Towards developing an international environmental AMR surveillance strategy

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Executive Summary

Antimicrobial resistance (AMR) is one of the major global human health threats, and infections caused by resistant pathogens are predicted to be the leading cause of death by 2050 if current trends continue [1]. This report focuses on AMR in bacteria (also commonly termed 'antibiotic resistance') and on the role the environment plays in its evolution, dissemination (spread within the environment) and transmission. Many of the resistance mechanisms acquired by clinical pathogens are of environmental origin having evolved over millions or billions of years to counteract antimicrobials naturally produced by bacteria and fungi [2]. The environment also plays an important role in person to person transmission through faecal contamination, as well as animal to human or environment to human transmission through direct contact with the environment or through consumption of water and food.

Systematic surveillance of AMR and antimicrobial usage (AMU) is crucial to tackling the silent AMR pandemic, enabling understanding of the complex dynamics of AMR within human, animal and environmental microbiomes. Reference to the emerging problem of antifungal resistance, which is in part driven by widespread antifungal use in agriculture, is also discussed in the appendix. Historically, bacterial AMR surveillance has been clinically focused; however, we need a global One Health approach as human, animal and environmental microbiomes are interconnected and antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs) are regularly exchanged between One Health compartments including human, animal and environmental microbiomes.

We aim to describe what a comprehensive and international environmental AMR surveillance strategy should encompass, considering potential scope and methodologies, as well as barriers to implementation, and we suggest solutions to overcome these. This report focuses on four key objectives that environmental surveillance can support: 1) tracking changes in the spatial and temporal patterns of AMR in the environment; 2) surveying risk factors that are known to contribute

to the amplification and emergence of ARGs from environmental bacteria in human and/or animal commensals and pathogens; 3) facilitating risk assessment of environmental exposure to, and transmission of, AMR in humans and animals; and 4) utilising wastewater to characterise AMR in human populations. An informative surveillance system will facilitate understanding of the evolution, dissemination and transmission of AMR within, to, and from the environment, thus enabling informed decision-making regarding the implementation and efficacy of mitigation strategies. Whilst the potential for ecological impacts caused by antimicrobials is likely, the ecological effect of AMR is less certain. Ecological effects are out of scope and are not considered in this report. Rather, the focus is on the environmental dimension of AMR that ultimately relates to AMR evolution and transmission, leading to drug-resistant infections in humans and livestock.

Understanding of which AMR targets should be used for environmental AMR surveillance is emerging, with a range of established methodologies to characterise and quantify AMR endpoints. Possible targets may be common to existing clinical and/or veterinary surveillance to facilitate comparisons across the One Health compartments, as is the case with the Tricycle Protocol that focuses on extended spectrum β-lactamase (ESBL) producing *Escherichia coli*. However, the environment constitutes an extraordinarily diverse reservoir of ARGs in environmental bacteria that can be mobilised to human, livestock, and wildlife associated bacteria. Therefore, standardised approaches tailored to environmental surveillance are necessary to not only quantify AMR across environmental compartments, but also to survey risk factors that contribute to the amplification and emergence of AMR in human- and animal-associated bacteria, including pathogens and commensals.

A comprehensive AMR surveillance programme should facilitate a systems understanding of AMR that enables the investigation of causal associations between high-level drivers and increased probability of AMR emergence and transmission. Surveillance targets should be balanced between priority pathogens with resistance to critically important antimicrobial drugs and those targets (bacterial and chemical) that have the potential to characterise enrichment of AMR within the

environmental microbiome and potentially transfer to human- and animal-associated bacteria including pathogens (i.e. the emergence of novel AMR in human pathogens). Clinical and veterinary surveillance currently focuses on bacterial targets, but environmental surveillance should consider culture-independent methodologies such as quantitative PCR and metagenomic sequencing to fully understand AMR dynamics within microbial populations. Moreover, recent proposals to implement wastewater-based AMR epidemiology offer a unique opportunity to integrate human and environmental surveillance.

Barriers to the implementation of a single global environmental AMR surveillance strategy include: 1) minimal awareness regarding the importance of the environment in contributing to AMR infections in humans and animals; 2) perceived uncertainty in the reliability of the generated data due to the complex nature of AMR and an incomplete evidence base; 3) lack of agreement on establishing standardised protocols for methods to be used in surveillance programmes that can be implemented globally, as well as methods for more comprehensive programmes; and 4) limited availability of resources and lack of prioritisation of environmental surveillance in different settings. Some of these challenges can be overcome through greater international, multi-sectoral collaboration and cooperation.

This report is intended to give a technical overview of the environmental dimensions of AMR, existing technologies and evidence that support the need and potential for a global environmental surveillance strategy.

Glossary of terms

Term (acronym)	Definition
Antibiotic resistant bacteria (ARB)	Bacteria that are resistant to antibiotic drugs
Antibiotic resistance gene (ARG)	Genes (sections of DNA) that confer an ability to a bacterium to tolerate the action of an antibiotic
Antimicrobial resistance (AMR)	The ability of a microorganism to survive or reproduce in the presence of drugs to which it was once susceptible
Antimicrobial use (AMU)	Data on antibiotic use in humans and animals
ESKAPE pathogens	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species
Extended-spectrum β-lactamase (ESBL)	A group of enzymes produced by antibiotic resistant bacteria to counteract the effects of a group of clinically important antibiotics, broad-spectrum β-lactams
Metagenomics	The study of the genetic material (DNA or RNA) extracted from samples containing a mixture of microorganisms.
Microbiome	The whole microbial community living in an ecosystem
Mobile genetic element (MGE)	Genetic elements that are capable of transfer between or within microorganisms, e.g., plasmids, integrons, transposons, insertion sequences, gene cassettes, etc. These may harbour multiple resistance genes
One Health	An integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems (WHO)
PCR/qPCR	Polymerase chain reaction/quantitative polymerase chain reaction. A molecular biology technique to amplify and detect specific, known genes in a sample
Resistome	The collection of resistance determinants in an ecosystem compartment e.g. environment, human, animal
Whole genome sequencing (WGS)	A technique to determine the DNA sequence in an isolated microorganism

Introduction

The emergence and spread of AMR in bacterial pathogens is one of the major global human health threats, acknowledged by the General Assembly of the United Nations in 2016 (A/RES/71/3/2016) [3]. Systematic surveillance of AMR and antimicrobial use (AMU) is crucial in all One Health compartments (human, animal and environment) to tackle this global health crisis, to inform policy and to promote successful AMR mitigation strategies. AMR requires a global (geographical) and holistic (multi-sectoral) One Health approach as human, animal, and environmental spheres are interconnected [4], and antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARGs), and their associated mobile genetic elements (MGEs) are regularly exchanged between One Health compartments and across geographical borders.

Historically, AMR surveillance has focused on resistance in key bacterial species that are important in causing infections in humans and animals (e.g. *Staphylococcus aureus*, *Clostridioides difficile*, *Escherichia coli* and *Salmonella* spp.) [5]. This has been undertaken by organisations such as the WHO [3], FAO [6], EFSA [7] and national organisations. In the EU, national scanning surveillance is performed by several countries on samples from diseased animals [8]. In the UK, for example, these data are reported in Veterinary Antibiotic Resistance and Sales Surveillance (VARSS) reports [9]. Such surveillance provides resistance trends in some bacterial commensals and pathogens of humans and animals. It also provides data on potential exposure risk to some food-borne pathogens, informs on the potential emergence of new resistance genes in target organisms and informs treatment options.

Implementing a One Health approach requires harmonised surveillance data across humans, animals (terrestrial and aquatic, livestock, wildlife and companion animals), crops and associated air, water and soil environments, with the use of output indicators to monitor AMR and AMU. Consequently, several joint national reports already publish AMR trends for key indicator bacteria and antibiotics (e.g. UK One Health report [10]; DANMAP [11]; Swedres-Syarm [12]; Scottish One

Health Antimicrobial Usage and Antimicrobial Resistance Report (SONAAR) [13]; Ireland One Health Report on Antimicrobial Use and Antimicrobial Resistance [14].)

The WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) [3] that promotes and supports a standardised approach to the collection, analysis and sharing of AMR data at a global level by encouraging and facilitating the establishment of national AMR surveillance systems that are capable of monitoring AMR trends and producing reliable and comparable data. The WHO has also supported the development of a One Health surveillance target, termed the Tricycle project, recognising the involvement of WHO, FAO and OIE (rebranded as the World Organisation for Animal Health (WOAH)), focusing on extended spectrum β-lactamase (ESBL) producing *E. coli* [15, 16].

The GLASS approach, relying on human blood, urine, and stool samples, provides information on culturable, abundant, and pathogenic (may also be commensal) bacteria in clinical and veterinary settings, so does not consider the environmental dimensions of AMR. To close this current major gap in the One Health approach there is an urgent need to fully understand the evolution and transmission of AMR within the wider environment encompassing human, animal, plant, and environmental microbiomes. However, at present, research into the environmental aspects of AMR has largely been confined to individual institutions or academic laboratories. National governments and international bodies (EU, UN, WHO) have recognised that effective environmental surveillance systems must be established to identify and monitor AMR in water, wastewater, soil, air, and wildlife in order to increase understanding of the natural environment's role in the emergence and transmission of AMR, including how the introduction of antimicrobials and resistant bacteria from human and animal sources into the environment contributes to AMR-related human health problems (e.g. EU One Health Action Plan 2017 [17], UK 5-year action plan for antimicrobial resistance 2019 to 2024 [18], US National Action Plan for Combating Antibiotic-Resistant Bacteria, 2020-2025 [19]). However, whilst environmental monitoring and surveillance systems for chemical substances and water quality are employed in many countries, few, if any, have environmental regulations regarding

similar monitoring for AMR. Wastewater-based epidemiology (WBE) of SARS-CoV-2 in wastewater has brought the utility of wastewater as a human-population-level surveillance target to the attention of governments and regulators. The utility of this approach for human AMR surveillance is gaining traction globally, with an example of its power illustrated by the Global Sewage Surveillance Project [20].

