Application of genomic technologies to measure and monitor antibiotic resistance in animals

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Abstract

Animal intestinal microbiota is one of the richest reservoirs of antibiotic-resistant bacteria and genes, contributing to the spread of antibiotic resistance in the environment and potentially into human pathogens. Both culture-based genomic technology and culture-independent metagenomics have been developed to investigate the abundance and diversity of antibiotic-resistance genes. The characteristics, strengths, limitations, and challenges of these genomic approaches are discussed in this review under the context of antibiotic resistance in animals. In addition, we discuss the advances in single-cell genomics and its potential for surveillance of antibiotic resistance in animals.

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Introduction

Since the introduction of sulfonamides and penicillin into medicine,^{1,2} antibiotics have contributed significantly to the control of bacterial infectious disease.³ However, the increasing emergence and spread of antibiotic resistance have hampered infection therapies by antibiotics, leading to numerous infectious death and huge costs of health care.⁴ Thus the World Health Organization (WHO) is developing a 5-year Global Antimicrobial Resistance Surveillance System (GLASS) to support the Global Action Plan on Antibiotic Resistance,⁵ with a major focus on antibiotic resistance in human pathogens. However, there are global concerns over the role of animal farms in the development and spread of antibiotic resistance among animals, environments, and humans.^{6,7}

Antibiotic resistance is ancient and ubiquitous, but undergoing rapid evolution and dissemination in the modern environment. Antibiotic resistance genes (ARGs) are the genetic determinants of antibiotic resistance. A collection of all the ARGs is referred to as the antibiotic resistome, which includes intrinsic resistance genes, acquired resistance genes, proto-resistance genes, and silent resistance genes.^{2,8–11} By using culture-based screening, quantitative polymerase chain reaction (qPCR), metagenomics, and functional metagenomics techniques, research efforts in the field of antibiotic resistomes have revealed the existence of diverse and abundant ARGs in various ecosystems, including humans,¹² animals,¹³ bodies of water,¹⁴ soils,¹⁵ and air,¹⁶ and even in extreme environments, such as glaciers,¹⁷ permafrost,¹⁸ and deep-sea sediments.¹⁹ Imprudent use of antimicrobial drugs in both human health care and the livestock industry is one of the major driving forces for the spread of antibiotic resistance.^{1,18,20} More than 50% of produced antibiotics have been administered in animal farms for preventing disease and promoting animal growth rather than treating infections,^{21,22} resulting in the enrichment of ARGs in animal microbiomes.^{13,23} The intestinal bacteria of animals have been recognized as one particular important reservoir contributing to the widespread dissemination of ARGs in the environment.^{24–27} ARGs in animal feces, animal farm wastewater, and associated environments were detected with comparatively higher diversity and abundance.^{24,27–29} ARGs can spread from animals to humans via various pathways.^{30–32} Although the increase in the number, diversity, and resistant phenotypes of clinical pathogens directly threatens human and animal health, many of the ARGs were recruited from environmental non-pathogenic isolates,^{33–37} and

exchange of ARGs was observed between environmental bacteria and human pathogens.^{36,38} These facts illustrate the potential human health risk posed by ARGs of animal origin and reinforce the urgent need for measuring and monitoring antibiotic resistance in animals. In recognition of the global threat posed by zoonotic pathogens, the Food and Agriculture Organization (FAO), the World Health Organization (WHO), and the World Organization for Animal Health (OIE) developed a tripartite collaboration framework to achieve One Health goals.³⁹

Antibiotic resistance is the phenotype of a given microbial organism, often but not always conferred by a specific gene product. The definition of antibiotic resistance can be either species-centric, including a clinical definition based on minimum inhibitory concentration (MIC) break points and an epidemiological definition based on common epidemiological cut-off values for resistance, or gene-centric, based on comparison of MIC values between an ARG-harboring strain and its parental strain that did not contain this ARG.⁴⁰ At the community level, antibiotic resistance of a given microbial community could be evaluated by microbial community-level growth and community tolerance.⁴¹ In the context of this review, we focus on the molecular genomic technologies that have been used or could be used for the characterization of ARGs in animals. Several reviews have described the diversity, ecology, and dispersion of ARGs in various environments, including animals, with both culture-based genomic and metagenomic approaches being discussed.^{1,2,6,7,42–50} The goal of this review is to complement previous reviews by extending the discussion of high-throughput quantitative PCR, microarray-based technology, and single-cell genomic technology applied in the investigation of ARGs in animals (Fig. 1).

