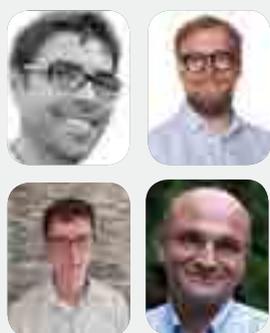


How next-generation sequencing can address the antimicrobial resistance challenge

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During the last decade, major improvements in sequencing technologies, referred to as next-generation sequencing (NGS), have considerably transformed the analysis of bacterial genomes and of complex bacterial communities, such as those found in the intestinal microbiota and the environment. As for antimicrobial resistance (AMR), NGS enables the identification of known resistance genes but also the prediction of novel ones, thereby opening new perspectives in AMR surveillance, risk assessment and in our understanding of the AMR dynamics between commensals and pathogens from the environment and our microbiota.

During the last decade, major improvements in sequencing, referred to as next-generation sequencing (NGS), have deeply transformed our capacity to address antimicrobial resistance (AMR). Together with bioinformatic tools and resources, NGS offers the possibility to sequence bacterial genomes (genomics) as well as the exploration of complex mixtures of species, such as the human microbiota or environmental samples (metagenomics). In the field of genomics, NGS has increased our capacity to track bacterial clones (such as the multidrug-resistant ones), to identify new antibiotic resistance genes (ARGs) and their genetic carriers, such as plasmids. As for metagenomics, it enables us to identify and track ARGs of clinical interest (such as the carbapenemases-encoding ones) as well as ARGs from commensals that can possibly emerge in pathogens. Besides, NGS applied on clinical samples (referred to as clinical metagenomics) appears to be a new perspective in the diagnostic of infectious diseases. Current challenges include the development of a standardized and curated database for ARGs and the need for high-throughput biological validation of new ARGs. Here, we propose to summarize the current state-of-the-art in the application of NGS for tackling AMR in strains, clinical samples and environmental samples (Figure 1).

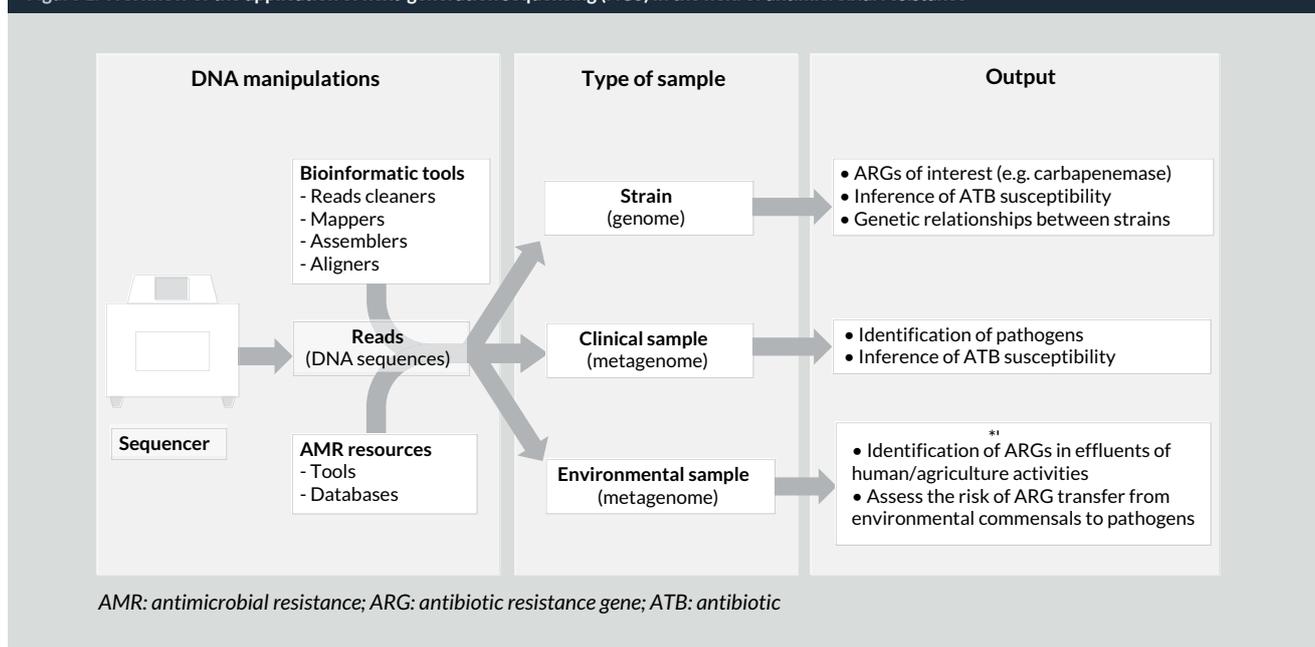
NGS tools: brief historical review, current standards and technical keypoints

