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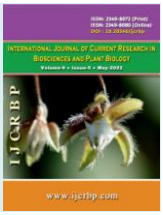

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Identification of mutant gene for antibiotics resistance by using bioinformatics tools

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Abstract

Bioinformatics analysis tools can enhance drug target identification and drug candidate screening and refinement and identify and condoling the mutant gene for antibiotic resistance. Identification of mutant genes for antibiotics resistance using bioinformatics tools is vital for understanding mechanisms and monitoring of mutant genes for antibiotics resistance. Antibiotic resistance has contributed immensely to the continuously growing concerns about the ineffective treatment against microbial infections. The bioinformatics tools play a key role in identifying and monitoring mutant genes for antibiotic resistance. Whole-genome sequencing is also useful to identify trends in antibiotic resistance, targeting the bacteria that are phenotypically sensitive but genotypically positive for a mutant gene for antibiotics resistance. The development and implementation of certain technologies such as whole-genome sequencing and, therefore the creation of national and international databases allowed bioinformatics to study the mutant gene for antibiotics resistance that allows fast, simplified, and accurate identification of the mutant gene for antimicrobial resistance. A web-based method, ResFinder uses BLAST for the identification of the mutant gene for antimicrobial resistance genes in whole-genome data. As input, the method can use both pre-assembled, complete, and partial genomes, and short sequence reads from four different sequencing platforms. A web server providing a convenient way of identifying mutant genes of antibiotic resistance in completely sequenced isolates was assigned. Therefore, Whole-genome sequencing-based bioinformatics tools (ResFinder (read-based), and Typewriter (BLAST-based) in terms of identification of the presence or absence of the mutant gene for antibiotics' resistance can be accessed at NCBI.

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Introduction

Background of study

Bioinformatics analysis approach can not only enhance drug target identification and drug candidate screening and refinement, but also identification and controling of the mutant gene for antimicrobial resistance. Antibiotic resistance has contributed immensely to the continuously growing concerns about the ineffective treatment against microbial infections (Shi et al., 2019). Antimicrobial resistance is a serious world health challenge, which is facilitated by the vertical or horizontal transfer of antibiotic resistance genes (Lal Ch

et al., 2020). Overuse of antibiotics and insufficient therapy are the main causes of making antibiotic resistance a world problem that leads to longer hospital stays, too costly treatments, and higher mortality rates (Elbadawi et al., 2019). Numerous genes can be responsible for antibiotic resistance. Identification of these genes is important to understand resistance epidemiology, for verification of non-susceptible phenotypes, and for identification of resistant strains, when genes are weakly expressed in vitro (Pieter-Jan Van C et al., 2020).

Microorganisms are categorized as antibiotic-resistant, either susceptible or non-susceptible to at least one antibiotic class. It is estimated that resistant infections may kill one person every 3 seconds by the year 2050, raising the death toll worldwide to 10 million annually (Sabino et al., 2019). In the late 60s, due to the presence of various antibiotics, most of the infectious bacteria remained sensitive to a great number of antibiotics being used to treat them. Since no new clinically useful structures have been discovered since 1961, the emergence of antibiotic resistance has escalated the ineffectiveness of the treatment. The reason, the current clonal spread of resistant bacteria is because they contain the resistant gene carrying plasmids that often dump their genes into the bacterial chromosome. To measure this, advances in whole genome sequencing and other high-throughput unbiased instrumental technologies to study the molecular pathogenic of infectious diseases enable the accumulation of large amounts of data that are amenable to bioinformatics analysis and the discovery of new signatures of AMR (Van Camp P et al., 2020).