Culture-based genomic identification of ARGs

Conventional culture-based techniques are classical methods to identify antibiotic resistance in the environment. Although culture-independent approaches have been used, the determination of break point values and resistance patterns is more frequently based on culture-dependent methods.^{51–53} The use of culture media with selective antibiotics allows the enumeration and isolation of specific bacterial groups. Antibiotic resistance of isolated strains can be measured using multiple tests, including broth and agar dilution assays for the estimation of MIC, the agar disk diffusion assay for the evaluation of the susceptibility of the bacterial isolate, and E-test assays.⁴⁴ The use of microplates could increase the throughput of MIC determination of isolates. ARGs in isolated resistant strains can be either identified by PCR amplifying specific target genes or via annotation of genome sequences.

In general, culture-dependent methods are laborious and time-consuming, and their major limitation stems from the small fraction of bacteria that can be cultivated from a complex environment.⁴⁵ Nevertheless, pathogens and indicator bacteria are easily grown on selective media using standard procedures, which facilitate the profiling of both resistance phenotypes and genotypes of these bacteria^{53–56} and further assessing their potential risks to human health.^{57,58}

Although culture-independent molecular approaches are promising for exploring ARGs from natural environments,⁴² the data provided by cultured-based technologies are still valuable and should not be underestimated. First, culture-based methods can establish direct links between the resistance phenotype and the genotype, providing phenotypic information about complete and active genes (Fig. 1), which is not always the case when using molecular methods. such as silent genes that express phenotypic resistance when mobilized or mutated to associated regulatory elements.^{8,45} Multidrug-resistant bacteria are of particular clinical concern;^{54,55,59} the culture-based method is the most direct way to test multidrug resistance in bacterial isolates.⁴⁴

Second, culture-based methods facilitate establishing links between ARGs and their bacterial hosts (Fig. 1).⁶⁷ Identifying the hosts of ARGs is particularly important to determine the origin of ARGs and to assess the dispersion of ARGs between environmental bacteria and pathogens.^{68,69} However, it is challenging to determine ARG hosts in complex environmental microbial communities using metagenomic technology, which requires increased sequencing depth and greater efforts and expertise in bioinformatics.^{15,43}

Third, whole-genome sequencing provides information about the arrangement of multiple ARGs and associated genes, such as metal-resistance genes.⁷⁰ With the identification of multiple ARGs from a bacterial genome, both the multidrug resistance phenotype and genotype of a bacterial isolate can be characterized.^{11,60–66} More importantly, it enables the identification of ARGs that reside on mobile genetic elements (MGEs). MGE-associated ARGs could be exchanged among bacteria and pathogens via horizontal gene transfer (HGT), which contributes largely to the spread of ARGs.^{71–73} In addition, comparative genome analysis provides evidence about the determination of acquired resistance and intrinsic resistance and the hosts of these MGEs and associated ARGs.^{8,35,65,66,74–76}

