Exploring next-generation sequencing in clinical microbiology

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Chapter 1

General introduction and scope of the thesis

General introduction

Infectious diseases are major causes of death, especially in low-income countries, making up six out of the top 10 causes of death reported in 2019 (1). One major task of clinical microbiology is to improve patient care and public health by rapidly and reliably identifying and characterizing pathogens in patient samples to make a correct diagnosis, as well as to ensure optimal treatment and infection prevention. Culturing has been the traditional method for microbiology diagnostics. However, it can take days to weeks for a microbe to be successfully cultured from a sample (Fig.1). This ensues mainly because some clinically relevant pathogens are slow-growing, fastidious or sometimes even non-culturable under routine conditions (e.g., intrinsically, or when the patient has previously received antimicrobials) (2,3). This leads to delay or even failure to detect infection-causing microorganisms. On the other hand, conventional molecular assays, used as a reflex-test (a follow-up test based on the results of the initially requested test) or an alternative to traditional culturing (Fig.1), are limited by a restricted panel of pathogen targets at a time.

Traditional diagnostic techniques in clinical microbiology hold the advantage of being relatively inexpensive and are still considered the gold standard for identifying pathogens. However, they often rely on a priori knowledge of what to expect from a particular clinical sample or patient to request the most appropriate test, such as multiplexed PCR-panels or specific culture media, which remains challenging in many clinical situations (4). For bacterial identification and as a complement to culture, Sanger sequencing of the 16S ribosomal RNA (rRNA) gene has been used (5,6). The 16S rRNA gene, ~1.5 kilobases (kb) in length, is present in all bacteria, either as a single copy or multiple copies. It has proven to be a valuable molecular target as it is highly conserved over time (6).

The The continuous advancements in sequencing technology have facilitated the development of high-throughput next-generation sequencing (NGS) technologies which allow sequencing of billions of nucleic acid fragments simultaneously and independently (7). And regardless of the differences in the NGS technologies, sequencing workflows in general consist of both wet-lab (from sampling to sequencing) and e-lab (data analysis and interpretation) steps (8). NGS has offered several advantages over Sanger sequencing (which utilizes specific primers for the amplification of each genomic region), e.g., the applicability of a single protocol for all pathogens for both identification and typing, a higher resolution and accuracy in identifying and typing microbial pathogens, and culture-independent testing from complex polymicrobial samples to detect and identify several pathogens in parallel (9-11).

