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Review Article

Harnessing eDNA Methodologies for Enhanced Surveillance of Antimicrobial Resistance Genes in Agricultural Soil: A Review

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ABSTRACT
Antimicrobial resistance (AMR) presents a notable catholic threat to animal and human health, with agricultural soil avowed as a major source of antibiotic resistance genes (ARG). The significant impact of anthropoid activities on the soil resistome is well-documented; a knowledge gap persists regarding certain microbial communities that confer resistance, along with the most efficacious methods for their monitoring. This review addresses the disparaging need for advanced, culture-based techniques to monitor ARGs in agricultural landscapes. We bring forth a broad-based overview of the methodologies and bioinformatic pipelines for using environmental DNA (eDNA) and metagenomics to snoop AMR. eDNA-based approaches are underscored for their cost-effectiveness, enhanced sensitivity, and non-invasive nature for detecting a wider scope of ARGs, as well as those from non-viable cells and mobile genetic elements, which repeatedly go undetected by traditional techniques. We moot how high-throughput sequencing of eDNA, incorporated with metagenomic analysis, permits a circumstantial assessment of the soil resistome, revealing both known and novel ARGs. We traversed the potential of these methodologies to bring forth subtle insights into ARG dynamics. This review rules that adopting a standardized eDNA metagenomics approach is key for effecting comparable, reliable data, through strengthening our understanding of AMR transmission via the soil-microbe-plant linkages and informing an effective public health blueprint to combat the post-antibiotic era.

Keywords: Antimicrobial Resistance; ARGS; eDNA; Metagenomics; Soil Resistome

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INTRODUCTION

Antibiotic resistance, a major global health concern that affects both human and animal populations, is brought on by the overuse and misuse of antibiotics (Cella *et al*., 2023; Patangia *et al*., 2022). Although other factors like environmental stress, unhygienic surroundings, and ignorance also play a role, antibiotic abuse in both humans and animals is known to be the primary cause of antibiotic resistance (Muteeb, 2023). Antimicrobial resistance genes having the ability to revert and regain resistance under suitable circumstances, their presence in bacterial communities complicates matters further (Deekshit and Srikumar, 2022). Additionally, the production of antibiotics can pollute the environment with active antibiotic residues, increasing the prevalence of microorganisms that resist antibiotics and the genes they carry (Bengtsson-Palme *et al*., 2019).

Antimicrobial resistance genes (ARG), which may have originated as a means of self-defense for microorganisms, are abundant in soil (Hernández *et al*., 2023). Due to the above, ARGs are found in all soil types, such as agricultural soil, forest soil etc. (Popowska *et al*., 2012; Willms *et al*., 2019; Zheng *et al*., 2021; Hwengwere *et al*., 2022).

However, little is known about the makeup of the soil microbiome community that is resistant to antibiotics (Hernández *et al*., 2023). The high concentration of eDNA in soil may be one of the primary causes, as it is unable to differentiate between active antimicrobial-resistant microorganisms and dormant or dead antimicrobial-sensitive microorganisms (Chen *et al*., 2019; Cerqueira *et al.,* 2020). This may be the cause of some studies' conflicting findings, which range from no change to a total alteration in microbiomes when antibiotics are added (Zheng *et al*., 2021). Monitoring microbiota may help determine the effects of restoration interventions because they are dependent on the resource and energy flows linked to aboveground biota (Van der Heyde *et al*., 2020). This technique can also be adopted for tracking of ARGs in agricultural soil.

These techniques are currently being widely used in a context of restoration. The advancement of high-throughput, inexpensive eDNA sequencing as made it possible to assess soil microbiota in an affordable, quick, and thorough manner (Breed *et al*., 2019; Mohr *et al*., 2022). eDNA high-throughput sequencing has become a potent instrument for ARG surveillance in various environmental matrices. This approach allows the possibility to identify and measure a variety of ARGs, providing valuable insights into their distribution and abundance across different ecosystems (Deshpande and Fahrenfeld, 2022).