Finally, genome sequencing of numerous resistant isolates from humans and animals followed by phenotype characterization and comparative genomic analysis enables molecular epidemiological research on antibiotic resistance in human and animal populations,^{52,75,77,78} which will contribute to the determination of the sources of antibiotic resistance.⁷⁹ Mather *et al.* proposed an ecological approach

to assessing the epidemiology of antimicrobial resistance in *Salmonella Typhimurium* DT104 and found that animals and humans have distinguishable DT104 communities with different diversity and phenotypic resistance profiles.⁷⁸ They further found distinguishable genotypic DT104 communities in human and animals, suggesting that other sources contribute to the diversity of the human resistance burden beyond the local animals.⁷⁷ Such large-scale studies of the molecular epidemiology of antibiotic resistance could provide useful data for human health risk assessment.⁸⁰ Martinez *et al.* determined that the ARGs residing on MGEs of clinical pathogens with validated resistant function to clinically important antibiotics exhibit the highest risk.⁴⁰ With the aforementioned advantages, culture-based technologies provide critical information for understanding the phenotype, genotype, dissemination, and epidemiology of antibiotic resistance, especially in clinically important organisms.

Culture-independent molecular genomics technologies are efficient at detecting microorganisms that cannot be grown in the laboratory or that multiply very slowly or persist⁸¹ in specific compartments, enabling the capture of antibiotic resistance profiles at microbial community level. These technologies, which have been widely used to explore ARGs in nature and to address the dissemination of ARGs in different environments,^{1,2,6,7,42,43} will be discussed in the following sections.

Metagenomic exploration of ARGs

Metagenomics is the study of all of the genome sequences of a community of organisms inhabiting an environmental sample.^{82,83} Metagenomic sequencing can provide a relatively unbiased view of not only the community structure (species richness and distribution) but also the functional (metabolic) potential of a community.⁸³ In contrast to microarray-based and PCR-based methods, which bear the limitations of primer or probe design and selection, sequence-based metagenomics analysis is a powerful tool to explore the entire antibiotic resistome, including the occurrence of unforeseen ARGs in diverse environments, enabling the discovery of novel ARGs and novel resistance mechanisms.^{6,46}

Metagenomic analysis of microbial communities from swine gut,^{23,84} cattle manure,²⁹ chicken ceca,⁸⁵ feline feces,⁸⁶ and mice gut⁸⁷ have revealed diverse and abundant ARGs and MGEs in animals. As a consequence, animal manure is considered as a hotspot for the spread of ARGs and MGEs to the environment.^{30,31} Antibiotic administration is one of the major factors influencing the abundance and

diversity of ARGs in animals.⁸⁸ Looft *et al.* investigated the effects of in-feed sublethal antibiotics (ASP250 including chlortetracycline, sulfamethazine, and penicillin) on swine intestinal microbiome ARGs.^{23,84} Analysis of the metagenomes revealed that, despite a high background of ARGs in non-medicated swine, ARGs increased the abundance and diversity in the medicated swine microbiome after 14 days of antibiotic treatment; indirect selection of aminoglycoside *O*-phosphotransferases conferring resistance to antibiotics that were not applied was also observed.²³ The segregation of ARGs is not solely due to the antibiotic usage in human and animals.⁸⁹ Application of metals can also affect the ARGs profiles in animals by changing the microbial community or co-selecting metal-resistance genes and ARGs, resulting in co-resistance to both metals and antibiotics.^{90,91} Metagenomic analysis of the mouse gut microbiome showed that exposure to metals without antibiotics resulted in alteration of the diversity and functions of gut microbiota and increased the frequency of ARGs and MGEs.⁸⁷

ARGs can be identified and annotated by homology search in the databases, such as the National Center for Biotechnology Information (NCBI) NR, the Antibiotic Resistance Gene Database (ARDB)⁹² and the Comprehensive Antibiotic Resistance Database (CARD).⁹³ Sequence-based homology search has an advantage for automatic annotation of ARGs in huge metagenomes. Regardless of this, homology search will become a limitation when no significant similarity to any sequences in the ARGs database occurred, and, consequently, novel types of ARGs present in a sample might be missed.^{46,94} More importantly, the function of most of the ARGs annotated by homology search was not verified, which could inevitably produce false-positive annotation of ARGs, resulting in a vicious feedback loop when these false-positive ARGs are included in the ARGs database.⁴⁰ Advances in bioinformatics, such as stringent similarity criteria, improved pipelines, and new algorithms for prediction of resistance phenotype from genotype, may improve the identification of true-positive ARGs.^{40,49,95}

