Towards the standardization of *Enterococcus* culture methods for waterborne antibiotic resistance monitoring: A critical review of trends across studies

Benjamin C. Davis, Ishi Keenum, Jeannette Calarco, Krista Liguori, Erin Milligan, Amy Pruden, Valerie J. Harwood

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**ABSTRACT**

Antibiotic resistance is a major 21st century One Health (humans, animals, environment) challenge whose spread limits options to treat bacterial infections. There is growing interest in monitoring water environments, including surface water and wastewater, which have been identified as key recipients, pathways, and sources of antibiotic resistant bacteria (ARB). Aquatic environments also facilitate the transmission and amplification of ARB. *Enterococcus* spp. often carry clinically-important antibiotic resistance genes and are of interest as environmental monitoring targets. *Enterococcus* spp. are Gram-positive bacteria that are typically of fecal origin; however, they are also found in relevant environmental niches, with various species and strains that are opportunistic human pathogens. Although the value of environmental monitoring of antibiotic-resistant *Enterococcus* has been recognized by both national and international organizations, lack of procedural standardization has hindered generation of comparable data needed to implement integrated surveillance programs. Here we provide a comprehensive methodological review to assess the techniques used for the culturing and characterization of antibiotic-resistant *Enterococcus* across water matrices for the purpose of environmental monitoring. We analyzed 117 peer-reviewed articles from 33 countries across six continents. The goal of this review is to provide a critical analysis of (i) the various methods applied globally for isolation, confirmation, and speciation of *Enterococcus* isolates, (ii) the different methods for profiling antibiotic resistance among enterococci, and (iii) the current prevalence of resistance to clinically-relevant antibiotics among *Enterococcus* spp. isolated from various environments. Finally, we provide advice regarding a path forward for standardizing culturing of *Enterococcus* spp. for the purpose of antibiotic resistance monitoring in wastewater and wastewater-influenced waters within a global surveillance framework.

**1. Introduction**

*Enterococcus* spp. are important members of the natural enteric microbiome of both humans and animals and have emerged as important antibiotic-resistant pathogens in clinical medicine (Arias and Murray, 2012). There are currently 60 classified *Enterococcus* species in the National Center for Biotechnology Information database, most of which are commensal microorganisms, although some act as opportunistic pathogens in humans. *E. faecalis* and *E. faecium* are among the most important etiological agents of nosocomial infections; including urinary tract infections (UTIs), central nervous system infections, endocarditis, bacteremia, neonatal infections, and surgical site infections (Moellering, 1992; Murray, 1990). From 2006 to 2017, *Enterococcus* spp. were responsible for approximately 14% of all healthcare-associated infections in the US, ranking second overall behind *Staphylococcus aureus* (Hidron et al., 2008; Sievert et al., 2013; Weiner-Lastinger et al., 2020; Weiner et al., 2016). *Enterococcus* spp. possess full or partial intrinsic chromosomal resistance to cephalosporins, aminoglycosides, lincosamides, trimethoprim-sulfamethoxazole, and penicillins (Hollenbeck and Rice, 2012). Their rapid development of multi-drug resistance has been attributed in part to their highly malleable genomes that lack CRISPR (clustered regularly interspaced...
Escherichia coli recipient and transmission pathway of antibiotic resistant bacteria into group known as enterococci, which are Gram-positive, catalase-negative. Control and Prevention, 2019; Tacconelli et al., 2018). To also consider other potential targets. namics has not been established. Thus, the present is a critical moment of water quality monitoring (Jang et al., 2017; Schoen et al., 2011; Sinclair et al., 2012). Enterococci (formerly classified within the larger group known as “fecal streptococci”) have been widely used to assess the microbiological safety of surface waters, drinking waters, recreational beaches, and as a target for assessing process removal efficiencies during wastewater treatment. Enterococci have also been found to correlate directly to public health outcomes; for example, across the US, the rate of gastrointestinal illness in swimmers has been correlated with Enterococcus spp. levels in recreational beach waters that are impacted by wastewaters (Prüss, 1998; Wade et al., 2006, 2003); however, these illnesses are assumed to be caused mainly by viral pathogens (Soller et al., 2010) rather than Enterococcus spp. Their importance as water quality indicators and their inclusion in governmental regulatory frameworks have led to a great deal of method development for isolation and enumeration from environmental samples (Boehm and Sassoubre, 2014; Health Canada, 2020).

Global and national action plans set in place to combat the spread of antibiotic resistance have generally embraced a One Health approach (humans-animals-environment) (European Commission, 2017; Hernandez-amado et al., 2019), but a better understanding of the role of environmental transmission and amplification of antibiotic-resistant Enterococcus and their genes to humans, animals, and aquatic environments is needed. Aquatic environments have been identified as a key reservoir of enterococci (Ekwanzala et al., 2020b; Virulence and antibiotic resistance determinants from clinical strains to environmental reservoirs of enterococci (Ekwanzala et al., 2020; Gouliouris et al., 2019, 2018) marks them as potentially comprehensive targets for antibiotic resistance monitoring. Like E. coli, they are members of the normal flora of human and animal gastrointestinal tracts (Byappanahalli et al., 2012) and are readily quantified in wastewater and environmental waters. Their status as normal human flora creates the disadvantage of extreme difficulty in tracking infection rates from human exposure to contaminated water, another characteristic they share with E. coli. A study of individuals engaging in surfing found that surfers were significantly more likely to be colonized with E. coli carrying blaOXYX compared to non-surfers (Leonard et al., 2018). Analogous studies of antibiotic-resistant Enterococcus spp. could shed light on aspects of direct transfer of these bacteria from aquatic environments to humans.

Recent progress has been made in applying culture-based methods for monitoring antibiotic resistant Enterococcus spp. in the environment, with emphasis on human and animal wastewater, and hospital wastewater-pollution (Gouliouris et al., 2019; Savin et al., 2020; Zabber et al., 2020). The phenotypic and morphological similarity of other Gram-positive organisms to the enterococci, however, makes isolation on selective media prone to false-positives, with cross-selectivity with other cocci (e.g., Streptococcus, Pediococcus, Weissella) (Harwood et al., 2001). False positives are especially problematic for environmental samples (Page and Hardy, 1980). Non-selectivity can actually be exacerbated with addition of antibiotics to media, because several members of non-target Bacillus and other genera are intrinsically resistant to clinically-relevant concentrations of certain antibiotics (Woodford et al., 1995).

The selection of any monitoring target or strategy entails consideration of the overarching purpose or questions to be addressed, and these may vary depending on the focus across the spectrum from wastewater to surface water. The following are examples of key monitoring goals and considerations addressed by this review:

• Monitoring antibiotic resistance among clinically-relevant strains of Enterococcus spp. in sewage as a means of assessing their levels carried in the human population
• Assessing whether clinically-relevant Enterococcus spp. or specific resistance phenotypes are effectively removed during wastewater treatment and if they persist in impacted aquatic environments
• Evaluating evidence that clinically-relevant Enterococcus spp. acquire antibiotic resistance genes from the environment
• Comparing resistant Enterococcus spp. in various water matrices both locally and globally to assess factors that may be contributing to antibiotic resistance in Gram-positive organisms

2. Literature review protocol

This systematic review was conducted in a four-tiered approach using search terms presented in Table S1 to collect studies published between January 2000 and December 2020 (Figure S1). Web of Science, PubMed, and Google Scholar were searched. Briefly, Tier 1 was designed to isolate topic relevant search terms for surface water, wastewater, recycled water, or reclaimed water (3,828,792 articles). Tier 2 was designed to select for those articles addressing antibiotic resistance (15,043). Tier 3 further narrowed the search to culturing techniques (5,439) and Tier 4 specifically to enterococci/Enterococcus (682).
Initially, these 682 articles were independently screened by two researchers for containing a complete workflow from environmental sampling through to characterization of individual isolates. Articles were excluded that only used previously collected isolates. Further exclusions were articles that exclusively investigated: biofilms, mesocosms, drinking water, sediments, or digested sludge (e.g., anaerobic digestion). Fecal source tracking articles based on antibiotic resistance analysis were also excluded (Harwood et al., 2000). Articles that used a non-selective media for initial isolation, such as R2A or TSA, were also excluded. Disagreements on article inclusion from the initial screening were presented to a larger group of five researchers to reach a consensus. The resulting 117 peer-reviewed articles were then subject to data extraction using parameters outlined in Table S2. All included articles are listed in Table S4.