ENVIRONMENTAL DNA (eDNA)

The genetic material present in environmental samples like soil, water, and air encompasses whole cells and extracellular DNA is referred to as environmental DNA, or eDNA (Barnes and Turner, 2016). Using this approach captures eDNA from environmental samples and preserves, extracts, amplifies, sequences, and categorizes based on its sequence (Deiner *et al*., 2017). The decomposing remains of larger species, skin, mucus, saliva, sperm, secretions, eggs, feces, urine, blood, roots, leaves, fruit, and pollen can all contain eDNA, whereas microorganisms can be obtained in their entirety. The amount of eDNA produced by an organism depends on its biomass, age, eating habits, physiology, life history, and space usage (Barnes and Turner 2016, Goldberg *et al*., 2016, Hering *et al*., 2018). This technique facilitates the detection of non-invasive microorganisms. eDNA is a relatively new surveying technique, it has exhibited great promise for biological monitoring. The traditional approaches to surveying richness and abundance may rely on techniques that make it difficult to find small or elusive species, have limitations due to taxonomic identification, and may disturb or destroy habitat (Ruppert, 2020). By concentrating on unique species, sampling a greater range of diversity, and enhancing taxonomic resolution, eDNA can support these methods (Deiner *et al*., 2017). In any case, it can be used to identify the initial appearance of uncontrollable species, the continued existence of indigenous species that are believed to be exterminated or threatened, and other elusive species that are found in low densities and would be challenging to find using conventional methods (Ruppert, 2020).

METAGENOMICS

The genomic analysis of microorganisms directly from their natural habitat, without preceding culturing, is termed as Metagenomics (Handelsman, 2004; Wooley and Ye, 2010). The revolutionization of soil microbiology research came through metagenomics by equipping the study of the entire genetic makeup within microbial populations in the environments. This approach not only provides direct access to the vast genetic diversity of the uncultivated soil microorganisms but also overcomes the constraint of traditional culturing and PCR-based methods (Garg *et al*., 2024; Semenov, 2021). Metagenomics techniques in soils include approaches like high-throughput sequencing and stable-isotope probing; these leverage on shotgun metagenomics, which can unveil the composition, function and diversity of soil bacteria influenced by land use and soil management (Garg *et al.*, 2024).

Functional metagenomics is one effective technique emerged for identifying ARGs in soil environments. This technique has revealed the soil as a repository of diverse ARGs, a large number of which are novel and differ from known resistance determinants (Su *et al*., 2014; Torres-Cortés *et al*., 2011). Several Studies have reported numerous new ARGs conferring resistance to abundant antibiotics, these include gentamicin, ampicillin, trimethoprim and chloramphenicol, (Torres-Cortés *et al*., 2011).

The identification of ARGs that are not found in the traditional database can be detected using functional metagenomics compiled in ResFinderFG v2.0 database (Gschwind *et al.,* 2023). These findings emphasize the importance of soil as a wellspring of antibiotic resistance and high spot the need for continued research to comprehend the dynamics of ARGs in the soil ecosystems.

METHODOLOGIES FOR eDNA ARGs SURVEILLANCE IN AGRICULTURAL SOIL

Soil Sampling

Addressing heterogeneity, larger volumes of samples of soil and sediment samples are required over larger spatial scales to detect diverse classes of organisms (Taberlet *et al.,* 2012, Creer *et al.,* 2016). Due to the consequential risk of contamination, supplies and equipment must be separated from the PCR, tissues, and organisms processed, and samples must be decontaminated (Goldberg *et al.,* 2016). Negative field controls are essential to validate the sample and identify contamination (Goldberg *et al.,* 2016, Hering *et al.,* 2018).

Soil Sample Preservation

Sample types can be preserved in a variety of simple ways, such as freezing (-20°C), drying, or storing in ethanol (absolute ethanol) or cell lysis buffer (Creer *et al*., 2016, Goldberg *et al*., 2016). Preserving samples immediately prevents degradation (Barnes et al., 2014, Strickler *et al*., 2015, Tsuji *et al*., 2017). This degradation sample can be influenced by the organisms and certain environmental factors such as temperature, pH, and light exposure.

Decontamination of Soil Sample

Although laboratory techniques vary from study to study, they are generally more consistent across sample types. It is recommended that eDNA be processed in a clean laboratory using non-removable equipment and supplies. Before entering the clean laboratory, personnel and supplies must be decontaminated (Goldberg *et al*., 2016). Filter pipet tips and clean gloves should be used during all procedures, and the lab's processing areas should be cleaned with bleach and UV light on a regular basis (Champlot *et al.,* 2010, Goldberg *et al*., 2016). Replicates, positive controls, and negative controls are key components of laboratory procedure (Ficetola *et al*., 2015, Deiner *et al*., 2017).

DNA Extraction Methods for Metagenomics Analysis

In metagenomics research, eDNA extraction methods for soil samples have been extensively researched, with diverse approaches showing varying levels of efficiency and quality.

Up to 100-fold increases in DNA concentration have been reported through direct extraction methods as compared to indirect methods (Roh *et al.,* 2006). The selection extraction methods can impact the observed microbial community structure, with statistical analyses showing variations in the recovery of genetic material for 24 out of 32 investigated phyla (Zielińska *et al*., 2017).

