# A REVIEW OF CURRENT WASTEWATER-BASED EPIDEMIOLOGY TO MONITOR ANTIBACTERIAL RESISTANCE AMONG GRAM NEGATIVE BACTERIAL SPECIES IN THE UNITED STATES

By

Sanchitha Meda

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## ABSTRACT

Antimicrobial resistance (AMR) is an increasing major public health problem in the United States. Therefore, monitoring the status of AMR is vital to protecting population health. While there are established surveillance systems in clinical and veterinary settings, there is a lack of well-developed surveillance in the environment. Wastewater-based epidemiology, with focus on wastewater treatment plants (WWTPs), has been proposed as a solution to this gap in observation. This kind of surveillance has many advantages, such as being able to receive results in real-time, and is needed in addition to clinical and veterinary surveillance for AMR. However, this system is not fully developed and is lacking in standardized gene targets and analysis methodology. This review aims to provide a broad and current perspective of how antibacterial resistance can be monitored in wastewater in the United States. It will assess the current literature on method standardization which will allow for the ease of research comparison and risk assessment. It will outline potential gene targets for gram-negative bacterial species, specifically *E. coli* and *Shigella spp.*, describe the advantages and disadvantages of the main analysis technologies (culture-based, amplification-based [e.g., qPCR], and metagenomics), and assess remaining knowledge gaps for the use of wastewater surveillance to monitor AMR. Wastewater-based epidemiology has the potential to be a low cost, passive surveillance method to help estimate the AMR status in a community and be used in conjunction with clinical and veterinary surveillance systems to aid public health officials inform policy and mitigation practices to slowing the spread of AMR.

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## INTRODUCTION

The World Health Organization (WHO) has declared that antimicrobial resistance (AMR) is one of the top ten global health threats to humanity (2021). The ability to treat common infections is being threatened by the emergence and spread of drug-resistant pathogens (WHO, 2021). According to a recent report from The Lancet, 1.27 million deaths worldwide were directly attributed to antimicrobial resistance in 2019 (Antimicrobial Resistance Collaborators, 2022). Globally, *Escherichia coli* is one of the leading antibiotic resistant bacteria (ARB) and responsible for over 600,000 deaths associated with AMR in 2019 (Antimicrobial Resistance Collaborators, 2022). In the United States, more than 2.8 million AMR infections occur each year (*National Infection & Death Estimates for AR*, 2022). Of those, drug-resistant *Shigella* accounts for about 77,000 estimated drug-resistant infections per year and has been labeled as a "serious threat," or a pathogen that requires prompt and sustained action in the Centers for Disease Control's 2019 AR Threats Report ("Antibiotic Resistance Threats in the United States, 2019," 2019).

To combat this drug-resistant crisis, it is essential to understand the status of AMR. The surveillance of AMR provides information on AMR's geographical and seasonal patterns, its incidence, as well as monitoring for new or rare resistance traits and emerging trends (Tiwari et al., 2022). Different sectors can utilize this information for mitigating AMR infections, prioritizing certain actions, evaluating interventions, informing empirical treatment guidelines, reducing adverse impacts, and developing new antimicrobial drugs (Tiwari et al., 2022). In the United States, AMR surveillance has been implemented in clinical and veterinary settings. However, AMR surveillance in the environment is currently not well established (Bengtsson-Palme et al., 2023). Not only is monitoring antibiotic resistance in the environment important for

providing information on the dynamics of AMR in the regional population, but it also is important since environmental pathogens can potentially disseminate AMR to clinical pathogens (Bengtsson-Palme et al., 2023; Tiwari et al., 2022).

More recently, wastewater-based epidemiology has been proposed for the surveillance of antibiotic resistance spread, especially since its successful application to monitoring SARS-CoV-2. Wastewater-based epidemiology is an epidemiological approach that is based upon the extraction, detection, analysis, and the interpretation of biomarkers, or chemical and/or biological compounds, in wastewater (e.g., sewage) (Sims and Kasprzyk-Hordern, 2020). These biomarkers can be linked to the community that is within the geographically defined water catchment areas, or watersheds (Sims and Kasprzyk-Hordern, 2020). Untreated wastewater, from places such as hospitals and municipal buildings, is usually disposed and collected by wastewater treatment plants (WWTPs). Typically, one WWTP serves a town or a city. Wastewater has been shown to have a high source of antibiotic pollution (Larsson and Flach, 2022). With the emergence of antibiotic resistant pathogens of clinical and veterinary significance over the past decades, WWTPs have been designated as a focal point in the fight against AMR (Nguyen et al., 2021). This type of surveillance can be utilized to predict the occurrence of ARBs and its respective genes at the population level (Tiwari et al., 2022).

Compared to traditional clinical surveillance, wastewater surveillance has been shown to be exceptionally resource and cost efficient, that is, one sample can embody pathogens from thousands of people and give comprehensive health information on communities (Larson et al., 2023; Sims and Kasprzyk-Hordern, 2020). With its ability to collect data in near-real time, it may have the potential to be utilized as a surveillance tool for early outbreak detection of rare forms of resistance (Larson et al., 2023; Sims and Kasprzyk-Hordern, 2020). Additionally, given that

information is given on a whole population and not on an individual level, there is minimal legal and ethical challenges and individual privacy concerns (Sims and Kasprzyk-Hordern, 2020). Some of the traditional surveillance programs for antibiotic resistance target sick populations; as such, the resistance rates in healthy populations are not well represented (Fahrenfeld and Bisceglia, 2016). In contrast, wastewater-based surveillance has the potential for providing variations in the levels of resistance for both healthy and sick human populations (Fahrenfeld and Bisceglia, 2016).