No clear consensus currently exists regarding the rationale for environmental AMR surveillance, or which AMR endpoints (bacteria, genes, MGEs, etc) should be targeted for the environmental sector, or how to successfully implement a global environmental AMR surveillance framework. In its most comprehensive form, environmental surveillance would be designed to facilitate understanding of the evolution, dissemination and transmission of AMR from specific sources to, in, or from the environment or to support implementation and report on the efficiency of mitigation and control measures. Environmental surveillance could also serve as an early warning system for the presence of important AMR pathogenic bacteria or ARGs, as well as alert us to the emergence of novel AMR determinants including those conferring resistance to new antimicrobials. This could inform the risk of these determinants spreading into human and animal commensals and pathogens. In addition, WBE focusing on characterising AMR in human populations could be included as the methods and capacity are similar to that required for environmental surveillance and would be of particular value in populations where clinical surveillance data are scarce. Interpretation of complex data sets is currently not within the scope of surveillance and monitoring agencies, however theoretical frameworks and tools are rapidly emerging to facilitate such analyses. Environmental surveillance needs to be integrated with clinical and veterinary surveillance (healthy and diseased animals) where possible, and therefore methodologies and quantitative data need to be comparable where targets are common across One Health compartments. Where surveillance seeks to understand the response of AMR within microbial communities and the impact of pollution on selection for ARGs and MGEs, specific methods applied to the environment may not currently be part of routine surveillance in humans and animals. However, these approaches will yield critical data facilitating

255 our understanding of AMR enrichment in the environment and the potential for the emergence of
256 novel ARGs in human commensals and pathogens.

257 Previous reports have provided economic evidence that a One Health AMR surveillance approach
258 is feasible and could broadly benefit society, animal welfare, and ecosystem health [21], while others
259 have provided viable surveillance goals, reviewed potential methodologies, and identified major
260 knowledge gaps to develop an environmental AMR surveillance strategy [22-24].

261 Based on this current knowledge base, we aim to describe what an environmental AMR surveillance
262 strategy should entail. In other words, what the minimum sampling framework should be to provide
263 reliable trends in AMR that are representative of the human, animal and environmental sectors. We
264 identify the current obstacles to implementation and provide potential solutions to overcome these
265 barriers.
266

267 Defining the scope of an environmental AMR surveillance strategy

268 Common, clearly defined terminology that is understood internationally and shared between diverse
269 stakeholders (policymakers, regulators, advisors, scientists, etc.) is needed to frame a global One
270 Health environmental AMR surveillance strategy. Here, we define what encompasses the terms
271 ‘environment’ and ‘surveillance’ in this context.

272 The environment is extremely diverse, and sample types needed for AMR analysis need to be
273 defined and standardised across time and space. For surveillance purposes, the environment not
274 only covers the classical compartments (water, soil, air, wildlife, etc.) but all compartments not
275 covered by human and livestock surveillance efforts. For example, crops, which are routinely
276 exposed to antibiotics as plant protection products globally, and farm environments (e.g. soil, and
277 water used for irrigation), as well as the edible parts of the plants themselves, may be enriched with
278 AMR bacteria and ARGs (bearing in mind some genetically modified plants may contain bacterial
279 ARGs) [25]. Box 1 lists which settings and samples may be considered ‘environmental’. Briefly, both

built environments and natural environments are included, although food processing and healthcare facilities are excluded, since these have their own specific surveillance efforts. However, liquid and solid wastes produced in these facilities and present in other settings (e.g. wastewater and sewage), should be included to fill the existing knowledge gaps in AMR surveillance between Source−Pathway−Receptor compartments.

Environment	The environments covered by the environmental AMR surveillance strategy include but are not limited to water, soil, and air associated with built (this includes waste entering the environment from all domestic, clinical, veterinary and industrial facilities), farmed (e.g. soil fertilised by animal manure vs. pristine soil, aquaculture ponds, natural surface waters) and natural environments (outdoor environments including outdoor air, surface water, groundwater and coastal water, land and soil, wildlife). Treatment processes to remediate waste streams such as wastewater treatment plants should be included to determine the efficacy of AMR removal and sewage itself can be used to inform AMR trends within human populations using a wastewater epidemiological approach. While clinical and food processing facilities are outside the scope of an environmental AMR surveillance programme (and have their own dedicated surveillance programmes), ecosystems receiving drug-resistant microorganisms or selective agents from these entities should be included (in some LMICs the distinction between compartments may not be well defined due to food production not taking place within defined facilities, for example). The environment needs to be considered as a recipient of drug-resistant microorganisms, antibiotic resistance genes (ARGs), mobile genetic elements (MGEs) and antimicrobials, as a reservoir of AMR and a location for AMR evolution and emergence.

Box 1: What encompasses 'the environment' in an environmental surveillance framework?

289 Surveillance is defined as the systematic and organised collection, analysis and interpretation of
290 data that can be related to human and animal health risk (Box 2). This can be extended to consider
291 impacts on the environment itself (e.g., functional microbial diversity, nutrient/geochemical cycling,
292 and ecosystem health). Although currently outside the scope of this document, this could be
293 considered in the future. Monitoring is considered a different activity, which involves comparison of
294 collected data against agreed thresholds or limits. Comprehensive AMR monitoring against such
295 criteria is currently unrealistic in most environments since defined standards or baseline levels for
296 acceptable AMR prevalence are lacking. This could be overcome if risk assessment efforts provide
297 the necessary data for setting quality criteria. Research is distinct from monitoring and surveillance
298 in that it aims to address hypotheses or develop novel methods, which may support surveillance
299 efforts. AMR surveillance, supported by hypothesis-driven research, is needed to record changes in
300 AMR prevalence and diversity, and to inform and evaluate policy.
301

Surveillance	Continuous recording, collection, and interpretation of observational data from a defined environment. Surveillance should ideally provide timely data on the resistance/residue trends for the purposes of identifying major changes to inform scientific understanding, policy and practice interventions. Surveillance programmes should also be robust, to minimise impacts of temporal, geographic, and socioeconomic factors.
Monitoring	Surveillance guided by a particular regulatory standard or metric, which is applicable for both high-, middle- and low-income countries, which can trigger action.
Research	Systematic collection of data to generate evidence to answer a research question or test a hypothesis. Research is needed to iterate and adjust surveillance frameworks, criteria, and monitoring.

302

303 **Box 2: Approaches to obtain data concerning the abundance, characteristics and trends in**
304 **environmental AMR**

A comprehensive global environmental AMR surveillance strategy needs to be based on our understanding of the hazards and risks that we aim to address. Considering the current AMR situation, this framework must be global and multi-sectoral so it can provide comprehensive data for use by public health entities, environmental protection agencies, and the wider scientific community.

A global environmental AMR surveillance framework could be implemented based on targets common to humans and animals, allowing comparability. However, different methodologies will be necessary to generate data on AMR dynamics within microbial populations or microbiomes (most of which cannot be cultured) to inform risk of amplification or emergence in human and animal pathogens. For example, metagenome analyses (the study of DNA extracted from a microbial community) in the environment focussing on relative abundance of ARGs in microbial communities gives important insights into AMR evolution. ARGs under enrichment may subsequently emerge in human pathogens and be detected through culture-based surveillance followed by whole genome sequencing (WGS) [26]. EFSA/ECDC are moving to implement WGS for reporting results for their AMR monitoring from phenotyping from 2021 (voluntary) and 2026 (compulsory) [27].

The WHO Tricycle ESBL project is one of the only global AMR surveillance efforts which integrates the environment as one of three spokes (alongside the human and animal compartments) [16]. In this programme, ESBL-producing *E. coli* are proposed as the global One Health surveillance indicator. The protocol uses a simple culture-based method, similar to published approaches used to study third-generation cephalosporin (3GC) resistance in *E. coli* in the gut microbiome [28] and in aquatic environments [29, 30]. This method has several advantages. First, the ESBL *E. coli* indicator is useful in identifying clinically important resistance mechanisms. *E. coli* itself is a well-established and widely used surveillance target due to associations with faecal contamination and negative health outcomes in exposed individuals [29, 31]. *E. coli* includes strains that are serious human pathogens, and are not simply considered as a proxy, marker or indicator, thus adding to its relevance [32]. Unlike molecular culture-independent methods, the ESBL *E. coli* target assesses bacterial viability, which is seen as a benefit by some stakeholders. The Tricycle method can facilitate

human exposure risk assessment and probability of environmental transmission, particularly if isolates from human, animal and environmental samples are subjected to further analyses to identify ESBL genes, genomic context, host sequence type, or indeed WGS. With downstream analyses of isolates, this method might also evaluate the emergence of novel ESBLs or other resistance genes in *E. coli*. Finally, the simplicity of the technique permits its widespread use, and generation of easy-to-interpret data.

The Tricycle method in its current form cannot provide data on AMR evolution or gene transfer dynamics within environmental bacterial populations, as it focuses on a single species which is the subject of regulatory controls in food and drinking water. These are processes that may result in the emergence of novel AMR, or the transfer of diverse ARGs to human pathogens, which a comprehensive AMR surveillance should aim to identify. The Tricycle method is appropriate in the very specific context of assessing the "circulation of AMR in the environment" highlighted in the WHO action plan [33] in environments with major human impact, but does not generate data that can be used to understand the impacts of human activity on AMR within the environmental microbiome. While the Tricycle method could be a logical starting point for global environmental AMR surveillance, further action is needed to collect the necessary data to address the remaining critical questions that environmental AMR surveillance can and should answer. These are outlined below.

Surveillance objectives and methodological approaches

The main objective of environmental AMR surveillance is to provide data on AMR prevalence, diversity, and dynamics in different environmental compartments over time. A comprehensive global environmental AMR surveillance framework should be informed by a systems-level understanding of AMR and encompass multiple environmental compartments to trace AMR from source to receptor. Such 'Sources−Pathways−Receptor' models are established in other areas of environmental risk assessment. The framework should include (as a minimum) standardised quantification of biological AMR targets, facilitating understanding of the conditions that affect AMR evolution, ecology, and

transmission. To provide insights into these aspects, we discuss surveillance targets, methods for their measurement, and sampling regimes. In addition, chemical components (i.e., antimicrobial residues, biocides, and heavy metals) can be considered to elucidate associations between antimicrobial selective (or co-selective) pressures and AMR.