3. Collection and analysis of published data

Data relevant to the species and phenotypic distribution of all isolated enterococci was collected from 117 peer-reviewed articles. First, the number of isolates per species was extracted from articles in which libraries were specified (91 articles) to reveal general population statistics across environments. Second, antibiotic susceptibility testing (AST) data were extracted from all articles that provided the percent of resistant isolates compared to total enterococci isolated in the absence of any antibiotic (77 articles). Studies that summed isolates with resistant isolates compared to total enterococci isolated in the absence of any antibiotic were excluded that only used previously collected isolates. Further exclusion criteria of studies included when using previously collected isolates, where the current gold standard for enterococci enumeration from the environment is considered the MF technique (Byappanahalli et al., 2012) and was used by over 90% of articles included in this review (Table 1).

The principal selective and differential solid media used in standard MF assays are Slanetz-Bartley (SB), mEnterococcus (mE), and membrane-Enterococcus indoxyl-β-D-glucoside (mEI). These media use various peptone and yeast extract-based nutrients with the addition of sodium azide and/or nalidixic acid. Sodium azide obstructs the growth of Gram-negative bacteria through the inhibition of cytochrome oxidase. Both SB and mE agars include 2,3,5-triphenyltetrazolium chloride (TTC), which dyes viable colonies red. Differentiated colonies grown on SB or mE are then confirmed as enterococci by their ability to hydrolyze esculin in the presence of bile using either bile esculin azide or esculin iron agars. The hydrolyzed esculin product, esculin, reacts with iron salt in the media to produce black to reddish colonies for enumeration. mEI is similar to mE medium, but contains the chromogen, indoxyl-β-D-glucoside. When cleaved by β-D-glucosidase positive enterococci, blue halos are formed around positive colonies. mEI is typically used as a standalone media as all colonies with blue halos are considered enterococci.

### Table 1: Published standardized methods for the detection and enumeration of enterococci in different water matrices

| Organization and Method | Recommended Matrix | Media | Number of Citations | Assay Turnaround (hours) | General Procedure |
|-------------------------|--------------------|------|---------------------|--------------------------|------------------|
| **Membrane Filtration (MF); (CFU/mL); Number of Studies Identified: 111/117** | | | | | |
| EPA Method 1600 | drinking water; source water; wastewater; marine and freshwater | mEI<sup>a</sup> | 18 | 24 | mEI (41.1°C for 24 hrs); Count blue halos |
| ISO 7899-2 | | Slanetz Bartley; Bile Esculin Azide | 15 | 48 | Slanetz Bartley (36°C for 44 hrs); Bile Esculin Iron Agar (44°C for 2 hrs) |
| EPA Method 1106.1 | marine and freshwater (not applicable to wastewater) | mEnterococcus; Esculin Iron Agar | 0 | 48 | mEnterococcus (41°C for 48 hrs); Esculin Iron Agar (41°C for 20 min); Count pink to red colonies |
| APHA SM 9230C.2a | drinking water; source water; wastewater; marine and freshwater | mEnterococcus; Esculin Iron Agar | 3 | 48 | mEnterococcus (41°C for 48 hrs); Esculin Iron Agar (41°C for 20 min); Count pink to red colonies |
| APHA SM 9230C.2b | drinking water; source water; wastewater; marine and freshwater | mEI<sup>a</sup> | 0 | 24 | mEI (41°C for 24 hrs); Count blue halos |
| APHA SM 9230C.2c | drinking water; source water; wastewater; marine and freshwater | mEnterococcus | 10 | 48 | mEnterococcus (35°C for 48 hrs); Count light and dark red colonies |
| **Multiple Tube Fermentation (MTF); (MPN/mL); Number of Studies Identified: 1** | | | | | |
| APHA SM 9230B | drinking water; source water; marine and freshwater (not applicable to wastewater) | Azide Dextrose Broth; Bile Esculin Azide Agar | 1 | 48-72 | Azide Dextrose Broth (35°C for 24-48 hrs); Bile Esculin Azide Agar (35°C for 24 hrs); Compute MPN |
| **Fluorogenic Substrate Test (MTF and MPN); (MPN/mL); Number of Studies Identified: 3** | | | | | |
| APHA SM 9230D | drinking water; source water; wastewater; marine and freshwater surface water; wastewater | Enterolert<sup>c</sup> | 3 | 24 | Enterolert Media (41°C for 24 hrs); Compute MPN |
| ISO 7899-1 | | MUD Media | 0 | 36-72 | MUD Media in Microtitre Wells (44°C for 36-72 hrs); Compute MPN |

<sup>a</sup> The ‘Number of Citations’ under Membrane Filtration do not correspond to the number of citations in the table as many articles did not follow or cite a standard method. Techniques not listed are direct plating after serial dilutions (8 articles).
<sup>b</sup> membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI)
<sup>c</sup> 4-methylumbelliferyl-β-D-glucoside (MUD), Environmental Protection Agency (EPA), International Organization for Standardization (ISO), American Public Health Association (APHA)
4.1. Performance of standard Enterococci culture assays

Several comparative studies have been conducted over recent decades to assess selectivity of culture media for Enterococcus (Table 2). Pagel et al. 1980 used pure cultures to assess PSE (Pfizer), KF Streptococcus, mE, and SB agars against over 100 pure cultures of clinical and environmental isolates of various cocci. The highest selectivity for enterococci was observed for PSE (94%) and mE (94%) agars, with the lowest being KF Streptococcus (80%) and SB (78%) (Pagel and Hardy, 1980). Compared to mE, however, PSE was found to yield lower recovery efficiencies from wastewaters with much higher rates of background colony growth. Other comparative studies found enterococci selectivity on PSE and KF Streptococcus agars as low as 86% and 54%, respectively (Brodsky and Schiemann, 1976). The original mE agar formulation study found a false positive rate of 10% and false negative rate of 11.2% for surface water isolates (Levin et al., 1975). Subsequent studies have confirmed false positive rates for mE agar as low as 2.5% when testing pure Enterococcus cultures (Oliveira and Watanabe Pinhata, 2008) and 1.7% in marine, riverine, and treated wastewater effluent (Adcock and Saint, 2001). The inclusion of the indoxyl-β-D-glucoside chromogen to mE agars resulted in an increase in specificity of Enterococcus to upwards of 99.7% in ambient freshwaters (Adcock and Saint, 2001). In a recent benchmarking study, ISO method 7899-2 (SB media) was found to have false positive rates as high as 18% and false negative rates as high as 57.1%, depending on the colony count on the filter membrane of recreational marine water (Tiwari et al., 2018). Differences in Enterococcus selectivity have also been documented between MF and defined substrate techniques, where E. faecalis is differentially selected for in wastewater using Enterolert, leading to the conclusion that these methods should not be used interchangeably for regulatory purposes (Ferguson et al., 2013, 2010; Kinzelman et al., 2003; Maheux et al., 2009).

