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ORIGINAL RESEARCH

Web-Based Tools Validation for Antimicrobial Resistance Prediction: An Empirical Comparative Analysis

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Abstract

Antimicrobial resistance (AMR) is a serious threat to global public health, necessitating rapid and precise diagnostic tools. The prevalence of novel antibiotic resistance genes (ARGs) has increased due to microbial sequencing, resulting in the need to extract vital information from vast amounts of data. Although many AMR prediction tools exist, only a few are accurate and scalable. We examined 20 widely used AMR prediction tools and chose 4 web-based tools for antimicrobial resistance surveillance over standalone software due to their easy accessibility, portability, and centralized data management, eliminating the need for complex installation and maintenance. CGE (Center for Genomic Epidemiology) provides bioinformatics tools and promotes open data sharing. At the same time, CARD (Comprehensive Antibiotic Resistance Database) is a valuable resource for antibiotic resistance gene information, collectively contributing to our understanding and management of antibiotic resistance. We highlighted web-based AMR prediction tools and performed a case study using the *Pseudomonas aeruginosa* complete plasmid sequence (CPS) to identify strengths and weaknesses in the system. Our study explored four web-based antibiotic resistance gene prediction tools: ResFinder, KmerResistance, ResFinderFG, and RGI. ResFinder excelled at finding acquired antimicrobial resistance genes as well as maintaining a database up to date. KmerResistance identified resistance genes using k-mer analysis. esFinderFG offered a unique perspective, excelling in detecting a broad range of resistant phenotypes, due to its inclusion of sequences discovered through functional metagenomics. RGI was versatile in detecting a wide range of resistance genes and provided extensive resistance mechanism information. Researchers must understand the capabilities and trade-offs of these tools to make well-informed choices for efficient resistance gene identification and surveillance as the antibiotic resistance landscape evolves.

Keywords Antimicrobial resistance (AMR) · Antibiotic resistance genes (ARGs) · ResFinder · KmerResistance · ResfinderFG

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Introduction

The world's most severe public health concern is the rapid growth of resistant superbugs, as well as the ongoing battle between bactericidal drugs and extensively drug-resistant bacteria. Antibiotic overuse in both medical and agricultural contexts has aided in the development of multidrug-resistant (MDR) bacteria [1]. Unfortunately, many antibiotics lack specificity, indiscriminately killing pathogenic and non-pathogenic bacteria and leading to antibiotic-associated illnesses [2]. The innovation of new antibiotics to treat resistant infections is a major goal in healthcare, yet no obvious answer to this problem has been identified. These antibiotic-resistant genes allow bacteria to resist antibiotics in a variety of ways, including the activation of efflux pumps, antibiotic molecule degradation by enzymes, and chemical alteration (ribosome and cell wall) to protect antibiotic-targeted cellular targets. These resistance mechanisms, when combined, represent a danger to the therapeutic effectiveness of antibiotics [3–5]. The World Health Organization (WHO) lists AMR as one of the top ten global public health threats to humanity [6, 7]. Drug-resistant diseases are also expected to kill ten million people every year by 2050 [8]. This indicates that drug-resistant diseases will result in more fatalities than road accidents, diabetes, and cancer combined [9]. Antibiotic formulation, testing, and screening are resource-intensive and expensive, limiting possible treatment choices for resistant bacterial species [2].

In addition to the challenges posed by antibiotic-resistant bacteria, antibiotic resistance genes (ARGs) can threaten public health [10]. ARGs are commonly present in transposons or plasmids, and they can be transferred from one cell to another by transduction, transformation, or conjugation. Resistance spreads rapidly within a bacterial population and among different types of bacteria due to gene transfer, a phenomenon known as horizontal gene transmission [11]. The detection of these genes is essential for identifying resistant strains, validating non-susceptible phenotypes, and better understanding resistance epidemiology [12].

The spread of antibiotic resistance genes (ARGs) among bacterial populations is greatly aided by plasmids, which are tiny DNA molecules distinct from chromosomal DNA. These mobile genetic components can easily travel across bacteria, allowing for the fast spread of resistance traits. Plasmids are like little vehicles holding ARGs, and they can transport these genes from one bacteria to another using methods including conjugation, transformation, and transduction [13]. This means that even if a bacterial strain develops resistance through mutation or other methods, the existence of plasmids allows it to rapidly share these ARGs with unrelated bacteria [14]. Plasmids can carry many ARGs at the same time, potentially generating a “reservoir” of resistance genes that can easily travel between bacteria. Understanding the role of plasmids in the spread of ARGs is essential in our efforts to prevent antibiotic resistance. Strategies for limiting the spread of ARGs must consider the role of these mobile genetic components, as targeting plasmids can be an effective strategy for limiting the spread of antibiotic resistance within microbial communities [15].

Phenotypic assays have traditionally been used to detect AMR. The criterion for determining antibiotic sensitivity is diffusion-based or standardized dilution in vitro antibiotic susceptibility test (AST), and much research and testing has been done to link AST findings with treatment response. Resistance surveillance and in some cases clinical therapeutic guidance are increasingly using molecular approaches. These approaches include everything from PCR-based resistance element detection to mass spectrometry-based methods [16]. Sequencing has become a viable approach for routine bacterial characterization due to the enhanced accessibility and cheaper cost of NGS [17]. Over the past few years, it has significantly improved our ability to combat AMR. Although NGS-based technologies can detect practically any known AMR gene or mutation and explore new variations of known AMR determinants, they have largely replaced traditional genotypic approaches for AMR identification [18–20]. Furthermore, as new phenotypic AMR determinants are discovered, sequence data can be continuously stored and re-analyzed. The primary drawback of any genotypic AST approach is that it can detect only proven AMR mechanisms, while resistance induced by novel mechanisms and/or gene expression regulation (hetero-resistance, increased efflux pump expression, etc.) cannot be detected [21].

Antimicrobial surveillance is therefore extremely crucial for forecasting antibiotic resistance developments and tracking the outcomes of medical interventions. The prediction of AMR in NGS data using a simple and quick method is essential for source tracking, infectious disease detection, diagnostics, and epidemiological surveillance, and additional research is highly required [22]. The development of tools for continuously monitoring AMR globally has become more important because these tools may provide useful information to assist healthcare professionals in developing stewardship programs and implementing public health measures. It is also required to create an infrastructure and network to support this huge amount of data [23].