Choice of surveillance targets

Current surveillance targets are largely driven by clinical and veterinary surveillance priorities such as the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species), plus some additional taxa identified as important infectious bacteria (*e.g.*, ESBL-producing *E. coli*), and food-borne pathogens (e.g., *Salmonella* and *Campylobacter* species). Resistance to antibiotics of clinical importance, as defined by the WHO and WOAH guidelines [34], also affects phenotypic and genotypic surveillance approaches in terms of antibiotic susceptibility and selective enrichment strategies. This can give useful information on existing AMR in relevant clinical and veterinary pathogens through phenotypic characterisation, while genome sequencing of these organisms gives information on ARG identity and may give insights into emerging resistance determinants. In addition*, Enterobacterales* such as *E. coli* used as water quality indicators (but also including important AMR pathogens) can be useful markers of AMR associated with faecal pollution in natural environments, particularly if enrichment using antibiotics with rarely occurring resistance in environmental bacteria are utilised. Resistant organisms can be quantified in different matrices using antibiotic containing plates that selectively enrich particular bacteria groups, allowing estimation of AMR prevalence in key taxa.

Over 3,000 ARG sequences, known to confer resistance to available antibiotics, are deposited in current databases [35-38]. This complexity produces obvious challenges, highlighting the fact that AMR surveillance is unlike comparatively simple pathogen surveillance. Correlations between ARGs and other genes has led to the use of proxy genes in AMR surveillance and research, e.g. the relative

and absolute abundance of the class 1 integron-integrase gene (*intI1*) [39]. Using this approach, resistance levels in sediments of the Thames River catchment in the UK were correlated with the number, proximity, size, and type of surrounding wastewater treatment plants based on the *intI1* marker validated against culturable resistance [40]. Not all ARGs conferring resistance to a particular class of antibiotics respond in the same way to selection [41] and ARG abundance and diversity varies by sample matrix and region [20], so it may not be possible to determine specific surveillance targets *a priori*. Depending on priorities, ARB/ARGs can be selected based on their clinical importance or on their abundance and utility as markers of anthropogenic pollution. Individual targets need to be above detection limits but not be found everywhere at similar densities. Because of the extremely high diversity of ARB and ARGs, approaches that target multiple endpoints such as high throughput qPCR and metagenomics analyses are valuable.

Methods to quantify biological AMR targets

Robust quantification of AMR in environmental microbial communities is crucial to achieve any surveillance goal. Data should be generated focusing on specific ARB, ARGs, MGEs, and ecosystem backgrounds. Currently, surveillance of prevalence, diversity, and abundance of AMR in the environment is being undertaken in disparate ways by different academic and regulatory agencies and institutions, monitoring different environments for several different resistance endpoints using a variety of methods including culturing and genome analyses of specific species, single strain genomics, quantifying genes with qPCR and high throughput qPCR, and metagenome sequencing of environmental DNA. The AMR targets and methodologies used in research-driven surveillance are not generally standardised and are therefore difficult to compare. A coordinated, standardised approach to environmental AMR surveillance across sectors, environmental compartments and countries is desirable, with regular evaluation of the evidence, including innovative methods and validity of collated data. However, coordination and standardisation does not preclude the use of distinct surveillance methods in the environment where existing methods utilised in human and

409 animal sectors are not able to reveal trends within microbial populations. Currently, AMR in the
410 environment can be investigated using a number of different approaches, including culture-
411 dependent methods, culture-independent-methods, and combinations of these. Below is a summary
412 of these approaches.
413

414 Culture-dependent methods

415 Culture-dependent approaches involve growing bacteria, or a particular target bacterial group, on
416 agar plates or in nutrient broth. Isolating and characterising microorganisms from complex
417 microbiomes has been a challenge since the early days of microbiology, when the link between
418 microorganisms and infectious disease was being established. There are well-established methods
419 for culture-dependent investigations of AMR in clinical and veterinary settings used by EFSA and
420 ECDC for selected pathogens. The first attempts at standardising protocols for testing AMR in single
421 strains for environmental samples were made by adapting methods used in clinical/food samples
422 based on EUCAST guidelines [42]. The Tricycle method [15, 16] falls into this category and is well
423 suited for detecting and quantifying resistance phenotypes in target bacteria that are identified using
424 specific or chromogenic, selective growth media. In the latter case, the medium includes the
425 antibiotic cefotaxime, which allows a large number of bacteria to be screened on plates when the
426 phenotype is relatively rare.

427 An additional approach that has been widely used to characterise bacteria causing infections (where
428 only one organism is usually the cause of a given infection) is to isolate target bacteria on non-
429 selective media and subsequently undertake susceptibility testing on selected isolates (e.g., using
430 disk-diffusion methods) revealing detail of resistance phenotypes in commonly occurring strains.
431 This latter approach is unlikely to detect rare (and slow-growing) phenotypes as they are likely to be
432 outcompeted by faster growing species, in addition it cannot be used for accurate quantification of
433 AMR prevalence in bacterial populations or communities. Approaches using antibiotic selection and
434 those that focus on isolates cultured on non-antibiotic plates are useful for different purposes as

described. While culture-dependent methods provide valuable information on the viability of target bacteria and their resistance phenotype they do not elucidate the molecular or genetic mechanisms responsible for the observed phenotype, although subsequent genome sequencing gives identity of resistance genes and mobile genetic element carriage allowing mobility of resistance genes to be determined. Further, a tiny proportion of microbiomes can be grown in the lab at present. Culture-independent methods, several of which are described below, allow identification of resistance genes in bacterial communities and unculturable bacteria, and the potential mobility of genes.

Culture-independent methods

Methods that can be used in the absence of culture-based steps to provide quantitative or semi-quantitative data on single or multiple ARGs, host bacterial identity, genetic context, and expression are described.

qPCR methods

Quantitative PCR-based methods have been widely used to determine the prevalence of AMR genes in complex microbial communities. As the process involves the amplification of target genes, qPCR is well suited for quantifying known ARGs, including those present at relatively low abundance. qPCR can be employed to detect single genes, but the recent development of multiplexed, high-throughput qPCR methods allows the simultaneous measurement of hundreds of genes [43, 44], albeit with an increase in detection limit due to the smaller amounts of DNA in miniaturised reactions. Like all PCR-based methods, qPCR is limited by the quality of available primers and can hence only be employed for known resistance genes. As the detection limit and sensitivity are reasonably low (around 200 copies per gram of soil for example), qPCR provides one of the most suitable methods to quantify ARGs in microbial communities and to monitor changes in ARG abundance over time. Limitations include DNA extraction efficiency and possible taxonomic biases, variation in ARG copy number per cell, the presence of extracellular (eDNA), variation in PCR efficiency for different targets, issues of

specificity which vary by primer, amplicon detection methodology which can be improved through use of additional probes (ie. TaqMan vs SYBRGreen), and inhibition caused by co-extracted compounds present in environmental matrices such as humic acids [45]. Careful optimisation of protocols, including efficiency and inhibition controls, and use of environmental PCR master mixes designed to reduce inhibition can be used to refine data generated. High-throughout qPCR is now available commercially, including bespoke gene panels for environmental applications. In terms of identifying qPCR targets, a pragmatic approach can be used including representative genes conferring resistance to each antibiotic class [23], where the focus can be on clinically important resistance genes and/or those conferring resistance to antibiotics used in livestock production or other criteria [46]. Alternatively, ARG choice can be informed by metagenome sequencing to identify those genes that are abundant (or vary in abundance as a function of pollution, for example) in a given environment as these will vary in relative abundance depending on locality, matrix and pollution sources among many other variables. qPCR can also be applied to mRNA transcripts to quantify gene expression in the presence of antibiotics or other stressors [47].

Metagenomic (and metatranscriptomic) approaches

Metagenomic approaches involve sequencing environmental (metagenomic) DNA using next-generation sequencing methodologies. The sequences produced can be annotated by comparison to similar sequences existing in the databases or assembled to create longer sequences [48, 49]. Interest in sequencing technologies and the information they can provide on microbial communities has led to diverse innovative methods, including using different commercial DNA extraction kits, sequencing methods, and downstream analysis pipelines. Therefore, data generated using such different approaches may not be directly comparable. Standardised protocols for generating surveillance data that include sample collection, DNA extraction, sequencing and sequence analysis methods would improve comparisons and meta-analyses. For example, protocols and pipelines

were created by the COMPARE network (https://www.compare-europe.eu/library/protocols-and-sops) as part of the Global Sewage Surveillance Project [50].

Sequencing technologies have advanced considerably in recent years. Much of the historical bacterial genome sequencing used short-read technologies, which permit quantification of known genes including ARGs. However, short reads cannot always elucidate the genetic context of such genes with high confidence, for example, whether they are borne on mobile genetic elements, and the proximity of other resistance genes that may be expressed simultaneously. This has meant that the possible transfer and transmission of AMR from the environmental microbiome to human and animal microbial communities has been obscured and under-appreciated. Long-read sequencing technologies, such as Oxford Nanopore Technologies [51] and Pacific Biosciences [52], can be used to create scaffolds that drastically reduce the probability of chimeras. Genomic approaches utilising long-read sequencing technologies to sequence genomes, plasmids and other non-chromosomal MGEs can extract this information [53]. Often combined with short-read methodologies, long-read sequencing can improve our understanding of the evolution and dispersion of resistance between microorganisms and ecosystems by improving our understanding of ARG genetic context and allowing molecular epidemiological analysis at the MGE level [54].

Sequencing depth as determined by the quantity of DNA sequenced compared to the quantity of DNA extracted from different taxa has a direct relationship to the statistical interpretations made. Most metagenomic projects sequence a very small subsample of the extracted DNA. For example, if one assumes an average bacterial genome is ~5 Mbp, a metagenome of 5 Gbp would include 1,000 genome equivalents and a metagenome of 50 Gbp would include 10,000 genome equivalents, assuming that there was no redundancy. In a complex community in soil or sediment of 10^9 cells per gram and around 10^6 different taxa, sequencing 5 Gbp would sample 0.0001% of the community, although fragments from genomes with relatively high abundance would be sequenced more frequently. Recent estimates suggest that over 200 million reads (>50 Gbp) are sufficient to explore the full diversity of the main AMR alleles within a given population [55]. Therefore, metagenomic

approaches are useful to quantitatively determine the prevalence and diversity of AMR genes in highly abundant organisms and to construct scaffolds and contigs (sequences longer than one gene) to evaluate the immediate genetic context of ARGs.