However, if utilizing wastewater-based epidemiology in general, there are some considerations. First, wastewater flow rates need to be tracked due to the wide variations in influent flows (e.g., rainfall causing dilution) (Sims and Kasprzyk-Hordern, 2020). This is conducted because when reporting upon the presence of a pathogen in a sample, it is reported as the daily loads in wastewater (mg/day) (Sims and Kasprzyk-Hordern, 2020). Second, it is challenging to extract specific targets from the abundance of chemical and biological targets that are present in wastewater (Sims and Kasprzyk-Hordern, 2020). However, the development of certain extraction methods such as solid phase extraction, immunoassay, and mass spectrometry have allowed for the analysis of numerous compounds (Petrie et al., 2015). Another situation associated with wastewater-based epidemiology is the problem constituted by dynamic populations (e.g., populations that fluctuate due to commuters or tourism) (Sims and Kasprzyk-Hordern, 2020). The current standard is to calculate the levels of certain endogenous biomarkers in humans (e.g., cortisol) as daily loads that have been normalized to the population (Sims and Kasprzyk-Hordern, 2020). Although, there are challenges in estimating the population size of individual WWTP catchment areas (Sims and Kasprzyk-Hordern, 2020).

While there are opportunities and challenges to utilizing wastewater-based surveillance for antibacterial resistance, there is a caveat; WWTPs are known for facilitating gene transfer and antibacterial resistance. WWTPs are an environmental reservoir for bacteria, antibiotics, and antibiotic resistance genes (ARGs) to persist, interact, and lead to the potential selection pressure for ARGs (Uluseker et al., 2021; Sambaza and Naicker, 2023). WWTPs contain antibiotic residues, ARGs, bacteria, rich supply of nutrients and other selectors which can facilitate pathogen interactions and potentially promote genetic transmission amongst pathogens through horizontal gene transfer of resistance genes via mobile genetic elements (MGEs), genetic mutations, or subsequent vertical transmission of these mutations (Tiwari et al., 2022). With this environment and other selection and stress factors, the production, transmission, and multiplication of drug-resistant pathogens can occur, and, subsequently, antibacterial resistance (Sambaza and Naicker, 2023). Furthermore, while WWTPs employ various treatment processes to remove a variety of contaminants from wastewater and sewage and have shown to be effective in reducing the ARB loads in effluent samples, some drug resistant pathogens and ARGs are still retained in the treated water by attaching to the organic matter in the wastewater and may be disseminated into the environment (Fouz et al., 2020; Sambaza and Naicker, 2023). These ARGs and drug resistant pathogens can then go on to cause untreatable or difficult-to-treat infections in humans (Sambaza and Naicker, 2023). Figure 1 illustrates the pathway of AMR in wastewater through a WWTP.

To combat antibacterial agents, bacteria are genetically encoded to use natural or acquired resistance mechanisms. Natural resistance may be intrinsic (always expressed in the bacteria) or induced (naturally occurring genes in the bacteria that are only expressed after exposure to an antibiotic) (Reygaert, 2018). Intrinsic resistance can be defined as a trait that is universal within a

bacterial species (Reygaert, 2018). This is a bacterial species' innate ability to resist activity of a particular antibiotic and allow tolerance of that particular antibiotic (University of Minnesota, 2023). One of the most common intrinsic resistance mechanisms is the reduced permeability of the outer membrane, specifically the lipopolysaccharide (LPS), in gram negative bacteria (Reygaert, 2018). As such, all gram negatives, which include *E. coli* and *Shigella spp.*, are intrinsically resistant to glycopeptides and lipopeptides (Reygaert, 2018). *E. coli* is also intrinsically resistant to macrolides (Reygaert, 2018). Acquired resistance occurs when a particular bacterium obtains the ability to resist the efforts of a particular antibacterial agent to which it was previously susceptible (University of Minnesota, 2023). Acquired resistance occurs through mutations of genes involved in normal physiological process and cellular structures, the acquisition of foreign resistance genes from horizontal or vertical gene transfer, or a combination of these mechanisms (University of Minnesota, 2023). There are four main categories of acquired mechanisms for antimicrobial resistance: (1) limiting drug uptake; (2) drug target modification; (3) drug inactivation; (4) drug efflux (Reygaert, 2018). Gram negative bacteria make use of all four main mechanisms (Reygaert, 2018). Thus, gram negative bacteria will be the focus of this review, specifically *E. coli* and *Shigella spp*.

While wastewater surveillance of AMR appears to be promising, it is not fully developed and there is a lot of work to be done before using the approach as a reliable surveillance tool (Tiwari et al., 2022). Specifically, there is a lack of agreed upon targets and no standardized methodical approach to monitoring AMR in the environment (Hu et al., 2018). This review aims to offer a broad and current perspective of how antibacterial resistance can be monitored among two gram-negative bacterial species, specifically *E. coli* and *Shigella spp.*, in wastewater in the United States. It will attempt to delineate potential ARG targets, analysis methodology, and assess

remaining knowledge gaps for the use of wastewater-based epidemiology for antibacterial resistance surveillance among the gram-negative bacterial species. The information from this review can aid in strengthening wastewater-based epidemiology for ARB surveillance among the aforementioned pathogens. Specifically, this review can benefit the creation of a wastewater-based surveillance system that assesses ARB status by determining which drug-resistant pathogens and ARGs are dominant or emerging in a certain population and to inform public health efforts. This can be a resource to provide context for government organizations that are considering implementing this kind of surveillance system.