Whole-genome sequencing can predict phenotypic resistance directly from a genotype, replacing laboratory-based tests (Mason A et al., 2018). Whole-genome sequencing is effective in tracking the onward transmission of bacteria or resistance plasmid transfer between bacteria. WGS is also useful to identify trends in antibiotic resistance, for instant targeting the bacteria that are phenotypically sensitive but genotypically positive for a resistance (Köser et al., 2014). However, the sensitivity of the populations and specificity of alleles to variants, causing different susceptibility phenotypes, sometimes remains lower than the detection method being used, making it even more challenging (Lanza et al., 2018). This new approach requires novel microbial informatics (for development of reference databases of molecular and clinical metadata), new algorithms (for prediction of resistome and resistance phenotype from genotype), and new protocols (for global collection and sharing of high-throughput molecular epidemiology data) (McArthur and Wright, 2015). Several scholars report concordance between genotypic predictions based on known or novel resistant determinants and phenotypic methods (McDermott PF et al., 2016).

Nowadays, the high-throughput sequencing tools and bioinformatics software, knowledge on high bacterial diversity in bacterial communities (metagenomics) is increasing **(**Hyunjo Kim and Jaehoon Song, 2019). So that, in this review, to identify the mutant gene for antimicrobial resistance, the role of sequencing tools and bioinformatics software, (approaches and knowledge) was analyzed. Therefore, Whole-genome sequencing-based bioinformatics approaches (ResFinder (read-based), and Typewriter (BLAST-based) in terms of identification of the presence or absence of different antibiotics resistance and the overall prediction of antibiotics susceptibility and the presence or absence of virulence genes were identified.

Objectives

General objective

To Identify Mutant Gene for antibiotics resistance by using bioinformatics Tools

Specific objectives

To review recent scholars on mutant gene identification by bioinformatics tools

To assign gene number of mutant genes for antibiotics resistance

To identify a specific gene mutant responsible for antibiotic resistance in bacteria by ResFinder (read-based) and typewriter (BLAST-based)

Gene mutation mechanisms for antimicrobial resistance

Evolutionary processes of the antibiotic resistance

Over millions of years, antibiotics and antibiotic resistance genes have co-evolved slowly. In this long period, the first transition was the acquisition of pre-

resistance genes by different bacteria (Hyunjo and Jaehoon, 2019). This genetic transference allowed the evolution toward true and more efficient antibiotic resistance genes. However, the great evolutionary transition was the discovery, mass production, and consumption of antibiotics. (Antibiotics accelerated dramatically the diversification of resistance genes and selection for reaching extraordinary efficient variants (Figure 1) (Patel et al., 2020).

Intrinsic resistance

Environmental changes like radiation, change in light or pH, and the bacteria have intrinsic resistance too and enzymes are used in intrinsic resistance to destroy or modify the drug (D'Costa et al., 2011). Bacteria can also produce inhibitors (acetylates, phosphorylates, and adenylyl) that reduce the drug's affinity for its target sites due to strict hindrance (Munita and Arias, 2016).

Mutation

The binding sites of antimicrobial can be altered by one or more point mutations resulting in the prevention of binding to the target by encoding abnormal target sites, which consequently increase the levels of resistance.

Point mutations in β-lactamase genes have assisted in the identification of over 300 enzymes linked with a range of β-lactam antibiotic resistance phenotypes (Harbottle et al., 2006).

Horizontal gene transfer

Horizontal gene transfer is the ability of bacteria to exchange genes, which is responsible for the spread and persistence of antibiotic resistance genes. There are three types of horizontal gene transfer; AMR gene linked with mobile genetic element, loss of gene loci in the host, and acquired AMR gene through genetic transfer (through transformation, transduction, conjugation) (Mullany et al., 2015; Pepper et al., 2018). Mobile genetic elements such as plasmids, transposing, integrals, and genomic islands harbor antibiotic resistance genes (Bennett, 2008).

Production of β-lactamases

Resistance to β-lactam antibiotics is frequently mediated through the production of β-lactamase enzymes, which break down β-lactam molecules. The β-lactamases bind to β-lactam antibiotics at a very fast deacylation rate, resulting in the opening and thus inactivation of the β-lactam antibiotic molecule.

It allows for the bacterial enzyme to return to the normal functioning of forming peptidoglycan polymers (Soraas, 2014) and the major mechanisms of antibiotic resistance encountered in clinical practice, providing specific examples in relevant bacterial pathogens.