Assembled sequences can also be used to refine the possible taxonomic affiliation of the microorganism carrying the identified ARG. This is performed either through the co-existence of a taxonomic marker (e.g., the gene that codes for the 16S rRNA) or by a taxonomic affiliation of several genes in the contig. The goal of many studies is to link functional AMR genes with their host bacteria using a phylogenetic anchor [56] or network analysis based on correlation of abundances. While recent advances using Hi-C genome and metagenome assemblies have been made [57, 58] that could be applied to achieve functional linkage of AMR and their host, short-read shotgun metagenomic sequencing remains a challenging data source for attributing resistance to specific taxa, which will be aided by more routine use of long-read methodologies. This will greatly aid the construction of metagenome-assembled genomes (MAGs) which are currently assembled from short-read sequences producing a representation of an isolate, or a chimera of closely related isolates [59], allowing the full detail of ARG/MGE/host relationships to be realised for difficult to culture taxa. A conceptual comparison of gene-based (metagenomic or PCR) versus isolate-based environmental surveillance with the objective of predicting the resistance situation in human population is presented by Larsson and Flach [46].

In addition to metagenomic approaches, metatranscriptomics can provide a snapshot of genes being expressed [60]. This requires the extraction of messenger (and possibly ribosomal) RNA from environmental samples. While the half-life of mRNA in microorganisms is in the order of hours, the detection of transcripts of ARGs provides immediate information about the resistance mechanisms employed at that moment.

Targeted metagenomic approaches

Targeted metagenomics approaches probe the diversity of single or multiple targets to identify genetic diversity, relative abundance, phylogenetic and/or genetic linkages in a given sample or environment. In targeted metagenomics, the gene(s) of interest are sequenced after amplification from extracted environmental DNA using PCR primers with a maximum level of degeneracy to still specifically amplify the greatest diversity of sequences for the gene of interest [61]. Consequently, targeted metagenomics is limited by the universality of primers chosen for the amplification step and has, due to the amplification step, a qualitative or relative quantification rather than a truly quantitative nature. The most common applications are for 16S rRNA to determine bacterial diversity, but applications have also focussed on resistance mechanisms and genetic platforms that contain ARGs [62].

Targeted metagenomic approaches can also utilise “emulsions, paired isolation and concatenation PCR (epicPCR)” [63] to investigate AMR gene context. In epicPCR, the target gene can be amplified while simultaneously linking it to a short fragment of the host cells 16s rRNA, which can be applied for ARG host identification from complex communities [64]. However, host identification in epicPCR is currently restricted to differentiation between genera rather than species, as only a short fragment of the highly conserved 16S rRNA gene is used for host identification. However, with technological advances allowing accurate sequencing of longer DNA fragments in the future, epicPCR could overcome these sequencing-based limitations and provide an accurate, quantitative, high-resolution surveillance tool to determine ARG host range and transmission from complex environmental communities.

Metagenomics can also be applied to DNA sequences from specific taxa or communities of bacteria using selective isolation. For example, Leonard et al. used a chromogenic medium containing cefotaxime to isolate ESBL-producing *E. coli* [29, 30] that were then sequenced to give a summary of all AMR genes in that specific *E. coli* sub-population.

Methods to detect unknown novel resistance genes

Environments at risk of pollution by selective agents are likely to be hotspots for the emergence of novel resistance genes. However, detecting the emergence and mobilisation of novel AMR genes is a difficult task, with most novel resistance determinants not currently recorded on metagenomic AMR databases. Hidden Markov Models (statistical methods also used in machine learning) are being developed to recognise resistance gene-like sequences and apply these to metagenomes for the identification of predicted novel resistance genes [65, 66]. In another approach, a novel AMR gene, present in clinical isolates, has been discovered for the first time by exploring the environmental microbiome, rather than through clinical emergence [67]. By taking advantage of a functional metagenomic approach (where metagenomic DNA is cloned into a susceptible experimental host to identify novel genes which may have no significant similarity to known ARGs) followed by sequencing of the cloned inserts and *in silico* filtering of known resistance genes to discover novel, mobilised AMR genes in class 1 integron cassettes, the garosamine-containing aminoglycoside resistance gene *gar* was detected in diverse clinical strains from wastewater samples across three continents [67]. Identification of emerging AMR from the environment before it reaches high prevalence in clinical settings could allow early surveillance, gene-based diagnostics and potentially inform mitigation strategies needed to limit spread.

Furthermore, metagenomic *in silico* strategies can be employed to identify AMR genes that are already highly mobile in environmental bacteria but are yet to emerge in human pathogens and might deserve specific attention. A framework to identify these AMR genes has recently been published which ranks ARGs based on anthropogenic enrichment, mobility, and host pathogenicity [68]. To achieve the goal of identifying emerging high-priority AMR genes from the environment, high-resolution surveillance (metagenomics, potentially including long-read sequencing and/or MAG assembly) is needed to distinguish homologous genes from within the same AMR gene family, as they pose different risks, and have varying host ranges and ecological distributions.

For high-resolution surveillance methodologies, it remains crucial to acknowledge that they are limited by the number of samples and tests that can be carried out. Those environments, such as anthropogenically impacted environments subject to microbial and chemical pollution, where we suspect there are high levels of AMR or where there is the greatest risk of the emergence of novel AMR (based on a probabilistic understanding of the drivers of AMR selection and evolution) should be sampled more frequently. Ideally, even less 'risky' environments should be subject to frequent surveillance to ensure that enrichment for AMR is not occurring through some unknown process. However, if only limited surveillance is possible, environments classed as high risk (high prevalence of AMR, highly mobile AMR, and/or subject to pollution by selective agents) should be prioritised.

Surveillance of risk factors for the emergence of AMR in the environment

The natural environment is an important reservoir and source of AMR determinants [69, 70] and genes conferring antibiotic resistance have been naturally present in microbial communities before anthropogenic antibiotic usage [71]. Research has shown a huge diversity of resistance genes in natural environments such as soil [72], but only a small fraction has emerged and been mobilised into human and animal pathogens. These genes can be mobilised from environmental to commensal and pathogenic bacteria [73], however an understanding of the conditions under which pathogens and commensals meet to exchange genes (whether this takes place in the human or animal gut or in the environment) is still poorly understood. Nevertheless, research has identified risk factors for the emergence and proliferation of AMR in different environments, which could be considered within an environmental framework to survey for the emergence of novel, or clinically important but rare resistance mechanisms before they mobilise and become fixed in human- or animal-associated pathogens.

Surveillance data can contribute to understanding of the processes that lead to the emergence of novel AMR genes, under which conditions they are enriched or persist in the environment, and where and when they are transferred from environmental bacteria to animal and human commensals and pathogens. Mobilisation of AMR genes into human and animal pathogens may be difficult to predict, with a small subset of these transfer events reaching fixation (stable maintenance within a bacterial strain, species or population). Even if we still do not understand many of the steps and processes leading to AMR emergence in human and animal pathogens, surveillance efforts will aid understanding of which drivers and conditions promote AMR acquisition in human pathogens.

Here we consider the risk factors shown to promote resistance, quantification of these known risk factors for the purpose of surveillance, as well as methods to detect unknown, novel resistance genes.

Antibiotics as selective agents

In most environments, introduction of AMR and pollutants with selective potential occur together, making it difficult to disentangle whether *in situ* selection occurs in the environment or if there is just simple dissemination of exiting AMR bacteria. Analysis of the relationship between AMR and human faecal pollution indicators, *intI1* or crAssphage, suggest that in many anthropogenically impacted environments, incidence of the dominant AMR bacteria can largely be explained by faecal pollution rather than selection in the environment [40, 74]. However, environmental selection of AMR has been shown to occur in environments receiving waste from antibiotic manufacturing. Such discharges can achieve antibiotic concentrations far above the minimal selective concentration or predicted no-effect concentration. In addition, existing risk assessment suggests that measured environmental antibiotic concentrations in environments impacted by municipal waste in HICs are above selective thresholds for some antibiotics in some environments [75, 76].

Laboratory studies on paired isogenic strains (identical strains with one carrying an antibiotic resistance determinant) have demonstrated that selection for AMR can occur at concentrations much lower than those preventing the growth of susceptible bacteria [77-79]. These studies highlight the importance of considering the minimal selective concentration in addition to the minimal inhibitory concentration for assessing risks associated with antibiotic concentrations in the environment. Further, predicted no-effect concentrations for resistance selection have been modelled using the lowest inhibitory values for single strains found in the public database of EUCAST [80]. In environmental settings, the presence of other microbial community members in high abundance [81, 82], spatial structure, predators or other compounds and metals affecting antibiotic efficacy [83-86] could significantly alter selection dynamics [87]. Recent experimental studies suggest that the prevalence of AMR in complex microbial communities [88-90] increases at concentrations as low as those previously suggested for single strain-based assays, which coincide with concentrations found in various, anthropogenically impacted environments (ranging from high ng/L to low µg/L). In addition, two very recent studies have characterised a new selective window that occurs below the minimal selective concentration [41, 91]. Although positive selection was not observed below the minimal selective threshold, a significant persistence (or a significant decrease in negative selection) of resistance genes [41] or resistant bacteria [91] was observed at certain antibiotic concentrations compared to when no antibiotics were present. Although this is less problematic than if positive selection occurs (i.e., enrichment), Stanton *et al.* (2020) recognised that the relative increase in total abundance of AMR in the environment might increase human exposure risk and probability of antibiotic gene transfer events driving increased risk of AMR evolution. The concentration where this phenomenon is first observed was defined as the minimal increased persistence concentration. Kraupner *et al.* (2018) proposed another argument for using this concentration as a basis for risk assessment; the minimal selective concentration represents the antibiotic concentration where benefit for the host negates the fitness costs of carrying the resistance factor. The minimal increased persistence concentration, on the other hand, represents the lowest concentration where a benefit is predicted or demonstrated, but where fitness costs are still larger. The critical aspect is that fitness

costs vary greatly with genetic context (including compensatory mutations), community composition and other biotic and abiotic factors, and the experimental system cannot consider more than a small fraction of those combinations (usually just one). The difficulty to predict fitness cost, and the apparently very low or close to insignificant fitness cost of widely circulating ARGs, could therefore motivate a risk assessment based on the lowest concentration shown to provide a reduced cost to resistant strains [91].