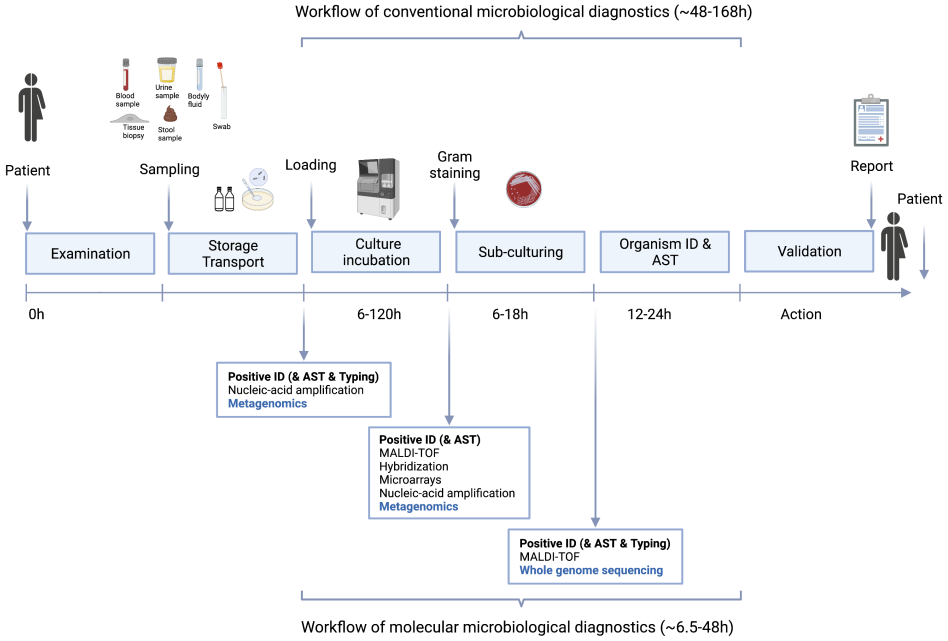

Fig.1: Workflow of microbiological diagnostics After physical examination of the patient, physicians may decide to take samples. Next, samples must be transported to the laboratory, where they will be cultured and incubated for 6-120 hours, usually at 35-37°C. After incubation, either molecular methods can be applied, a MALDI-TOF analysis may be performed to identify the microorganism and their antimicrobial resistance genes, or sub-culturing may be started, after which identification takes place by MALDI-TOF analyses and antimicrobial susceptibility testing, and further characterization through whole-genome sequencing. Metagenomics and nucleic acid amplification-based molecular techniques can be applied directly to clinical samples without incubation and after nucleic acid extraction. The NGS techniques addressed in this thesis are indicated in blue. ID, identification (Positive ID, specimens positive for identification of pathogens); AST, antimicrobial susceptibility testing [Figure modified from Peker N. et al., 2018, CMI (11)].

I. NGS in clinical microbiology

Recent advances in NGS technologies and bioinformatics tools have expanded sequencing as a diagnostic tool guiding the treatment of infectious diseases, infection control and prevention, and outbreak management in clinical microbiology. NGS, which allows the detection and identification of several pathogens in parallel, can help to improve the microbial diagnosis by a) expediting pathogen detection; b) decreasing time to antimicrobial resistance (AMR) and virulence detection; c) enabling pathogen typing with high discriminatory power.

a. Expediting pathogen detection

Several NGS approaches can be used for pathogen detection and identification, including i) whole-genome sequencing (WGS) after bacterial/fungal culture,

ii) targeted metagenomics after enrichment of targeted microbes from clinical samples or iii) shotgun metagenomics (SMg) directly from clinical samples. WGS has high discriminatory power for pathogen identification based on the whole genome. Unfortunately, often it can only be applied after culture of the pathogen. Amplicon sequencing (e.g., 16S rRNA gene sequencing) can be applied directly to patient material or after pathogen culture (Fig.1). Although NGS of the 16S rRNA gene is now widely used in clinical microbiology, species identification and discrimination are not always possible for bacteria as their 16S rRNA genes sometimes have high sequence homology (e.g., species of the *Streptococcus mitis* group; *Escherichia coli* and *Shigella* spp.) (6,11,13,14). NGS of the 16S-23S rRNA encoding region (~4.5 kb) directly from patient samples has been recently shown to provide a higher resolution for species identification than the sequencing of only the 16S rRNA gene (15,16). Its high-resolution power, combined with the possibility of pathogen detection directly from clinical (non-sterile) samples, makes it a convenient diagnostic tool. However, this approach is limited to species identification. On the other hand, SMg can also be applied directly to clinical samples and provides information on all pathogens present (7). A significant drawback is a lower sensitivity due to the host background DNA.

b. Decreasing time to detection of antimicrobial resistance (AMR) and virulence

NGS NGS is currently helping to improve the laborious and time-consuming procedure of various bacteriological, biochemical, and molecular assays for typing and characterization of pathogens (17). NGS may serve as an ideal one-step tool to study a broad range of pathogen characteristics from sequence data and can be applied on a wide range of pathogens for a rapid and improved i) molecular surveillance of pathogens both at regional and national scale, ii) prediction of disease severity through virulence profiling of pathogens, iii) prediction of AMR profiles for optimising antimicrobial treatment (11,18). Rapid and accurate diagnosis, which is essential for timely implementation of appropriate antimicrobial therapy, also helps prevent transmission and emergence/spread of AMR pathogens.

For slow-growing pathogens, for which time to results of the conventional phenotypic drug susceptibility testing is considerably delayed, rapid determination of AMR is of utmost importance. Specifically, in the case of tuberculosis (TB), one of the top-ranking causes of death from infectious diseases worldwide (19), decreasing time to AMR detection is highly valuable. Hence, WGS has been implemented in some countries, e.g., the UK and the Netherlands, for antimicrobial resistance profiling to predict susceptibility to first-line drugs as a diagnostic tool guiding TB treatment (20). However, translating genetic information into drug susceptibility phenotypes requires

sophisticated and robust bioinformatics pipelines, next to sufficient curated phenotypic validation data.