Diagnostic measures mutant gene for antimicrobial resistance

Antibiotic susceptibility testing

Antibiotic susceptibility testing finds a dynamic antibiotic dosage and develops a form of diagnostics for protection against bacterial infections. Minimum inhibitory concentrations of various antimicrobial susceptibility testing are classified by various international agencies.

The susceptibility of microorganisms towards the antibiotic is interpreted as susceptible (S), intermediate (I), and resistant (R) (Jan Hudzicki, 2016). Most countries follow the epidemiological MIC cut-offs (ECOFFS) determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020) and/or the Clinical and Laboratory Standards Institute (CLSI, USA) (Khan et al., 2019). Presently, AST is performed using either classical manual methods or growth-dependent automated systems based on BMD testing. Other AST methods (manual and automated), commonly performed by clinical laboratories, are conventional disk diffusion, agar dilution, antimicrobial gradient (e.g. the E-test, AB Biodisk), and automated instrumentation (Schofield, 2012).

PCR

Apart from culture as the standard for diagnosing infection, sequence-based approaches and quantitative PCR offer a selective and sensitive way to identify numerous Antibiotic Resistance Genes.

However, PCR requires a prior selection of targets which can overlook many important Antibiotic Resistance Genes in a particular environment (Lindgreen et al., 2016) but is helpful to capture the non-cultivable section of non-clinical antibiotic resistors.

Whole genome sequencing

Whole Genome Sequencing technology has made it possible to determine and evaluate the whole DNA sequence of a bacterium at low costs in just a few days (Punina et al., 2015). WGS not only allows *in silico* prediction of antimicrobial resistance (including resistance to compounds not routinely tested phenotypically) but also the early detection of outbreaks or their epidemiological investigation (Köser et al., 2014). Since the *in silico* prediction of resistance needs to be validated by phenotypic antimicrobial testing (Zankari et al., 2013), the combined use of phenotypic assays and techniques allowing the identification of genetic determinants of resistance can be helpful in epidemiological surveillance. Bacteria show similar resistance patterns, but different mechanisms can also be identified with WGS (Gordon et al., 2014).

The unprecedented level of details of assays obtained from WGS for microbial typing and AMR surveillance can describe current trends and differentiate between emerging tendencies (Ellington et al., 2017). Moreover, Multidrug restance (MDR) patterns are defined with much greater precision with DNA sequence-based surveillance as compared to phenotypic tests. The reason is that bioinformatics analysis goes beyond the concept of MDR as resistance to compounds from three or more drug classes, as it considers the co-carriage of particular genes behind different MDR patterns, allelic trends, their potential for horizontal transfer, and their distribution by source (Magiorakos et al., 2012).

Bioinformatics tools gene for antibiotics resistance identification

Sequencing platforms

First-generation technology has remained the leading technology for the past twenty-year for DNA sequencing (Sanger et al., 1977), using a traditional shotgun technique that produced long low throughput read sequences (500-1000 bp) at a relatively higher cost.

Second-generation sequencing technology was fast and high throughput, generating short reads of 25-100 bp length (HiSeq from Illumina, Life sciences from Roche, Solexa, and SOLiD. They were able to run over a few million reads in a single run with high coverage depth, cutting short the cost for DNA sequencing significantly (Butler and Grimme, 2010).

Sequencing by synthesis approach used by Illumina has made it dominate the industry in recent years (Bentley et al., 2008), using fluorescent-labeled reversible minatory nucleotides, on clonally amplified DNA templates (immobilized on acrylamide coating on the surface of glass flow-cell). In 2011, MiSeq was released, which is suitable for smaller laboratories and the clinical diagnostic market (Quail et al., 2012).

One other generalization on sequencing platforms; PacBio (Biosciences, 2014) has enabled single molecules timeline sequencing (SMART). Here, DNA polymerase molecules, which are bound to the DNA template, are attached to the bottom of 50 nm wide wells (zero-mode waveguides (ZMWs). The second strand is synthesized by each polymerase in the presence of γ-phosphate fluorescently labeled nucleotides.