Significant media-dependent differences in Enterococcus concentrations have also been reported. For instance, several studies were conducted in the wake of the advent of Enterolert assays in the mid-1990s to compare its efficacy against established MF techniques for water quality monitoring (Fricker and Fricker, 1996). Significant differences in concentrations were reported between Enterolert and SB agar in marine and recreational freshwaters (Valente et al., 2010), while no significant differences were found between mE agar and Enterolert concentrations across surface water, wastewater, or marine waters (Abbott et al., 1998; Budnick et al., 1996; Ecker, 1998; Fricker and Fricker, 1996). No significant differences in enterococci concentrations were identified between mE and mEI agar (Adcock and Saint, 2001). Importantly, no studies were identified that directly compared the specificities and concentrations derived from SB and mE or mEI across water matrices. Such a comparison should be considered in future studies that assess their utility for regulatory frameworks for antibiotic resistance monitoring internationally. Any biases in species distributions and total enterococci concentrations originating from the selective media could skew downstream distributions in resistance frequencies and introduce bias if the data are used for risk assessment.

5. Workflows for antibiotic-resistant Enterococcus monitoring

A useful method for culturing and enumerating both generic and antibiotic resistant environmental Enterococcus would strike a balance between sensitivity (i.e., detect all Enterococcus spp. that are present), specificity (i.e., avoid detecting other genera), and the high-throughput needed for large-scale environmental monitoring. Aquatic matrices display a large and dynamic range of enterococci concentrations, and a method for their enumeration would also need an appropriately low limit of detection for “cleaner” samples and a sufficiently high limit of quantification for matrices like wastewater where enterococci are concentrated. Specific logistical considerations are also warranted, such as the ability to perform the assay in low-tech laboratories using materials, techniques, and media that are economically feasible for AMR monitoring in low- and middle-income countries. In this instance, standard methods that have been developed for enumerating generic enterococci can be leveraged for their extensive vetting with respect to quality assurance/quality control and adapted to the increased throughput needs of AMR monitoring projects.

Culture-based approaches for investigating antibiotic resistance amongst environmental Enterococcus must be modified based on the aquatic matrix being investigated and the purpose of the assessment. Here we delineate these approaches into three general categories: population-level surveys, targeted monitoring for specific antibiotic resistant phenotypes, and recovery of low concentration or viable but non-culturable (VBNC) populations (Figure 1), each with their own benefits and limitations.

For population-level monitoring (73 articles) (Figure 1A), where the objective is to achieve an unbiased snapshot of the distribution of resistance phenotypes, a collection of isolates can be generated using an Enterococcus selective method (e.g., Table 1). After colonies have been counted, isolates with the specified morphologies can then be selected randomly off plates for phenotypic antibiotic susceptibility testing, generating an antibiotic resistance profile as a function of the total number of isolates subsampled. The disadvantage of this approach is

| Table 2 | Performance of Enterococcus selective media used in standard membrane filtration assays. |
|---------|-----------------------------------------------------------------------------------------|
| Medium  | Matrix Tested | Presumptive Colonies | Specificity (%) | Selectivity (%) |
| mE      | Marine        | 1361                  | -              | 82.4           |
| mE      | Pure Cultures | 101                   | 97.3           | 100            |
| mE      | Surface       | 54                    | -              | 100            |
| mE      | Surface; Wastewater; Marine | 1279 | -                  | 94.9           |
| mEI     | Surface; Wastewater; Marine | 641 | -                  | 94.5           |
| mEI     | Surface; Wastewater | 361 | -                  | 93.9           |
| mEI     | Marine        | 80                    | -              | 97.5           |
| mEI     | Marine        | 624                   | -              | 94.2           |
| mEI     | Pure Cultures | 93                    | 91.0           | 88.2           |
| mEI     | Surface       | 2231                  | -              | 88.5           |
| mEI     | Surface; Wastewater; Marine | 1043 | -                  | 90.2           |
| Slanetz-Bartley | Marine | 97 | - | 93.8 |
| Slanetz-Bartley | Marine | 234 | - | 92.7 |
| Slanetz-Bartley | Pure Cultures | 82 | 78 | 74.4 |
| Slanetz-Bartley | Surface | 321 | - | 95.3 |
| Slanetz-Bartley | Surface; Wastewater | 385 | - | 93.8 |

1. Specificity = (True Negatives)/(True Negatives + False Positives)
2. Selectivity = True Positives/(True Positives + False Positives) or (Colonies Confirmed to Enterococcus Genus)/(Total Presumptive Enterococcus Colonies in Collection)
that most colonies screened may not be antibiotic resistant and finding colonies with the resistant phenotypes of interest, and to achieve required statistical power, can be akin to “searching for a needle in a haystack”. The advantage to this approach is that it provides a denominator for total Enterococcus in the sample and an unbiased distribution of both enterococcal species and their genotypes and phenotypes, resulting in an ecologically-relevant analysis (Cho et al., 2019).

If the phenotype of interest is already known (e.g., high-level VRE), targeted monitoring approaches may be more efficient for in-depth characterizations of sub-populations of Enterococcus. Such approaches use an antibiotic at clinically-relevant breakpoints to select for specific resistance phenotypes (Figure 1B). The use of low/intermediate breakpoints of antibiotics may be useful for capturing a broad range of phenotypes in the environment but will frequently capture clinically-irrelevant organisms, especially in the case of glycopeptide resistance (Figure 1C). The sample can also be plated in tandem on the selective media without the antibiotic (Figure 1D), thus allowing the quantification of the resistant population as a fraction of the total enterococci population (WHO Tricycle program). E) Interest in low concentration/injured cells (e.g., disinfected water). 1Note that pre-enrichment for resistance phenotypes (injured or VBNB cells) prevents their quantification. 2Antibiotic susceptibility testing of subsampled colonies often includes original selective antibiotic to confirm full “resistant” classification. 3Whole genome sequencing is recommended for the most accurate speciation and comprehensive genotyping for global isolate comparisons. AntR = antibiotic resistance, VBNB = viable but non-culturable, ADB = azide dextrose broth, BEA=bile esculin azide, EIA=esculin iron agar, ARGs = antibiotic resistance genes

5.1. Antibiotic susceptibility testing methods

Several techniques and automated platforms exist for generalized antibiotic susceptibility testing (AST) or the determination of minimum inhibitory concentrations (MICs) of isolate libraries; including Kirby-Bauer disk diffusion assays (~ 47% of articles), various commercial automated systems (24%), manual broth or agar dilutions (20%), or strip methods (9%) (Figure 2A). Over 90% of antimicrobial sensitivity tests (AST) were performed on either Mueller-Hinton agar or in Mueller-Hinton broth. Concentrations of antibiotics chosen for AST were predominantly determined by referencing the Clinical and Research Standards Institute (CLSI; 70%) breakpoints for Enterococcus, according
to the most currently available guidelines. Other standardized breakpoint concentrations were specific to a particular nation or governmental body, e.g., including the European Committee on Antimicrobial Susceptibility Testing (EUCAST; 7%), the National Antimicrobial Resistance Monitoring System (NARMS; USA; 2%), and the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS; 1%). Approximately 15% of studies utilizing AST that also differentiated degrees of resistance (i.e., “susceptible”, “intermediate”, or “resistant”) did not cite a justification for antibiotic concentrations nor a standardized method for determining the level of resistance observed.