## STANDARDIZED METHODS FOR AMR MONITORING OF WASTEWATER

There have been calls to standardize targets and methods for environmental AMR monitoring (Berendonk et al., 2015; Pruden et al., 2018). With AMR being quite complex, a multitarget and adaptable approach would be required with emphasis on quality assurance and quality control practices (Pruden et al., 2018; Liguori et al., 2022). There is a consensus that there needs to be an agreement on targets for monitoring and a standardization of the methods for AMR surveillance (Berendonk et al., 2015; Pruden et al., 2018; Liguori et al., 2022). With method standardization, it is easier to share research findings and compare risks (Tiwari et al., 2022). The occurrence of ARGs is frequently detected in WWTPs and studies have illustrated that the ARGs found in wastewater often reside in clinically relevant pathogenic bacteria (Uluseker et al., 2021). This section will attempt to shed light on potential ARG targets and methods that could be used to monitor AMR among *E. coli* and *Shigella* spp. in humans to inform public health efforts.

### *Resistance Mechanisms and Antibiotic Resistance Gene Surveillance Targets: E. coli*

*Escherichia coli* is a gram-negative bacterium that can cause severe infections and is a major reservoir of resistance genes that may be responsible for the failure of clinical treatments in humans (Poirel et al., 2018). During the last decades, there has been an increasing number of resistance genes acquired by horizontal gene transfer identifies in *E. coli* isolates (Poirel et al., 2018). *E. coli* acts in a dual manner as a donor of genetic material to other bacteria and as a recipient of resistance genes from other microorganisms (Poirel et al., 2018). As such, the *Enterobacteriaceae* family can develop resistance to many classes of antibiotics (Galindo-Méndez, 2020). Currently, *E. coli* is resistant to many major classes of antibiotics including β-lactams, quinolones, aminoglycosides, sulfonamides, fosfomycin, as well as last resource antibiotic classes such as the polymyxins and carbapenem (Galindo-Méndez, 2020). The

following will focus on the resistance mechanisms developed by *E. coli* against the β-lactam antibiotic group.

β-lactams inhibit the synthesis of peptidoglycan, a component of a microorganism's cell wall by inactivating penicillin-binding proteins (PBPs) via hydrolysis (Galindo-Méndez, 2020). With regards to *E. coli*'s resistance to the β-lactams, they produce a group of enzymes referred to as β-lactamases (Galindo-Méndez, 2020). These enzymes are ancient compounds with over 2,800 unique proteins that emerged from environmental sources (Bush, 2018). The β-lactamase genes are usually found in MGEs such as plasmids and can be transferred horizontally (Nzima et al., 2020). The two clinically relevant β-lactamases that are of public health concern are extended spectrum β-lactamases (ESBL) and the carbapenemases.

While most members of the *Enterobacteriaceae* family can produce these enzymes, *E. coli* is one of the predominant ESBL-producing genera (Galindo-Méndez, 2020). Of the ESBL-producing *E. coli*, the most common types during the 1980s were $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ (Higgins et al., 2023). However, the $bla_{\text{CTX-M}}$ has become the dominant ESBL in *Enterobacteriaceae*, associated with human and animal infections (Higgins et al., 2023). A recent meta-analysis reported that the prevalence of ESBL-producing *Enterobacteriaceae* in wastewater, with the highest being *E. coli*, has been increasing over time in the United States (Zaatout et al., 2021). The meta-analysis also confirmed that among the ESBL genes, $bla_{\text{CTX-M}}$ had the highest prevalence in wastewater, followed by $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ (Zaatout et al., 2021). Another study asked survey participants to select three targets from a list of ARGs for AMR monitoring of water environments and $bla_{\text{CTX-M}}$ was one of the five most frequently selected targets (Liguori et al., 2022). Based on the literature, it is acceptable to utilize these genes as wastewater

surveillance targets for β-lactam resistance. More examples of the ARGs detected in wastewater that could potentially serve as surveillance targets are provided in Table 1.

Another clinically relevant β-lactamases are the carbapenemases that decreases a bacteria's susceptibility to carbapenems. Carbapenems are a group of antibiotics that are considered the last line of drugs for the treatment of severe infections (Murugan et al., 2019). They bind to PBPs and induce spheroplast formation and cell lysis without filament formation (Galindo-Méndez, 2020). With the rise in ESBL-producing *E. coli*, there has been an increase in the carbapenem usage, which has resulted in the spread of carbapenemase-producing *E. coli* (Murugan et al., 2019). Some studies report that the carbapenemases in *E. coli* mainly include KPC, MBL, including the NDM type and OXA; however, different reports illustrate the predominant types in *E. coli* are NDM-1 and OXA-48 (Galindo-Méndez, 2020). As far as wastewater detection, a study, funded by the U.S. Environmental Protection Agency (EPA), conducted a survey from different U.S. WWTPs that confirmed the presence of carbapenem-resistant *E. coli* and found that the most commonly detected carbapenemase gene was $bla_{VIM}$, followed by $bla_{KPC}$ (Hoelle et al., 2019). Another study found that $bla_{\text{OXA-363}}$, $bla_{\text{OXA-309}}$, and $bla_{\text{OXA-371}}$ were prominent β-lactam ARGs in US/Europe sewage (Prieto Riquelme et al., 2022). Overall, it can be concluded that these genes can be targeted for surveilling carbapenem resistance in wastewater. More examples of the ARGs detected in wastewater that could be potential surveillance targets are provided in Table 1.

***Resistance Mechanisms and Antibiotic Resistance Gene Surveillance Targets: Shigella spp.***

*Shigella* is a genus consisting of four species, including *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*, and is considered as a major pathogen responsible for the increasing rates of morbidity and mortality caused by dysentery (Ranjbar & Farahani,

2019; Shahin et al., 2019). In addition, about half of the strains of *Shigella* in many parts of the world are now resistant to multiple drugs (Ranjbar & Farahani, 2019). The CDC found that between 2015 and 2023, the percentage of multi-drug resistant *Shigella* was largely made up by *Shigella sonnei* (66%), followed by *Shigella flexneri* (34%) (2023). There is multiple resistance mechanisms *Shigella spp.* utilize to evade the effects of an antibacterial agent.