Functional metagenomic screening of ARGs can alternatively complement the limitation by providing direct evidence of resistance phenotypes when screening metagenomic libraries on selected media with target antibiotics (Fig. 1). Although functional metagenomics is relatively time consuming, laborious, and suffers from bias in molecular cloning and heterologous gene expression,⁴⁸ it is powerful at identifying novel ARGs with validated function in the environment.^{38,96–100} Using this technology, a novel tetracycline resistance gene was identified and the association between tetracycline-resistance genes and MGEs as observed in the pig gut microbiome.¹⁰¹ Functional screening of metagenomic libraries coupled with Illumina sequencing of inserts increases the

throughput of this technology and extends our knowledge of the ecology of the ARGs.¹⁵ By combining functional metagenomic and third-generation sequencing technology (PacBio sequencing), diverse ARGs were identified in dairy cow manure, including a novel clade of chloramphenicol acetyltransferases.¹³

One particular challenge of metagenomics is to determine the host bacteria of identified ARGs (Fig. 1). The host of ARGs can be inferred indirectly by network analysis based on the co-occurrence pattern between ARGs and bacterial populations.²⁸ Sequence analysis of the ARGs and flanking DNA provides direct information about the ARG host. The metagenome can be assembled, and the taxonomy affiliation of contigs containing annotated ARGs can be assigned. By using similarity-based methods, Ma *et al.* inferred the host of ARGs from diverse samples at different taxonomic levels, with the *Escherichia* genus exhibiting high correlation with multidrug-resistance genes.⁷⁰ The taxonomic origin of ARGs can also be predicted using RAIphy, a composition-based classifier that uses the relative abundance index metric and an iterative classification method for taxonomic classification of DNA contigs with a large range of lengths assembled from metagenome data.¹⁰² By using this algorithm, the source phyla of functional selected ARGs were predicted, with Proteobacteria and Actinobacteria being the most prevalent predicted phyla in soil.¹⁵ Although the phylogeny of ARGs was predicted at lower-resolution taxonomy (i.e., the phylum level),¹⁵ and the percentage of taxonomically identified contigs is relatively low at the genus level (i.e., less than 30%⁷⁰), these studies successfully established the linkage between ARGs and their host, supporting further analysis of the origin of ARGs.

Another major challenge of metagenomics is to capture the genetic context of ARGs, which could not only aid in the taxonomic classification of annotated ARGs but also provide information on the co-occurrence of ARGs and ARGs with MGEs, which is of particular interest for assessing multidrug resistance and potential HGT. The detachment of genetic context largely is largely attributed to the detection limits at the current sequencing depth and cost and the challenge in efficient assembly of metagenomic sequences.⁶ This limitation could be ameliorated by decreasing sequencing costs and the development of bioinformatics. The third-generation sequencing can also help increase the efficiency for obtaining the genetic context of ARGs. With much longer reads than 454 pyrosequencing and Illumina sequencing, PacBio sequencing was used to sequence the large insert in the resistant clone from a cow manure metagenomic library, showing that the identified ARGs originated from diverse species and frequently clustered together, providing the genetic basis for the prediction of multidrug resistance.¹³