The panel of antibiotics used to screen isolates varied across studies, but overarching trends were apparent. VRE was mentioned in the title of ~35% of articles identified, and vancomycin was included in the screening panel in over 75% of the articles (Figure 2B). The number of observed phenotypes among clinical and environmental isolates of *Enterococcus* are wide-ranging, which warrants a diverse range of antibiotics included in the panels. The antibiotics tested were further categorized into twelve distinct classes by activity. The most prominent classes across all studies were glycopeptides (76%), macrolide-lincosamide-streptogramin (MLS; 72%), tetracyclines (72%), beta-lactams (especially penicillins) (70%), and aminoglycosides (64%). Interestingly, antibiotics that are either approved by the FDA to treat VRE infections or are commonly used to treat VRE (Arias and Murray, 2012) were less commonly included in panels. These compounds include linezolid (27%), quinupristin/dalfopristin (21%), daptomycin (8%), and the synthetic glycopeptide, teicoplanin (28%). High-level aminoglycoside resistance (e.g., gentamicin, streptomycin, and kanamycin) in enterococci isolated from the environment was the focus of small subset of studies.

5.2. Multidrug resistance profiling

Because enterococci are intrinsically resistant to several antibiotics; including cephalosporins, penicillins, clindamycin, and aminoglycosides, resistant phenotypes are commonly found in environmental samples. Plasmid- and transposon-mediated resistance to tetracyclines, erythromycin, chloramphenicol, trimethoprim, vancomycin, and clindamycin; however, have further allowed the genus to become a leading cause of multidrug resistant nosocomial infections, particularly in the US (Murray, 1998). Modern nosocomial *E. faecium* isolates, for example, are commonly resistant to ampicillin, vancomycin, and high levels of aminoglycosides (Miller et al., 2014). Recently, the emergence of multidrug-resistant VRE to newer, last-resort antibiotics; including oxazolidinone-linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline, have caused frequent treatment failures and are of global concern (Ahmed and Baptiste, 2018). Thus, screening for multidrug resistance amongst isolated environmental enterococci is essential for monitoring the evolution of the genus over time, specifically as a function of anthropogenic pollution, as well as assessing the relative hazard posed by the isolate. The choice of antibiotics to include in screening panels is crucial, as most are not useful from a risk-based monitoring framework, although some may highlight ecological relevance. For instance, screening for phenotypes that are intrinsic to the genus may not have clinical relevance but may be useful in determining the distribution of endemic phenotypes to contextualize the relative frequency of VRE detection.

5.3. Biochemical and molecular confirmation and speciation

Because the virulence and resistance characteristics across different *Enterococcus* spp. vary substantially, speciation of resistant *Enterococcus* isolates is desirable. A suite of biochemical tests has been developed by clinical microbiologists to confirm *Enterococcus* to the genus level. These tests include Gram staining, catalase testing, thermal growth range/thermotolerance (growth at 10 and 45 °C), halotolerance (6.5% NaCl), growth at pH 9.7, pyrrolidonylarylamidase activity, and the ability to hydrolyze esculin in the presence of bile salts (e.g. growth on bile esculin agar) (Facklam and Collins, 1989; Teixeira et al., 2015). These tests were common features of nearly three quarters of articles and served as a prerequisite for inclusion in downstream characterization, including further speciation (Figure S2). Studies that did not confirm isolates to the genus level either relied on chromogenic agar (e.g., mEI,

![Fig. 2. Summary of antibiotic susceptibility testing (AST) methods applied across the reviewed articles. A) Distribution of assays and commercialized platforms used for AST. B) Distribution of antibiotics used for screening enterococci isolate collections.](image-url)
CHROMagar VRE) to select presumptive enterococci or specified their library without screening for characteristic metabolisms or morphologies. Genus-specific primers based on the 16S rRNA gene (Deasy et al., 2000), 23S rRNA gene (EPA Method 1611) (EPA, 2012), or the elongation factor EF-Tu (tuf) (Ke et al., 1999) gene have also been used for rapid identification of the genus Enterococcus. However, 16S rRNA primer sets are known to fail to capture all Enterococcus spp. (Botina and Sukhodolets, 2006), while EPA Method 1611 has proven to be very reliable in our experience.

Speciation of enterococci libraries was common and performed in 91/117 articles, the most common approach of which was PCR. There are several conserved proteins and corresponding genes that are targeted in these assays. The simultaneous detection of enterococcal species and glycopeptide resistance was the first molecular approach to improve diagnostic speeds for clinical enterococci and was based on the detection of genes encoding D-alanine:D-alanine (D-Ala-D-Ala) ligases and other glycopeptide resistance determinants. A reduced affinity for glycopeptides in VanA- and VanB-type resistance in enterococci are due to the integration of D-alanyl-D-lactate into peptidoglycan precursors by the chromosomally-encoded D-Ala-D-Ala ligases (Dutta-Malen et al., 1995b). The D-Ala-D-Lactate enzymes in E. faecium (ddl, fucam) and E. faecalis (ddl, fael) are conserved, and in resistant strains, these enzymes are present in addition to vanA or vanB. Similarly conserved ligases, vanC1 and vanC2-3 are highly specific for E. gallinarum (Dutta-Malen et al., 1992) and E. casseliflavus (Navarro and Courvalin, 1994), respectively. The primers published by Dutta-Malen et al. (1995) for ddl, fucam, dfael, vanC1, galinarum, and vanC2-3 casseliflavus have been the most widely used for the speciation of environmental enterococci, as many researchers are specifically concerned with the identification of these four most common and clinically-relevant species (Table S3). (Kariyama et al., 2000) and (Depardieu et al., 2004) provided additional multiplex PCR assays for more high-throughput approaches to VRE surveillance. A multiplex PCR assay based on species-specific superoxide dismutase (sodA) genes developed by Jackson et al. (2004) includes primers for 23 different enterococcal species (Jackson et al., 2004).

The PCR primers described above were in part developed due to a lack of consensus between commercially-available systems and kits, such as the Analytical Profile Index (API; bioMérieux), PhenomePlate (PhPlate Microplate Techniques AB), Phoenix Microbiology Systems (BD Phoenix), VITEK (bioMérieux), Micronaut-Strept2 (MERLIN), MicroScan Walk Away (Beckman Coulter), and BBL Crystal (MG Scientific) manual or automated rapid identification systems. The principles behind these higher-throughput systems are derived from conventional biochemical phenotyping of enterococci which involve differentiating carbohydrate fermentation of mannitol, sorbitol, sorbose, inulin, arabinose, melibiose, sucrose, raffinose, trehalose, lactose, glycero, salicin, and maltose, among others (Facklam and Collins, 1989; Teixeira et al., 2015). The commercial methods employ a panel of biochemical tests in parallel to reduce the labor costs of manual phenotyping. However, if atypical species are present, these systems will struggle to identify the organism with acceptable levels of certainty (Castillo-Rojas et al., 2013). This issue is especially problematic in matrices outside of the clinical setting, as these systems were developed and validated targeting common clinical strains and reference cultures with distinguishable biochemical characteristics and not the wide phenotypic diversity of environmental samples. A comprehensive survey of these systems has been reviewed previously (Emery et al., 2016). An emerging technology for the rapid identification of microbial organisms is matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Singhal et al., 2015). This method uses lasers to generate singly protonated ions from analytes in the sample. In the process of identifying unknown microbes, these analytes are primarily housekeeping and ribosomal proteins, given that they constitute a large portion of the dry weight of microbial cells. The ionized proteins are then separated by their mass-to-charge ratio and depending on their time of flight through a channel, a peptide mass fingerprint is generated that can be compared to openly sourced databases. MALDI-TOF MS for the purpose of enterococci speciation emerged in the literature in 2017, as this is an emerging technique in environmental studies. MALDI-TOF MS systems are considerably more expensive than most molecular or phenotypic methods, but the throughput is generally larger (thousands of isolates) and can better accommodate the demand in clinical laboratories.