Being that *Shigella spp.* belongs to the family *Enterobacteriaceae*, it is closely related to and shares many common characteristics with *E. coli* (Devanga Ragupathi et al., 2017). As such, *Shigella spp.* also has the ability to acquire resistance to β-lactams via ESBLs and carbapenemases (Ranjbar & Farahani, 2019). Common ESBL resistance enzymes found in *Shigella* and detected in wastewater are TEM, SHV, and CTX-M (Ranjbar & Farahani, 2019; Rizzo et al., 2013). For carbapenem resistance genes found in wastewater, VIM and IMP genes were detected in *S. sonnei* and *S. flexneri* isolates (Ranjbar & Farahani, 2019; Rizzo et al., 2013). Attributing the ESBL resistance enzymes in either *E. coli* or *Shigella spp.* in wastewater may be difficult as they are the same. Other targets or indicators may be needed to overcome this problem. On the other hand, the carbapenem resistance genes listed above would be adequate wastewater targets to monitor. More examples of the ARGs related to β-lactams that are detected in wastewater and could potentially be utilized as surveillance targets are provided in Table 1.

*Shigella spp.* also has acquired resistance to fluoroquinolones through several mechanisms including mutations in the quinolone resistance-determining region (QRDR) and plasmid-mediated quinolone resistance region (PMQR) (Teimourpour et al., 2019). One form of fluoroquinolone-resistance is by targeting the two bacterial enzymes that play a role in DNA replication and are encoded in the QRDR area are DNA gyrase (*gyrA* and *gyrB* genes) and topoisomerase IV (*parC* and *parE* genes) (Teimourpour et al., 2019). While *gyrA* has been

detected in wastewater, the more commonly detected gene is of the *qnr* genes (Pazda et al., 2019). *qnr* genes are plasmid genes found in PMQR regions that encode proteins protecting DNA gyrase and topoisomerase IV against quinolone compounds and are located on MGEs (Pazda et al. 2019). These genes are the main reason for resistance to fluoroquinolones among *Shigella* isolates (Ranjbar & Farahani, 2019). As such, these would be adequate target genes for the surveillance of fluoroquinolone resistance. More examples of the ARGs related to fluoroquinolones that are detected in wastewater are provided in Table 1.

Another class of antibiotics that *Shigella spp.* has acquired resistance to is the tetracyclines (Ranjbar & Farahani, 2019). Tetracyclines' main mechanism of resistance is due to the efflux pump and ribosomal protection system (Shahsavan et al., 2017). The efflux pump genes encode membrane proteins which export tetracycline from the cell, making it ineffective (Shahsavan et al., 2017). Five efflux genes –*tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(G) — and one ribosomal protection protein encoded by *tet*(M) have been identified among *Shigella* isolates (Ranjbar & Farahani, 2019). All these resistance genes have been found in wastewater, with tetA and tetB more commonly found (Nguyen et al., 2019; Pazda et al., 2019; Rizzo et al., 2013; Szczepanowski et al., 2009). In fact, many studies have found that the *tet* resistance gene is one of the most commonly occurring ARGs in wastewater treatment systems in many countries (Uluseker et al., 2021). As such, it would be acceptable to utilize these genes as wastewater surveillance targets to monitor tetracycline resistance. More examples of the tetracycline related ARGs that are detected in wastewater are provided in Table 1.

***Other Considerations***

It is important to note the difference in concentration of ARGs in influent and effluent sources. In a review of ARGs in WWTPs, Pazda et al. reported 98% of ARGs were removed

from the effluent (2019). However, they also reported that there was enrichment of some resistance genes in the effluent (Pazaa et al., 2019). This illustrates that WWTPs are not designed to remove ARGs (Pazda et al., 2019). If using WWTPs as a surveillance source, the location of where the samples are taken should be considered. With the objective of building a surveillance system that determines which forms of AMR are dominant or emerging in human populations to information public health efforts, samples should be taken from influent wastewater, as opposed to effluent. This source is more relevant to the community that provides the sewage to the WWTP.

At the heart of this is determining which ARGs should be used for the surveillance of antibacterial resistance among humans and be used to inform public health efforts. There are many different ideas that have been proposed. One is the "ResCon" risk ranking system in which individual ARGs are assigned a risk value between 1 and 7, depending on a variety of considerations (Martínez et al., 2015). ARGs that are more recently evolved, encode resistance to new antibiotics, or that are associated with MGEs score higher on the risk scale (Martínez et al., 2015). Others have argued that ARGs that are well-known and have been around for decades pose a lesser risk (Vikesland et al., 2017). For emerging ARGs, Bengtsson-Palme et al. have suggested to create a ranked watchlist for upcoming potential AMR threats (2023). They describe this list to include latent ARGs that are of concern for various reasons, including high level resistance to critical antibiotics observed in experiments, indications of broad-spectrum activity or poor clinical outcomes when the gene is detected in pathogens, the gene is located on a highly transferable MGE, and more (Bengtsson-Palme et al., 2023). They argue that this environment-based watchlist for ARGs would provide an early warning about emerging AMR before these genes are widespread (Bengtsson-Palme et al., 2023). This could potentially be one method of

determining ARG wastewater surveillance targets. A more obvious choice would be to select ARG targets based on the geographical location of the WWTP.