Pseudomonas aeruginosa is a well-known opportunistic pathogen with inherent resistance to several antibiotics, making it a serious problem in clinical settings [24]. Due to its unique combination of features, *Pseudomonas aeruginosa* is distinguished in the field of plasmid transmission and antibiotic resistance genes. It poses considerable difficulty in

healthcare settings due to its broad host range of plasmids, innate and acquired resistance mechanisms, biofilm-forming properties, and high clinical significance [25, 26]. The ability of this bacteria to rapidly transfer resistance genes via plasmids within clinical settings highlights its significance in the wider field of antibiotic resistance research and management efforts [27].

In this context, the significance of understanding the resistance profile of *Pseudomonas aeruginosa* cannot be overstated. Using several ARG identification methods on *Pseudomonas* plasmid sequences can provide useful information into the specific resistance genes prevalent in this bacterium and their ability to spread to different strains or species. This understanding is essential for efficient treatment and infection control measures in healthcare settings, making it an important component of the overall effort to battle antibiotic resistance [28–31].

AMR is detected computationally by querying input DNA or amino acid sequence data for the existence of a pre-determined set of AMR determinants provided in AMR reference databases using a search algorithm (Fig. 1) [22]. Numerous drug resistance prediction tools have been developed and made publicly available online over the last couple of years [32]. There is, however, a requirement for the standardization of tools. In this study, we conducted a thorough evaluation of several commonly available web-based drug resistance prediction tools, including ResFinder, ResFinderFG, KmerResistance, and Resistance Gene Identifier (RGI). We chose these tools primarily because of their user-friendly interfaces, which make them accessible even to people with no background in bioinformatics. These tools have many advantages over standalone applications, including convenience and access from any location with an internet connection. Real-time updates and scalability are other advantages, as web-based systems can easily adapt to expanding data quantities. Data backup and security features offer peace of mind, while collaboration tools promote teamwork and information exchange. Furthermore, because of their cross-platform portability and low cost, they are perfect for research projects with various technology setups and restricted resources. Using these benefits, we improve the efficiency and dependability of our research, resulting in more meaningful outcomes and advancing our understanding of drug resistance.

Materials and Methods

The framework employed in this study is described in the subsequent sections, and the comprehensive methodology is depicted in the figure below (Fig. 2).

Validation Dataset

Pseudomonas aeruginosa is a pathogenic Gram-negative bacterium that is becoming more common in infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains, limiting available effective treatments. Plasmids contribute significantly to antibiotic resistance because they are the means by which resistance genes are captured and then disseminated. *Pseudomonas aeruginosa* plasmid nucleotide sequences were acquired using the key phrase "*Pseudomonas aeruginosa*" from the nucleotide database of the NCBI (https://www.ncbi.nlm.nih.gov/nuccore/). The organism was then chosen as *Pseudomonas aeruginosa*, the species as bacteria, the molecular type as genomic DNA/RNA, the sequence type as nucleotide, the genetic compartment as plasmid, and the length from 900 to 1100 bps. We picked this range of plasmid lengths for our investigation to compare the effectiveness of various antibiotic resistance gene prediction methods on shorter sequences, which are more difficult to annotate than longer