16S rRNA gene sequencing was less frequently used than other methods due to its non-specificity. Other genes may be sequenced, including the sodA, rpoA, and pheS genes, which have shown to be more discriminatory than the 16S rRNA gene for closely-related species, such as E. casseliflavus and E. flavescens (Naser et al., 2005; Poyart et al., 2000). In a recent comparative study of various loci to differentiate closely-related Enterococcus spp., it was determined that the alpha subunits of ATP synthase (atpA), chaperonins (groESL), and phenylalanyl-tRNA synthase alpha subunits (pheS) performed equally well or better than 16S rRNA gene sequencing against 308 enterococci isolates from untreated urban wastewater (Sanderson et al., 2019). The rate of false identification of consensus reference strains based on loci sequencing was approximately 2%, much lower than the parallelized carbohydrate phenotyping systems discussed above, such as RapID STR, which had error rates of 15.9% for E. faecalis, 21.5% for E. faecium, and 56.9% for E. casseliflavus/gallinarum. The gold standard for speciating enterococci, and any organism for that matter, is WGS (Sanderson et al., 2019).

5.4. Genotyping resistant Enterococci

Numerous genetic determinants confer antibiotic resistance across the genus Enterococcus. Co-occurrence of resistance genes and virulence factors is critical of concern from a clinical standpoint and is common among nosocomial strains (Guzman Prieto et al., 2016; Pöntinen et al., 2021). Most genetic determinants of antibiotic resistance in Enterococcus spp. are intrinsically encoded, i.e., they exist on the chromosome within the core genome of the genus. Typically, acquired resistance, i.e., a product of horizontal gene transfer, is of greater interest for monitoring, where the purpose is to examine trends in resistance patterns and if they are changing in time and space. Acquired resistance genes are of primary concern as drivers of failure of antibiotic treatment in clinical infections. Canonical mobile resistance determinants within enterococci include those that confer resistance to glycopeptides (van gene clusters), aminoglycosides (aac(6’)-Ie-apo(2’)-Ia and ahp(3’)-IIIa), MLS (ermB), and tetracyclines (tetM and tetL) (Figure S3).

Just over a third of analyzed articles (41/117) genotyped colonies for antibiotic resistance determinants after they had been isolated on antibiotic-containing media, of which 37 used PCR and 4 used WGS. The van operon was commonly targeted, with an emphasis on vanA and vanB within VRE isolates themselves (Figure S3). There are nine distinct gene clusters conferring glycopeptide resistance in enterococci (VanA, B, C, D, E, G, L, M, N) (Hancock et al., 2014; Teixeira et al., 2015) and these determinants differ both genetically and phenotypically based on their physical location (encoded on mobile genetic elements or chromosomal), whether resistance is inducible or constitutive, the type of peptidoglycan precursor that is produced, and ultimately the level of resistance conferred. VanA gene clusters are the most common in clinical isolates and are typically found on Tn1546-like transposons, are frequently integrated into a wide range of plasmids, and produce clinical levels of resistance to vancomycin (MIC 64-1,000 µg/mL) and teicoplanin (MIC 16-512 µg/mL) (Teixeira et al., 2015). Similar to VanA, VanB gene clusters are also typically found in clinical isolates and are present on transposons (Tn1547 or Tn1549 to Tn5382), but differ from vanA due their inability to recognize teicoplanin, allowing strains with the VanB phenotype to remain susceptible (Miller et al., 2014). These two gene clusters are the most significant genetic determinants in clinically-resistant enterococci and several PCR assays have been developed for their detection (Dutta-Malen et al., 1995b; Kariyama et al., 2006).
Enterococci displaying susceptible to intermediate resistance are typically attributed to chromosomally encoded van clusters, like vanCI in *E. gallinarum* and vanC2/3 in *E. casseliflavus*, which are commonly detected in environmental samples. The much more rare vanD-N genotypes were not detected in any articles that screened for them (Roztamanidis et al., 2009; Tauber-Kapteijn et al., 2016; Zdragas et al., 2008).

### 5.5. Virulence factors and pathogenesis

The pathogenesis of infections caused by enterococci is still poorly understood (Teixeira et al., 2015). However, several PCR assays have been developed for the detection of virulence factors common to *Enterococcus*, including: surface adhesion proteins (esp), aggregation substrates (agg), cytolsin (cly) and hemolysin (hyl) secretion operons, collagen adhesion (ace), and gelatinase secretion proteins that are predominantly found in endocarditis isolates (gelE) (Eaton and Gasson, 2001; Mannu et al., 2003; Vankercikhooven et al., 2004). These virulence factors were primarily screened for in the studied articles due to their implications in pathogenesis routes from environmental reservoirs back into humans and animals. Only 18 of the studies identified screened for virulence factors, the most common being esp (all 18 articles), cly, and gelE (Figure S3). The first vancomycin-resistant *E. faecalis* strain documented in the US was revealed to carry a large, transmissible pathogenicity island containing both esp and a complete cly operon, and several other functions that are non-essential to commensal behavior of the organism (Shankar et al., 2002). The *E. faecalis* pathogenicity island is an integrative conjugative element that can be mobilized between plasmids and chromosomes in *E. faecalis* and *E. faecium*, transferring virulence factors and antibiotic resistance determinants (Laverde Gomez et al., 2011; Manson et al., 2010). The co-occurrence of virulence with antibiotic resistance is a key consideration when screening the environment for the emergence of potentially hyper-virulent strains. Rathnayake et al. (2012) found significant correlations between the presence of virulence factors and phenotypic antibiotic resistance among both *E. faecium* and *E. faecalis* isolates in surface waters and regional clinical isolates in Australia (Rathnayake et al., 2012). Similarly, Lata et al. (2016) documented widespread co-occurrence of vanA and vanB genotypes with gelE, ace, efaA, and esp virulence factors in both *E. faecalis* and *E. faecium* in impacted surface waters in northern India (Lata et al., 2016). Such studies demonstrate the value of monitoring both antibiotic resistance and virulence, particularly for gaining insight into ecological factors at play in observed resistance patterns.

### 5.6. Multilocus sequence typing

Multilocus sequence typing (MLST) is a technique by which multiple loci, or specific internal DNA fragments within an organism’s genome, are amplified by PCR, sequenced, and then compared across multiple isolates of that species. The loci are typically housekeeping genes common to the genus, and the allelic composition of the set of targets determines the “sequence type”, allowing for the determination of clonal complexes (CCs; isolates with differences of no more than 2 loci) and potential source attribution of isolates. Sequence types can then be compared to publicly available and curated databases, such as PubMLST (Jolley et al., 2018), where global isolate comparisons can be made. Pulse-field gel electrophoresis is a similar technique to MLST and was previously known as the “gold standard” for source attribution and epidemiological linkages of bacterial isolates, but low interlaboratory reproducibility and inability to perform phylogenetic or population structure studies makes it unsuitable for global, long-term epidemiological studies (Nemoy et al., 2005). MLST profiles of *Enterococcus* have only been developed for *faecium* (Homan et al., 2002) and *faecalis* (Ruiz-Garbajosa et al., 2006) and are therefore the only two species present in the PubMLST database. The two profiles are derived from a mixed set of 11 housekeeping genes: *gdh* (glucose-6-phosphate dehydrogenase), *purK* (phosphoribosylaminomimidazo carboxylase ATPase subunit), *psaS* (phosphate ATP-binding cassette transporter), *atpA* (ATP synthase, alpha subunit), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *adk* (adenylyl kinase), *ddl*, *gki* (glucokinase), *aroE* (shikimate 5-dehydrogenase), *xpt* (shikimate 5-dehydrogenase), and *yqIL* (acetyl-coenzyme A acetyltransferase), which were chosen for their low ratios of nonsynonymous to synonymous mutations and their dispersed locations on the chromosomes. MLST is useful when exploring potential clonal relationships between *Enterococcus* spp. isolated from the environment and those from critical AMR monitoring points such as hospital wastewaters and pharmaceutical production waste. The clonal complex 17 (CC17) of *Enterococcus faecium*, for example, is a nosocomial strain associated with outbreaks worldwide and is generally ampicillin and quinolone resistant and contains the esp surface adhesion protein (Top et al., 2008). CC17 has been detected in several environmental samples and is an indication of the interconnectedness of the environment and clinical wastewater streams (Caplin et al., 2006).

