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GENOME SEQUENCE AND ASSESSMENT OF THE VIRULENCE AND ANTIBIOTICS RESISTANCE OF A NOVEL *PAENIBACILLUS* SP. 6A ISOLATED FROM A PATIENT WITH ACUTE OTITES MEDIA IN DIYALA CITY, IRAQ

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Abstract

Bacterial infection is a major cause with acute/chronic inflammatory diseases. Otitis media is one of the most common reasons for children to visit a physicians and its important cause of hearing loss. A section prospective study with in ENT clinics in Diyala city from October 2018 to June 2019. A total 50 ear samples were collected from children up to 12 years old and cultured according to the standard microbiological procedures. In this study, we reported the draft genome sequence of *Paenibacillus* sp. 6A, isolated from a patient with Acute Otites Media (AOM). The genome consists 5, 941, 665bp, with a GC 35.5%. It has 2335 predicted protein-coding genes encoding multidrug resistance transporters, virulence factors. The draft genome sequences project was deposited in Gen Bank under accession no. SAMN14163233. The draft genome was annotated using RAST tool. Furthermore, to estimate the phylotypes in the selected genomes, the 16S rRNA gene sequences were retrieved from the RAST annotation and used as a query against the SILVA reference database with the threshold set to above 97%. CARD tool was used to characterize the features of antibiotic resistance and virulence factors. Bacterial susceptibility to antibiotics was determined using E-test method. Our result showed that the tested strain harbors several antibiotic resistance genes. These genes were compared with the data of antibiotic susceptibility test, *Paenibacillus* sp. 6A resisted to macrolides, Trimethprime-Sulfamethoxazole and Chloramphenicol.

Key words: Otites Media, *Paenibacillus* spp., Antibiotic resistance, Whole genome sequences

Introduction

Acute Otits Media (AOM) is a very common childhood disease. It affects more than 80% of children at 10years old (Hoberman *et al.,* 2011).

Many factors could play animportant role in spreading of otitis media infection throughout the populations, including poor hygiene, limited formal education and overcrowding (Binks *et al.,* 2011). A novel spore-forming *Paenibacillus* sp. strain VT-400, was detected from the saliva of patients with acute lymphoblastic leukemia (Tetz *et al.,* 2015). *Paenibacillus* spp. was reported as a human pathogenin the recent reports identified *P.alvei, P. thiaminolyticus* and *a P. sputi* in the respiratory and urinary tract infection (Tetz and Tetz, 2017). Notably, spore-forming bacteria are poorly studied and only a few such bacteria have been described and associated with the human microbiota (Tetz *et al.,* 2016). This study comes to shed the light on the bacterial genus *paenibacillus* sp. which is a Gram-positive filamentous bacterium characterized by a complex morphological growth and differentiation space. In generall, the genome of *Paenibacillus* sp. is closed to the genome of *Mycobacterium* genus (Bentley *et al.,* 2002). The genetic properties and composition of *Paenibacillus* spp. (chromosome and its replication) have been extensively explored and showed the guanine/cytosine (G+C) nucleotide pairs is more than 50% (Hopwood, 2006). Genome mining for new natural products is an area of great interest *Paenibacillus* spp. produces several antibiotics which have been used for industrial applications (Nolan and Walsh, 2009, Katz and Baltz, 2016). Bacterial susceptibility to antibiotic could be impacted by the environmental stress, which promotes adaptive and protective responses that alter cell physiology

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and potentially generate resistance to antibiotics. Antibiotic polluted soils often harbor unique and phylogenetically diverse bacterial communities including *Actinomycetes*, *Firmicutes* or *Protebacteria*, as the key elements for bioremediations systems (Ug and Ceylan, 2003).