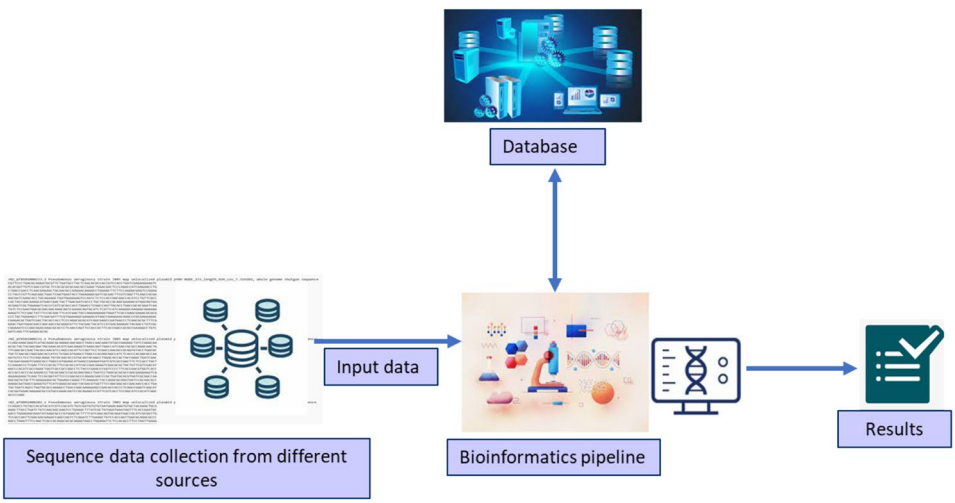

Fig. 1 Working principle of AMR prediction tools

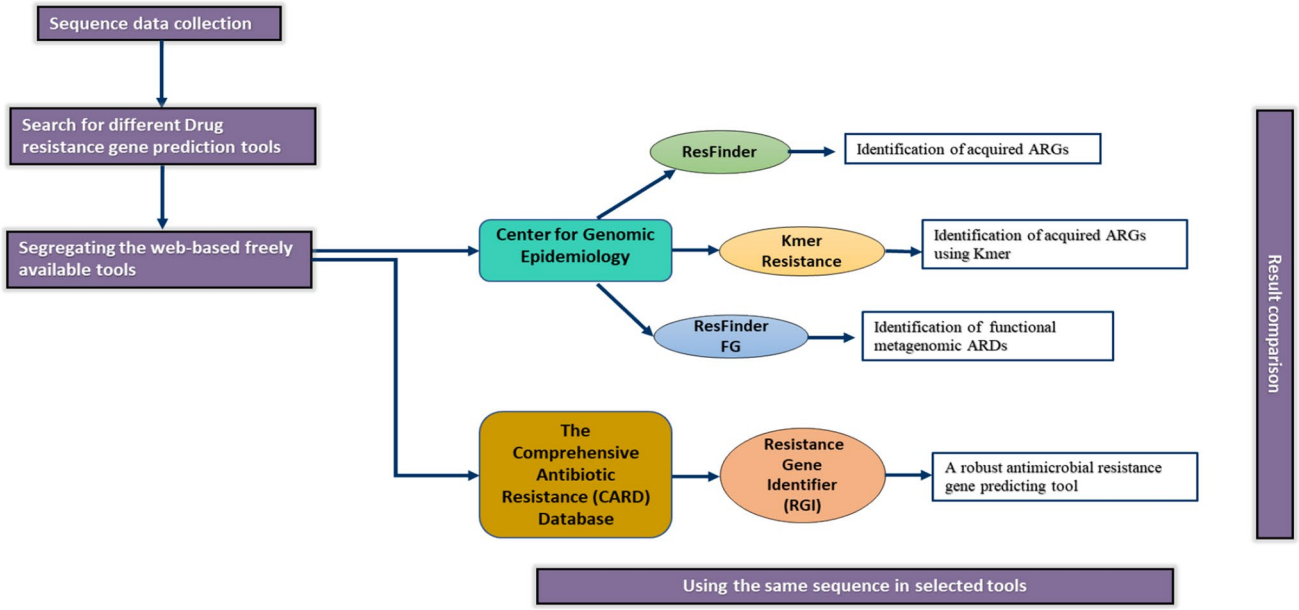

Fig. 2 An overview of the AMR prediction pipeline

ones. This option was chosen to test the tools' versatility and robustness in detecting antibiotic resistance genes in plasmids of varied lengths, particularly in cases involving relatively short plasmids. A thorough evaluation of these prediction tools requires assessing their performance in challenging scenarios. To obtain a standard range, the length of the sequence was taken into account by the Romaniuk et al., 2019 article [33]. We obtained 26 *Pseudomonas aeruginosa* plasmid sequences from the aforementioned screening, which are further considered for the AMR prediction. Table 1 includes NCBI accession numbers, plasmid length, and species of the retrieved sequences.

Segregation of In Silico AMR Determination Tools

The development of online databases and bioinformatics tools has been required for drug-resistance gene prediction. After conducting a scientific literature search from 2012 to 2022, 47 freely accessible bioinformatics tools for identifying AMR determinants were discovered, including the most commonly used tools listed below (Table 2).

Center for Genomic Epidemiology (CGE) is fully non-profit and provides a variety of free online bioinformatics services. The Technical University of Denmark (DTU) provides core funding, as well as funding from a variety of public and commercial sources [51]. The Center for Genomic Epidemiology offers 38 services, including nine phenotyping tools: ResFinder, ResFinderFG, LRE-finder, KmerResistance, PathogenFinder, VirulenceFinder, Restriction-ModificationFinder, SPIFinder, and ToxFinder (Fig. 3). From the mentioned nine phenotypic tools, we selected four

Table 1 Length and accession numbers of the plasmid sequences used

Sl no	Accession no.	Plasmid length (bps)
1	NZ_WTXS01000233.1	929
2	NZ_WTXS01000231.1	988
3	NZ_WTXR01000281.1	1038
4	NG_070900.1	1001
5	NG_049735.1	995
6	NG_049605.1	1031
7	NG_049577.1	1001
8	X60321.1	919
9	D78374.1	1014
10	NZ_CP033773.1	1089
11	MN013162.1	1022
12	CP033773.1	1089
13	NG_065882.1	912
14	KU881625.1	912
15	AY027589.1	957
16	NG_049776.1	983
17	NG_049393.1	1001
18	NG_049343.1	928
19	NG_049267.1	983
20	NG_048742.1	1067
21	NG_048082.1	1040
22	NG_047483.1	1082
23	NG_047415.1	1004
24	NG_047360.1	969
25	NG_047329.1	1034
26	NG_049223.1	938

Table 2 Details of tools for the prediction of drug resistance genes (2012–2022)

Source	Description	Accessibility	Approach	Type	Year	Availability Status	Refs.
ARG-ANNOT	It identifies current and potentially new antibiotic resistance (AR) genes in bacterial genomes	Standalone	Assembly-based tools	Tool and database	2014	Archived last update in May 2018	[34]
ARIBA	It is a tool for rapid genotyping of antimicrobial resistance based on sequence reads	Standalone	Assembly-based tools	Tool	2017	Active	[35]
DeepArg	A deep learning framework for identifying antibiotic resistance genes using metagenomic data	Web	Read-based tools	Tool	2018	Archived	[36]
GROOT	A tool for identifying Antibiotic Resistance Genes (ARGs) from metagenomic samples	Standalone	Read-based tools	Tool	2018	Active	[37]
KmerResistance	KmerResistance links mapped genes with identified species of Whole Genome Sequencing (WGS) samples, allowing for gene identification in badly sequenced samples or highly accurate predictions for contaminated samples	Web	Read-based tools	Tool	2016	Active	[38]
NCBI-AMRFinder	Using protein annotations and/or assembled nucleotide sequences, this tool can discover AMR genes, resistance-associated point mutations, and other kinds of genes	Standalone	Assembly-based tools	Tool	2018	Active	[39]
ResFinder	It identifies acquired genes and/or chromosomal alterations mediating antimicrobial resistance in bacteria's complete or partial DNA sequence	Web and standalone	Assembly-based and read-based tool	Tool and database	2012	Active updated regularly	[12]
ResFinderFG	Based on a functional metagenomic antibiotic resistance determinants database, it identifies a resistance phenotype	Web	Assembly-based tool	Tool and database	2016	Active	[40]
RGI	It can be used to identify resistomes utilizing protein or nucleotide data using homology and SNP models	Web and/or standalone	Assembly-based tools	Tool	2015	Active	[12]
SEAR	Create full-length, horizontally acquired Antibiotic Resistance Genes (ARGs) from sequencing data. It is intended for use in environmental metagenomics and microbiome research where the diversity and relative abundance of ARGs must be determined fast and conveniently	Web and/or standalone	Read-based tools	Tool	2015	Archived	[41]
ShortBRED	ShortBRED is a high-specificity approach for profiling protein families of concern in shotgun meta-omic sequencing data	Standalone	Read-based tools	Tool	2015	Active	[42]

Table 2 (continued)

Source	Description	Accessibility	Approach	Type	Year	Availability Status	Refs.
SRST2	Rapid genomic monitoring for public health-care and clinical microbiology laboratories	Standalone	Read-based tools	Tool	2014	Active	[43]
SSTAR	SSTAR offers rapid and accurate antimicrobial resistance (AR) surveillance using data from WGS. It can identify known AR genes as well as possible novel variations and shortened genes caused by internal stop codons. SSTAR can also detect changes and/or truncations in outer membrane porin gene sequences	Standalone	Read-based tools	Tool	2016	Active	[44]
ARGDIT	It is an Antimicrobial Resistance Gene Database Validation and Integration Toolkit	Standalone	Read-based tools	Tool	2019	Active	[45]
LRE-Finder	Finding the genes and mutations responsible for linezolid resistance	Web	Assembly-based tool	Tool and database	2019	Active	[46]
IRIDA plugin AMR detection	It is a pipeline that detects antimicrobial resistance genes using the RGI/CARD and staramr tools	Standalone	Assembly-based tool	Tool	2019	Active	[47]
PARGT	Software for predicting bacterial antimicrobial resistance	Standalone	Read-based tools	Tool	2020	Active	[48]
HMD-ARG	Annotating antibiotic resistance genes using hierarchical multi-task deep learning	Standalone	Read-based tools	Tool	2021	Active	[49]
YunxiaoRen/ML-iAMR	Antimicrobial resistance prediction using whole-genome sequencing and machine learning	Standalone	Read-based tools	Tool	2022	Active	[50]