The fate of AMR bacteria and ARGs in the environment will also be a function of the physiology and ecology of the bacteria themselves, with different species behaving in very different ways in terms of growth, persistence, and horizontal gene transfer, with indigenous and introduced human- and animal-associated bacteria having very different fates. By combining metagenomic data including metagenome-assembled genome data (MAGs) with anthropogenic markers (*e.g.*, *intI1*, crAssphage) and chemical data, it may be possible to evaluate in which environments and under which conditions *de novo* AMR evolution occurs. However, this is still challenging, and currently experimental approaches are needed to determine causal relationships between AMR prevalence and low concentrations of selective compounds. As part of surveillance efforts, it is therefore imperative to evaluate antibiotic and other potentially selective compounds to determine where minimal-selective-threshold (or minimal persistence threshold) concentrations are exceeded as statistical association of AMR and antibiotic residues may not be sufficient to determine the potential for *in situ* selection.

Non-antibiotic selective agents

Pollution with non-antibiotic selective agents and materials such as heavy metals, biocides, disinfectants, plastic, non-antimicrobial pharmaceuticals, or plant protection products including fungicides, herbicides, and pesticides can contribute to environmental selection and transmission dynamics of AMR [92-99]. Non-antibiotic selective agents may indirectly select for AMR through cross-resistance or co-resistance (co-selection and co-regulation) [100-102]. Further, transfer dynamics of AMR plasmids can be immediately altered through exposure to non-antibiotic stressors

687 [103-106]. The co-existence of biocide and metal resistance genes together with ARGs (co-
688 resistance) primarily on plasmids isolated from bacteria colonising humans or domestic animals (not
689 wild animals or other environments) suggests antibiotics rather than biocides or metals are the main
690 driver for this co-localisation [101]. Regardless, when co-localised, selection pressures from either
691 metals, biocides or antibiotics would suffice to provide a selective advantage to such strains.
692 Pollution with metals is especially problematic as metals are highly persistent and toxic even at low
693 concentrations [107], providing conditions which are suitable for long-term AMR enrichment. In
694 certain environmental settings, heavy metals such as copper (Cu) and zinc (Zn) may exert stronger
695 selection than antibiotics for the selection of antibiotic resistance, as antibiotics are more degradable
696 and more likely to sorb to particulates so are less bioavailable in many environmental matrices such
697 as soil [108]. Conversely, Zn has been shown to reduce selection for ciprofloxacin resistance in
698 experimental evolution experiments, potentially through reduced availability of the antibiotic [109].
699

700 ## Methods to identify environments at risk of pollution by selective agents

701 Environments receiving pollution from human communities or activities that utilise large amounts of
702 selective agents are likely to receive complex mixtures of chemicals. Indirect measurement of
703 selective agent use, such as antibiotic prescribing, may be possible in some settings where antibiotic
704 use is regulated and documented. Global surveillance of AMU is needed to support policies to reduce
705 antibiotic consumption and the spread of AMR. Data on the anthropogenic usage of antimicrobials
706 and other selective agents (biocides, heavy metals, etc.) for clinical and agricultural applications is,
707 however, limited. Klein *et al*. estimated that antimicrobial consumption increased from 21.1 to 34.8
708 billion defined daily doses in humans per year between 2000 and 2015, taking advantage of the
709 IQVIA MIDAS database of antibiotic sales for 76 countries [110]. Currently, IQVIA is the only provider
710 of harmonised data on global antibiotic consumption, but it remains questionable how well sales data
711 reflect AMU globally and it is unlikely data will be as useful in estimating AMU in LMICs where data
712 are less accessible, and antibiotics may be donated in addition to being sold. An alternative AMU

surveillance system to confirm if global sales and usage trends correlate is needed to improve our understanding on how usage relates to changes in the human, animal and environmental resistome. Surveillance of AMU and AMR prevalence in the human microbiome at a population level can be achieved as part of an environmental AMR surveillance strategy through the use of sewage as a human AMR surveillance proxy. It is also possible to estimate AMU based on residue concentrations found in sewage. This would have the benefit of being independent of the collection of robust sales data, which is currently missing for a range of countries [111].

Direct measurement is also possible, though complex. Recently a novel method to support large-scale monitoring campaigns of pharmaceuticals on a global scale has been validated and employed to quantify 61 active pharmaceutical ingredients and their metabolites in aquatic environments [112, 113]. This powerful approach, taking advantage of a miniaturised sampling and shipping approach with a high-throughput and fully validated, direct-injection High-Performance Liquid Chromatography-Tandem Mass Spectrometry method, can overcome the high costs and limited accessibility to necessary equipment that currently stifle global data collection.

Temporal and geographic patterns in AMR/Sampling locations

Determining environmental AMR dynamics over time and by geographical region will be key to the implementation and assessment of potential mitigation strategies for AMR [22]. An adequate surveillance framework should recommend the frequency of testing, sample type, and geographic coverage to successfully survey critical changes in AMR in the environment, which will vary based on spatial and temporal variation in demographics, land use, climate, soil and water physicochemical properties, pollution, waste treatment, and variability of drivers within a given environment. Baseline surveillance studies will be needed to determine the optimal strategy to ensure surveillance produces meaningful data relating to potential drivers rather than simply describing natural variation over space and time. These sampling strategies are likely to be variable and may need to be established

739 locally by establishing baselines that are geography- and environment-specific. Strategies must be
740 informed and developed by starting surveillance activity and developing optimal strategies in an
741 iterative manner.
742

743 Choice of sampling location and rationale for focusing on high-risk
744 environments

745 One strategy in the selection of environments to include in surveillance is to identify those at greatest
746 risk of selecting for novel, or clinically important ARGs. Another strategy is to prioritise surveying
747 environments that pose the greatest risk to human health in terms of exposure and transmission.

748 The likelihood of acquiring ARB/ARGs from the environment by humans, animals, and their
749 microbiomes is an important and ongoing area of research. Recent evidence demonstrates that
750 humans are exposed to ARBs in natural environments and highly exposed populations are more
751 likely to be colonised by those ARBs [29]. The evidence on the probability of AMR transmission to
752 humans and/or animals depends on four main factors: i) the abundance of AMR in environments to
753 which humans are exposed; ii) the rate of bacterial uptake (ingestion, inhalation or skin/wound
754 contact) by humans in these environments; iii) the likelihood of AMR bacterial colonisation or survival
755 in the human host; and iv) the likelihood of ARG transfer to bacteria within the human microbiome.
756 There are complex processes governing these dynamics, and there are still data gaps in terms of
757 our understanding of the drivers of environmental transmission. However, effective mitigation
758 strategies may be implemented at key control points to prevent or minimise exposure and
759 transmission based on our understanding of probability of exposure and transmission relating to
760 environmental prevalence and identity of ARBs and ARGs.

761 A key component in assessing the risk of human exposure and colonisation from the environment
762 could be to integrate an AMR parameter in quantitative microbial risk assessment (QMRA) guidelines
763 [114]. However, this is hindered by a lack of empirical dose-response data for opportunistic

764 pathogens, let alone ARGs currently borne by commensals or environmental bacteria. Currently,
765 QMRA integrates absolute bacterial numbers rather than genes as a measurable marker. However,
766 strategies aimed at reducing AMR transmission require an understanding that AMR itself can
767 constitute a transmissible entity in its own right via mobile ARGs. Transmission is not, therefore,
768 necessarily tied to the life history of the pathogen or host bacterium of concern. Human and
769 veterinary medicine, related epidemiology and consequently policy documents have historically
770 relied on strain-based epidemiology, which has been invaluable in terms of understanding pathogen
771 transmission. Evidence suggests that strain-based epidemiology, even genomic approaches using
772 short-read sequencing technologies, has limited power in aiding understanding of AMR gene transfer
773 between bacteria and microbial populations, particularly the emergence of AMR from environmental
774 bacteria before becoming widely associated with key lineages of human and animal pathogens [115].
775 Recent efforts are focused on the use of long-read sequencing to resolve genomes and complete
776 plasmid architectures.

777 The fact that most human infections caused by AMR bacterial pathogens occur through human-to-
778 human transmission of a relatively small number of epidemic, human-adapted strains has often been
779 used to counter arguments considering the importance of the environment. However, this view
780 ignores the fact that the ARGs and MGEs are mobilised between bacterial strains, species, genera
781 and even phyla, and, in many cases, the ARGs carried by these previously susceptible clinical
782 pathogens are likely to have an environmental origin. In addition, some methods used to infer
783 pathogen transmission are based on strain identity, with human-to-human transmission assumed for
784 human adapted strains. However, sewage and treated wastewater contain high numbers of human
785 adapted pathogens. Environmental transmission of human faecal pathogens and commensals is
786 likely to be included within human-human transmission so underestimating the magnitude of
787 environmental AMR transmission.

788 Rare AMR emergence events (analogous to emergence of zoonotic infection) are difficult to predict.
789 However, as for emergence of zoonotic infections such as SARS-CoV-2, there are processes that

increase the probability of emergence. These include the selection for AMR in specific ecosystem compartments and processes that disseminate or transmit AMR between environmental and human or animal microbiomes. It may not be possible to predict when or from where a specific AMR gene will emerge in a human or animal pathogen, but we do know that the magnitude of selection and the opportunities for transmission will determine the probability of emergence events.

Sample substrates

Water

Depending on the specific objective(s), water sampling could focus on water environments most likely to contain ARB/ARGs and antibiotic residues, and/or on water environments likely to be involved in large-scale transmission of AMR to humans and animals. Those environments most likely to contain anthropogenic AMR include effluents from wastewater treatment plants, hospitals, pharmaceutical production sites, farms, abattoirs, run-off from agricultural land with grazing animals or fertilised with manure/sewage sludge-based fertilisers, and water around aquaculture facilities. Receiving waters, especially those where people or animals are likely to be exposed, should be sampled to assess environmental AMR as part of understanding exposure risks. This includes groundwater, irrigation water, surface water (near informal settlements or slums which are regularly set up near surface waters in LMICs), human/animal drinking water abstraction points, recreational bathing waters, and aquaculture sites including shellfish production areas which might present increased transmission risk. Drinking water should also be subject to AMR surveillance as even in HICs with up-to-date drinking water treatment, bacteria can enter treated water through faults in distribution systems and through regrowth after treatment, leading to exposure. These high-risk water types should also be viewed in the context of unbiased baseline surveillance data including unimpacted localities and samples, although resource constraints may lead to prioritisation based on probable risk.