Co-occurrence of ARGs and MGEs can be investigated by directly sequencing the mobilome in the environment, which is defined as a collection of all mobile genetic elements in a metagenome.^{7,103} The transposon-aided capture (TRACA) system has been employed to isolate novel plasmids from the environmental metagenome, revealing the prevalence of ARGs and MGEs for HGT in microbial communities of sewage treatment plants.¹⁰⁴ Gillings *et al.* found that the class 1 integron–integrase genes (*intI1*) recovered from clinical contexts have a homogenous and conserved DNA sequence and suggested that the relative abundance of these “clinical” *intI1* genes could serve as a genetic marker for anthropogenic influence owing to their emergence in human-dominated ecosystems and close links with ARGs.¹⁰⁵ Sequencing the integron pools revealed a high proportion of integrons in hospital effluent, with distinct gene cassette diversity and gene cassette arrays.¹⁰⁶ Sequencing a murine fecal phage population revealed that the phageome is a potential genetic reservoir of ARGs and that antibiotic treatment has resulted in the enrichment of ARGs for both administered drugs and unrelated antibiotics.¹⁰⁷ Characterization of the mobilome in animals would provide insights into the mobile pools of ARGs that could be easily spread in the environment via HGT.

Microarray-based detection of ARGs

Microarray-based approaches are also high-throughput technologies for analysis of microbial phylogeny and functional genes in complex environments that enable simultaneous detection of up to thousands of well-designed biomarker genes. According to the types of targeted biomarkers, developed microarrays can be grouped into two major categories (i.e., phylogenetic and functional gene arrays¹⁰⁸). Phylogenetic gene arrays were widely used for the characterization of microbial taxa in environments by targeting phylogenetic marker genes, including rRNA¹⁰⁹ and *gyrB* genes.¹¹⁰ Various types of phylogenetic gene arrays have been developed targeting microbial communities in different habitats, including PhyloChip, which targets broad taxa;^{111,112} COMPOCHIP, which targets bacterial communities of different composts;¹¹³ and a microarray targeting human intestinal microbiota.¹¹⁴ In addition, microarrays based on *gyrB* have been developed for fine-level phylogenetic analysis of pathogenic species, including *Escherichia coli*, *Shigella*, *Salmonella*, *Mycobacteria*, and *Legionella pneumophila*.^{110,115–118}

Functional gene arrays are designed on the basis of genes involved in the genomes of various bacterial species,¹¹⁹ pan-genomes (the complete set of genes of all strains in one bacterial species),¹²⁰ and metagenomes¹²¹ for comparative genomic study, genotyping, or investigation of microbial

functional composition and structure.^{122,123} Previously identified ARGs can be used for designing oligonucleotide probes that hybridize with genomic/metagenomic DNA for the detection of targeted ARGs in animals. Different types of ARG microarrays have been developed for detecting specific ARGs since at least 2003^{124,125} and have identified broad ranges of ARGs in pathogenic isolates^{126–129} and environmental DNA.¹³⁰ These ARG microarrays have been used for genotyping and profiling ARGs in pathogens from livestock and poultry.^{131–136} Virulence factor genes are often included in microarrays with ARGs to achieve a fine-level characterization of both resistant and pathogenic genotypes in bacterial isolates of animal origins.^{126,131,132,137} A virulence factor gene–based microarray, PathoChip, containing 3715 probes targeting 13 virulence factors from 1397 microbial species (2336 strains), has been developed to investigate the pathogen population dynamics and pathogenic properties in the environment.¹³⁸ ARGs are also targeted in other functional gene arrays. A microbial source–tracking microarray targeting pathogens, fecal indicator bacteria, source-tracking markers, and ARGs was designed for high-throughput detection of fecal contamination and pathogens in the environment and was tested against sewage and animal fecal samples.¹³⁹ GeoChip, a functional gene array targeting genes involved in diverse biogeochemical processes, including virulence, metal resistance, and antibiotic resistance,¹²² has also been used to characterize the ARG profiles in environmental samples.^{130,140}

As compared to metagenomic technology, which could provide new sequences, thus enabling the discovery of novel ARGs and resistant mechanisms, microarray-based technology requires existing sequences for probe design and does not provide new molecular information about ARGs or detect novel ARGs. Nevertheless, microarrays are characterized with high throughput, low detection limits, high reproducibility, and potential quantification,¹⁰⁸ which is promising in rapid profiling of ARGs in the environment. The challenges of microarray-based technology are (1) the design of specific probes for ARGs that are highly homologous, incomplete, or both; (2) occasional large variations between or within technical replicates; (3) distinguishing true hybridization signals from nonspecific background noise;¹⁰⁸ and (4) relatively lower sensitivity compared with a PCR-based assay.