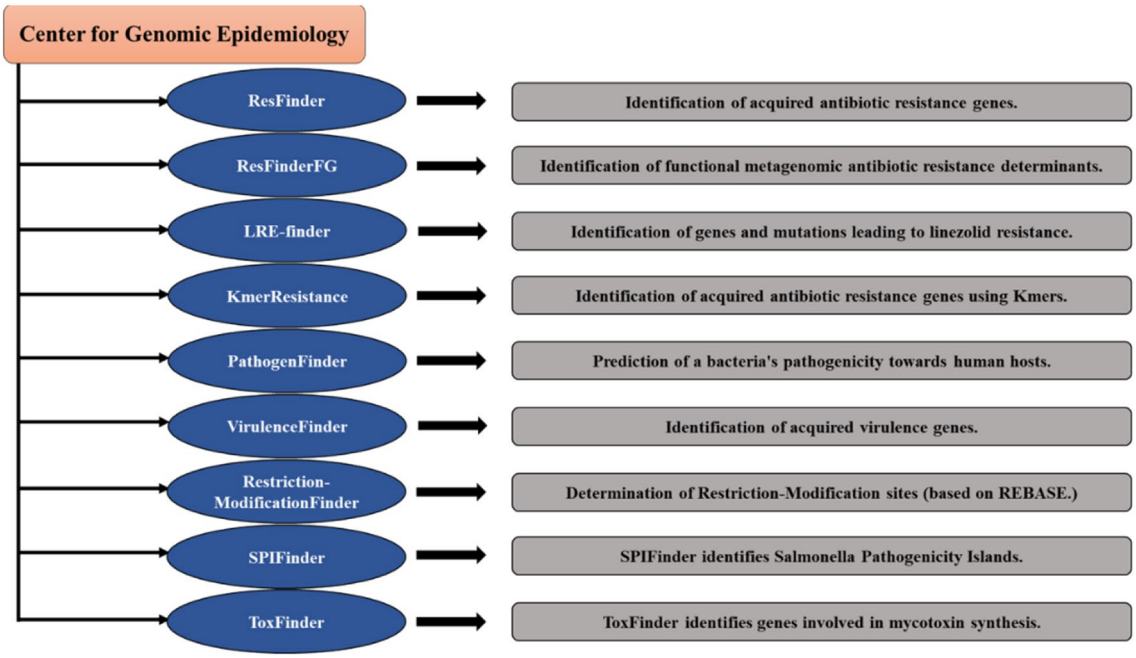

Fig. 3 Center for Genomic Epidemiology based phenotyping tools

tools with versions (ResFinder 4.1, KmerResistance 2.2, ResFinderFG 2.0, and RGI 6.0.2) based on similarities of their objective i.e., to find out the resistance factors. Here we did not include LRE-Finder in our study because the input data format is FASTQ sequence, it only predicts AMR for a single species i.e., *Enterococcus faecalis*, and only identifies acquired linezolid resistance genes without considering other antibiotics.

The comprehensive antibiotic resistance database (CARD) contains a large collection of rigorously curated antibiotic resistance genes that serve as the foundation for our study into antibiotic resistance mechanisms. The resistance gene identifier (RGI), one of the several tools offered by CARD, is especially significant for our research. RGI is an important part of our research because of its unique capacity to detect and identify resistance genes.

Steps and Parameters Setup for Segregated Tools

ResFinder 4.1 searched the database for all seventeen classes of antibiotic drugs, regardless of the target region. The sequences were entered into the database, and the acquired resistance gene testing parameters were adjusted to predict resistance genes for all seventeen drug classes provided by the server. The minimum percentage identity was set to 90%, with perfect alignment set to 100%. The percentage of identity was computed by counting the number of identical nucleotides between the best-matching resistance gene in the database and the equivalent sequence in the plasmid. The tool was run with the aforementioned parameters, and the results were recorded.

The scoring method in KmerResistance 2.2 was species identification on maximum query coverage. Similarly, all sequences were entered into the database. The host database was set to the bacterial plasmid. The gene database was set to resistant genes, and the identity threshold was left at 70%, with a depth correction threshold of 10%. The AMR genes identified in the resulting output were recorded, and the host organism and template sequence were also noted.

The functional genomics database ResFinderFG 2.0 uses functional metagenomic antibiotic resistance determinants to identify resistance phenotypes. Here again, all sequences were entered into the database. The percentage identity setting was set to 98%, along with the minimum query length was set to 60%. The read type used was 'assembled contigs/genomes,' and the sequences were screened for all the thirteen antibiotic resistance determinant (ARD) families present in the database.

The RGI 6.0.2 tool was executed with the default parameters. These default settings were used by the tool to guarantee a thorough and consistent analysis. All sequences were uploaded into the tool, and the analysis was carried out using the default settings. The outcomes produced with these default settings were meticulously recorded and analyzed.

Results

This study provides better insights into the advantages and drawbacks of the tools, such as user-friendliness, to determine which tool has the best visualization and which tool offers the maximum information.

Results have been discussed with the following aspects:

- Comparison based on the output-resistant sequence IDs
- Comparison based on the output antibiotic resistance genes (ARGs)
- Comparison based on the amount of information provided and result visualization
- In detailed results of each tool

Comparison Based on the Output-Resistant Sequence IDs

In this analysis, four distinct resistance prediction tools were used to find and evaluate the presence of antibiotic-resistance genes in 26 plasmid DNA sequences. The data from each tool showed diverse patterns of shared and unique elements. ResFinder and KmerResistance, interestingly, shared 2 elements. In contrast, ResFinderFG featured a unique element that was absent in the other tools. In addition, all four tools uniformly identified 5 elements. ResFinder, KmerResistance, and RGI additionally shared 11 elements in common (Fig. 4).