Whole genome sequencing (WGS) of novel environmental bacteria using Next Generation Sequencing (NGS) technologies can open new perspectives in microbial ecology studies (Mardis, 2008). The introducing of NGS technology has a major impact on the understanding and comparing the role of microbial evolution and metabolism in the environment (Brown, 2015). Illumine Mi Seq technology produces around 400 bp reads via paired-end sequencing (Buermans and Den Dunnen, 2014). The use of *de novo* assembly software and annotation algorithms is a powerful approach that allows bacterial genomes to be assembled with automated annotation, which allow vast quantities of sequence results to be processed into meaningful data that are useful for general comparison of novel bacterial strains (Kisand and Lettieri, 2013). The draft whole genome sequences (WGS) assembly and annotation aids the description of core genomes and different metabolic features of novel environmental bacteria, e.g. genome size and basic metabolic pathways.

Furthermore, 16S rRNA sequencing-based methods help to identify many bacterial isolates as a possible pathogen of AOM (Ederveen, 2019).

Microbes produce a wide variety of bioactive natural products, such as polyketides and non-ribosomal peptides, which are derived from large biosynthetic gene clusters (BCGs) encoding regulatory, biosynthetic, post-production modification and immunity or resistance genes (Ohnishi *et al.,* 2008). In this study, we described *Paenibacillus* sp. strain 6A, a novel bacterium isolated from ear infection and investigated the draft genome prosperities and the resistance genes.

Materials and Methods

Bacterial strains

A sectionprospective study with in ENT clinics in Diyala city from October 2018 to June 2019. A total 50 ear samples were collected from children up to 12 years old and cultured according to the standard microbiological procedures. Twenty five bacterial isolate was obtained from middle ear of infected children with AOM that hospitalized at Al-Zahraa hospital, Diyala, Iraq. The selected isolate was grown on Columbia agar supplemented with 5% sheep blood (BioMerieux, France) and then, stored at -80ºC into Columbia broth (BioMerieux, France) containing 50% glycerol. Only one isolate (6A) was chose for further study.

Determination of minimal inhibitory concentration (MIC) of antibiotics via E-test

The minimal inhibitory concentration (MIC) of 20 antimicrobial was identified against the selected isolate using E-test strips (Himedia laboratories Pvt Limited, India). Each isolate was inoculated into 5 ml of Muller Hinton Broth (MHB) and incubated aerobically for 18 hours at 37°C at 200 RPM. The overnight culture was adjusted to 0.5 McFarland stander which is equal to bacterial count 10^6cfu/ml. A100 µl of the standardized bacterial inoculum suspension was added and spread overall lMHA plates (Pruesse *et al.,* 2007, Di Bonaventura *et al.,* 1998).

A relevant antibiotic E-test strip was then placed at the center of each plate and incubated at 37°C for 18 haerobically. After incubation, the MIC was then determined by measuring the inhibition zones according to manufacturer's guidelines. The MIC breakpoint for susceptibility was defined as the lowest antibiotic concentration that exhibited complete inhibition of microbial growth. The experiment was performed twice and in triplicate.

Extraction of genomic DNA and genome annotation

The whole genome DNA sequencing and genome assembly was out sourced to the Swansea Genome Sequencing facility upon provision of genomic DNA samples. The whole genome of isolate 6A was aligned using Rapid Annotation Subsystems Technology (RAST) (Aziz *et al.,* 2008).

Taxonomic affiliation

The assembled genomes in multi-counting format were used "at species level" and the isolates were assigned (the isolates number based on their soil origin). Initially, the strains were given isolate numbers when the taxonomic identity of the species was unknown. To estimate the phylotypes of the selected genomes, the 16S rRNA gene sequences were retrieved from the RAST annotation and used as a query against the SILVA reference database with the threshold set to above 97% (Pruesse *et al.,* 2007).

Statistical analysis

R statistical programming language was used for multivariate statistics, moreover R is used for several types of graphical representations (Team, 2013).

Resistance *Gene prediction*

*Paenibacillus*sp. 6A draft genomes were analyzed

using the Comprehensive Antibiotic Resistance Database (CARD; version 1.2.0) to estimate the total number of antibiotic resistance genes ARGs (https://card.mcmaster.ca/analyze/rgi) (McArthur *et al.,* 2013).