### 5.7. Whole genome sequencing

Despite the development of several sophisticated molecular biological assays for the complete characterization of enterococcal isolates over the last few decades, the advent and proliferation of next-generation sequencing techniques has allowed for comprehensive and high-throughput functionality of all the previous assays in a singular method (Figure 1). WGS of isolates allows for the simultaneous detection of ARGs, virulence factors, plasmids, bacteriophages, insertion sequences, transposons, and the sequence type and cladal relatedness of isolates that can be compared with enterococcal libraries globally. Only four articles reviewed here performed WGS (Ekwanzala et al., 2020a; Gouliouris et al., 2019, 2018; Zaheer et al., 2020), indicating that comprehensive epidemiological analysis of antibiotic resistant, virulent, hospital-adapted enterococcal clades is largely absent from the environmental literature. In WGS workflows, resistant enterococci are initially screened for on selective media supplemented with antibiotics (in this case ampicillin or vancomycin) and then isolated and speciated before they are subject to sequencing. Genomic surveillance of *E. faecium* isolates from retail meat, patients with bloodstream infections, and wastewater treatment plants revealed distinct clades with limited sharing of ARGs between livestock and humans in the UK (Gouliouris et al., 2018). There was, however, extensive overlap between isolates from bloodstream infections and those from the influents and effluents of 17 different wastewater treatment plants (WWTPs) in the region, which could indicate the emergence of new lineages of *E. faecium* that are both hospital-adapted and persist in the environment (Gouliouris et al., 2019). Similarly, a South African study investigated the prevalence of VRE in hospital wastewater, municipal wastewater, and the receiving surface water (Ekwanzala et al., 2020a). Thirty-five percent of the enterococci exiting the wastewater treatment plant were vancomycin resistant, leading to the greatest VRE loadings in the downstream sediment. Subsequent comparative genomics found that ST40, a human pathogenic *E. faecalis* sequence type, and CC17 of *E. faecium* were found persisting in downstream sediments, posing a risk to human health, and demonstrating the need for more advanced wastewater treatment in this scenario. Although WGS is more expensive and difficult to perform than PCR-based genotyping, its high-throughput and robust analysis is quickly becoming commonplace as sequencing costs continue to fall. Also, the storage and sharing of sequenced genomes to public databases allows for longitudinal, phylogenetic tracking of problematic clones as they are transmitted globally (van Hal et al., 2021).

### 6. Trends in total and antibiotic-resistant *Enterococcus* found in water environments

A comprehensive data extraction and analysis was performed to
identify overarching trends in the species distribution of generic and antibiotic-resistant *Enterococcus* spp. to illuminate general trends and inform sampling priorities, extracting data from all articles that reported resistance as a percentage of the total number of isolates in a collection. Together, this collection consisted of 42,459 isolates extracted from 95/117 articles. To reduce the amount of bias introduced by sampling and enrichment procedures, only *Enterococcus* AST data that was generated in the absence of an initial selective antibiotic were used. This reduced the collection size for AST data to 18,729 isolates extracted from 77 articles but allowed for an estimation of the “true” phenotypic diversity of environmental antibiotic-resistant *Enterococcus*. This approach also allows for the empirical prioritization of monitoring targets for *Enterococcus* resistant to critical antibiotics as well as a baseline for further studies across different water matrices without a bias towards resistant populations.

### 6.1. Total Enterococci

Only 62 of the 91 articles in which libraries were speciated were the data provided in a format that could be extracted unambiguously (e.g., in tabular format) or detailed population statistics reported, resulting in a collective of 28,343 speciated isolates for analysis (Figure 3). The three most common *Enterococcus* spp. identified across the 62 articles were *E. faecium* (34.0%), *E. faecalis* (29.0%), and *E. hirae* (13.0%). Minor species such as *E. raffinosus*, *E. avium*, and *E. pseudointermedius* (Table S3) each represented less than 2% of the total isolates, although this is likely influenced by underrepresentation of these species in common PCR confirmation assays. In fact, in many articles, only *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus* were screened, as these species represent the most encountered resistant clinical isolates. Despite the uneven representation of the number isolates from each water matrix, there were clear trends among enterococcal populations. In WWTP and hospital wastewater samples, the combined proportions of *E. faecium*, *E. faecalis*, and *E. hirae* were nearly identical, making up approximately 90% of all isolates (Figure 3). These proportions are consistent with the natural distributions of *Enterococcus* spp. in the gastrointestinal tract of healthy human adults and animals (Silva et al., 2012). The proportions of non-fecal related or undefined *Enterococcus* spp. were greater in surface water and coastal water samples, consistent with dilution of fecal contamination with environmental strains. A large proportion *E. hirae*, the dominant species excreted by cattle (Jackson et al., 2011), was found in cattle feedlot drains (Zaheer et al., 2020). The even proportions of species across comparable water matrices (e.g., WWTPs and hospital wastewater) observed, despite extensive heterogeneity in methodology, suggests that there is a general lack of systematic errors or biases in either media selectivity or speciation techniques.

### 6.2. Surface water

The majority of isolates obtained across studies were derived from surface water (fresh water), which included both urban and rural watersheds and riverine sites directly impacted by municipal or hospital wastewater discharge (7,146 isolates) (Figure 4). Compared to the other environments examined, surface waters displayed some of the lowest percentages of resistant isolates to all 18 antibiotics and antibiotic classes. It should be noted that, despite dilution effects and environmental attenuation, 3.5% of 7,146 *Enterococcus* spp. isolates were fully resistant to vancomycin. The vast majority of these isolates were either *E. faecium* or *E. faecalis*. By contrast, full vancomycin resistance was virtually undetected in *E. casseliflavus*, *E. gallinarum* and the other less dominant species (Figure 4; Table S3). Despite intrinsic resistance of *E. casseliflavus* and *E. gallinarum* via vanC genes, their phenotypes rarely exceeded CLSI breakpoints for full clinical resistance. Łuczkiwicz et al. (2010) examined *Enterococcus* resistance to 13 different antibiotics in an urban river system in Poland in the absence of wastewater treatment plant discharge and found that resistance to erythromycin, ciprofloxacin, and tetracycline was common among all isolates (Łuczkiwicz et al., 2010). They also found multidrug resistance (some to all 13 antibiotics tested), including vancomycin and high-level aminoglycoside resistance, among *E. faecalis* and *E. faecium* isolates in the two main tributaries feeding the coastal waters. The authors suggested that riverine enterococci should be considered as a potential risk for downstream recreational bathers, even in the absence of point-source wastewater pollution. In contrast, studies of rural watersheds (<1% urban) in Ontario (Canada) and Georgia (US) found that the diversity and distribution of antibiotic resistance among *Enterococcus* were strikingly different than in more anthropogenically-impacted water bodies (Cho et al., 2019; Lanthier et al., 2011). These two studies, together comprising 2,195 isolates, indicate that the *Enterococcus* species and their phenotypes were stochastically distributed and sparse, with few multidrug (<6 antibiotics) resistant strains and no isolates reaching the CLSI breakpoints for vancomycin, teicoplanin, or linezolid. They...
attributed the dispersion of the resistant fecal indicators to domesticated animal and wildlife fecal pollution and their potential for dissemination to be limited. The highest rates of resistance among *Enterococcus* isolates from surface water studies came from the North West province of South Africa where 86/124 *Enterococcus* spp. were phenotypically resistant to vancomycin. These were isolated in the absence of a selective antibiotic and displayed multidrug resistance to ampicillin, amoxicillin, penicillin, ciprofloxacin, erythromycin, and tetracycline. Interestingly, a single isolate of *E. sulfureus* was found to be resistant to ampicillin, amoxicillin, penicillin, streptomycin, vancomycin, chloramphenicol, ciprofloxacin, erythromycin, and tetracycline (Molale and Bezuidenhout, 2016).