High-throughput quantitative PCR quantification of ARGs

PCR-based technologies are highly sensitive and relatively rapid in detecting ARGs in either bacterial genomes or metagenomes.⁴³ Traditional PCR is qualitative and can only provide evidence for the presence of ARGs in environments. In contrast, quantitative PCR (qPCR) provided both qualitative

and quantitative information in addition to the benefits of PCR, thus providing a powerful means for rapid detection and enumeration of the abundance of ARGs, supporting comparative study on antibiotic resistance. Quantitative PCR has been successfully established to quantify ARGs and MGEs markers in environmental samples, including soil, water, and sewage sludge,^{105,141–143} and in animals^{16,23,101,144–151} to investigate the distribution of ARGs and to assess the anthropogenic impacts on the ecology of ARGs.

Advances in genomic and metagenomic surveys have resulted in an increase in the number of ARGs in ARGs database, which facilitates the design of multiple specific primers targeting a variety of ARGs or MGEs markers.^{105,148,149,152–154} With conventional qPCR, Li *et al.* showed the potential spread of plasmid-mediated quinolone-resistance genes from swine feedlots by investigating the distribution of eight plasmid-mediated quinolone-resistance (PMQR) genes from swine feedlots and their surrounding environment.²⁵ McEachran *et al.* detected several ARGs in the particulate matter from 10 beef cattle feed yards, raising concerns about the spread of ARGs via the air pathway.¹⁶ Our previous work demonstrated the prevalence of antibiotic residue and ARGs by quantifying 15 tetracycline-resistance genes in soils near nine swine feedlots.¹⁵⁵ A total of 46 ARGs conferring resistance to several types of antibiotic were tracked by PCR and qPCR to evaluate the impact of manure fertilization on ARGs in soil and on vegetables at harvest.¹⁵⁶ Despite the successful detection of ARGs by qPCR, conventional qPCR approaches suffer from low throughput design via 96/384-well microplates; therefore, numerous reactions are needed to detect and quantify diverse ARGs in environmental samples. Therefore, expanding the number of target ARGs and samples of interest will inevitably lead to dramatically increased cost and labor.

High-throughput qPCR (HT-qPCR), with the capability to perform thousands of nanoliter qPCR reactions, has been developed to simultaneously assess the expression and abundance of hundreds of genes,^{157–160} such as virulence and marker genes of pathogens.^{161,162} ARGs are diverse and abundant; a limited number of targeted ARGs is inadequate to represent the overall antibiotic resistance level in complex communities. For example, the tetracycline resistome comprises more than 40 resistance genes, including genes encoding efflux pumps, ribosomal protection proteins, mosaic elements, and inactivation enzymes.¹⁶³ To address this limitation, HT-qPCR has been used to investigate the abundance and diversity of ARGs in environmental samples, including wastewater treatment plants,¹⁶⁴ drinking water treatment plants and distribution systems,¹⁶⁵ soils,¹⁶⁶ compost,¹⁶⁷ rivers,¹⁶⁸ animals, and associated environments.^{23,24,169,170}

Although HT-qPCR is promising for rapid detection and quantification of hundreds of ARGs, similar to microarray-based technologies, the major limitation of HT-qPCR is that it depends on the ARG sequences in a database for primer design, and thus is unable to detect unknown distantly related ARGs in the environment.⁶ One major challenge of HT-qPCR is primer design and optimization for amplification conditions for each ARG, since the amplification reactions and thermal cycles are uniform throughout the assay.⁴³ In addition, the detection limit of HT-qPCR is relatively higher due to the smaller reaction volume at nanoliter level rather than microliter level for conventional qPCR.