Comparison Based on the Output Antibiotic Resistance Genes (ARGs)

ResFinder, KmerResistance, and RGI were three different tools that were used in the analysis of antibiotic resistance gene predictions. Notably, these three techniques had 2 resistance genes as common elements. 13 more common resistance gene sequences have been identified from ResFinder and KmerResistance. RGI, on the other hand, provided 12 distinct resistance gene sequences. Additionally, ResFinder only identified 1 distinct resistance gene, whereas KmerResistance identified 2 exclusive elements (Fig. 5). ResFinderFG uses gene names that are distinct from those used by the other two tools since they are based on functional annotation of gene products, which is another important point to take into consideration.

Comparison Based on the Amount of Information Provided and Result visualization

The results of all three CGE tools are made available through email, whereas the results of RGI can be downloaded directly from the webpage. The outcomes of the analysis were displayed in a tabular manner in ResFinder, with the first table containing Antimicrobial, Class, WGS-predicted phenotype, and Genetic background. Other tables in ResFinder are organized by drug class, with each table containing various drug class information, such as Resistance gene, Identity, Alignment Length/Gene Length, Position in reference, Contig or Depth, Position in contig, Phenotype,

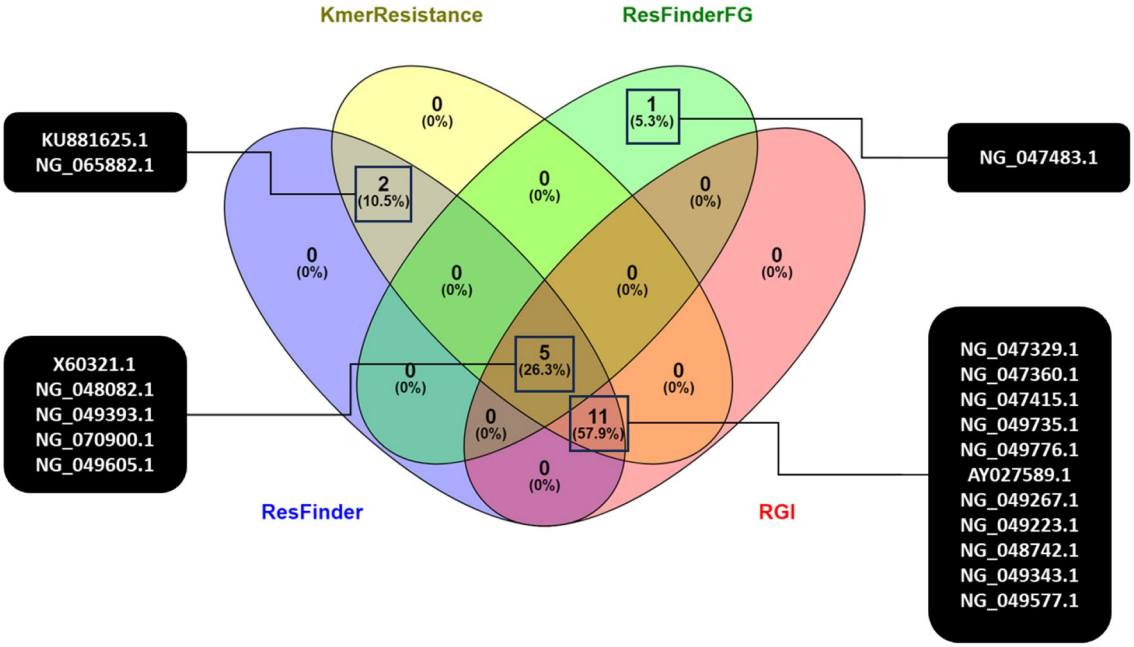

Fig. 4 Venn diagram comparing the resistance sequence IDs results of ResFinder 4.1, KmerResistance 2.2, ResFinderFG 2.0 and RGI 6.0.2

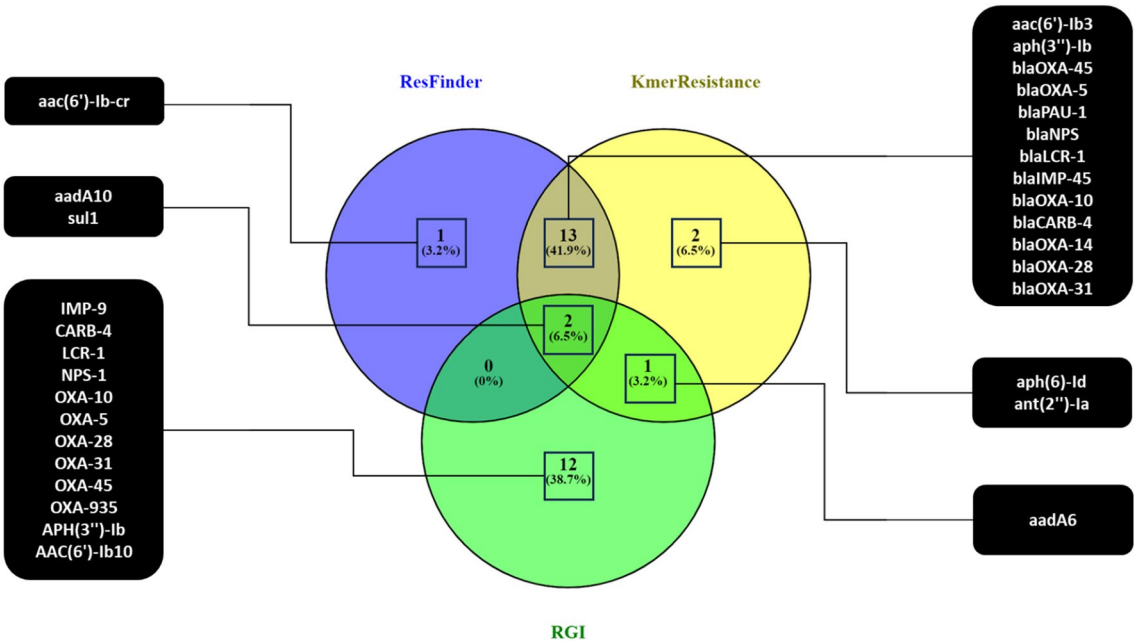

Fig. 5 Venn diagram comparing the genes results of ResFinder 4.1, KmerResistance 2.2 and RGI 6.0.2

PMID, Accession no., and Notes. There is an extended output option at the bottom of the result page that displays the alignment result. The results for KmerResistance are provided in a single table, which contains the following columns: Template, Score, Expected, Template Length, *Q* Value, *P* Value, Template Id, Template Coverage, Query Id, Query Coverage, Depth, and Depth Corr. The ResFinderFG result is displayed as a tabular box with numerous columns containing Hit name, Identity, Query/HSP, Contig, Position in contig, Drug treatment, and Accession no. Each of the three CGE tools offers a variety of downloadable files containing various types of information (Table 3). The results for RGI are provided in two tables: the 1st table contains the following columns: Filename, Date (UTC), RGI Criteria, Perfect Hits, Strict Hits, Loose Hits along with the download option, and the 2nd table consists of the following columns: RGI Criteria, ARO Term, SNP, Detection Criteria, AMR Gene Family, Drug Class, Resistance Mechanism, % Identity of Matching Region, % Length of Reference Sequence.