Results and Discussion

To shed light on the bacterial diversity in the microbiological community reside in the ear of patients having otitis media, the selected isolate (6A) was sequenced using whole genome sequencing Technique (WGS).

The whole genome sequences was performed in Illumina Miseq. The assembly generated 2157 counties spanning 5,941, 665 bp, with GC content 35.5% and 112 RNAs. table 1.

Nucleotide sequence accession number

The draft genome was deposited in GenBank under the accesses no.AMN14163233. In this paper, the first version was described.

Taxonomic

Taxonomic classification, which based on 16S rRNA gene sequence analysis using the SILVA database as reference, revealed that the sequenced isolates was belonged genus, *Paenibacillus* spp. An earlier study performed by (Brown, 2015) detected *Paenibacillus*sp. strain VT400 from the saliva of patients with acute lymphoblastic leukemia (Brown, 2015). The WGS of the tested isolates were selected for further comparative genomic analyses that aid in identification of genetic elements associated with differential resistant or tolerant phenotypes. For estimating the phylotypes of the selected genomes, the 16S rRNA gene sequences were retrieved from the RAST annotation and used as query against the SILVA reference database with the threshold set to above 97%.

Genes encoding virulence factors

The genome analysis of isolated bacteria revealed a large number of genes encoding for virulence factors that contributing in the pathogenicity. The genes encoding for degradative enzymes was mostly identified followed by putative antibiotic resistance genes (ARGs). The

Table 1: Draft Genome assembly matrix for genome sequences ofsix environmental bacteria isolated from archived metal-polluted soils sorted by GC% content.

Isolate ID	Total number of reads	Genome size (Mb)	GC (%)	N 50	Number of contigs (>= 1000 bp)	Largest contig	Number of RNAs
6A	321617	5,941,665	35.5	730	36	1956809	112