Single-cell genomic technology

Microbial genomes among a population of cells within an ecosystem are highly heterogeneous and far beyond what bulk approaches, such as metagenomics and HT-qPCR, can resolve.¹⁷¹ Recognition of cellular heterogeneity is very important considering that biological processes can be driven by individual cells with dynamic genomic variation. For example, heteroresistance within a clonal bacterial population under antibiotic stress is observed in a wide range of microbes and accounts for treatment failure; however, the exact genetic mechanism and its roles in adapting to antibiotic resistance remains obscure at the single-cell level.^{172,173} Single-cell sequencing aims to understand genomics at the single-cell level by dissecting the contribution of individual cells.^{171,174}

Single-cell genomics can be used to identify and assemble the genomes of uncultivated bacteria or viruses (microbial dark matter) directly from environmental or clinical specimens (Fig. 1), enabling identification of vast numbers of novel genes encoding specific functions or metabolic pathways from microorganisms of interest that lack available sequence in the database.¹⁷⁴ The captured genome can be further applied in a wide range of contexts, such as screening and enhancing biofuel production by bacteria,¹⁷⁵ obtaining therapeutics from environmental bacteria,¹⁷⁶ promoting the development of culture methods by correlating genome with the nutritional needs and metabolic processes of the organism,¹⁷⁴ and facilitating design of a fluorescence *in situ* hybridization (FISH) probe for analyzing a microbial community and high-throughput screening of a microorganism of interest.¹⁷⁷ Single-cell sequencing can also be combined and cross-validated with metagenomic methods to investigate shifts in microbial community structure by providing genetic linkage within individual cells and the overall genome of a community.¹⁷⁸

There are technical challenges involved in obtaining high-quality single-cell genomes.¹⁷¹ Efficient physical isolation and collection of individual cells are the first steps in single-cell genomics. Approaches for isolating single cells have advanced from low-throughput manual operations to high-throughput ones that are more automatic. The low-throughput methods include micropipetting,¹⁷⁹ nanoliter microwell dilution,¹⁸⁰ optical tweezers,¹⁸¹ and laser ejection.¹⁸² The high-throughput methods mainly include flow cytometry and microfluidic devices combined with droplet or micromechanical valves or both.^{183–187} The combination with other techniques, such as fluorescence and Raman spectroscopy, can improve efficiency by screening cells of interest or biological importance.^{182,188–191} Whole-genome amplification (WGA) of single cells with acceptable bias and sufficient content for downstream analysis is another critical challenge in obtaining single-cell genomes. Multiple displacement amplification (MDA) is the most commonly used approach for WGA. Finally, single-cell genomics requires cost-effective sequencing, related bioinformatics, and appropriate data interpretation.¹⁷¹

Regardless of these challenges, single-cell genomics has competitive advantages in monitoring antibiotic resistance. Theoretically, emergence of antibiotic resistance in a single cell could allow the resistant strain to survive, proliferate, and eventually outcompete others under the selective pressure of antibiotics.^{80,192} The frequency of resistance mutation and HGT could be very low in the environment (e.g., 10^{-8} for resistance mutation¹⁹³ and 10^{-5} for HGT^{194,195}); tracking these rare events could be laborious using conventional culture methods. However, a single-cell genomic approach could be employed to sensitively and quantitatively detect these rare events (Fig. 1). The compartmentalization of single microbes ensures proliferation of both slow-growing and fast-growing bacteria without competition (Fig. 1) and thus produces diverse resistant phenotypes that might be missed via bulk culture. An agarose droplet-based microfluidic emulsion PCR was established with the ability to detect a single pathogenic *E. coli* O157:H7 cell in a high background of 100,000 excess normal K12 cells.¹⁸⁵ By using a novel microfluidic-based picodroplet platform, fusidic acid-resistant strains could be screened and isolated in a high-throughput manner at an estimated frequency of resistance at 10^{-8} ; further sequence analysis of *fusA* detected novel single-nucleotide polymorphisms of *fusA* in isolated mutants.¹⁹⁶ Single-cell genomics can also help reveal the genomic mechanisms of heteroresistance and lend insight into the changes of bacterial metabolism under selection pressure.^{172,197,198}