A major challenge with focusing on ARGs as surveillance targets is that they are inherently difficult to detect in the environment (Vikesland et al., 2017). Prior to the broad antibiotic use in modern medicine, antibiotic resistance has been a natural phenomenon (Li et al., 2020). In fact, genes encoding resistance to β-lactams, tetracycline and glycopeptide antibiotics have been detected in natural soil and even in 30,000-year-old permafrost sediments (Li et al., 2020). Therefore, it is vital for ARG source tracking that autochthonous and allochthonous ARG in an environment are differentiated (Li et al., 2020). The quantification and report of ARGs needs to be interpreted based on the significance of their presence and how it relates to the rapid evolution and spread of MDR bacteria (Nguyen et al., 2021). Seasonality changes can also impact surveillance efforts through diluting or enriching ARGs in a certain area (Li et al., 2020). As of late, the class 1 integron - integrase gene, *intl1*, has been proposed as an indicator for AMR and HGT because of its common linkage with ARGs and its quick response to diverse environmental pressures (Li et al., 2020; Rumky et al., 2022). Overall, it is clear that to conduct AMR surveillance in wastewater, other indicator or control factors are needed to differentiate target bacteria or genes from background bacteria and environmental conditions.

***Wastewater Analysis Methodology for ARGs and ARBs***

As mentioned previously, to utilize wastewater-based epidemiology for monitoring AMR, the standardization of methods is needed. Currently, there is no universal monitoring method available (Miłobedzka et al., 2022). It is important to consider the objective of the analysis (i.e., studying either the diversity of ARGs and ARBs or to measure the abundance (per mass/volume of sample) or prevalence (per total bacteria) in a given environment) when determining which

method is appropriate (Manaia et al., 2018). There are three main wastewater analysis methods that are being considered for monitoring antibacterial resistance: culture-based methods, amplification-based methods, and metagenomic-based methods (Liguori et al., 2022).

***Culture-based methods***

Culture-based methods have been considered for AMR monitoring because specific clinically relevant targets can be selected, the methods are well standardized for defining clinical resistance levels and not technically difficult to conduct, and the recovered target is viable (Scott et al., 2020; Liguori et al., 2022). Its main appeal is being able to determine phenotypic traits, which is the basis to assess the propagation or gene transfer potential of specific ARB under environmental conditions (Manaia et al., 2018). Those isolated can be subject to further analysis including multidrug-resistance testing, sequence-based testing, or whole genome sequencing (i.e., can help in identification MGEs carrying ARGs, identifying sources of outbreak strains, or delineate phylogenetic relationships among strains) (Liguori et al., 2022; Tiwari et al., 2022). In addition, since the current AMR surveillance approach is based on clinical isolates, a culture-based method can be relatively convenient at the local level (Tiwari et al., 2022). A key distinction is that culture-based methods have been better able to inform human health risk assessments (Liguori et al., 2022).

However, with the large number of microorganisms found in wastewater, the likelihood of interference to isolate the target is increased (Liguori et al., 2022). One study attempted to address this concern by adapting and improving already standardized tests originally developed for human medical purposes (Schreiber et al., 2021). While the researchers were able to provide an appropriate culture-based approach for the microbiological investigation of environmental water samples for ESBL-producing bacteria, they recommended that for the determination of

carbapenem-resistant bacteria, gene detection via PCR would be better suited since numerous environmental bacteria harbor intrinsic carbapenemase genes (Schreiber et al., 2021). Furthermore, only less than 1% of environmental microorganisms can be cultured, therefore those that harbor resistance can be overlooked by culture-based methods (Tiwari et al., 2022). Moreover, certain pathogens may be overlooked because they have concentrations that are too low for detection by culture, but that concentration may be high enough to cause infection (Girones et al., 2010). Additionally, after prolonged exposure to water, bacteria might enter a viable but non-culturable state, while retaining their infective potential (Girones et al., 2010). Another practical challenge with culture-based methods is that it is a costly and laborious procedure that involves enrichment and different selective media to be able to isolate pathogens from background bacteria (Girones et al., 2010; Manaia et al., 2018). Furthermore, attempting to achieve appropriate enrichment is often difficult and time-consuming (Girones et al., 2010). It also does not provide any information of the mechanisms of resistance which may disseminate to other bacterial species via MGEs (Anjum, 2015). These limitations are important to consider, especially in cases when critical and timely intervention for infectious diseases is required. With all that in mind, it is clear that a different methodology is needed in combination with cultivation for the detection and quantification of ARGs and pathogens in wastewater. Table 2 describes the strengths and weaknesses in using culture-based methods to detect ARGs and bacteria in wastewater.

***Amplification-based methods***

As technologies have advanced, the analysis of ARGs have started to move towards culture-independent methods (Nguyen et al., 2021; Liguori et al., 2022; Miłobedzka et al., 2022). PCR techniques, more notably real-time quantitative PCR (qPCR), are considered to be highly

specific and sensitive (Girones et al., 2010; Liguori et al., 2022). qPCR is quickly becoming established in the environmental sector and is currently the preferred method for the identification and quantification of ARGs (Girones et al., 2010; Scott et al., 2020). This cost-effective and rapid technique uses specific probes to gather a significant amount of information on the presence, quantity and distribution of pathogens in water (Girones et al., 2010). With its high sensitivity, qPCR has the power to nearly doubling the gene target every thermal cycle, allowing for a lower limit of detection (Borchardt et al., 2021). In addition, qPCR can simultaneously analyze a large number of genes (Manaia et al; 2018; Loguori et al., 2022; Miłobedzka et al., 2022). This circumvents the limitations of culture-based methods, e.g., the requirement to choose a single species or genera of bacteria to study, including the limited ability of selective media to isolate and quantify targets against background bacteria (Keenum et al., 2022). qPCR, in combination with epidemiological surveys, can also be useful for risk assessment (Girones et al., 2010). Furthermore, it is advantageous that qPCR uses DNA, as it can detect ARGs in non-culturable bacteria (Abramova et al., 2023).