The first methods of DNA sequencing were invented in the mid-

70s and decoded hundreds of bases in one day. Two methods were widely accepted at that time: the chain terminator procedure developed by Sanger et al. and the chemical cleavage procedure developed by Maxam and Gilbert (1,2). Both methods resolved the DNA fragments by electrophoresis on polyacrylamide gels for each base-specific reaction, which enabled the single-base resolution. The Sanger method formed the basis for the first automated DNA sequencers using fluorescence chemistry, resulting in the completion of the first complete bacterial genome, *Haemophilus influenzae*, in 1995 (3). The Sanger sequencing remained the primary sequencing technology until 2005. The first-generation sequencing was low throughput, but produced relatively long, high-quality DNA sequences. Sequencing of multiple samples was possible by including several capillaries in the same instrument, each allowing the sequencing of an individual sample.

The key technical change of NGS was multiplexing. The introduction of massively parallel DNA sequencing technologies revolutionized the field and allowed hundreds or thousands of samples to be analysed simultaneously. A typical NGS workflow includes DNA extraction and fragmentation, ligation of adaptors, amplification and sequencing. Intense competition between different companies resulted in a drastic reduction of per-base cost of DNA sequencing. Compared to Sanger sequencing, read lengths are still shorter in the low hundreds of bases. This feature gave its name to this second-generation sequencing, so called short-read sequencing.

Figure 1: Workflow of the application of next-generation sequencing (NGS) in the field of antimicrobial resistance



Short-read sequencing requires template amplification with its intrinsic drawbacks, such as copying errors, sequence-dependent biases and a loss of information. Conversely, the third-generation sequencing is real-time and single molecule-based long-read sequencing. The approach developed by Pacific Biosciences relies on the optical observation of a single polymerase and its template DNA in real time (4). Reads over 10kb are typical and have allowed de novo genome assembly and direct detection of DNA methylation sites. The second approach, developed by Oxford Nanopore Technologies, relies on the movement of DNA molecules through a nanopore and measurement of an electrical signal altered according to the base currently passing the pore (5). The channels can be controlled independently in real-time by reversing the voltage across the pore, rejecting undesired DNA molecules and enabling selective sequencing of fragments of interest (6). These new sequencing technologies have paved the path for sequencing single genomes, but also complex communities of microorganisms, and identify the antibiotic resistance determinants.

AMR resources

There exists a number of databases for ARGs and other factors related to AMR. Importantly, many of those are no longer being updated and maintained. The most comprehensive sources for ARGs that are still being curated are the Comprehensive Antibiotic Resistance Database (CARD) (7) and ResFinder (8). The foci of these two resources are somewhat different. CARD aims to collect information on a wide range of ARGs and it does not explicitly state that they need have been experimentally confirmed to be included, although most ARGs deposited

in CARD are. ResFinder, on the other hand, only includes genes that have been shown to be present on mobile genetic elements and therefore can be horizontally transferred between bacterial species. There are also resources taking fundamentally different approaches, such as FARME DB and ResfinderFG, which collect inserts from functional metagenomics studies that have been shown experimentally to confer antibiotic resistance (9,10). In addition, there are databases such as PATRIC (11), which collects information specifically on pathogens, VirulenceFinder (12) and VFDB (13), which contains known virulence factors, and the Mobile Genetic Element Database (14), containing plasmids and other markers of mobile genetic elements.

These databases can be queried using a variety of software tools. A more in-depth review of those has recently been published (15). These bioinformatics tools generally take raw DNA sequence reads or longer DNA sequences assembled from NGS data – either from genomes or metagenomes – and match them to a database of the user's choice. While software such as BLAST (16) is able to perform this task, these older algorithms are grossly inefficient on the large datasets generated by NGS. Instead, tools such as Bowtie2 (17), BWA (18), Vmatch (19), Usearch (20) and Diamond (21) are vastly more suitable for this task. These tools trade sensitivity for speed, but this is seldom a great concern for ARGs, as they tend to be relatively well conserved, especially those that have been mobilized and circulate among pathogens (15).

Besides bioinformatic resources, Lanza and colleagues developed ResCap, a sequence-cap targeted sequence capture platform based on SeqCap[®] EZ (Roche) technology (22). ResCap includes probes for 8,667 canonical resistance

genes (7,963 antibiotic resistance genes and 704 genes conferring resistance to metals or biocides), 2,517 relaxase genes (plasmid markers) and 78,600 genes homologous to the previous identified targets (47,806 for antibiotics and 30,794 for biocides or metals). Applied to human faecal samples, ResCap was able to recover more ARGs than conventional shotgun metagenomics.