Upstream and downstream sampling of municipal wastewater discharge was carried out in several studies. The detection of enterococci with resistance to clinically-relevant antibiotics downstream of municipal wastewater discharge was regionally dependent and linked to the degree of treatment employed by the WWTP. A Tunisian study found no ampicillin-resistant *Enterococcus* (ARE) or VRE in the influent, effluent, or receiving water, suggesting the regional variation in their distribution (Ben Said et al., 2015). Another study (Bessa et al., 2014) found vanA positive *E. faecium* mostly upstream of WWTP discharge in Portugal, a region where VRE is prevalent in the population.

### 6.4. Municipal and hospital wastewater

Municipal and hospital wastewater were commonly sampled across the identified articles. Together they accounted for 7,400 isolates in the collection and displayed high percentages of clinically-relevant antibiotic resistant phenotypes (Figure 4). *Enterococcus* spp. isolates from untreated hospital wastewater displayed the highest percentages of resistance to fluoroquinolones, ampicillin/penicillin, macrolides, and vancomycin.

WWTPs, which encompassed raw influent, activated sludge, secondary effluents, and variably treated final effluents across 15 different countries, yielded *Enterococcus* spp. isolates with the highest percentages of resistance to cephalosporins, lincosamides, nitrofurantoin, and teicoplanin (Figure 4). Within the “WWTP” category, only 2.7% of 6,057 isolates were resistant to vancomycin in the collection. The highest percentages of full vancomycin resistance across studies of WWTP influents came from Germany (19%; 16/84 isolates) (Gallert et al., 2005), Portugal (3.4%; 17/499 isolates) (Araújo et al., 2010), Iran (3.3%; 19/577) (Talebi et al., 2007), and Poland (3.2%; 6/185 isolates) (Luczkiwicz et al., 2013). The 2005 paper (Gallert et al., 2005), is the oldest of the group, and reveals the challenges of accurately assessing antibiotic-resistant *Enterococcus*, particularly VRE, whose phenotypic characteristics are mimicked by genera such as *Leucostoc*, *Pediococcus* and *Weisella* (Harwood et al., 2001). In this case bacteria were isolated on relatively non-selective bile esculin agar, and were confirmed only by ability to grow at 6.5% NaCl and by microscopy for cell morphology. Furthermore, the method for assessing antibiotic resistance relied on a set zone of inhibition of 2 mm for all antibiotics, rather than standardized zone diameters.

Although they represented a smaller fraction of isolates, hospital wastewater environments showed the most definitive contributions of phenotypic resistance to receiving waters. For example, Novais et al. (2005) found statistically significant increases in phenotypic resistance exceeding CLSI breakpoints among *E. faecium* and *E. faecalis* for vancomycin, teicoplanin, ciprofloxacin, and ampicillin in urban sewers receiving hospital wastewater discharge in Portugal (Novais et al., 2005). Clonal analysis, Tn1546 typing, and virulence factor assays were also consistent with local clinics being the source of downstream surface

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**Fig. 4.** Distribution of percent of *Enterococcus* isolates resistant to each antibiotic by sample matrix. Isolates were only included if they were initially isolated in the absence of a selective antibiotic. The number of unique isolates representing each matrix is in parentheses in the headers but note that not every isolate was tested for every antibiotic. The number of resistant isolates per antibiotic tested is represented adjacent to each bar in each panel. CAFO = concentrated animal feeding operation, WWTP = wastewater treatment plant. QD = quinupristin/dalfopristin; SXT = sulfamethoxazole/trimethoprim.
water isolates. Similar observations were made along a medical center-retirement home-wastewater treatment-plant-river continuum in France in 2013, where the hospital-adapted clonal complex, CC17 Enterococcus faecium, was culturable along the continuum and into receiving waters (Leclerq et al., 2013). The CC17 concentration, though, was attenuated by the WWTP and the proportion of CC17 became outweighed by environmental strains. Further, epidemiological source tracking using WGS of VRE isolated from 20 WWTPs in the UK in 2019 determined that there was widespread dissemination of hospital-adapted Enterococcus faecium in WWTP effluents across eastern England (Gouliouris et al., 2019). They found that WWTPs receiving hospital wastewater had significantly higher VRE and ARE concentrations than non-hospital associated treatment plants and found highly similar isolates shared between the local teaching hospital and those emitted from surrounding WWTPs. Together, these studies provide strong support of Enterococcus spp. as both a clinically- and environmentally-relevant target for waterborne monitoring. Hospital wastewaters, the municipal wastewater infrastructure responsible for treatment, and receiving waters are key monitoring points for tracking their dissemination.

6.5. Pharmaceutical wastewater

Pharmaceutical wastewater is a critical monitoring point in the dissemination of resistant microorganisms and is currently understudied in the field. Only two such articles focused on resistant Enterococcus were encountered in this review. Guardabassi et al. (2002) documented intermediate resistance of presumptive Enterococcus to 20 ug/mL vancomycin and evidence of vanA and vanD isolates in the waste biomass from the fermentation tanks used in the production of vancomycin (Guardabassi et al., 2002). The WWTP treating the waste was also enriched with presumptive, intermediate-level VRE, and quantifiable CFUs were routinely emitted from the plant after secondary clarification without disinfection. PFGE analysis found identical VRE patterns between vancomycin production fermentation waste and the final effluents of the WWTP, suggesting that pharmaceutical production waste can be a direct contributor to the dissemination of VRE into the environment. Further, Guardabassi et al. (2004) investigated the relationship of Tn1546-like elements in Enterococcus spp. isolated from municipal sewage, activated sludge, vancomycin production waste, human feces, mussels, and soil using long PCR-restriction fragment length polymorphism and found indistinguishable elements shared across the ecologically distinct locations and between enterococcal species, suggesting ready transferability of the vanA genotype between clinical and environmental strains (Guardabassi and Dalsgaard, 2004).

6.6. Recycled water

Due to the increased pressures on freshwater around the world, wastewaters are increasingly being treated and reintroduced into water and food cycles as the recycled water is used for crop irrigation, groundwater recharge, and even direct potable reuse. Only three studies in the current review examined resistant Enterococcus in wastewaters intended for reuse. Goldstein et al. (2014) sampled two WWTPs in the Mid-Atlantic and two from the Midwest regions of the US that reuse their treated effluents and detected VRE in 27% of wastewater samples, with higher rates in the Mid-Atlantic plants. VRE were only detected in final treated effluents when there was lack of chlorination (Goldstein et al., 2014). Subsequent studies from the same WWTPs found that VRE are detectable at low concentrations at the point of use after recovery from UV disinfection, although other phenotypes are more prevalent (Carey et al., 2016). Both WWTPs studied receive hospital wastewater and their effluents were used for spray irrigation (Goldstein et al., 2012).