Single-cell genomics have advanced rapidly in recent years and have resulted in new discoveries that bulk genomics cannot achieve. Application of single-cell genomics to address antibiotic

resistance in animals has been rarely reported to date, but holds promising potential. For example, single-cell whole-genome sequencing of a large majority of uncultivated resistant bacteria facilitates the identification of new genes encoding novel resistance mechanisms and pathways. Bacterial species of interest in a given environment could be labeled, sorted out, and sequenced for comparative genomic analysis. Isolated single cells could be incubated in various antibiotics with different concentrations for determination of resistance, and the obtained resistance phenotypes could be sequenced for elucidation of their resistance mechanisms. The correlation of phenotype with genotype at the single-cell level will unambiguously link the resistance genes with its host bacteria, reaching a much higher resolution compared with community-level analysis of resistance genes and microbial structure. In addition, single-cell stable-isotope probing (SIP) coupled with Raman spectroscopy can directly reveal bacteria that subsist on antibiotics.¹⁹⁹

Concluding remarks and perspectives

Antibiotic resistance in animals is an important and urgent problem posing direct risks to animal and human health, owing to the diversity and abundance of ARGs detected and the potential exchange of antibiotic resistance genes among animals, the environment, and humans. Culture-based, culture-independent, and single-cell genomic approaches can be employed to measure and monitor antibiotic resistance in animals; each has its advantages and disadvantages, and should be selected depending on scientific hypotheses and the objectives of a given study. The goal of investigating antibiotic resistance in animals is to delineate the origin, evolution, development, dissemination, and risk of antibiotic resistance within animal-farming environments, and further within the general environment and humans. The integration of cultivation and molecular technologies provides a more comprehensive understanding of the ecology of antibiotic resistance (Fig. 1). Genome-based studies will also facilitate the identification of new drug targets that could be used for the development of novel antimicrobial agents and therapies.

Presently, most diagnostic tests for antibiotic resistance are based on the phenotypic characterization of isolated bacteria and thus require extended time from specimen collection to results; as a result, patients may be initially treated with broad-spectrum antibiotics. Molecular-based methods may provide a promising means for point-to-need diagnostics of antibiotic resistance and facilitate treatment decisions. Several PCR-based tests have been approved by the U.S. Food and Drug Administration for the detection of antibiotic resistance.²⁰⁰ Given that the numerous identified

resistance mechanisms, high-throughput PCR is a possible solution for comprehensive detection of antibiotic-resistance profiles. Genomic analysis would provide information not only about antibiotic resistance determinants, but also their associated expression-regulatory mechanisms. Standard protocols for molecular diagnosis tests for measuring and monitoring antibiotic resistance, other than the culture-based approach, should be developed for the establishment of a global surveillance system and large-scale comparisons, which would also provide basic information for human health risk assessment of antibiotic resistance.

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Conflicts of interest

The authors declare no conflicts of interest.

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Figure legend

Figure 1. Genomic technologies for studying ARGs in animals. Culture-based genomic and metagenomic methods have been commonly employed, and can be complemented by single-cell genomic methods to investigate the abundance and diversity of ARGs. Integration of these technologies will support modeling and prediction of the evolution, dynamics, and dissemination of ARGs within animals and further to environments and humans. Red dashed rectangles: metagenomics should be coupled with culture-based method for functional validation of annotated ARGs; culture-based genomic analysis would provide information for host identification of ARGs identified in metagenome. Green dashed rectangles, the compartmentalization of microbes in single-cell genomics would reduce the complexity of the microbial community, thus increasing the opportunity of obtaining complete genomes from the metagenome. Blue dashed rectangles: compartmentalization of microbes in single-cell genomics would enable growth optimization of various microbes without competition with other bacteria compared with a culture-based method.

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