The combination of chemical (CTAB and $CaCl_2$) and enzymatic (lysozyme and proteinase K) approaches have ensured efficient cell lysis and production of high-quality metagenomic DNA from diverse soil samples (Verma *et al*., 2017). The Use of polyethylene glycol (PEG) and isopropanol for precipitation aids in the removal of humic impurities (Verma *et al*., 2017). For saline soils, a protocol that utilizes mechanical (beads and sonicator) and soft lysis (SDS and enzymes) methods has been developed to extract DNA suitable for metagenomic analysis (Purohit and Singh, 2009).

It is reported that the optimum soil sample preservation and DNA extraction techniques for eDNA metagenomic analysis depend on practical limitations, research goals and costs (Guerrieri *et al*., 2020). It is recommended to test several extraction kits at the start of each study to select the one that produces the most representative results (Zielińska *et al*., 2017).

To identify and measure eDNA-associated ARGs in soil samples, several methods have been developed. In environmental samples, magnetic bead-based techniques have demonstrated potential for the separation of intracellular DNA (iDNA), adsorbed eDNA (a-eDNA), and free eDNA (f-eDNA). Compared to more conventional techniques like alcohol precipitation, CTAB-based extraction, and commercial DNA extraction kits (<10%), this strategy has shown higher recovery rates (>85.3%) (Yuan et al., 2019).

In order to accurately partition ARGs between soil and sediment samples, a novel internal standard method for extracting eDNA and iDNA has been developed (Mao *et al*., 2013). This technique has

been applied in assessment of several sediment, underling the importance of eDNA reservoir. ARGs were detected for more than 20 weeks in sediment samples, whereas chromosomally encoded 16S rRNA genes were undetectable after 8 weeks, indicating higher persistence of plasmid-borne ARGs (Mao *et al*., 2013). eDNA concentrations were found to be higher than iDNA in sediment samples, probably due to enhanced persistence when associated with clay particles and organic matter (Mao *et al.,* 2013). This technique utilizes binding eDNA with sediment particles to create a protective environment which will subsequently reduce degradation.

Different kits exist and their effectiveness differs depending on the target taxa, especially between eukaryotes and prokaryotes. kit-based extraction procedures are likely the most popular and generally effective (Deiner *et al*., 2017, Creer *et al.,* 2016). The efficient approaches are phenol chloroform extractions, which in some cases may isolate more genetic material than other techniques (Creer *et al*., 2016, Deiner *et al*., 2017).

The ability to detect ARG reservoirs in soil environments has greatly improved due to the advent of efficient eDNA extraction methods and advanced quantification methods like qPCR arrays and shotgun metagenomic sequencing (Wang *et al*., 2015). These methods help learn more about the abundance, diversity, and potential for horizontal gene transfer of ARGs. This is important for stopping the spread of antibiotic resistance in the environment (Takeda-Nishikawa *et al*., 2023).

Library Preparation for eDNA Sample

Metagenomic library preparation is a pivotal step toward studying microbial communities across a variety of different environmental samples. Several methods and kits are available for constructing metagenomic libraries, each with certain benefits and drawbacks.

Library preparation methods may affect the metagenomic data and further analyses from the very first experiment. The allowability of library preparation with low DNA input remains controversial. For example, some studies describe the construction of libraries with 1 pg to 1 ng of input DNA using KAPA Hyper Prep and Nextera XT kits (Hirai *et al*., 2017). The lower bias limit in libraries was found to be about 10 pg input DNA. However, libraries made from very small amounts of DNA, such as 1 pg, are expected to be more biased in GC content, k-mers, or Small Subunit rRNA Gene composition (Hirai *et al*., 2017).

Furthermore, different library preparation methods can introduce varying biases. A comparison of the Nextera XT DNA Library Preparation Kit and a standard 454 FLX Titanium protocol indicates that GC content bias was largely attributable to the Nextera protocol rather than the sequencing technology itself (Marine *et al*., 2011). The TruSeq kit has been shown to produce more uniform data quality than Nextera XT for Mycobacterium tuberculosis sequencing, regardless of input DNA quality **(**Tyler *et al*., 2016). Some methods, like the NEBNext Enzymatic Methyl-seq and Swift Accel-NGS Methyl-Seq kits, have demonstrated better performance for whole-genome DNA methylation sequencing (Morrison *et al.,* 2021).

