using Prokka 1.11, NCBI 'prokaryotic genome annotation pipeline', and the PATRIC/RAST pipeline. A comparative circular genome map was built in PATRIC circular genome viewer. The genome assembly comprised 479,6141 base pairs from 753,595 reads distributed across 273 contigs, with a G + C content of 50.7 %.

In-Silico Analysis of Whole Genome Sequence

A total of 8 *Enterobacter cloacae* genomes were used for pan-genome analyses (7 from GenBank and the new genome from this study). BPGA (Bacterial Pan-genome Analysis tool) was used to estimate core, pan and species-specific genomes. Initial clustering was done through Usearch34 algorithm and output processed into pan, core, and accessory gene categories. Resistance genes, and plasmids were predicted using ResFinder (Zankari *et al*., 2012) and PlasmidFinder (Carattoli *et al*., 2014). Default settings were used in all software unless otherwise mentioned. Variant calling was performed on Snippy 4.6.0.

Genome Accession Number

The accession number of the sequence of *Enterobacter cloacae* MB649 determined in this study can be found in GenBank (http://www.ncbi.nlm.nih.gov) under the accession number JABWTM000000000, BioProject number PRJNA639985, and BioSample number SAMN15296398. Raw sequencing reads were deposited in the Sequence Read Archive (SRA) under accession number SRR12052856.

Overview of the Sequenced Genome

Genomic libraries containing of 5297873 base pairs and 239462 reads distributed in 104 contigs, with a G + C content of 54.46 % were constructed giving 16.9-fold coverage of the genome (Figure 1). PGAP annotation revealed that the genome comprised of 5,131 genes, out of which 4,913 were coding DNA sequences and 118 were RNA genes including 6, 10, 17 (5S, 16S, 23S) 23S rRNA copies. 80 tRNAs coding for all 20 amino acids and 5 ncRNAs were predicted.

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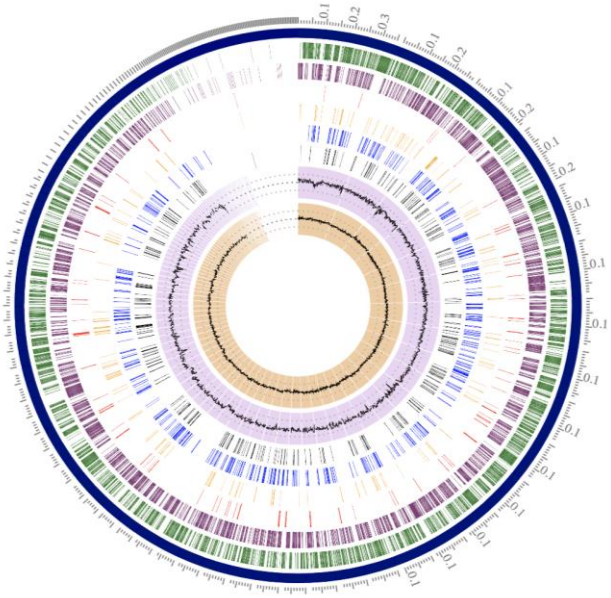

Figure 1: Circular genome map of *Enterobacter cloacae* MB649. Moving inwards from the outer ring, contigs are represented in blue; CDS-Forward in green; CDS-Reverse in purple; AMR related genes in red; virulence factors in orange, transporter in blue; drug targets in black, GC content in violet and GC skew in brown

MLST

A multi-locus sequence typing (MLST) scheme was established using PubMLST database, strain MB649 is represented by sequence type ST167.

Metal and Antibiotic Resistance Profile

Multiple sets of virulence determinant and heavy-metal resistance genes were mined in the genome. In *Enterobacter cloacae* MB649, we catalogued Type II, IV, V, VI, VIII secretions systems along with several heavy metal resistant genes such as systems for copper, nickel, tellurium, and tellurite resistance. The organism carries genes for several multidrug efflux proteins, suggesting its broad range of antibiotic resistance. Multiple resistance genes responsible for resistance to antibiotics such as fosfomycin, trimethoprim, β-lactams, erythromycin, and tetracycline, were identified in the strain, enabling it to tolerate and thrive in the presence of antimicrobial compounds. *Enterobacter cloacae* MB649 harboured two plasmids IncHI1A(NDM-CIT) and IncHI1B(pNDM-CIT) carrying genes encoding the metallo-β-lactamase NDM-1. Type I–F CRISPR–Cas system including proteins cas1, cas3, cys1, cys2, cys3 was also mined in the genome.