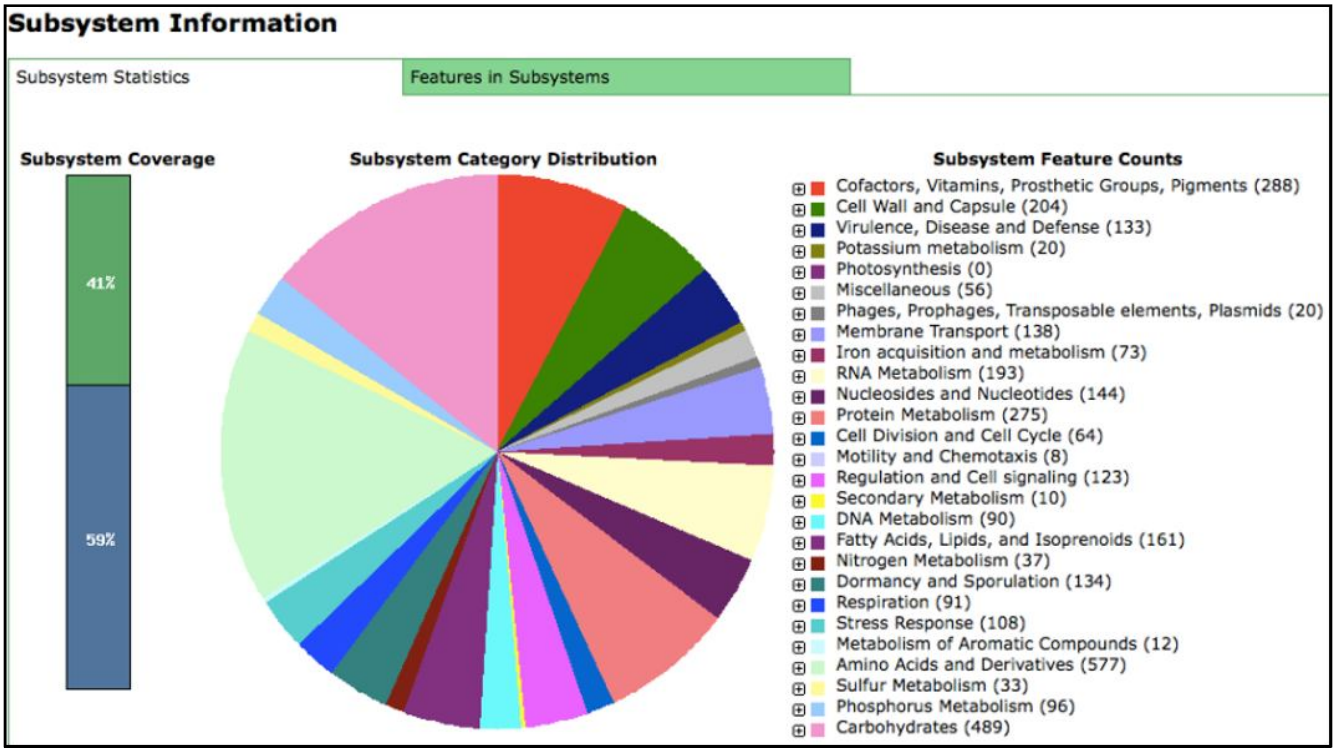

Fig. 1: An overview of the subsystem categories of antibiotic and metal resistance genes using RAST server.The number of the genes with associated functional categories of *denovo* assembled *Paenibacillus*space. 6A genome was explained.

Table 2: Functional categories of putative genes that predicted by theRAST annotation tool; key of antibiotic resistance genes in the *Paenibacillus* sp. 6A.

Subsystem of putative protein	Functional role
Copper homeostasis	Copper-translocating P-type ATPase (EC 3.6.3.4)
Copper homeostasis	Multidrug resistance transporter, Bcr/CflA family
Copper homeostasis	Copper resistance protein D
Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein
Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein CzcD
Cobalt-zinc-cadmium resistance	Transcriptional regulator, MerR family
Resistance to Vancomycin	Vancomycin B-type resistance protein VanW
Multidrug Resistance, 2-protein version Found in Gram-positive bacteria	Multidrug resistance protein [function not yet clear]
Multidrug Resistance, 2-protein version Found in Gram-positive bacteria	Membrane component of multidrug resistance system
Multidrug Resistance, 2-protein version Found in Gram-positive bacteria	TetR family regulatory protein of MDR cluster
Tetracycline resistance, ribosome protection type	Ribosome protection-type tetracycline resistance related proteins
Tetracycline resistance, ribosome protection type	Translation elongation factor G
Aminoglycoside adenylyltransferases	Aminoglycoside N6'-acetyltransferase (EC 2.3.1.82)
Resistance to fluoroquinolones	DNA gyrase subunit B (EC 5.99.1.3)
Arsenic resistance	Arsenate reductase (EC 1.20.4.1)
Fosfomycin resistance	Fosfomycin resistance protein FosB
Multidrug Resistance Operon mdtRP of Bacillus	Multidrug efflux transporter MdtP
Multidrug Resistance Operon mdtRP of Bacillus	MdtR transcriptional regulator, MarR family
Tetracycline resistance, ribosome protection type, too	Ribosome protection-type tetracycline resistance related proteins
Tetracycline resistance, ribosome protection type, too	Translation elongation factor G
Beta-lactamase	Beta-lactamase class A
Beta-lactamase	Beta-lactamase class C and other penicillin binding proteins
Beta-lactamase	Beta-lactamase (EC 3.5.2.6)
Beta-lactamase	Metal-dependent hydrolases of the beta-lactamase superfamily I
Cadmium resistance	Cadmium efflux system accessory protein
Resistance to chromium compounds	Chromate transport protein ChrA
Multidrug Resistance Efflux Pumps	Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps
Multidrug Resistance Efflux Pumps	Acriflavin resistance protein
Multidrug Resistance Efflux Pumps	Multidrug-efflux transporter, major facilitator superfamily (MFS) (TC 2.A.1)

number of predicted antibiotics and metalresistance genes were obtained by browsing the annotated genomes in SEED viewer based on homology to genes in the RAST database Fig. 1.