When the fluorescence appears with a distinctive pulse, it means that fluorophores attached to the nucleotides are excited by the energy penetrating the waveguide at the time of addition of a new base. It produces a relatively small number of longer reads (> 10 kbp) as compared to numerous short reads <200 bp like Illumina. However, higher cost per base and higher sequencing error rate (15-20%) have limited their use ingenome assembly (Schadt et al., 2010).

Oxford Nanopore Technologies (ONT) MinION8 uses a new technique where native DNA molecules are pulled through nanoscale pores that accept only one DNA molecule at a time. As the DNA molecule moves through the pore, followed by sensors detecting changes in the ionic current produced by each passing nucleotide. This information can be visualized in a 'squiggle plot' and provides the signal used for base calling. Resulting l among reading lengths significantly improves *de novo* genome assemblies and the detection of structural variations in large genomes (Deamer et al., 2016). ONT is the first technology that can deliver sequencing data from clinical samples in a timeframe that allows early de-escalation and refinement of antimicrobial treatment (Schmidt et al., 2016).

Antimicrobial resistance gene detection tools

Antimicrobial resistance gene databases with comprehensive and accurate gene records are needed to assess antimicrobial resistance prevalence. Different approaches used are BLAST (Peirano et al., 2014),

Hidden Markov Model (HMM) (Gibson et al., 2015), and nucleotide or protein-based differentiation, web interface, or operation on local servers. The researchers have to choose between the collections of resistance genes for use in HMMs (Gibson et al., 2015), or collections of nucleotides or protein sequences of individual resistance genes or resistance-related mobile elements (McArthur et al., 2013). Some databases focus on allele variation of housekeeping genes and their contribution to resistance, and some focus on acquired resistance mechanisms (Feldgarden et al., 2019). Another important factor to be considered is the bias of antibiotic Resistance Genes databases towards experimentally validated genes. Thus, the selection of stringent cutoffs (≥ 90% per reading/config) increases the probability of targeted functional genes, but it also omits environmentally relevant Antibiotic Resistance Genes that can be more diverse.

ResFinder is a highly cited tool among the established tools for antibiotic Resistance Genes characterization in WGS data. It accepts both short reads and assembled genomes/configs, using BLAST and/or KMA (Khmer alignment) based approaches to detect the acquired resistance, except for the resistance due to chromosomal mutations. To avoid ambiguous results, it is recommended to use 90% identity and 60% query coverage (Zankari et al., 2012). Methods, principles, and other methods of mutant gene identification tools for antimicrobial resistance are discussed in table 1.

In silico plasmid detection tools

Plasmids primarily contain the genes related tothe environmental fitness of the host, catabolism, and resistance (Carattoli, 2013; Zhang et al., 2011), leading them to contribute to horizontal gene transfer between different species (Thomas and Nielsen, 2005). However, the assemblies generated using Illumina sequencing do not produce complete genomes, which affects the efforts to characterize the plasmid content of samples. This happens because the plasmids tend to contain repeat sequences with sizes greater than sequences generated by Illumina technology (Arredondo-Alonso et al., 2017).

The need for *in silico* plasmid detection also emerged from the difficulty of plasmid DNA purification if they are longer than 50kbp (Smalla et al., 2015). Moreover, since the metagenomes are usually biased towards chromosomal content as compared to plasmids, many plasmid sequences remain unidentified in sequenced metagenomes, making it a complex process (Dib et al., 2015). Most of the *in silico* plasmid detection methods are recovering circular contigs from de Bruijn assembly graphs (Rozov et al., 2017). However, even if plasmids are assembled directly from WGS by a short-read sequencing platform, still they have repeat region sequences that prohibit a complete assembly of the plasmids, and they rely on laborious and computationally intensive methods (De Toro et al., 2015; Kristiansson et al., 2011).