Conversely, there are some limitations to utilizing qPCR. First, it is incapable of distinguishing living from dead cells (Manaia et al., 2018; Scott et al., 2020; Liguori et al., 2022). There have been studies that illustrate overcoming this limitation, for instance, using propidium monoazide to distinguish membrane injured cells from intact cells (Manaia et al., 2018). However, there are other mechanisms of cell inactivation that those studies do not address (Manaia et al., 2018). In addition, qPCR is designed to follow the amplification of a specific gene fragment through the use of primers that have been previously described, therefore, for unknown/new genes, creating primers would be virtually impossible (Manaia et al., 2018; Scott et al., 2020). Furthermore, if the primer is designed based on a specific ARG variant, it may not

universally capture all versions of these variants (Keenum et al., 2022). Therefore, if the objective is to identify emergent resistance threats, qPCR may not be the best method (Abramova et al., 2023). It is also important to keep in mind that qPCR is susceptible to factors such as the reaction components, the analytical equipment, master mixes, the type of sample (e.g., wastewater) (Miłobedzka et al., 2022). This can produce significant difference in results (Girones et al., 2010). Additionally, it is important that the personnel have expertise in molecular biology, which isn't as needed with culture-based methods (Liguori et al., 2022). Moreover, some protocols may have limited details with regards to key quality assurance aspects for environmental samples, including positive and negative controls, limit of detection, and limit of quantification, etc. (Borchardt et al., 2021; Keenum et al., 2022). Overall, if the objective is to detect high-risk ARGs for monitoring resistance, qPCR would be an appropriate method to use because of its sensitivity. Table 2 describes the strengths and weaknesses of using amplification-based methods in detecting ARGs and bacteria in wastewater.

***Metagenomic-based methods***

While qPCR is limited to only conducting targeted analysis, metagenomics is suitable for non-targeted analysis (Manaia et al., 2018). Metagenomic methods sequence the whole metagenome present in the sample (Manaia et al., 2018). Utilizing next-generation DNA sequencing (NGS) allows for the possibility of profiling all ARGs, unculturable bacteria, and other genes in a sample without prior knowledge of gene targets (Liguori et al., 2022; Miłobedzka et al., 2022). This allows for the possibility to provide an overview of not only the already known ARGs, but also of their variants or possible new ARGs that may exist in an environment (Manaia et al., 2018). Wastewater surveillance via metagenomics could potentially prove to be useful for identifying emergent resistance threats (Miłobedzka et al., 2022). In fact,

there has already been an attempt to monitor and predict the occurrence of AMR in a global healthy human population using metagenomics (Hendriksen et al., 2019). Not only can metagenomics provide broad contextual information, but it can also provide a large amount of information on the diversity of ARGs and MGEs in different environments (Rice et al., 2020). It is also being utilized for examining shifts in the resistome thorough WWTPs and identifying MGEs to determine the extent of HGT events (Liguori et al., 2022). The information can also be stored and reused later to allow for retrospective analysis of resistance genes after the initial run (Bengtsson-Palme et al., 2017).

Currently, there are some challenges with using metagenomics for wastewater surveillance for AMR. One major challenge is that there are multiple ways to generate, analyze, and interpret the data, making it difficult to compare metagenomic data across studies (Liguori et al., 2022). If there are differences in sampling, storage conditions, DNA extraction methodologies, and sequencing depths, it can bias the generation and comparison of metagenomic libraries, influencing the abundance and diversity of ARGs detected in a sample (Liguori et al., 2022; Miłobedzka et al., 2022). Additionally, the obtained genes, or close variants of them, are present in a reference database to assign them to a resistance phenotype (Bengtsson-Palme et al., 2017). These databases are inconsistent and vary in completeness and nomenclature (Liguori et al., 2022). Additionally, regarding measuring specific gene abundances, metagenomic-based methods are less sensitive, or require higher detection limits, than qPCR (Bengtsson-Palme et al., 2017). Furthermore, because metagenomics has a non-targeted approach, there is a higher representation of genes that are not of interest, and thus very rare genes or targets of interest will likely not be detected (Liguori et al., 2022). To overcome this limitation, deeper sequencing is required, however that is very expensive (Miłobedzka et al., 2022; Liguori et al., 2022). It also

does not assess bacterial viability (Scott et al., 2020; Liguori et al., 2022). Another limitation of metagenomics is that it requires extensive technical knowledge to analyze the data and is laborious and time consuming (Scott et al., 2020; Miłobedzka et al., 2022). Overall, metagenomics is a promising method to utilize for monitoring AMR in wastewater, but because of its cost and other challenges, it should be used in conjunction with other methods, such as qPCR. Table 2 describes the strengths and weaknesses of using metagenomics in detecting ARGs and bacteria in wastewater.

***Future Directions for Method Standardization***

With each method having different strength and weaknesses, it leaves the question of which method would be best for AMR monitoring in wastewater. Pruden et al. have suggested that the standardization of methods should be employed in a tiered fashion (2021). The first tier would be the most accessible to all and should be carried out by all participating locales (Pruden et al., 2021). This would include sample collection and concentration, culture and storage of the samples, and nucleic acid extraction (Pruden et al., 2021). The second tier would be carried out in-house or by centralized facilities (Pruden et al., 2021). This is where phenotypic and genotypic antimicrobial resistance profiling via qPCR would occur (Pruden et al., 2021). The final third tier would be conducted by centralized facilities and would be the least accessible due to cost (Pruden et al., 2021). This is where whole genome sequencing and metagenomic methods would occur (Pruden et al., 2021). All of the samples and data would be collected in a centralized sample archives and would then be used to create public-facing dashboards to further facilitate standardization of data analysis, reporting, and sharing (Pruden et al., 2021). This seems like an excellent option in implementing a surveillance system for monitoring AMR in wastewater. With standardization and centralized sample archives, sharing and comparing findings will be easier.