While metagenomic library preparation has advanced significantly, allowing for the analysis of low biomass samples and the use of portable equipment (Acharya *et al*., 2020), researchers must carefully consider the impact of their chosen method on the resulting data. Factors such as input DNA quantity, GC content bias, and the specific research objectives should guide the selection of library preparation protocols to ensure accurate representation of microbial communities in metagenomic studies.

Sequencing of eDNA Sample for Metagenomics

Whole genome sequencing (WGS) and metagenomics, has revolutionized our understanding of microbial diversity and genes functions in various environments. The rapid evolvement of omics results in advances in DNA sequencing technologies, moving from shotgun sequencing to high-throughput next-generation sequencing (NGS) and third-generation sequencing (TGS) **(**Oulas *et al*., 2015; Zhang *et al*., 2021). These technologies have significantly improved our ability to study uncultured microorganisms, novel enzymes, and microbe-environment interactions in diverse settings such as aquatic, terrestrial, and human biomes (Ininbergs *et al*., 2015; Ju and Zhang, 2015). De novo assembly and reference-based assembly are the two approaches documented to assembling short shotgun sequence reads into longer contiguous genomic sequences (Ng and Kirkness, 2010). The de novo method compares and overlaps

sequence reads, while the reference-based approach maps reads to a reference genome sequence.

The development of targeted approaches, such as stable isotope probing combined with whole genome shotgun sequencing, allows researchers to link specific microbes to ecological functions in complex communities (Kalyuzhnaya *et al.,* 2008). As sequencing technologies and bioinformatics tools continue to advance, metagenomics will play an increasingly important role in environmental monitoring, biotechnology applications, and our understanding of microbial ecology (Bragg and Tyson, 2014; Ininbergs *et al.,* 2015; Ju and Zhang, 2015).

WGS methods continue to advance, offering improved resolution for genetic analyses across various applications. While challenges remain, such as direct sequencing from clinical specimens (Mcnerney *et al.,* 2017) and standardization of typing tools (Uelze *et al.,* 2020), ongoing research aims to optimize WGS methodologies. The development of streamlined sample preparation platforms, like Digital-WGS for single-cell WGS (Ruan *et al*., 2020), and the comparison of different sequencing approaches contribute to the continuous improvement of WGS techniques (Chen *et al*., 2021), paving the way for broader applications in genomics research.

Bioinformatics Tools for eDNA Metagenomics

Environmental DNA (eDNA) serves as powerful tools for biodiversity assessment and ecological monitoring. These techniques rely heavily on bioinformatics for data processing, analysis, and interpretation. Several software packages and pipelines have been developed to handle the complex computational tasks associated with eDNA studies (Curd *et al.,* 2019; Sprague *et al.,* 2018).

The eDNA shotgun metagenomics can be utilized for monitoring ARGs in various environments. For ARG detection and quantification, several bioinformatics pipelines and databases are available. These include ARGs-OAP, which uses the Structured ARG reference database (SARG) for fast annotation and classification of ARG-like sequences (Yang *et al.,* 2016). Other databases like ARDB and CARD are also commonly used (Gupta *et al*., 2020). The choice of database and pipeline can significantly impact results, so it's essential to select tools appropriate for the specific analysis being conducted (Gupta *et al.,* 2020).

Different bioinformatics approaches can yield varying results. A report by Deshpande and Fahrenfeld, (2022) on comparison of network analysis of raw reads versus assembly for host assignment of ARGs showed that the raw reads pipeline identified more ARG hosts (247) compared to the assembly pipeline.

R packages like ranacapa provide user-friendly interfaces for exploratory biodiversity analyses and visualizations of eDNA results (Sprague *et al*., 2018). The Shiny web app within ranacapa allows users to interact with eDNA data through interactive figures and simple community ecology analyses. In the R/Bioconductor ecosystem, the lefser package offers functionality for metagenomic biomarker discovery, improving upon the widely-used LEfSe algorithm in terms of performance, accuracy, and reproducibility (Khleborodova *et al*., 2024).

Python-based tools are also prevalent in eDNA metagenomic analysis. InSilicoSeq, for instance, is a Python package designed to simulate metagenomic Illumina sequencing data, which is crucial for benchmarking bioinformatics tools and experimental design (Gourlé*et al*., 2018). Several online tools are available for metagenomic data analysis, with MG-RAST, IMG/M, and METAVIR being among the most cited (Dudhagara *et al*., 2015).

The field of eDNA metagenomic analysis benefits from a diverse array of bioinformatics tools and software packages in both R, Python, BASH, Perl, etc. These tools cater to various aspects of metagenomic analysis, from data simulation and quality control to biodiversity assessment and visualization. As the field continues to evolve, more specialized and user-friendly tools will likely emerge to address the complex challenges of eDNA metagenomic data analysis.