De Bruijn graph-based plasmid prediction is done by Recycler (Rozov et al., 2017) and PlasmidSPAdes (Antipov et al., 2016). PlasmidSPAdes first calculates the median coverage from the SPAdes assembly graph to estimate a chromosome coverage, then it builds a second assembly graph which considers only those contigs which have a read contig coverage differing from chromosome coverage (Antipov et al., 2016; Bankevich et al., 2012).

These second assemblies are regarded as putative plasmids after repeat resolution by ExSPAnder (Prjibelski et al., 2014). However, the read contig coverage dependency of Plasmid-SPAdes makes large and low copy plasmids nearly indistinguishable from the chromosome. This dependency is not applied by the databases like PlasmidFinder, and MOB-suite for resistance analysis (Page et al., 2018).

PlasFlow is a neural network model, that is trained to separate chromosomal and plasmid sequences (short-length) (Vollmers et al., 2017) from different phyla by finding hidden structures in highly complicated biological data (Angermueller et al., 2016). A total of 9565 FASTA sequences were used to compile it, including 1961 chromosomes and plasmids 7604 of organisms from the kingdom Bacteria (Krawczyk et al., 2018).

Unlike Plasmid Spades and Recycler, which output full-length plasmid sequence predictions, based on their circularity or differential sequencing coverage, PlasFlow can predict the plasmid origin of the contigs even if it does not cover the whole plasmid sequence.

That clarifies PlasFlow usage in the type of analysis that does not require full plasmid sequences with precise taxonomic information (Arredondo-Alonso et al., 2017; Krawczyk et al., 2018).

Table 1. Representative Bioinformatics Approach for antimicrobial resistance gene analysis and identification.

Bioinformatics Tool	Type of Tools	Database-link	Approach	Input description	Requirements	References
AMRFinderPlus	Detection database-based	Reference Gene Catalog	NA; EA; Assembly based and read based tool	Protein search, protein, and nucleotide	HMMER, BLAST+, Linux, and Perl	Bortolaia Vet al., 2020
ResFinder	Detection database-based	Resfinder	NA; EA; Assembly based and read based tool	Whole-genome sequencing, isolate or annotated genome, preassembled partial, complete genomes, reads		Zakaria Eet al., 217
PointFinder	Detection database-based	Pointfinder	NA; EA; Assembly based and read based tool	The sequence file in FASTA	BioEdit platform	Gupta SKet al., 2014
Antibiotic Resistance Gene Annotation (ARGANNOT)	Detection database-based	ARG-ANNOT	NA; EA; Assembly based and read based tool	Analyzing genomes, genomes assemblies, metagenomics contigs or proteomes	Prodigal, DIAMON	Alcock BP et al., 2020
The Resistance Gene Identifier (RGI)	Detection database-based	CARD	NA; EA; Assembly based and read based tool	Metagenomics	R	Yin X et al., 2018
Online Analysis Pipeline for Anti-biotic Resistance Genes Detection	Detection database-based		NA; EA; Assembly based and read based tool	DB	python	Inouye M et al., 2014
Search Engine for Antimicrobial Resistance (SEAR)	Detection database-based		NA; EA Read-based Tool	Sequence reads	Linux	Rowe W et al., 2015
Antimicrobial Resistance Identification By Assembly (ARIBA)	Detection database-based	ARG-ANNOT, CARD, MEGAres and ResFinder	NA; EA; Assemblybased and read based tool	Sequence reads	Linux	Hunt M et al., 2017
AdaBoost (PATRIC)	Detection and Classification		Read-based Tool	Whole-genome sequencing reads	NA	Wattam AR, et al., 2014
PART	Detection database-based		NA	Protein sequences	R and Python	Chowdhury AS, et al., 2020
Resfams	Databased and AMR protein predictor	Resfams	NA	NA	None	Gibson M et al., 2015
Antibiotic Resistance Genes Database (ARDB)	Database	ARAB	NA	NA	None	Liu B, et al., 2015
RefSeq	Database	Refseq	NA	NA	None	Tatusova T, et al., 2014

Note: NA:Not applicable EA: Exploratory approaches, HMMER; Hazardous Materials Management and Emergency Response