Aquatic pollution is weather and season dependent. High rainfall is associated with major pollution events from land run-off and subsurface flow, and untreated sewage discharges will increase with sewage overflow discharges. Low rainfall is associated with lower dilution and thus higher concentrations of pollutants in rivers, with increased probability of *in situ* selection due to higher concentrations of selective compounds. Temperature is also important in terms of bacterial growth which may be greater for human- and warm-blooded-animal-associated bacteria at high temperatures, although persistence may be greater at lower temperatures.

Sewage

Wastewater-based epidemiology (WBE) is an emerging field, and the power of interrogating untreated sewage to determine levels of AMR in human populations at a global scale has recently been demonstrated by members of the Global Sewage Surveillance Project [20, 50]. This project coordinated standardised sewage sampling across 79 locations globally and developed standard DNA extraction and metagenomic analyses protocols in their surveillance efforts to quantify AMR gene diversity and abundance in human communities. They concluded that ARG diversity and abundance are highly variable by region (N.B. one reason why it's difficult to identify a universal panel of AMR surveillance targets) and that socio-economic, health, and environmental factors, rather than AMU and bacterial diversity, were the best predictors of AMR prevalence and diversity. There has been much discussion that WBE capacity developed for SARS-CoV-2 surveillance can be adapted for AMR; although the latter is more complex, the methodologies utilising qPCR and metagenomic approaches to characterise variants overlap with approaches used to characterise AMR.

Whilst sewage is thought of as an environmental matrix, it is of human origin: AMR WBE should not be conflated with environmental surveillance and is not a substitute for surveillance described within this report. However, as it utilises the same approaches and can contribute understanding of AMR

842 in sewage which enters the environment in many regions, including where treatment is widespread,
843 it is important to consider alongside environmental surveillance.
844
845 ## Soil/sediment
846 Sediment samples should, like the water body they are part of, be chosen based on their exposure
847 to anthropogenic pollution through AMR bacteria or selective compounds. Bacterial communities in
848 sediment and the water column differ, with numbers of bacteria in sediment orders of magnitude
849 greater per ml or gram of substrate than water. In addition, AMR in sediments may more accurately
850 represent the pollution characteristics of that specific site, as sediment is less transient than water.
851 For soils, those directly impacted through fertilisation with animal manure or sewage sludge from the
852 wastewater treatment process deserve increased attention. Due to sorption of antibiotics in soils and
853 sediments it is more difficult to establish bioavailable fractions of antimicrobials which might drive
854 selection. This should be considered in the interpretation of chemistry data and methods should be
855 further developed to establish bioavailable fractions of selective compounds.
856
857 ## Air
858 Microorganisms in the atmosphere are critical signatures of local environments and wind conditions
859 [116]. Air samples, especially those taken near environments with high AMU (i.e. healthcare facilities,
860 farming operations) or high AMR levels (such as wastewater treatment plants) could reveal
861 information on exposure risk to airborne AMR in surrounding populations [117]. The Environment
862 Agency in the UK recently published a review on airborne antimicrobial resistance [118].
863
864 ## Wildlife
865 Wild animals are well known to carry ARB and ARGs, with many studies focusing on ESBL and
866 carbapenemase-producing Enterobacterales, and there are several recent reviews [119, 120]. There
867 is also an indication that AMR carriage appears more associated with animals concomitant with

urban environments and that it mirrors the occurrence in human populations. Particular focus has been given to migratory birds, but also to other animals with large migratory ranges, as these might have the potential to spread AMR globally [121-124]. Meanwhile, animals with restricted or fixed-living habitats have been suggested to give insights into local environmental levels of AMR [125, 126]. A JPIAMR surveillance network focused on wildlife, the environment, and AMR: "Wildlife, Agricultural soils, Water environments and antimicrobial resistance - what is known, needed and feasible for global Environmental Surveillance (WAWES)". A high-profile recent example of the role of wildlife in AMR evolution and transmission is represented by the discovery that a fungal skin commensal in hedgehogs selected for specific strains of methicillin-resistant *Staphylococcus aureus* (MRSA), which have subsequently been transmitted to domesticated animals and humans [127].

Recommendations for a comprehensive/minimum surveillance strategy

A comprehensive environmental surveillance strategy could:

- Address questions that can be informed by surveillance using appropriate methodologies and technologies enabling risk characterisation and management (e.g., culture-independent methods to study population level trends vs culture-dependent pathogen surveillance to estimate human exposure risk to extant AMR pathogens).
- Determine optimal sampling strategies to determine baseline and trend data in given geographies/environments to ensure surveillance produces meaningful data relating to potential drivers rather than simply describing natural variation over space and time
- Consider transmission routes via water, soil, air, crops, and wildlife.
- Link to existing environmental surveillance and monitoring where synergies exist.
- Leverage cross-disciplinary techniques to bring increased value in methodology and application of novel technologies.

- Use culture-based methods aligned with clinical and veterinary surveillance for priority pathogens with resistance to clinically important antimicrobials, e.g., Tricycle ESBL *E. coli* recommended by the WHO.
- Undertake DNA culture-independent surveillance of metagenomes/microbiomes including, but not limited to, characterising bacterial diversity, ARGs, and MGEs to inform drivers of AMR dynamics and emergence.
- Include the surveillance of antimicrobial residues (and potentially other pollutants that may select for AMR).
- Include comprehensive metadata and physicochemical sample analyses.
- Be adaptable to the local/regional conditions, while also using a similar set of tools, to be globally comparable.

A minimum strategy could include:

- Surveillance of AMR hotspots as potential sources and areas with a high probability of transmission from the environment to humans and livestock to assess transmission risk.
- Sample sewage, wastewater effluent, water, sediment, soil, and wildlife.
- Include targets common to existing clinical and veterinary surveillance (e.g., ESBL-producing *E. coli* in the Tricycle project), together with limited markers of anthropogenic pollution/AMR (e.g., class 1 integrase).
- If resources allow, baseline culture-independent approaches should be used to assess a specified panel of locally relevant AMR targets.
- Include spatial and temporal metadata and available sample analysis e.g., temperature, pH, and water chemistry variables.

N.B. Wastewater-based epidemiology (WBE) has received increased attention because of its use for population level surveillance of SARS-CoV-2 during the COVID-19 pandemic. Whilst analysis of

sewage can be considered part of an integrated AMR surveillance programme, WBE focusing on analysis of treatment plant influent should not be considered a substitute for environmental AMR surveillance as it does not inform on risk of environmental exposure/transmission or the potential for AMR evolution in natural environments.

Barriers and Solutions to Implementation of global environmental AMR surveillance

When developing a global, environmental AMR strategy, there are several different barriers to implementation to consider. These barriers, discussed in more detail in the following section, can be financial, logistical, ethical, political, academic, cultural, or legal in nature. Barriers will differ by nature and extent in different countries, and any recommended surveillance strategy that wishes to overcome these barriers would require a clear and strong case for the proposed approaches, articulating the benefits of using them and the drawbacks of not using them.

Creating awareness for environmental surveillance of AMR and integrating it into policy and regulation

Despite the relatively short history of clinical use (~80 years), the availability of antimicrobial drugs to treat infections is taken for granted, making AMR a low-priority issue in terms of public and stakeholder perception. With the risks involved in infectious disease outbreaks currently highlighted through the COVID-19 pandemic, it would be valuable to underpin in policy briefs to policymakers that untreatable drug-resistant infections could similarly a) pose severe risks to societal health and wealth, being a ‘slow-motion pandemic’, and b) emerge from the environment before transmission to humans. A One Health systems approach to the AMR crisis is urgently needed, as most existing national action/surveillance plans do not adequately incorporate the environmental dimension of AMR. Due to the lack of legislation and regulation, major actors such as environmental regulators and water companies are not always aligned to current thinking around AMR and are in turn

constrained by their existing duties, powers, and funding. Most environmental legal and regulatory frameworks deal with safe limits of pollution by coliforms, substances, or quality parameters (e.g., nitrogen, phosphorus, BOD) rather than microorganisms or ARGs that may be amplified in the environment. However, there is an urgent need to integrate AMR into existing policy or regulation across sectors avoiding traditional silos around health, agriculture, and environment. This would avoid new primary legislation, which could cause serious delays. As jurisdiction differs between countries and sometimes even within countries in federal states, policy briefs on environmental AMR surveillance strategies to stakeholders should be adapted based on the individual local realities and be provided in multiple different languages.

Tackling AMR in a multidisciplinary One Health approach

Tackling or mitigating global problems of the magnitude of AMR requires expertise in a variety of different scientific, technical and political fields. To develop a global, environmental AMR surveillance strategy, experts from these distinct technical areas need to work together. These involve, but are not exclusive to, clinicians, veterinarians, public health experts, microbiologists, molecular biologists, bioinformaticians, mathematical modellers, environmental scientists, engineers, epidemiologists and data scientists, but also policymakers and representatives from service delivery bodies. At present, research into environmental aspects of AMR has been largely confined to individual institutions or academic laboratories with single areas of expertise. This has led to surveillance efforts being the by-products of research projects. Accordingly, establishing multidisciplinary networks that facilitate these currently rare interdisciplinary, collaborative efforts is crucial to further develop environmental AMR surveillance and translation to policy. Moreover, integrating different areas of expertise allows development of a common level of communication and expert language in strategic and policy documents. This will allow development of surveillance programmes that are cost effective, feasible and action-driven where costs and benefits are estimated facilitating investment into One Health

AMR surveillance. These proposals can then be scrutinised through global public consultation. This could be facilitated through activities such as the forthcoming JPIAMR One Health AMR Partnership.