Application for genomics

In 1995, the first bacterial genome ever sequenced was considered as a scientific achievement (3) and no one would have thought of using whole bacterial genome sequencing (WGS) as a routine diagnostic technique. Yet, with the tremendous development of next-generation sequencing (NGS, see dedicated section) and the numerous bioinformatic tools freely available online, bacterial genomics has become a realistic option for a series of indications. First of all, WGS has emerged as the ultimate technique for bacterial outbreak analysis. It builds on the largest possible nucleotide diversity (way more discriminant than the previously used, technically challenging, pulse-field gel electrophoresis (PFGE) (23), provides numerical data within a relatively short time span and is –apparently– ideal for that purpose (24–26). However, this very fine typing capacity has a scientific cost, driving in itself additional financial costs: one can now appreciate that bacterial carriage is not perfectly monoclonal (e.g., for *Neisseria meningitidis* (27)), nor is the composition of a single bacterial colony on a plate (e.g., *Staphylococcus aureus* (28)). These findings raise the issue of defining how many strains and samples per patient should be considered in order to capture the dominant genome(s) (29). The assessment of the relationship via the number of genetic events (SNVs, for example) which differ between strains is also a complex question, as the time for occurrence of genetic events appears to be exquisitely species-dependent, even possibly strain-dependent. One is therefore exposed to the risk of missing the detection of an outbreak in highly genetically mobile strains, or referring to an outbreak in genetically very stable strains. A second application of bacterial WGS consists of defining whether the isolation of two invasive isolates in the same patient has to be considered as a reinfection or a relapse due to the persistence of the same clone, prompting for the active search of a hidden infection.

The third application might become more and more important in the future, when antimicrobial susceptibility prediction will become more reliable, based on the sequence detection of antibiotic resistance determinants (see the AMR resources section above). This will become of paramount importance in those cases where no bacteria can be cultivated, due to the presence of fastidious organisms or the previous

use of antimicrobial agents. One can expect such indications to become useful in infective endocarditis or bone and joint infections (30) (see section below). Finally, the only application that has now become routine is the prediction of antimicrobial susceptibility for *Mycobacterium tuberculosis*, an achievement that was made possible due to the highly clonal nature of the organism, the absence of horizontal gene transfer and the very large number of strains sequenced (31,32), providing actionable results much faster than with conventional methods, due to the slow growth of *M. tuberculosis*.

Application for metagenomics (human): Basics in clinical metagenomics, promises and hurdles

Clinical metagenomics (CMg) refers to as the application of NGS on clinical samples in order to recover information of clinical relevance, such as the identification of pathogens and the prediction of their susceptibility to antimicrobials (33). Indeed, CMg offers the potential to directly detect all microorganisms present in a sample or even detect RNA viruses, if coupled to a previous reverse-transcription step. This approach could therefore provide unbiased detection of all microorganisms present in the sample, including those organisms that are fastidious or even cannot be cultivated. By skipping the cultivation phase, CMg could constitute a rapid and generic approach providing all medically-actionable information: the presence or the absence of microorganisms (detection), its identification to the species level or even beyond (speciation, and potentially some genotyping capabilities), the detection of antimicrobial resistance determinants (i.e., ARGs and mutational events associated with resistance) with the potential to guide antibiotic therapy and virulence-associated genes. In theory, sequencing-based diagnostics could compete and maybe replace conventional methods. CMg has now been successfully applied to a wide range of clinical situations, including bloodstream infections (34–36), bone and joint infections (30,37), pneumonia (38), central nervous system infections (39) and urinary-tract infections (40,41). In many situations, CMg was able to detect the pathogen(s) identified with conventional methods. However, CMg was also able to identify other microorganisms for which the pathogenic role remains unknown, such as obligate anaerobic bacteria. In order to assess whether those bacteria could be involved in an infectious process, considering the host's response (as measured by the host's cells transcriptome) could be an option (38,42).

CMg could address the AMR challenge by the fast identification of pathogens and parallel prediction of susceptibility to antimicrobials. While the current turn-around time of culture-based conventional methods is 48 hours, that of CMg could be reduced to 6–8 hours (41,43). Hence, CMg could

help in decreasing the duration of inappropriate treatment due to probabilistic antibiotic regimen, i.e., when the antibiotic susceptibility of the pathogen is not known.

Nonetheless, several hurdles currently remain to be addressed before CMg could enter the routine diagnostic laboratories, namely its high cost (typically hundreds of US dollars per sample), lack of automation and standardization for both wet-lab and dry-lab steps, lack of demonstration of clinical impact, the difficulty to report actionable information to the clinician, the lack of reimbursement, not to mention the various remaining wet-lab and dry-lab technical challenges raised.