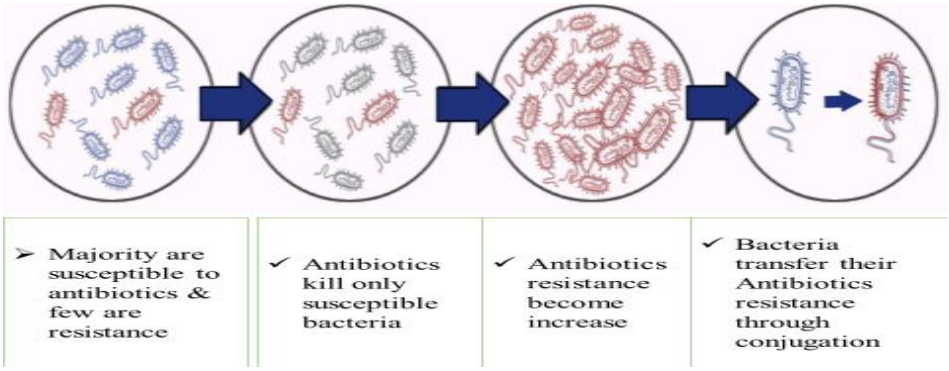

Fig. 1: Evolutionary processes of the antibiotic resistance.

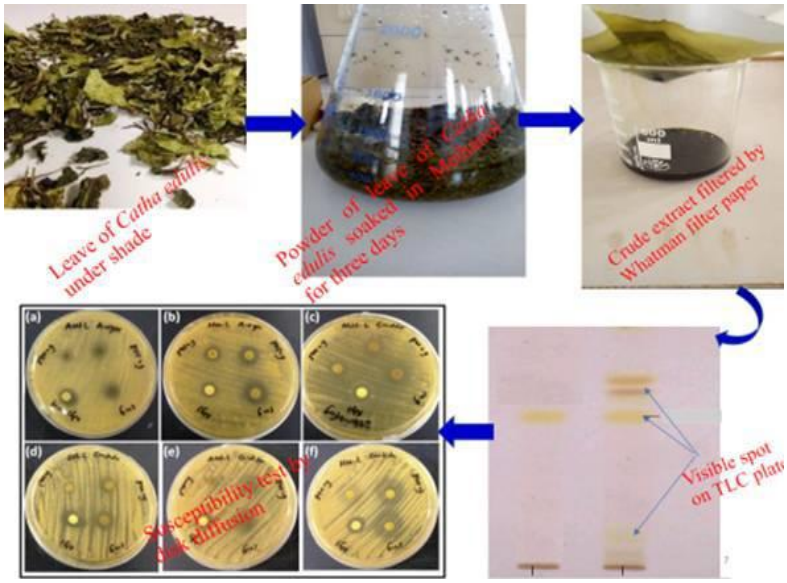

Fig. 2: Antibiotic susceptibility testing by Abu F. and Jedala R (2020),

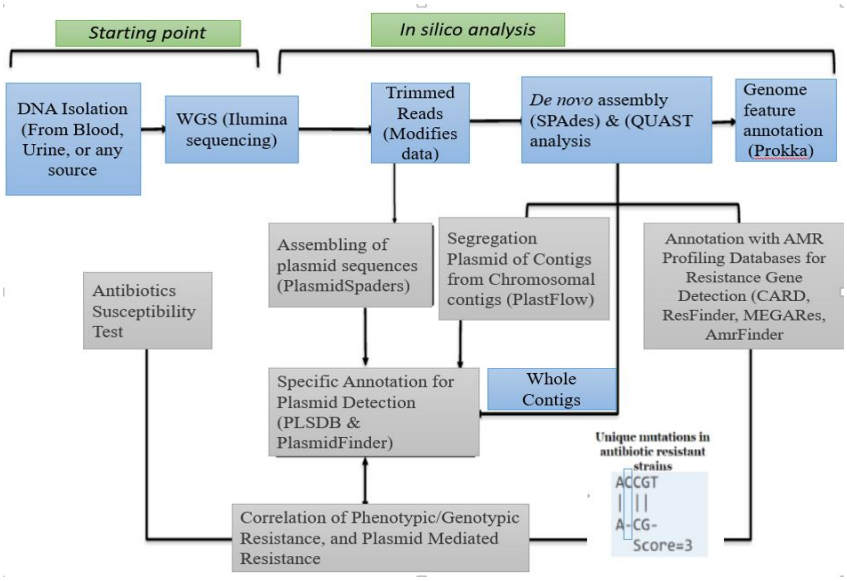