In Detailed Result of Each Tool

As a case study, we took 26 plasmid sequences and executed them through four different prediction tools to identify AMR factors.

ResFinder 4.1

ResFinder 4.1 predicted that plasmid X60321.1 contains the majority of AMR genes. It carries two AMR genes, aac(6')-Ib3 resistance to aminoglycosides and aac(6')-Ib-cr resistance to fluoroquinolone classes of antibiotics. The presence of AMR genes was found in 18 of the 26 plasmid sequences tested in this study, with no resistance gene found for thirteen of the drug classes: Colistin, Disinfectant, Fosfomycin, Fusidic acid, Glycopeptide, Macrolide, Lincosamide, and Streptogramins (MLS), Nitroimidazole, Oxazolidinone, Phenicol, Pseudomonic acid, Rifampicin, Tetracycline, and Trimethoprim. A

Table 3 CGE AMR tools and provided downloadable files

Tools	Output downloadable files
ResFinder	Phenotype table, species-specific phenotype table, results as text (acquired AMR gene results), resistance gene sequences, hit in genome sequences, results as tab-separated file (acquired AMR gene results), results as tab separated file (Chromosomal point mutation results), and results as a text file (Chromosomal point mutation results)
ResFinderFG	Hit in genome sequences, results as text, resistance gene sequences, and results as tab separated file
KmerResistance	Resistance results, species results, full resistance results, resistance alignment results, resistance consensus results, not-sam file and log file

fair number of AMR gene homologs were identified in the other four drug classes (Supplementary Table 1). The drug classes in which AMR genes were detected were beta-lactams (13 out of 19; 68.42%), followed by aminoglycosides (21.05%), fluoroquinolones (5.2%), and sulfonamides (5.2%). There were 16 number of different acquired AMR genes found to be resistant to the four drug classes, with aminoglycosides and beta-lactams showing the highest frequency (Table 4). The most common genes are aadA10 (2/19), blaNPS (2/19), and blaPAU-1 (2/19), each of which was found to be present in 10.52% of the outcomes observed (Fig. 6).

KmerResistance 2.2

The sequences were uploaded to the KmerResistance database. 8 plasmid template genes were linked to other Gram-negative bacteria, while one plasmid template gene was linked to a Gram-positive bacterium, indicating that they could be the source of acquisition (Supplementary Table 2). Two *Escherichia coli*, one each of *Klebsiella pneumoniae*, *Comamonas testosteroni*, *Acinetobacter* sp., *Pseudomonas putida*, *Achromobacter xylosoxidans*, and *Providencia* sp. linked with Gram-negative strain. *Glutamicibacter nicotianae* Gram-positive strain is associated with one of the

Table 4 Resistance to specific antimicrobial drug classes was identified in the input plasmid sequences

Sl. no.	Accession no.	AG	BL	FQ	SM	Total
1	NG_070900.1		✓			1
2	NG_049735.1		✓			1
3	NG_049605.1		✓			1
4	NG_049577.1		✓			1
5	X60321.1	✓		✓		2
4	NG_065882.1		✓			1
7	KU881625.1		✓			1
8	AY027589.1		✓			1
9	NG_049776.1		✓			1
10	NG_049393.1		✓			1
11	NG_049343.1		✓			1
12	NG_049267.1		✓			1
13	NG_048742.1		✓			1
14	NG_048082.1				✓	1
15	NG_047415.1	✓				1
16	NG_047360.1	✓				1
17	NG_047329.1	✓				1
18	NG_049223.1		✓			1
Total (%)	–	4 (21.05%)	13 (68.42%)	1 (5.2%)	1 (5.2%)	19

The presence of various numbers of AMR genes in four drug classes was observed in the 18 plasmids listed
AG aminoglycoside, *BL* beta-lactam, *FQ* fluoroquinolone, *SM* sulfonamide

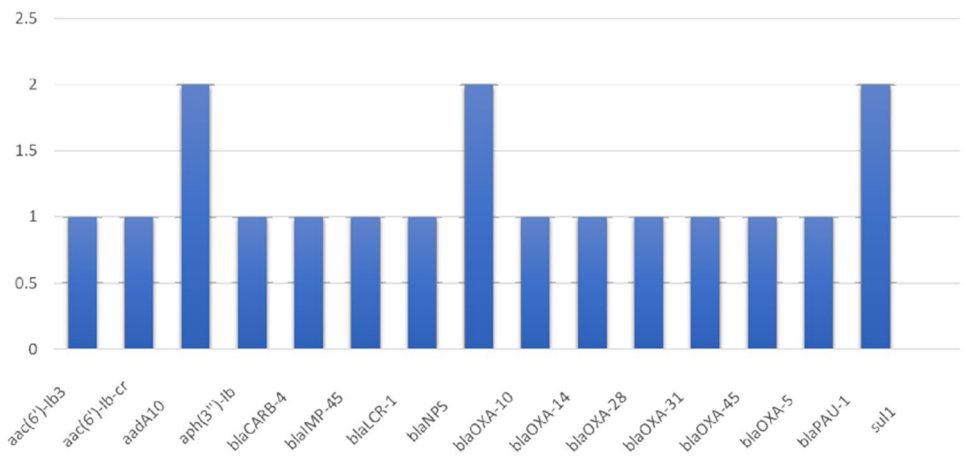

Fig. 6 ResFinder 4.1 revealed prevalence of 16 AMR genes found in 18 *Pseudomonas aeruginosa* plasmids

plasmids. Out of 26 input plasmid sequences, it predicted 19 ARGs in 13 plasmids.

ResFinderFG 2.0

In ResFinderFG 2.0, three distinct ARD families were identified in six input plasmid sequences. The most common was beta-lactamase, which was found in four different input plasmid sequences. Three of the four plasmids with beta-lactamase ARDs conferred resistance to ampicillin and one conferred resistance to piperacillin. Others include one dihydropteroate synthase (dpr) and one aminoglycoside acetyltransferase (AAC), as both are resistant to Sulfamethazine and Amikacin respectively (Supplementary Table 3).