Table 1. Summary of methodologies for eDNA ARGs Surveillance in Agricultural Soil

Category	Description
Soil Sampling	Larger quantities from various sites to prevent heterogeneity
Soil Preservation	Preserving in cell lysis buffer or 100% ethanol, drying, or freezing at -20°C
Soil Decontamination	Use of controls and duplicates, clean lab processing, bleach and UV cleaning, and separate field and lab equipment
DNA Extraction	Direct techniques produce more DNA; enzymatic and chemical strategies for effectiveness; techniques for saline soils; testing multiple kits for best results; and techniques based on magnetic beads for sorting different types of DNA
Library Preparation	Effects on metagenomic data, difficulties with low biomass samples, possible biases, and the significance of choosing the right method for accurate representation
Sequencing	Developments from shotgun to NGS and TGS, reference-based and de novo assembly, and the connection between microbes and ecological roles
Bioinformatics Tools	Pipelines and databases for ARG detection (e.g., ARGs-OAP, SARG, ARDB, CARD), R packages (e.g., ranacapa), Python-based tools (e.g., InSilicoSeq), and online tools (e.g., MG-RAST, IMG/M, METAVIR) are used for data processing, analysis, and interpretation.

CHALLENGES OF eNA METAGENOMICS

eDNA metagenomics has many obstacles to overcome, it also offers exciting new avenues for ecological monitoring and biodiversity assessment. The absence of standardized procedures and methods for eDNA sampling, preservation, and analysis is one of the main obstacles (Pawłowski *et al*., 2021). The problem is made more difficult by the lack of universal standards for guaranteeing that data is FAIR (findable, accessible, interoperable, and reusable) (Shea *et al*., 2023). The robustness and comparability of findings across studies may be impacted by this variation in methodology. The different sources and states of DNA in environmental samples, along with its persistence in sediments, can further complicate the interpretation of eDNA data (Pawłowski *et al*., 2021).

Numerous biotic and abiotic factors affect eDNA concentrations in natural environments, making it difficult to determine the accuracy of abundance estimates from eDNA. This weakens the relationship between eDNA and actual organism abundance (Yates *et al*., 2021).

While eDNA metagenomics has a lot of promise for monitoring biodiversity, resolving these issues is essential to enhancing the accuracy and usefulness of these methods. To overcome these constraints and fully utilize the potential of eDNA-based approaches in ecological research and conservation, efforts must be made toward standardization, reference database expansion, and a deeper comprehension of eDNA dynamics in various ecosystems.

FUTURE DIRECTIONS OF EDNA METAGENOMICS

eDNA metagenomics has a bright future. Advancements in sequencing technologies, bioinformatic algorithms, pipelines, and methods for converting biosynthetic gene clusters into small molecules are expected to increase the rate of antibiotic discovery from metagenomes.

Combining advanced molecular biology methods with eDNA analysis is a crucial step forward. This includes the application of single-cell technologies, genomics, and epigenomics, which are expected to change eDNA analysis from a method for identifying species distribution to one that can clarify an organism's physiological condition and behavior. As reported in a study by Yates *et al*., 2021, the integration of eDNA dynamics and physiological

factors into models could improve abundance estimates.

The development of environmental protein analysis, environmental sample processors, and ecogenomic sensors offers new opportunities for eDNA technology (Wang *et al*., 2019).

In conservation and spatial planning, eDNA analysis is poised to play a crucial role in informing biodiversity conservation decisions. The integration of eDNA data with spatial planning processes could provide valuable information on biodiversity over spatial-temporal scales that are currently prohibitive in traditional studies (Bani *et al*., 2020). This could significantly enhance our ability to make informed decisions about ARGs areas and other conservation efforts.

As these technologies continue to evolve, eDNA metagenomics is set to become essential tools in ecological monitoring, global conservation studies, and drug discovery efforts.

CONCLUSION

This essay aims to review the methodologies to harness eDNA assay for the surveillance of soil microbiome and antimicrobial resistance genes in agricultural soil, assess the effectiveness of the assay, and review the literatures of eDNA metagenomics in order to design a research plan for implementing the best molecular methods for the project. Despite the considerable potential of eDNA research as reported in several studies, tracing ARGs through eDNA metagenomics is a challenging endeavor, one of the predominant issues is a targeted approach utilizing mainly on bioinformatics tools, and the patchiness of bacterial eDNA on soil. Further complications include the generally limited persistence of eDNA, considerable differences between systems regarding physicochemical conditions which may affect eDNA decay rates and detectability, and the incompleteness of reference databases.

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