Fig. 3: Sep-by step of Mutant Gene Identification.

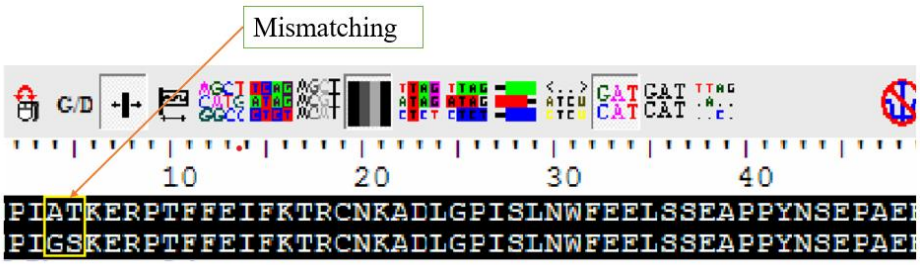

Fig. 4: Result of ResFinder shows mismatch or Malignant Mutation

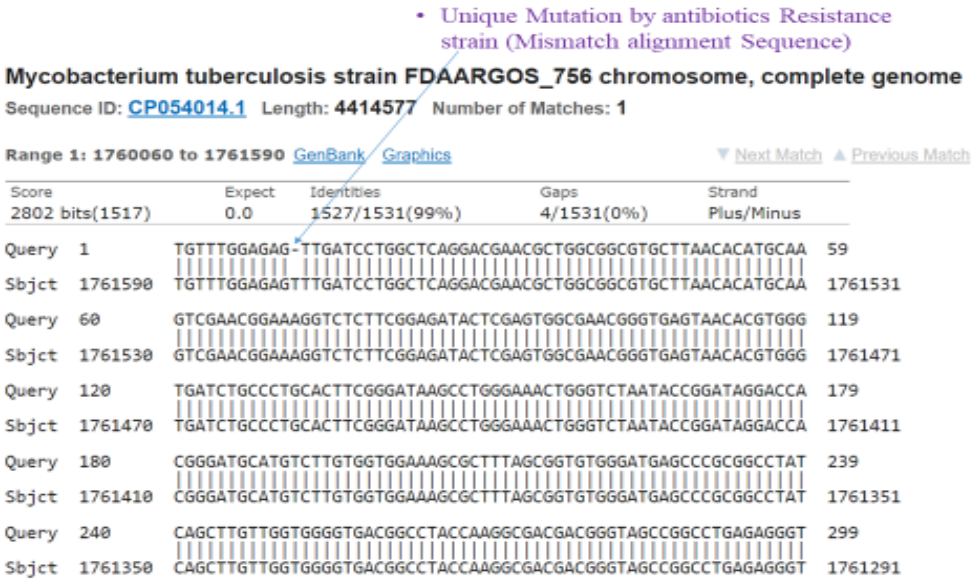

Fig. 5: A mismatch between protein gene and patient's protein which means there is a malignant mutation.