Overcoming a limited evidence base

The complexity of the processes involved in AMR evolution and transmission dynamics, including gene transfer and emergence in and mobilisation from the environment, makes it difficult to distinguish between correlation and causation between environmental and clinical/veterinary settings. While studies have been able to demonstrate correlation of environmental exposure events with colonisation by AMR organisms [30, 128, 129], and sequence analyses of environmental bacteria and clinical pathogens have provided evidence of an environmental origin of many clinically relevant ARGs, this is often regarded as circumstantial evidence. Despite these limitations, the major global organisations dealing with human and animal health (WHO, UN, ECDC, EFSA) appreciate the environmental dimension of AMR as one of the crucial pillars to mitigate the global AMR crisis. This is illustrated by the inclusion of UNEP in the Quadripartite Collaboration for One Health in 2022. However, without the emergence of an irrefutable evidence base linking the environment to clinical and veterinary treatment failure due to AMR pathogens, the environmental dimension of AMR and hence the set-up of a global, environmental surveillance framework may not receive the high-priority levels needed to engender local (national/regional) policy, infrastructure investments, and research funding. Consequently, a higher degree of risk assessment and basic research studies are still needed to provide such an evidence base, although debates around the necessary levels of evidence versus the precautionary principle are emerging. Funding for these risk assessment studies and their communication through policy briefs should be made a priority investment to further the development of a truly One Health focused, global, AMR surveillance strategy that includes the environmental dimension.

Inadequate resources for environmental surveillance of AMR

At present the global, national, and regional economic budgets and funding for environmental AMR surveillance are limited. If funds are available for tackling AMR, they rarely prioritise the environmental dimension of the problem. It has been suggested that environmental surveillance should utilise locally (national or regional) available capacities in, for example, veterinary labs. However, this may represent an oversimplified view of what environmental surveillance should and can deliver, since the methods needed (e.g., genomic and metagenomic techniques) to answer some of the questions outlined above far exceed those mainly utilised in current veterinary surveillance programmes targeting AMR in clinically relevant indicator species. To generate the necessary resources and funding for environmental surveillance, its purpose, importance, urgency and need for prioritisation need to be clearly explained in policy briefs to the relevant stakeholders in governmental and budgetary positions. New and existing programmes will need to be leveraged to create a resource base for an environmental AMR surveillance programme. An example of this is the UK government AMR surveillance pilot project, PATH-SAFE https://food.blog.gov.uk/2021/11/23/path-safe-tracking-foodborne-pathogens-and-antimicrobial-resistant-microbes/, which was in part informed by discussions within this JPIAMR network that included government representatives leading the PATH-SAFE project. Environmental AMR surveillance could, for example, be included in funded programmes on water, hygiene, and sanitation (WASH, good animal husbandry or biosecurity) and contribute to delivery of Sustainable Development Goals associated with AMR.

Including low- and middle-income countries in environmental AMR surveillance

For an environmental AMR surveillance strategy to gain global traction, it needs to consider LMICs which may not have well-developed clinical and veterinary surveillance programmes. This includes financial and technical limitations that hinder successful implementation as well as taking into account the different environmental routes of AMR proliferation, for example, lack of infrastructure

for the collection and treatment of sewage. In these countries, surveying sewage treatment plants might be far less impactful compared to sampling direct environmental compartments where much of the human faecal load is disposed. However, wastewater-based epidemiological approaches focusing on sewage would inform on AMR in the human population, including in clinical and veterinary settings, whereas surveillance of environments receiving untreated sewage would inform on transmission risk. Hence, to globally implement a surveillance strategy, country-, economy-, and region-specific surveillance and research priorities need to be considered, rather than an attempt at a one-size-fits-all approach. This will require international engagement with the development process to ensure an integrated, system-based approach to surveillance is achieved.

When settling on standard surveillance targets and methods, it remains important to consider the trade-offs that might be needed to align with country capacity, although it is possible that some approaches could be delivered in collaboration with other countries where capacity exists. While there might be a best-possible set of targets and methods, these need to align with a potentially lower technical and financial feasibility and/or capability in LMICs. Technical restrictions on recommending the best methods might however be naturally overcome as the increase in technical capacity in LMICs will most likely progress faster than translation of an environmental surveillance strategy into policy and regulatory documents. To set up one reference laboratory capable of producing the surveillance data based on the recommended protocols per country, significant investment in human and technical capital development on environmental surveillance in LMICs is needed. This should include increased opportunities for mutually beneficial networking of scientists at all career stages in LMICs to foster the critical knowledgebase around the topic and the importance of environmental AMR surveillance.

Standardising surveillance protocols across countries

Environmental surveillance efforts currently lack systematic protocols on the environments tested, methodology, targets and frequency of sampling, data analysis and interpretation, and though

surveillance systems with standardised protocols exist for clinical and veterinary settings, these do not translate well to environmental matrices or surveillance questions. The JPIAMR Embark project includes protocols for elements of AMR surveillance in the environment (https://antimicrobialresistance.eu/).

To attain global surveillance coverage across different countries, a standardised environmental AMR surveillance framework and toolkit needs to be developed, consisting of protocols and SOPs for different methods encompassing sample sites, sample sources, laboratory processing, data analysis and data interpretation for action. This requirement should be balanced against availability of specific reagents or facilities within countries and not be so restrictive that it impacts participation or innovation. These tools should not only be appropriate to achieve surveillance goals but also be robust to potential logistical challenges of transporting samples over long distances at high temperatures without access to cold storage. Investment in infrastructure to facilitate storage and cold-chain transport might be necessary, as otherwise the number of environments, sampling frequency, and performed analysis methods would be limited. Alternatively, complementary methods to stabilise or analyse samples in the field are needed.

To promote implementation of environmental AMR surveillance globally, surveillance protocols need to clearly communicate environmental, societal, and health benefits as well as potential actions that can be taken based on surveillance data. Ideally, recommended protocols need to be specific enough for straightforward implementation into regulatory documents and guide, for example, water treatment practices, prescription guidelines, and agricultural practices.

Conclusions

There is evidence that the environmental dimension of AMR is increasingly being recognised globally [130], with recent inclusion of UNEP in the Quadripartite Collaboration for One Health alongside the WHO, FAO and WOAH, and a commitment at UNEA 5 to establish a Global Science-Policy Panel

on Chemicals and Waste which would include antimicrobials and AMR within its remit. There is growing interest in environmental AMR surveillance and this report attempts to provide a rationale for surveillance, describing how it can inform on multiple processes from wastewater-based epidemiology focusing on AMR within human populations, to AMR evolution and emergence in polluted environments and assessing AMR transmission risks through different environmental matrices and associated human activities. Whilst it is not possible to recommend a concise panel of AMR surveillance targets due to geographical variability, environmental heterogeneity, differences in microbial diversity, and variation in pollution source and fate, we have summarised current knowledge in the field and approaches that can be used to undertake surveillance that can inform our understanding of AMR dynamics within and across One Health compartments. Elements of the toolkit described can be used to investigate the complex role of the environment in the AMR pandemic as and when resources allow. Over time this would generate a comprehensive surveillance strategy that, when combined with clinical and veterinary surveillance, would reveal a full picture of AMR across One Health sectors.

There is an urgent need to continue to communicate the importance of the environmental dimension of AMR to the wider AMR scientific community and beyond to stakeholders and policymakers. Only when the fundamental contribution of the environment to the emergence and transmission of AMR is appreciated will resources be prioritised for environmental AMR surveillance. There is a call for further evidence on the role of the environment in contributing to AMR infection in the clinic, and data should continue to be generated to address knowledge gaps. However, it should be remembered that there is also great uncertainty regarding the drivers of AMR human infections, with a poor correlation between AMU and AMR in many parts of the world [131]. We know that transferable resistance genes did not necessarily evolve in the clinic and also that the human microbial resistome is intimately linked to human and environmental microbiomes. Smith *et al.* commented on the nature of evidence surrounding AMR in agriculture and links with human health, concluding that AMR is inherently complex and that decision-making should be informed by uncertainty generated by

biological complexity [132]. The same is true of the environment, and this knowledge should underpin understanding of the value of environmental surveillance to facilitate understanding of AMR as a One Health phenomenon.

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Competing interests

Sabiha Essack is Chairperson of the Global Respiratory Tract Infection Partnership (GRIP) and member of the Global Hygiene Council (GHC), both sponsored by unrestricted educational grants from Reckitt, UK.

Appendix 1: antifungal resistance

Fungal infections account for more than a billion skin infections, hundreds of millions of mucosal infections, tens of millions of serious allergies and well over a million deaths due to invasive infections each year [133]. Collectively, fungal infections kill more people than malaria and similar numbers as those succumbing to tuberculosis and HIV. *Candida* spp., *Aspergillus* spp., and *Cryptococcus neoformans* are the main fungal pathogens causing life-threatening invasive infections. Delayed clinical presentation and diagnosis is driving mortality rates. On the other hand, the high mortality and the historic difficulties in diagnosing these infections have led to an overuse of empirical and prophylactic antifungal therapy associated with increased antifungal resistance development. Acquired resistance to antifungal drugs has become an increasingly important clinical problem and some fungi are inherently resistant to commonly used antifungals [134-138]. Unpredictable,

emergent resistance is mostly due to induced changes and mutations that lead to over-expression in drug efflux pumps or drug targets, or due to mutations that result in target modification. Changes in chromosome karyotype – aneuploidies or loss in heterozygosity can also lead to changes in drug sensitivity by increasing the copy number of drug pump transcriptional regulators as has been particularly noted in *Cryptococcus*. In some cases the fungus can adapt to the challenge of an antifungal by modifying its cell wall composition and a multitude of different factors have been implicated in the greater resistance that yeast cells embedded in biofilms display as compared to planktonic cells. However, unlike for bacteria, there are no recorded instances of plasmid-mediated transfer of antifungal resistance genes. It is also recognised that fungal strains may exhibit drug tolerance, whereby some cells remain susceptible to the drug but induce mechanisms that enable them to survive and grow slowly, thereby retaining the ability to seed recurrent infections after drug treatment has ended and to survive to acquire mutations leading to drug resistance [138].