## KNOWLEDGE GAPS IN THE UTILIZATION OF WASTEWATER SURVEILLANCE FOR AMR

When developing a surveillance system, arguably one of the main concerns that should be addressed is the human health risk assessment. For the wastewater surveillance of AMR, that is unclear. To evaluate human health risk, there needs to be more research done on AMR characterization and the findings of wastewater-based ARB studies need to be compared with clinical evidence (Nguyen et al., 2021; Tiwari et al., 2022). However, with wastewater, this comparison and characterization is not straightforward.

To start, the ARB detected in wastewater may not just come from symptomatic human individuals, but it could also be from asymptomatic carriers or from animal sources (Tiwari et al., 2022). To overcome this hurdle, this would require comprehensive expert interpretation and having knowledge on the current events around the community (Tiwari et al., 2022). To supplement, monitoring ARBs with host-specific primers and using microbial source tracking methods may help in differentiating the source (Tiwari et al., 2022). Without this information, the prevalence estimation of ARBs reported at the population level would be inaccurate. More research into how to determine what source the ARB came from is vital in being able to assess risk.

Additionally, there is no current threshold for wastewater surveillance of ARBs with regards to how much diversity and abundance of bacteria is too high or result in elevated exposure and risk of acquiring a resistant infection (Liguori et al., 2022; Tiwari et al., 2022). Interpreting the results from molecular methods can be challenging and not being able to accurately interpret the results of wastewater surveillance of ARBs and relay that to health risk is of concern. Standards need to be put in place for this AMR characterization, as well as, when to declare an outbreak or

emergency state (Tiwari et al., 2022). There also needs to be more research on how to determine what an accurate representation of the extent of ARB/ARG exposure is and to be able to correlate that to associated health risks (Larsson et al., 2018; Nguyen et al., 2021). This would require more knowledge on exposure levels and to what extent the exposure leads to disease or colonization (Larsson et al., 2023). Overall, there needs to be extensive research done on AMR characterization and human health risk assessment before AMR surveillance in wastewater can be utilized on a grander scale.

## CONCLUSION

Wastewater-based surveillance is an up-and-coming surveillance system that has great potential to be used as a tool to monitor AMR in the United States by estimating the extent to which AMR might be circulating in a given community in real-time. However, wastewater-based epidemiology for AMR monitoring has not been fully developed for quantitative surveillance. Specifically, the literature assessed in this review illustrates that while there has been progress in method standardization, there needs to be a consensus on which ARGs to target and what analysis methods should be used for this system to be a reliable surveillance tool. The current position on ARG target standardization has been to focus on clinically relevant and/or high-risk genes as potential targets. With regards to analysis technologies, each of the three main methods currently used have their own advantages and disadvantages. Cost, the amount of expertise needed, and human health risk assessments are essential factors that should be taken into consideration for public health departments when implementing an analysis method. There is not one single method that can fulfill all the requirements of wastewater analysis for AMR monitoring. Additionally, there are many knowledge gaps with regards to interpreting wastewater results and determining the prevalence of the source of AMR that need to be addressed. Future directions should focus on assessing human health risk by comparing wastewater findings with clinical evidence. There needs to be a coordinated effort among public health departments, laboratories, hospitals, and other organizations in employing established standard methods at the local, state, and national level to slow the spread of AMR. Once assessed to be consistent and reliable, wastewater surveillance can help provide AMR burden data for a community. AMR surveillance in the environment via wastewater is just one component of being able to capture the complete spectrum of AMR. Wastewater surveillance should be used in conjunction with

clinical and veterinary surveillance to monitor AMR status and together these systems can provide essential information to public health officials to inform policy and mitigation practices to help maintain the effectiveness of antibiotics in the future.