The functional sub-categories system revealed that similar numbers of resistance genes in all genomes were recognized. There were five genes that encode for putative beta-lactamase enzymes and four genetic markers for fluoroquinolone and tetracycline resistance genes. Only two predicted genes encode resistance to vancomycin, while only one genetic marker was detected for streptothricin resistance. The subsystem feature toxic compound resistance displayed five predicted genes that putatively encode for arsenic resistance, whereas four predicted genes encoded resistance to cobalt-zinc-cadmium and enable copper homeostasis. For copper tolerance, three putative genes were detected and finally only one gene is putatively encodeda mercuric reductase. The total number of resistance genes was calculated from the annotated genomes of *Paenibacillus* sp. 6A to the SEED subsystem and computing the number of predicted genes under functional subcategories / virulence, disease and defense window Fig. 1.

The evolution of antibiotic resistance genes in bacterial strain could be of concern in different environments. Bacterial resistance could be influenced when interact with the antibiotic-producing competitors (Martínez, 2008). However, many bacterial strains have 'intrinsic' activity of broad-spectrum efflux pumpsystems which are mediated by several genes (D'costa *et al.,* 2006).

Identification and characterization of putative antibiotic resistance genes with RAST/SEED and CARD tool

Genome analysis revealed that *Paenibacillus* sp. 6A harbors various antibiotic resistance genes. The analysis showed major facilitator superfamily (MFS), multidrug drug resistance (MarR), multidrug ATP–binding cassette (ABC), tetracycline resistance (TetR), aminoglycoside, resistance to fluoroquinolones, beta-lactamase, multidrug resistance efflux pumps, fosfomycin resistance (FosB). The number of predicted antibiotic and metalresistance genes was obtained by browsing the annotated genomes in the SEED viewer based on homology to genes in the RAST database. Five genes encode putative beta-lactamase enzymes and four genetic markers of fluoroquinolone and tetracycline resistance. Only two

Table 3: The total coverage of similarity for *Paenibacillus*sp.6Agenome usingCARD reference amino acid sequen**ces** in CARD tool.

Gene type	Resistance profile
Listeria monocytogenesmprF, Bacillus subtilismprF	antibiotic target modifying enzyme; peptide antibiotic resistance gene
bcrA	efflux pump conferring antibiotic resistance; peptide antibiotic resistance gene
Bifidobacteria intrinsic ileS conferring resistance to mupirocin	mupirocin resistance gene
Bifidobacteria intrinsic ileS conferring resistance to mupirocin	mupirocin resistance gene
blt, emeA	efflux pump conferring antibiotic resistance; fluoroquinolone resistance gene
Escherichia coli EF-Tu mutants conferring resistance to kirromycin	antibiotic resistant gene variant or mutant; elfamycin resistance gene
lmrD	efflux pump conferring antibiotic resistance; lincosamide resistance gene
mecC, mecB	antibiotic resistance gene cluster, cassette, or operon; beta-lactam resistance gene; antibiotic inactivation enzyme; antibiotic target replacement protein
mecC, mecB	antibiotic resistance gene cluster, cassette, or operon; beta-lactam resistance gene; antibiotic inactivation enzyme; antibiotic target replacement protein
PBP1b	antibiotic inactivation enzyme; beta-lactam resistance gene; antibiotic target replacement protein
rifampinphosphotransferase	rifampin resistance gene; antibiotic inactivation enzyme
sav1866	efflux pump conferring antibiotic resistance
Staphylococcus aureusrpoB mutants conferring resistance to rifampicin	rifampin resistance gene; antibiotic resistant gene variant or mutant
tetB(P)	antibiotic target protection protein; tetracycline resistance gene
vanSF	glycopeptide resistance gene; antibiotic resistance gene cluster, cassette, or operon; gene conferring antibiotic resistance via molecular bypass

Table 4: Antibiotic susceptible of *Paenibacillus* sp. 6A.