The plasmid detection programs that try to determine the plasmid origin of contigs include Plasmid Finder and cBar. cBar predicts plasmid-derived sequences (using self-organizing maps, based on genomic signatures (k-mer composition) in full-length sequences (Zhou and Xu, 2010), while the Plasmid-Finder tool detects the plasmid replicons and assigns the query plasmids to the respective Inc., a group in *Enterobacteriaceae* (Orlek et al., 2017). Since two plasmids sharing the same replication mechanism cannot co-exist within the same cell, the plasmids are put into different incompatibility groups (Carattoli et al., 2014). However, the size of the Plasmid-Finder database and its limitation only to *Enterobacteriaceae* replicons limits its usage for metagenomics studies. In Plasmid Constellation Network (PLANET), BLAST is used to compare sequences against reference databases to reconstruct plasmids through network analysis. Plasmid prediction by PLACNET depends on the expertise of the researchers because it needs scaffold linking and coverage information, replication initiator proteins (Rip) and relaxase proteins, and similarity of the sequences with non-redundant plasmid sequences from NCBI. In addition, it relies on manual curation of obtained sequence clusters, which prevents its use in an automatic annotation pipeline (Lanza et al., 2014).

Another plasmid database; PLSDB has an extensive set of complete bacterial plasmids from the NCBI database covering records from RefSeq and INSDC (DDBJ, EMBL-EBI, and Gen-Bank). All the plasmids present in the database are annotated using ARG-ANNOT (Gupta et al., 2014), CARD (Jia et al., 2016), Res-Finder (Zankari et al., 2012), and VFDB (Chen et al., 2005), and characterized by Plasmid-Finder (Carattoli et al., 2014).

Required Databases and Methods of Mutant Gene Identification

Data on acquired resistance genes were collected from databases and published papers, including reviews. All

sequences were collected from the NCBI nucleotide database and used to build the ResFinder database. The assigned gene number, the largest collection of the mutgenesgene for antimicrobial resistance, and corresponding gene or protein sequence were found in a common file of computers can access. All genes from the ResFinder database were blasted against the assembled genome, and the best-matching genes were given as output.

The ResFinder and BLAST tools were used to identify antibiotic resistance genes in the pathogen microbessets. ResFinder and BLAST are based on a database of more than 2,000 resistance genes covering 12 types of antimicrobial resistance agents. Resfinder (read-based) and Typewriter (BLAST-based), search for the DNA Sequence and Protein sequence have been assigned. Then click on "APPLY or BLAST". Click on "ResFinder Gene Resistance or Nucleotide BLAST or Protein BLAST" shown by the arrow or position if there is a nucleotide sequence code resistance gene; protein BLAST if there is a sequence of amino acids code resistance gene. Paste the sequence into the search box, and click on "BLAST or APPLY" at the bottom of the page. The next step is to compare these sequences to all known pathogenic microorganisms' sequences to identify the mutant gene for antimicrobial resistance.

Using BioEdit package which can accept Notepad file containing the standard (normal) gene sequence to checkup whether mismatch among them or not. Figures 2-4 depicts the antibiotic resistance genes and their identification; Figure 5 shows when scrolling down on the results page, an alignment of the sequence searched indicates different gene mutation is at position 11, where the normal nucleotide 'T' is not correctly aligned with 'T'in the mutated query 1.

Bioinformatics analysis approach can not only enhance drug target identification and drug candidate screening and refinement, but also identification and condoling of the mutant gene for antimicrobial resistance. Antimicrobial resistance has contributed immensely to the continuously growing concerns about the ineffective treatment against microbial infections. The bioinformatics approach plays a key role in identifying and monitoring mutant genes for antimicrobial resistance. Whole-genome sequencing is also useful to identify trends in antibiotic resistance, targeting the bacteria that are phenotypically sensitive but genotypically positive for a mutant gene of antimicrobial resistance. The development and implementation of certain technologies such as whole-genome sequencing and the creation of national and international databases allowed the application of bioinformatics in the study of the mutant gene for antimicrobial resistance that permits fast, simplified and accurate identification of the mutant gene for antimicrobial resistance. Therefore, Whole-genome sequencing-based bioinformatics approaches (Resfinder (read based), and Typewriter (BLAST-based) in terms of identification of the presence or absence of the mutant gene for antimicrobial resistance and the overall prediction of antimicrobial susceptibility and the presence or absence of virulence genes can be analyzed by bioinformatics approaches.

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

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