RGI 6.0.2

The sequences were submitted to the RGI database, revealing the presence of AMR genes in 16 out of the 26 plasmid sequences examined in this study. The results show that the observed plasmids have a wide range of resistance profiles. Several plasmids were shown to be a perfect match to certain AMR gene families, including IMP beta-lactamase, ANT(3") aminoglycoside resistance, sul1 sulfonamide resistance, and several beta-lactamase types, including CARB, LCR, NPS, OXA, and OXA-935. These plasmids had a high identity and closely matched the reference sequences. Some plasmids, on the other hand, showed a strict match to resistance genes, such as APH(3")-Ib and AAC(6')-Ib10, showing their participation in aminoglycoside resistance. Furthermore, some plasmids did not match any known resistance genes in the database (Supplementary Table 4).

Discussion

Currently, the use of sequencing technology is revolutionizing practically every component of biological study. In the field of infectious diseases, scientific discoveries, as well as diagnostic and outbreak investigations, are developing at a rapid pace. The ability to interpret sequencing data and the benefit of quick development, on the other hand, is not evenly distributed between institutions and countries [52, 53]. We choose CGE tools because its goal is to give access to bioinformatics tools to those with limited knowledge, allowing all countries, institutions, and individuals to benefit from new sequencing technology. It is believed that doing so, will encourage more open data sharing around the world and give equal advantages to all. CGE is fully non-profitable and provides a variety of free online bioinformatics services [51].

We next opted to explore the CARD and its RGI tool. CARD is well-known for its large collection of curated antibiotic-resistance genes, which makes it a useful tool for understanding antibiotic resistance in bacterial genomes [54]. The RGI tool, which is part of CARD, is specifically designed for identifying and characterizing antibiotic resistance genes, providing detailed information about their distribution and processes.

The AMR prediction methods have been built using DNA or amino acid sequence data. The presence or absence of software for searching within an AMR determinant database, which can be precise to a tool or replicated from other resources, the type of input data accepted, and the search approach used, which can be alignment or mapping, are all factors that distinguish bioinformatics resources. Each tool has its own set of capabilities and limitations when it comes to AMR prediction, which includes the identification of AMR sequences, ARGs, the volume of information produced, and visualization. The web-based application uses a website as its interface or front-end. It has the potential to provide competitive benefits over traditional software-based systems by allowing researchers to streamline data and information at a lower cost, time, and maintenance [51]. Using a regular browser, users can quickly access the application across any computer with internet access. Functionality and features were the two main elements that were prioritized. From this study, a non-bioinformatician or a non-technician can gain subject-specific knowledge as well as determine which tool is appropriate for their specific work. The table below compares the three tools used for identifying drug resistance genes and resistance sequence IDs as well as the amount of information they provide and the way their results are displayed (Table 5).

Description and Significant Observations of Each Selected Web-Based Tools Have Been Discussed Below

ResFinder is a web-based tool that finds chromosomal alterations that promote antibiotic resistance in bacteria's whole or partial DNA sequence and identifies acquired antimicrobial resistance genes in the whole-genome data using BLAST. As input, this tool accepts pre-assembled, whole, or partial genomes, along with fragmented sequence reads from four distinct sequencing technologies. It is accessible at (https://cge.food.dtu.dk/services/ResFinder/). It is also constantly being updated whenever different resistance genes are discovered [55]. Here, we found that the database showed a restriction that stated it only looked for acquired genes and did not detect chromosomal mutations. Since new resistance genes are continuously discovered, it may be necessary to confirm the presence or absence of identified AMR genes phenotypically. According to a study [56], genotyping using aligned whole-genome sequences is a practical substitute for surveillance based on phenotypic antimicrobial

Table 5 Empirical evaluation of four different AMR prediction tools

susceptibility due to the high concordance (99.74%) between phenotypic and predicted whole-genome sequence antimicrobial susceptibility. One of the three most common genes, the aadA10 gene, was found to be resistant to the drug class aminoglycoside, while the other two genes, blaNPS and blaPAU-1, were found to be resistant to the beta-lactam drug class. AadA10 is a class 1 integron containing gene cassette, which suggests that rather than transposition, the resistance determinants from one plasmid to another plasmid were moved by recombination between two class 1 integrons [57]. The ability of integrons in bacteria to acquire new cassettes and recombine cassette rows emphasizes the adaptability of integron diversity. It is necessary to be aware of what other integron-mediated traits, such as increased resistance to antimicrobials, virulence, or pathogenicity, might affect human health in future given their capacity to quickly spread resistance phenotypes. There is an urgent need for control integrons and cassette formation [58]. Tauch et al., 2003 found that blaNPS can rapidly transfer from one species to another [59]. According to Subedi et al., 2018, environmental resistance gene pools contain blaNPS, which can be acquired and maintained in clinical isolates [60]. In a prior study, it was discovered that a clinical isolate of *Pseudomonas aeruginosa* contained a transferable plasmid containing the gene known as blaPAU-1, which is connected to the mobile genetic element [61]. Considering the high ubiquity of beta-lactam resistance genes, it is preferable to monitor the level of antibiotic resistance and resistance genes in patients with *Pseudomonas aeruginosa* infection [62]. In this study, we found that *Pseudomonas aeruginosa* plasmids contain many beta-lactam resistance genes, several of which are found in a single plasmid. This leads us to the conclusion that beta-lactam resistance genes are rapidly spreading through plasmids, and the need of the hour is to control their spread.

KmerResistance (https://cge.food.dtu.dk/services/KmerResistance/) is primarily based on KmerFinder, which has been developed for the typing of bacteria with raw WGS data. KmerResistance and KmerFinder search for co-occurring k-mers between such a query genome and a resistance gene database. KmerResistance, like KmerFinder, biases the threshold based on the quality of the data, as shown by the coverage as well as the depth of the detected species genome. Since these k-mers in this scenario are dispersed across the total sample, we can estimate both depth and coverage. Unlike KmerFinder, KmerResistance may generate an outcome for species prediction in addition to getting acquired antimicrobial resistance genes [38]. This database improves on poor-quality assembly using k-mers to map raw whole-genome sequence data against reference databases and species. (Fragments of a DNA sequence of length k) [12]. Additionally, it can find host or template genes. The KmerResistance database displays the resistance genes but not the drug classes as an analysis output, even though it is claimed to be more precise than ResFinder. As a result, comparisons were restricted to resistance genes that were present in both databases rather than an overall assessment of their sensitivity. ResFinder, ResFinderFG, and RGI accept input sequences in a single input file and provide results based on each sequence, while KmerResistance requires us to provide the sequence file separately and perform separate executions. If we provide the input sequence to KmerResistance in a single file, the results are very perplexing and unrelated to the input sequence.