Antifungal agents in clinical practice

Several classes of antifungal agents are used clinically but the arsenal of drug options to treat invasive fungal infection is relatively limited and cross-resistance within some drug classes is common [139, 140]. The first antifungals to be developed were the polyene macrolide antibiotics, exemplified by amphotericin B, which is a broad spectrum antifungal used in the treatment of systemic fungal infections, and nystatin and natamycin used as topical agents. Amphotericin deoxycholate is nephrotoxic but lipid carrier formulations such as liposomal amphotericin B (AmBisome) offset this toxicity.

Flucytosine or 5-fluorocytosine (5-FC) is a chemically synthesised inhibitor of fungal RNA and DNA synthesis. Emergent resistance is commonly associated with the use of 5-FC monotherapy and therefore it is normally used in combination with amphotericin B, which is the first-line treatment for cryptococcal meningitis.

The imidazoles and triazoles (known as the 'azoles') are the largest class of antifungals used in the treatment of fungal infections. These agents inhibit fungal sterol biosynthesis by inhibiting cytochrome P450–Erg11p (Cyp51p) that is encoded by the *ERG11* gene. These are chemically synthesised and modifications of the core triazole ring has created a family of antifungals with differences in their spectrum of activity and bioavailability. Fluconazole is active against yeasts, and used in the treatment of candidiasis and cryptococcosis but is not usually effective against filamentous fungi, and intrinsic or acquired resistance is common in some species such as *C. glabrata, C. krusei* and *C. auris*. Itraconazole, voriconazole, posaconazole, and isavuconazole have activity against both yeast and Aspergillus and other filamentous fungi, including the endemic mycoses. Voriconazole and isavuconazole are first-line treatment options for invasive aspergillosis. The azoles have the advantage of being available both in an oral and IV formulation. Multiple drug-drug interactions (azoles are metabolized by CYP450 isoenzymes) and the need for therapeutic drug monitoring complicates the use of these antifungals.

The third major class of antifungal are the echinocandin antibiotics, of which caspofungin, micafungin and anidulafungin are well established in clinical use and rezafungin and ibexafungerp are in final phase clinical trials. Echinocandins (IV only) are first-line treatment for invasive candidiasis. In vitro, echinocandins are fungicidal against *Candida* spp., resulting in lysis of the fungal cell due to cell wall weakening, usually only fungistatic against *Aspergillus* spp., and have no activity against *C. neoformans*. Currently available echinocandins have to be administered daily by IV infusion but rezafungin has a substantially longer half-life and therefore requires less regular dosing, and ibrexafungerp is active after oral administration. These are natural products and they target β, 1,3 glucan synthase (Fks1p) – an essential enzyme required for the synthesis of β, 1,3 glucan − a highly conserved component of the fungal cell wall. Resistance occurs by mutations in known hotspots of the *FKS1* gene.

Griseofulvin (a mycotoxic product of *Penicillium* spp. that inhibits microtubules and thereby mitosis) and the allylamine terbinafine (a squalene epoxidase inhibitor that targets ergosterol synthesis) are used topically against dermatophyte infections.

Drug resistance in the clinical setting

Clinical resistance to antifungal drugs can be related to both intrinsic and acquired resistance following patient exposure. There are well-established and predictable patterns of innate *in vitro* resistance to one or more antifungal agents associated with many pathogenic yeast and mould species [141-144]. Emerging, newly recognised fungal pathogens such as *Lomentaspora prolificans*, *C. auris* and a number of the so-called black fungi have shown resistance to all three classes of systemically available antifungals, resulting in some infections being essentially untreatable [143, 145]. Acquired resistance to amphotericin B is extremely rare, although intrinsic reduced susceptibility of different species can exist. For example, *Aspergillus terreus, C. auris, Scedopsporium* and *Arthroderma* strains are often less susceptible to this antifungal due to lower levels of ergosterol or mutations in the ergosterol biosynthetic pathway.

Clear examples of acquired resistance include the selection of antifungal resistance in *Candida* species due to the extended use of echinocandins, and the development of azole resistance in about 20% of *Aspergillus fumigatus* isolates during prolonged treatment for chronic aspergillosis [137, 146]. Clinically associated azole resistance mutations are predominantly described to be within *cyp51A*. Specific non-synonymous point mutations in *cyp51A* have been shown to confer azole resistance by altering the structure of the ligand entry channel, which impacts azole docking [136]. Transcription factor mutations can lead to the upregulation of drug efflux pumps such as *MDR1*, *CDR1,* and *CDR2* or to upregulation of *ERG* genes in the ergosterol biosynthetic pathway [134, 135, 138].

Fungal AMR and multidrug resistance is recognised in some species such as *C. glabrata, C. auris* [147], and some filamentous fungi (below), and there are examples of infections that are currently

essentially untreatable. However, there is some hope from two new classes of fungal agents with unique fungal targets currently in clinical trials: orlorofim, an orotomide, which targets a key enzyme in the pyrimidine biosynthesis pathway, and fosmanogepix, which inhibits the fungal Gwt1 enzyme (a GPI-anchored wall transfer protein 1). Both are active against organisms that have developed resistance to other drug classes.

Well-recognised, difficult-to-treat organism groups include the *Mucorales* that cause infections that are rapidly progressive and responsible for high mortality; all are resistant to fluconazole, voriconazole, flucytosine and the echinocandins, and they show variable species-specific susceptibility to the remaining systemic agents [143, 148]. *Fusarium* species, which cause sight-threatening keratitis in contact lens wearers and less frequently disseminated infection in haematology patients, are often resistant to amphotericin B (34% isolates), demonstrate resistance to most azole drugs and variable susceptibility to voriconazole, which nevertheless currently remains the best systemic treatment option [143, 149]. The list of potential pathogens has increased in line with the increase in the pool of susceptible patients, as has the finding of reduced susceptibility or resistance to one or more classes of agent in these emerging pathogens. Amongst them is *Lomentospora prolificans,* for which 100% of isolates are resistant to most of the currently available systemic agents [143] and infection is associated with very high mortality. In addition there has been the emergence of new, previously unrecognised species, such as the yeast *Candida auris,* which has caused numerous, difficult-to-control nosocomial outbreaks globally and is almost always resistant to one, and often several, classes of antifungal agent. A recent publication on isolates of *C. auris* from candidaemia cases in COVID-19 patients reported resistance to fluconazole (100%), flucytosine (60%) and amphotericin B (40%), with 70% of isolates classed as multidrug resistant (resistant to two or more classes of agent) [145].

A recent worrying development has been the emergence of resistance in strains of *A. fumigatus* to azole drugs widely used in agriculture, leading to cross-resistance to one or more of those used in clinical practice, thus potentially compromising a first-line treatment option for invasive aspergillosis

[150]. Environmentally acquired resistance is predominantly due to a single resistance mutation in the target of azoles Cyp51A, TR34/L98H. This mutation is a 34 bp tandem repeat in the promoter region of *cyp51A* with L98H within *cyp51A*. This confers itraconazole resistance by inducing overexpression of *cyp51A* [151, 152]. In addition, other tandem repeats have been reported in association with a range of *cyp51A* single nucleotide polymorphisms including L98H, Y121F, M172I, T289A and G448S [153]. Sexual reproduction is hypothesised to play a role in the development of these resistance mechanisms, by the generation of genetic diversity via meiotic recombination [150]. Azole-fungicide-containing compost heaps are thought to be one site for the development of resistance [153].

In a recent study in COVID-19 patients with suspected pulmonary aspergillosis, 3/46 (6.5%) isolates were resistant to voriconazole and displayed the relevant mutation associated with development of resistance to environmental azoles [154]. Moreover, there are alarming reports of extensive superficial cutaneous infections due to multidrug-resistant strains of the dermatophyte *Trichophyton mentagrophytes* rapidly spreading through India and beyond [155].

Environmental surveillance for antifungal resistance

Because some of the recent resistance in clinical *A. fumigatus* strains appears to have been driven by the use of azole antifungals in the environment, or more speculatively environmental accumulation of environmental or clinical azoles in the case of fluconazole-resistant yeast such as *C. auris*, it may be helpful to perform environmental surveillance. It is possible that some yeasts have an aquatic reservoir as do *Scedosporium, Exophiala* and *Lomentospora* species, and it is well known that some of the harder-to-treat infections such as those with *Fusarium* species are primarily plant pathogens.

One of the best methods for the high-throughput screening required for large numbers of environmental isolates has been the introduction of commercially prepared plates in which dilutions

of the test agents are incorporated into an agar base and then inoculated with the test strains straight from environmental isolation plates without the need for sub-culture. Plates with four wells containing different breakpoint concentrations of three antifungal agents, itraconazole 4.0 mg/L, posaconazole 0.5 mg/L, and voriconazole 1.0 mg/L, and a control well have been developed and validated in a multicentre study (VIPcheck, Nijmegen, Netherlands) [156]. Growth on only the control well suggests an azole-susceptible isolate, whereas specific resistance to one or more agents can be detected by growth on drug-containing wells. Such methods have already been employed in several studies of the incidence of azole-resistant *A. fumigatus* in the environment and could equally be applied to other moulds and yeast provided the correct antifungal breakpoints are employed.

Molecular methods using *Aspergillus*-specific commercial PCR methods such as AsperGenius™ and MycoGENIE™, which detect the known resistance mutations associated with environmental azoles, can be used as a quick confirmatory test on cultures [157]. Because fungi do not acquire resistance genes through HGT as bacteria do, sequencing-based gene-targeted methods widely used in research on antibiotic resistance in natural and farmed environments are not applicable.

A recent paper documented approaches used to investigate antibacterial resistance evolution from an environmental perspective that could be applied to the understanding of antifungal resistance evolution in the environment and whether environmental antifungal residues are likely to drive selection for antifungal resistance [158].

Conclusion

Antifungal drug resistance is a significantly problematic aspect of patient care. Resistance exists to all of the major classes of antifungal agents although resistance to polyene macrolide antibiotics is rare. The use of agricultural azoles is likely to be driving the emergence of drug resistant strains of *Aspergillus* in the clinic and emerging species with multiple intrinsic or acquired resistance traits is leading to examples of invasive infections that are highly recalcitrant or resistant to multiple classes

of antifungal agents. Therefore there is a great need for the development of new classes of antifungal agents to tackle these emerging threats and for more focused research on the problem of antifungal AMR and drug resistance including environmental surevillance strategies.

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