6.7. Marine waters and recreational beaches

Freshwater and marine water environments used for recreational bathing are at the direct interface between environmental fecal pollution and human exposure and are therefore important monitoring points. Studies of enterococcal populations of marine and freshwater beaches from Spain, Puerto Rico, Poland, Greece, Malaysia, Brazil, Italy, and Michigan confirmed that many drug resistant strains are readily culturable in recreational marine water and sand (Alm et al., 2014; Arvanitiou et al., 2001; Dada et al., 2013; de Oliveira and Watanabe Pinhata, 2008; Monticelli et al., 2019; Sadovy and Luczkiwicz, 2014; Santiago-Rodriguez et al., 2013; Tejedor Junco et al., 2001). High phenotypic and phylogenetic diversity was observed across all studies, and many environmental-associated enterococci dominated local populations, including Enterococcus casseliflavus (Monticelli et al., 2019), Enterococcus hirae (Sadovy and Luczkiwicz, 2014), and E. avium and E. raffinosus (Arvanitiou et al., 2001). Beach sands are of particular interest as they represent a niche environment for enterococci where horizontal gene transfer can occur at higher frequencies than among planktonic bacteria, accelerating the rate at which fecal microbiota exchange genes with pathogens and facilitating human-pathogen interactions (Alm et al., 2014; Oravcová et al., 2017).

6.8. CAFOs and irrigation water

Studies of the effects of cattle and swine concentrated animal feeding operation (CAFO) wastewater on downstream environments were also prevalent in the literature. The enterococcal isolates from CAFO studies in the collection showed the highest resistance prevalence to tetracyclines, which is not surprising given that tetracyclines make up nearly 40% of all antimicrobials used in animal husbandry in the US. (CDC, 2013). As many CAFOs contain and treat their wastewater on-site, their direct impact on groundwater or downstream surface water (e.g., due to runoff or unintentional discharge) was a concern. The animal products themselves also represent a potential direct line of exposure from animals to humans through the food chain. Sapkota et al. (2007) sampled upstream and downstream of a high-density swine operation in the Mid-Atlantic region of the US and found higher MICs for clindamycin and tetracycline amongst the Enterococcus spp. in both downstream groundwater and surface water (Sapkota et al., 2007). Similarly, Stine et al. (2007) sampled the waste lagoons, surface waters, and well water of a swine CAFO in the US that had been administering tetracycline-containing feed for over 20 years and found that 68% of all enterococci were resistant to tetracyclines, and a total of 60 different species displayed phenotypic resistance to tetracyclines across the sites (Stine et al., 2007). Further evidence for the direct dissemination of clinically-relevant enterococci into ambient surface waters by CAFOs was documented by Jahne et al. (2015) (Jahne et al., 2015). They documented a cattle CAFO and its on-site wastewater treatment system comprising of an infiltration basin with subsequent sequestration by a constructed wetland. Enterococci that displayed co-resistance to vancomycin, linezolid, and daptomycin were common in the wastewater and, during rain events, the increased hydraulic loading on the infiltration basin and constructed wetland resulted in the direct emission of these organisms into downstream surface waters.

7. Conclusion and recommendations

In this review we recounted the last 20 years of research assessing antibiotic-resistant enterococci in various water environments. Because Enterococcus has been shown to be a reliable indicator of fecal contamination of water bodies, several nationally and internationally recognized standard culture methods have been developed for their enumeration. Various Enterococcus spp. are both clinically-relevant and survive and persist in the environment. The studies surveyed here lay the groundwork for considering Enterococcus spp. as a standardized target for waterborne monitoring of antibiotic resistance.

Recently, the WHO put forward a standardized, comprehensive surveillance program for One Health-inspired monitoring, i.e., the
Tricycle protocol (WHO, 2021), which targets the Gram-negative ESBL E. coli. Here, Enterococcus spp., as Gram-positive organisms, represent a compelling target to consider as a complement to such monitoring programs. Enterococcus spp. display resistance to critically-important antibiotics that would not be captured by an E. coli-targeted monitoring program alone. As observed in this critical review, Enterococcus spp. also display sensitive responses to anthropogenic pollution, including hospitals and CAFOs, that are apparent in their distinct geographical occurrence patterns.

The recommended path forward for standardizing environmental antibiotic-resistant Enterococcus monitoring should ensure the comparability of monitoring points, methods employed, and reporting metrics. Accessibility and ease of application are also important considerations. Ideally, Enterococcus spp. monitoring for the purpose of antibiotic resistance surveillance could be incorporated into existing monitoring programs, especially considering the high level of existing standardization and regulatory requirements. The existence of nationally and internationally recognized standard culture methods is of great value in ensuring comparability of the data gathered in space and time, however, further standardization is needed for the purpose of antibiotic resistance surveillance specifically. The conclusions and recommendations based on this critical review are as follows:

- mEI is a prime candidate for a standard selective media, given that it yields the highest selectivity over mEnterococcus and Slanetz-Bartley and is integrated into existing regulatory recommendations in the US. Still, the findings here were encouraging that studies are generally consistent, even if different media were employed, but confirmation of the genus is critical because no Enterococcus media is 100% selective. The addition of a selective antibiotic to the media can decrease specificity by selecting for intrinsically-resistant, non-target genera.

- In line with recommendations made in the WHO Tricycle protocol, plating environmental samples on the selective media with and without a primary selective antibiotic produces both a percent resistance of the enterococci population and a CFU/unit volume measurement (e.g., CFU/ml). A CFU/mL measurement represents a universally comparable magnitude of antibiotic-resistant bacteria.

- Depending on the research question, vancomycin and other antibiotics used for primary selection may be added to media at the breakpoint for full, intermediate, or low resistance. The full resistance breakpoint is the most useful for clinical relevance, but in some environments, one may be interested in intermediate resistance. Where one expects bacteria to be compromised (injured), use of low-level antibiotic in enrichment cultures may be useful for lowering detection limits. The use of low/intermediate breakpoints may complicate human-health risk assessments due to the isolation of intrinsically-resistant species that are common in the environment unless further testing is done.

- A defined set of key antibiotics aimed at treating VRE infections could also be employed for monitoring emerging phenotypes and multidrug resistance. These include ampicillin, teicoplanin, oxazolidinone-linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline. The emergence of multidrug resistant VRE that are co-resistant to these 6 antibiotics are of great concern.

- Antimicrobial sensitivity testing using disk diffusion on Mueller-Hinton agar or agar dilutions in Mueller-Hinton broth should be followed according to the most up-to-date CLSI guidelines.

- Speciation and virulence typing of resistant enterococci is of interest for risk assessment and longitudinally tracking changing genotypes and phenotypes across the genus. PCR-based approaches are appropriate for low-tech labs, specific loci sequencing, and emerging technologies such as MALDI-TOF MS will increase throughput without sacrificing accuracy.

- Key monitoring points to consider for the dissemination of resistant enterococci are the hospital-municipal wastewater continuum and their receiving water bodies, especially where they impact recreational waters. Pharmaceutical wastewater and recycled water are critically understood for resistant Enterococcus. The surrounding areas of CAFOs are of particular concern after storm events.

- Whenever possible, isolation of antibiotic-resistant colonies for WGS will aid in determining the sequence type, virulence genotype, plasmid type, acquired AMR genes, and chromosomal point mutations. Public sharing of WGS data will help to advance understanding of the ecology, epidemiology, and global transmission of this important pathogen.

Declarations of Competing Interest

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Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.100161.

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