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## APPENDIX

**Figure 1**
Pathway of AMR via wastewater through a wastewater treatment plant.

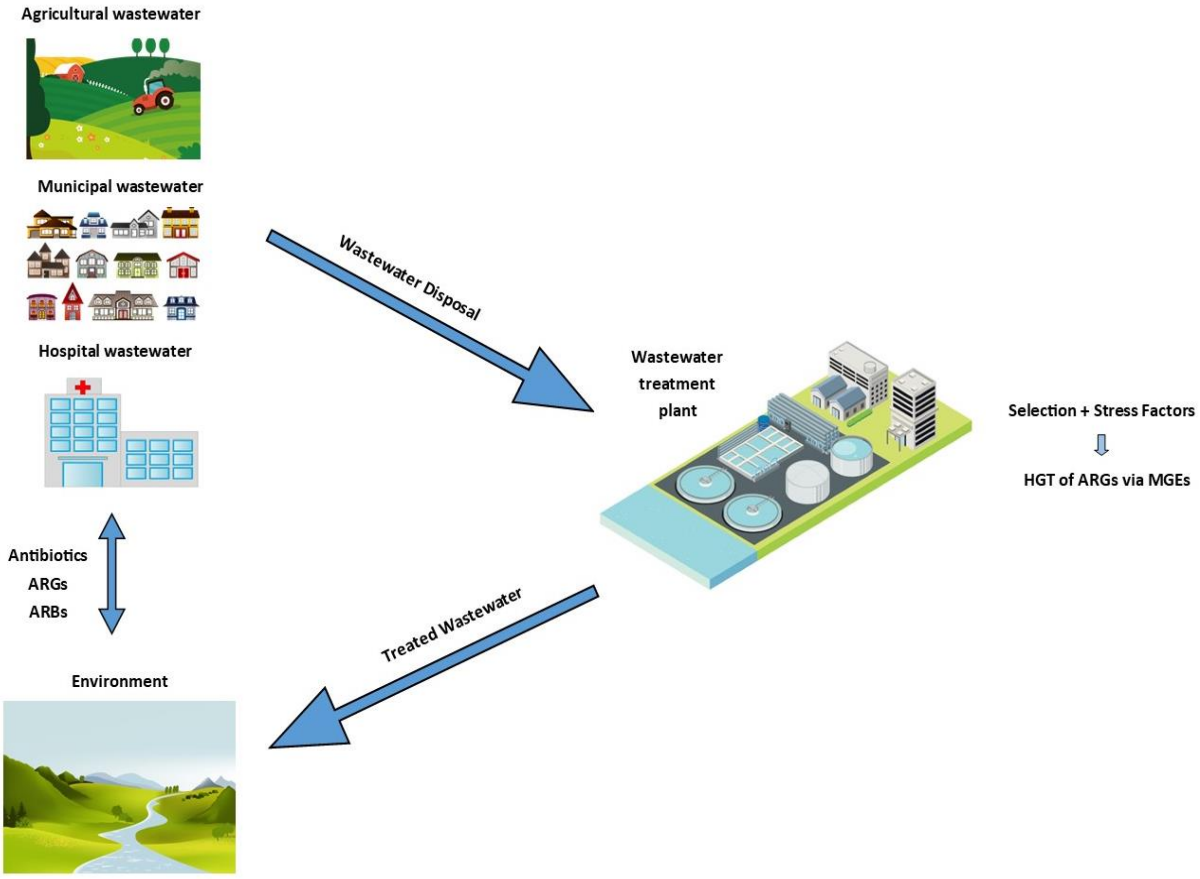


Image Source: Meda, S. (2023). *AMR Pathway in Wastewater via a WWTP* [Graphic Image].

**Table 1**
Examples of antibiotic resistance genes detected in wastewater that could serve as potential surveillance targets.

| Antibiotic Class | Mechanism | Example of genes [a] | Reference |
|---|---|---|---|
| β-lactams | Hydrolyze narrow ESBL antibiotics | *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{GES}$, *bla*$_{TLA}$ | Ranjbar & Farahani, 2019: Szczepanowski et al., 2009; Zhang et al., 2019 |
| | Hydrolyze carbapenems | *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{KPC,}$ *bla*$_{OXA-363}$, *bla*$_{OXA-309}$, *bla*$_{OXA-371}$ | Ranjbar & Farahani, 2019: Szczepanowski et al., 2009; Zhang et al., 2019; Prieto Riquelme et al., 2022 |
| Fluoroquinolones | Plasmid-mediated | *qnr* (A, B, C, D, S) | Nguyen et al., 2021; Pazda et al., 2019; Rizzo et al., 2013 |
| | Chromosomal target-site mutations | *gyr* (A B), *parC* | Pazda et al., 2019; Szczepanowski et al., 2009 |
| Tetracyclines | Efflux pumps | *tet* (A, B, C, D, E) | Nguyen et al., 2019; Pazda et al., 2019; Rizzo et al., 2013; Szczepanowski et al., 2009 |
| | Ribosomal protection protein | *tetM* | Nguyen et al., 2019; Pazda et al., 2019; Rizzo et al., 2013; Szczepanowski et al., 2009 |

[a] This is not an exhaustive list; Gene variations are within parentheses for simplicity.

**Table 2**
Strengths and weaknesses of methods used for detecting ARGs and bacteria in wastewater.

| Methods | Strengths | Weaknesses | References |
|---|---|---|---|
| **Culture-based** | • Not technically difficult.<br>• Highly standardized.<br>• Confirms bacterial viability for human health risk assessment.<br>• Able to determine phenotypic traits for clinically relevant targets.<br>• Already established infrastructure. | • Difficult to isolate pathogens with background bacteria.<br>• Expensive and time-consuming procedures.<br>• <1% of environmental bacteria can be cultured.<br>• Hard to detect low concentrations of bacteria.<br>• Provides no indication of the mechanisms of resistance.<br>• Requires additional testing for further analysis. | Girones et al., 2010; Anjum, 2015; Manaia et al., 2018; Scott et al., 2020; Schreiber et al., 2021; Liguori et al., 2022; Tiwari et al., 2022 |
| **Amplification (qPCR)** | • High sensitivity and specificity.<br>• Rapid and cost-effective tool.<br>• Allows for specific genes or mutations to be targeted.<br>• Can simultaneously analyze multiple ARGs targets.<br>• In combination with epidemiological studies, can carry out risk assessment studies.<br>• Can detect ARGs on non-culturable bacteria. | • Does not assess bacterial viability.<br>• Impossible to design primers for new/unknown genes.<br>• Protocols may lack quality assurance, especially for environmental samples.<br>• Requires personnel to be knowledgeable in molecular biology. | Girones et al., 2010; Manaia et al., 2018; Scott et al., 2020; Borchardt et al., 2021; Nguyen et al., 2021; Keenum et al., 2022; Liguori et al., 2022; Miłobedzka et al., 2022; Abramova et al., 2023 |

**Table 2 (cont'd)**

| | | | |
|---|---|---|---|
| **Metagenomics** | • Non-targeted approach allows for comprehensive overview of unknown and variety of targets in a microbial community.<br>• Wealth of knowledge on the taxonomic and functional genetic diversity in a sample.<br>• Provides information on AMR prevalence, distribution, and routes of transmission.<br>• Sequence libraries can be stored and retrospectively analyzed.<br>• Can identify unculturable or emergent microorganisms. | • Lack of standardization for data generation and analysis.<br>• Non-targeted approach ultimately hinders detection limit.<br>• Expensive.<br>• Laborious and time-consuming.<br>• Does not assess bacterial viability.<br>• Less sensitive than qPCR.<br>• Only allows detection of known genes present in a reference database, that of which are inconsistent.<br>• Requires extensive technical knowledge to analyze data. | Bengtsson-Palme et al., 2017; Manaia et al., 2018; Hendriksen et al., 2019; Rice et al., 2020; Scott et al., 2020; Liguori et al., 2022; Miłobedzka et al., 2022 |