Antibiotic	Susceptibility
Cefoxitin	R
Benzylpenicillin	R
Oxacillin	R
Gentamycin	S
Ciprofloxicin	R
Erythromycin	R
Clindamycin	S
Daptomycin	S
Vancomycin	S
Tetracycline	S
Fosfomycin	R
Fusidic acid	I
Chloramphenicol	R
Trimethprime-Sulfamethoxazole	R
Ampicilline	S
Ampiclox	S
Azithromycin	R
Neomycin	S
Kanamycin	S
Meropenem	S

S – Susceptible; I – Intermediate; R – Resistant (CLSI guidelines, Cockerill *et al.,* 2012).

predicted genes encode resistance to vancomycin (VanW) and only one gene marker was detected for streptothricin resistance table 2. The subsystem feature toxic compound resistance displayed five predicted genes that putatively encode for arsenic resistance, while four predicted genes encoded resistance to cobalt-zinc-cadmium and enable copper homeostasis. For copper tolerance, three putative genes were detected and finally only one gene putatively encodes a mercuric reductase.

The subsystem technology (RAST) version 4.0 and SEED viewer program (Aziz *et al.,* 2008) was used to annotate the genes on each county In this process, version 11 of the genetic code was selected for bacteria. Then, the name of genus, species and strain was given, in addition, the organism's details were viewed under "Browse annotated genomes in SEED viewer".

Furthermore, the CARD tool was used to explore the diversity and abundance of antibiotic resistance genes (ARGs). The resistance gene identifier (RGI) hits 15 predicted resistance proteins which have the same distribution among all draft genomes. Most of putative resistance proteins were related to the following: efflux pump system, rifampin resistance genes, putative resistance to mupirocin, lincosamide, elfamycin, fluoroquinolone and glycopeptide resistance genes table 3.

The whole genomecomparis on was performed using CARD and RAST tools to scan the functional profiling and evaluate the genetic variations of resistance determinants in the draft genome. Recently, (Stepanauskas *et al.,* 2005) reported that microorganisms isolated from coal combustion ash settling basins were resistant to kanamycin, gentamycin, tetracycline, ciprofloxacin and streptomycin. Another study found that many efflux pump systems contribute to antibiotic resistance in *Acinetobacter baumanii* strains (Lin and Lan, 2014).

Antimicrobial susceptibility testing and antibiotic resistance genes

The antibiotic susceptibility of *Paenibacillus* sp. 6A was evaluated against 20 antimicrobial agents which are commonly prescribed for treatment of ear infection. Table 3 showed that, the bacterial isolate was resistant to macrolides (erythromycin and azithromycin), chloramphenicol and Trimethprime-Sulfamethoxazole. However, it was sensitive to a few types of ²-lactams antibiotics, including : tetracycline, lincosamides and fluoroquinolones table 4.

Identification of spore-forming pathogens in the clinical samples could explain the microbial tolerance to high temperature, antibiotics, metals and toxic chemicals (Bloem *et al.,* 2017). Early identification of these pathogens might help to reduce the harmful effects of the bacterial infections. However, many resistance genes of *Paenibacillus* sp. 6A might not be expressed due to a mutation that led to change of genetic structure of the resistant phenotype. Therefore, we used the antimicrobial databases, such as CARD and RAST tools for detection of the resistance genes.

Conclusion

In conclusion, this study comes to demonstrate the virulence and antibiotic resistance data-based on draft genome and antimicrobial susceptibility testing of *Paenibacillus* sp. 6A. The obtained data could be used to assess the bacterial community (as microbial flora) and detect bacterial infections that have an important application in the clinic and disease diagnosis. However, many resistance genes of *Paenibacillus* sp. 6A might not be expressed under the idea that many mutations do not lead to resistant phenotype. Therefore, our present study used antimicrobial databases, such as CARD and RAST tools to detect the resistance genes which could be hazardous to immunocompromised patients.

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