The ResFinderFG (https://cge.food.dtu.dk/services/ResFinderFG/) approach is based on databases containing sequences detected by functional metagenomics but not represented in existing databases constructed mostly from antibiotic-resistant genes in clinical isolates. It identifies a resistant phenotype in general [63]. Here this tool provides valuable insights into the presence of these beta-lactamase genes. One of the main causes of beta-lactam resistance in *Pseudomonas aeruginosa* is the increased prevalence of beta-lactamase [62]. Beta-lactamase enzymes render beta-lactam antibiotics ineffective by hydrolyzing the peptide bond of the characteristic four-membered beta-lactam ring. The bacterium gains resistance after the antibiotic is rendered inactive. Over 300 beta-lactamase enzymes have been described so far, with numerous kinetic, structural, computational, and mutagenesis studies. The threat posed by more and more powerful beta-lactamases to antimicrobial therapy is only going to increase [64]. The scientific and medical communities are only one step ahead and must continue to

put forth diligent effort to prevent being overcome by the difficult and rapidly rising nosocomial pathogen resistance.

The RGI is a powerful tool for identifying antibiotic resistance genes [65]. The results show that the plasmids analyzed by RGI contain a variety of antibiotic resistance genes (ARGs) that confer resistance to numerous antibiotic classes, including carbapenems, cephalosporins, penams, penem, aminoglycosides, and sulfonamides. The bulk of ARGs identified by RGI is based on protein homolog models, which means they have a high degree of sequence similarity to existing ARGs in the CARD database. RGI observed ARGs from several families and resistance mechanisms, including beta-lactamases, aminoglycoside-modifying enzymes, and sulfonamide-resistant sulfonamides. These ARGs can inactivate or alter the target antibiotics, as well as replace the target enzymes with resistant ones [54].

The results also show that RGI can detect both homology-based and SNP-based ARGs, which is critical for understanding the diversity and evolution of resistance mechanisms. RGI discovered IMP-9, a variation of IMP-1 with a single amino acid change (Glu140Gly) that gives enhanced imipenem resistance [66]. RGI additionally identified OXA-5, OXA-28, OXA-31, OXA-45, and OXA-935, which are OXA-1 variations with distinct SNPs that give varying amounts of carbapenem resistance [67].

RGI can also handle complete and partial genes, which is useful for analyzing low-quality or low-coverage assemblies, metagenomic contigs, or small plasmids. For example, RGI identified OXA-5 with a reference sequence length of 109.74%, indicating that the input sequence contains some additional nucleotides at the ends of the gene. RGI additionally detected OXA-28 with a reference sequence length of 103.76%, indicating that the input sequence contains some insertions inside the gene. RGI identified OXA-935 with a reference sequence length of 112.41%, indicating that the input sequence contains some duplications within the gene.

The results also reveal that RGI can offer thorough annotations and classifications of the predicted ARGs, such as gene name, family, mechanism, drug class, and ontology. RGI, for example, identified APH(3")-Ib as an APH(3") family aminoglycoside-modifying enzyme that confers resistance to aminoglycosides via phosphorylation [54]. CARB-4 was also identified by RGI as a beta-lactamase from the CARB family that imparts resistance to penams via hydrolysis [54]. RGI also identified sul1 as a sulfonamide-resistant sul that belongs to the sul family and confers sulfonamide resistance by replacing the target enzyme dihydropteroate synthase [54].

However, the findings highlight some of RGI's limitations and concerns. RGI, for example, cannot directly analyze raw metagenomic reads, it only accepts data in FASTA format. It requires an additional process of assembly or mapping before using RGI. Depending on the quality of the assembly or mapping techniques, this can result in errors or biases in the output. Some plasmids in the data, for example, had no ARGs discovered by RGI, which could be owing to poor assembly or mapping quality [21]. RGI also relies on CARD reference data, which is updated and curated regularly. This means that depending on the version of CARD used, the RGI information could vary over time. This also implies that RGI may overlook some novel or rare ARGs that are not yet listed in CARD. Some plasmids in the results, for example, have no ARGs detected by RGI, which could be the result of rare or novel ARGs not found in CARD [21]. RGI additionally employs a defined cut-off for homology-based ARG detection based on curated bit scores. This may not be optimal for a wide range of sequences or organisms, resulting in false positives or false negatives. For example, some plasmids in the data had ARGs discovered by RGI using strict criteria rather than perfect criteria, implying that their bit scores are lower than the cut-off. This could be due to a low identity or a short matching region between the input and reference sequences [21].

Conclusion

Finally, our study compared four different web-based antibiotic resistance gene prediction tools: ResFinder, KmerResistance, ResFinderFG, and RGI. ResFinder is a web-based application that specializes in detecting chromosomal changes in bacterial DNA sequences that increase antibiotic resistance. It excelled at identifying acquired antimicrobial resistance genes in whole-genome data and kept its database up to date with newly found resistance genes. ResFinder, on the other hand, is confined to acquired genes and does not include chromosomal alterations. Based on the KmerFinder approach, KmerResistance presented an alternative solution. It searched for co-occurring k-mers in query genomes and resistance gene databases, revealing information on species predictions and acquired antimicrobial resistance genes. The ability of KmerResistance to overcome poor-quality assembly concerns using k-mers to map raw WGS data is an important feature. ResFinderFG brought a unique perspective to our study. It excelled in detecting resistant phenotypes in general, due to databases including sequences discovered by functional metagenomics. As already stated RGI proved to be a strong technique for discovering antibiotic resistance genes. It excelled in detecting a wide range of resistance genes, both homology-based and SNP-based, revealing important information about resistance mechanisms. Furthermore, RGI's capacity to handle full and partial genes made it a versatile candidate for analyzing various types of sequences. RGI distinguishes itself by providing detailed information regarding gene name, family, mechanism, drug class, and ontology in its annotations and classifications of

predicted ARGs. The four tools were empirically evaluated in this study, with their features, limitations, and sensitivities examined. As sequencing technology advances the area of infectious disease research, enabling fair access to bioinformatics tools is critical for global pathogen surveillance and effective tracking of antibiotic resistance based on genomic data. Researchers can make informed selections on which tool most effectively achieves their study goals by understanding the capabilities and trade-offs of different tools. The constantly evolving landscape of antibiotic resistance needs continuous attempts to improve tools and methods for more efficient resistance gene identification and surveillance.

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Declarations

Conflict of interest The authors declare no competing interests.

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