Toxin–antitoxin (TA) systems are small genetic elements composed of a toxin gene and its cognate antitoxin. Currently, TA systems are assigned to five classes (I–V) according to their genetic structure and regulation. (Unterholzner et al., 2013). Our genome also encompassed four classes Type I, II, IV and V of the TA systems. Among these four

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types of the TA system, Type II toxin-antitoxin (TA) systems are widespread in bacterial and archeal genomes. These modules are very dynamic and participate in bacterial genome evolution through horizontal gene transfer (Jurėnas *et al*., 2017). Our strain carries ten super-families of type II toxin-antitoxin system RelE/ParE, VapC, RatA, HicA, HicB, CcdA, PemK/MazF, HigB, HipA and RelB/DinJ family.

Pan Genome Analysis

The pan-genome forms the repertory of all the genes present in a specific species including core genome, accessory genome and singletons which includes the unique genes. This type of analysis has been used to determine the genetic diversity in a group of species relating phenotypic traits, ecological adaptation, new host colonization, virulence, and antibiotic resistance. Ecological adaptations, pathogenic and phenotypic traits could, therefore, be sieved out through this approach. In past pan-genomic information have been used for developing therapeutics to help combat the bacterial infections (Ali *et al*., 2015)· The pan-genome analysis of *Enterobacter cloacae* MB649 showed that most of the unique genes are related to information storage and processing while most of the core genes are associated to metabolic functions. KEGG annotation was classified into five functional categories (cellular processes, environmental information processing, genetic information processing, human diseases, and metabolism). Results indicated that a total of 1824 genes were involved in the metabolic activities, with the most abundant category represented by carbohydrate and amino acid metabolism. In addition, 567 genes participated in environmental information and processing.

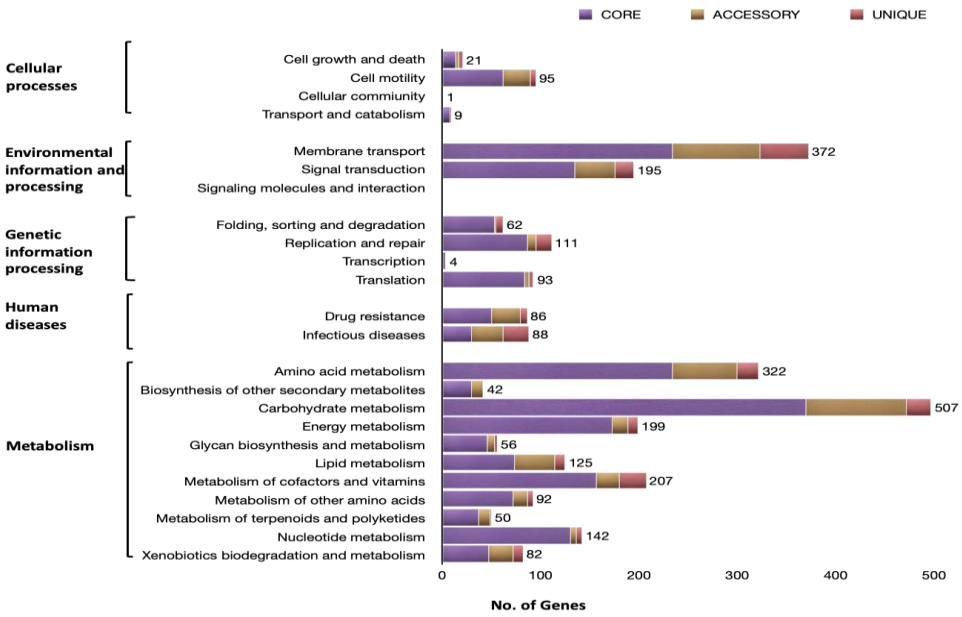

Figure 2: The gene functional annotations based on Kyoto Encyclopaedia of Genes and Genomes (KEGG) database of the genes forming core, accessory, and unique portion of genomes under study

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CONCLUSION

We studied the phenotype and sequenced the strain *Enterobacter cloacae* MB647 isolated from infected orthopaedic implant samples collected from a hospital in Islamabad, Pakistan. A multi-locus sequence typing (MLST) scheme was established using PubMLST database, strain MB649 is represented by sequence type ST167. The genome sequencing of strain MB649 provided a glimpse into its pan genome and antibiotic resistance capability of the multidrug resistance strain. The identified strain harboured several genes for antibiotic resistance and also carried two plasmids IncHI1A (NDM-CIT) and IncHI1B (pNDM-CIT) carrying genes encoding the metallo-β-lactamase NDM-1. The transmission these multidrug resistant strains in the healthcare facilities can be restricted by active surveillance, raising awareness, regular screening, and implementation of infection control strategies.

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Disclaimer: None to Declare

Conflict of interest: None to Declare

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