Application for the environment

The environmental applications for NGS largely mirror those used for human health purposes (15). However, in environmental settings the risk picture looks somewhat different and therefore other information is considered the most relevant. The environmental risks associated with AMR can basically be divided into risks for spreading resistant (often opportunistic) pathogens and risks for selecting for novel types of ARGs that could later be recruited into human pathogens (44). To understand and control the spreading of resistant bacteria, culturing and/or PCR approaches are often used in environmental resistance surveillance (45).

Here, NGS – and particularly metagenomics – can contribute by offering the possibility to investigate a much larger set of ARGs at once. This makes identification of rare, but rising, resistance threats possible to detect. Thereby, NGS could provide a cornerstone for early warnings of future resistance hazards. Since NGS analysis allows easy investigation of archived data from environmental samples, it is also possible to trace the origins of certain ARGs and how they have spread between different environments. This allows insight into whether a certain ARG may have had a single origin or was present virtually everywhere before ending up in a human pathogen (46). Such retrospective analyses aid the understanding of what mitigation strategies would have been effective to prevent spread of AMR and how we can improve interventions in the future. A recent example of this use for NGS data is the discovery of the mobile colistin gene *mcr-1* (47), which was quickly identified to already be present in the human microbiome, despite having gone undetected (48,49).

Furthermore, NGS has important uses in identification of novel ARGs that could be recruited to human pathogens, as it enables very sensitive bioinformatic methods to be used to detect previously undescribed ARGs, which could become important threats to human health in the future (50,51). It also aids our understanding of the genetic context in which ARGs

appear, such as if they are located on a genetic element that can be transferred between bacteria and what species they are present in (15), as well as the identification of human and natural selective drivers for AMR in the environment (52,53). Finally, recent results suggest that the diversity of known ARGs can be used to predict the diversity of unknown ARGs (44), which would greatly aid in prioritizing risk environments (54).

Conclusion and perspectives

During the last decade, the NGS capacities have skyrocketed and several sequencing options have now been made available to scientists and clinicians at a cost which keeps decreasing. Downstream sequencing, the bioinformatic community has been very active in developing numerous solutions to take the best out of the DNA sequences. Initially aimed for bioinformatic specialists, those tools are becoming more accessible to non-specialists via point-and-click interfaces (e.g., Galaxy) or web-based interfaces (e.g., the center for genomic epidemiology <https://cge.cbs.dtu.dk/services/>).

Control of AMR is benefitting from the NGS revolution at various levels. At the genomic scale, NGS has now become the reference method to establish relationships between strains, even if standards are currently lacking. Whole genome sequencing has proven to be a powerful tool when it comes to assessing the connections between strains both at healthcare structures and worldwide levels. It has also become the reference method for inferring the antibiotic susceptibility in *M. tuberculosis* and perhaps will it become so for other species in the coming years (55,56).

The application of NGS to clinical samples, referred to as clinical metagenomics, is a vibrant field as witnessed by the sustained success of the yearly International Conference on Clinical Metagenomics (ICCMg) that started in 2016 (57–59) in Geneva. CMg is widely accepted as a potent framework that can change the paradigm of the diagnostic of infectious diseases, but several technical, clinical and regulatory challenges have to be overcome before CMg could enter the routine diagnostic laboratories.

As for environmental samples (i.e., potentially everything outside human and animal samples), NGS could help in tracking how multidrug-resistant bacteria from effluents interact with environmental commensals, and conversely, how ARGs from environmental commensals can be transferred to human and animal pathogens.

Nonetheless, it will take time for scientists and clinicians to understand all the information found in genomic and metagenomic data. NGS-based tools have solved issues, but have concomitantly raised several others. Hence, we believe that the story about NGS and AMR is just at its beginning. ■

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Dr Johan Bengtsson-Palme holds a PhD in Medicine and did his doctoral studies on antibiotic resistance in the environment. He was a postdoctoral fellow in the lab of Professor Jo Handelsman at University of Wisconsin-Madison, investigating antibiotic effects on bacterial colonization and invasion ability. He now leads a research group at the University of Gothenburg studying effects of antibiotic exposure on microbial communities in the human body

and the environment.

Dr. Yannick Charretier holds a PhD in Chemistry. He developed mass spectrometry-based methods to characterize microorganisms during his doctoral studies. Next, he moved to the Genomic Research Laboratory at Geneva University Hospitals, supervised by Professor Schrenzel, where he studied antibiotic resistance mechanisms in Gram-negative bacteria as a postdoc. Concurrently, he developed molecular methods (isothermal amplification, metagenomics) that could be implemented in the routine diagnostic laboratories.

Professor Jacques Schrenzel obtained his medical degree in 1989 at the University of Geneva. Since 2000, he has been head of the Genomic Research Laboratory (GRL) and since 2004, the director of the Bacteriology Laboratory of the Geneva University Hospitals. He was appointed Associate Professor of the University of Geneva in 2010. The GRL is involved in the development of metagenomics and its application for medical research and diagnosis.

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