Rapid AMR detection through, e.g., an amplicon-based NGS approach can be advantageous also for directed screening during outbreaks. This approach bears the potential to rapidly clear the vast majority of negative samples and allow for selectively screening for the target of interest. As mentioned above, SMg, which can be applied directly to clinical material, provides different levels of diagnostic information: pathogen identification, detection of virulence factors, identification of AMR markers and typing (4). A relatively short turnaround time of the SMg method, compared to conventional diagnostics workflow (Fig. 1), allows obtaining clinically actionable results in a reasonable time frame (19,21,22). This approach has been tested for TB, decreasing the time from clinical specimen to antimicrobial susceptibility testing from 69.5 days with culture to 45.65 hours with Illumina sequencing and 6.28 hours with ONT sequencing (23). Another study using ONT sequencing directly on blood cultures detected and identified pathogens after 10 minutes of sequencing and AMR-encoding genes and plasmids within one hour (24).

c. Enabling pathogen typing with high discriminatory power

Typing pathogens is necessary for surveillance purposes and public health management. The early days of microbial typing relied on phenotypic methods, e.g., phage typing and serotyping (25), for which the typing and discrimination of the pathogens was complicated due to cross-reactions (26) or by the presence of infections caused by highly fastidious bacteria, which could not be confirmed by culture (27). Thus, the phenotypic methods have mainly been replaced by molecular typing methods as, e.g., the restriction fragment length polymorphism (RFLP), amplification-based typing methods as, e.g., variable number of tandem repeats [VNTR)], multi-locus variable tandem-repeat analysis [MLVA]), and the often used amplification fragment length polymorphism (AFLP), and sequencing-based methods which are based on characterization of single gene, called single locus sequence typing (e.g., *spa* typing for Staphylococcus aureus/ methicillin-resistant S. aureus (MRSA)), or several genes, called multi locus sequence typing (MLST)(26,28). As there is a need for speed in detecting outbreaks to implement accurate and timely infection control measures, typing directly from patient samples is preferred. SMg does not require culturing of pathogens and is faster than WGS- typing of cultured isolates. However, it comes with several challenges. This is true, especially if multiple isolates of the same species are present in a sample. Next to speed, outbreaks require high discriminatory typing methods. Genome analyses for molecular pathogen typing such as core genome multi-locus sequence typing (cgMLST) and core genome single nucleotide polymorphism (cgSNP) analysis provide such high typing resolution and reveal additional

detailed genetic characterization for outbreak isolates compared to other typing methods (e.g., MLST and MLVA that target only selected genomic regions (7).

II. NGS technologies: short-read and long-read sequencing

Currently, short-read sequencing platforms of Illumina (San Diego, CA) and Thermofisher (Waltham, MA), and long-read sequencing platforms of Oxford Nanopore Technologies (ONT) (Oxford, United Kingdom) and PacBio (Pacific Biosciences) (CA) are used in clinical microbiology. The currently available short-read sequencing technologies, offering high sequence read accuracy, are limited to 50-400 bp read lengths. The presently available long-read sequencing technologies offer longer read sizes of 1-100 kb at the expense of read accuracy (Table 1) (29).

The sequencing approach and platform of choice depends on the laboratories' capacity and need. For instance, most Illumina platforms require batching to be cost-effective and are primarily used in reference laboratories or for surveillance and are less applicable in routine diagnostics where selected samples need to be processed on demand (4). The most widely used sequencing platform in diagnostics and research is Illumina. By contrast, ONT platforms, such as the MinION, offer low to medium throughput flexible operation. With cost-effectiveness (provided by on-demand processing of individual samples), portability in resource-limited settings and the ability to provide same-day diagnostic results (Table 1), ONT platforms have become more popular for clinical diagnostics and outbreak investigations (21,22,30).

Table 1. Characteristics and strengths of the sequencing platforms (11,29,31) used in this thesis.

	Short-read sequencing		Long-read sequencing
	Illumina (e.g., MiSeq, NextSeq)	**ThermoFisher** (IonTorrent PGM™)	**Oxford Nanopore Technologies Limited** (e.g., MinION, GridION)
Read length	Up to 600 pb	200 bp-400bp	Up to 100 kbp
SNV error rate	< 0.1 %	< 0.1 %	1%-5%
Indel error rate	< 0.1 %	<1 %	5%-10%
Advantages	Read accuracy High throughput Scalable	Read accuracy High throughput	Read length Portability

Abbreviations: SNV, single nucleotide variant; Indel, insertions deletions

III. Bioinformatics in clinical microbiology

The rapid development of sequencing technologies has already transformed microbiological diagnostics in clinical settings. Bioinformatics is needed to interpret the data obtained by sequencing devices, starting with converting raw signals (e.g., squiggles; signal data of ONT sequencing) to DNA sequences (Fig. 2: base calling of sequence reads). After, reads are trimmed to get the highest quality reads, and to discard the low-quality reads that might interfere with the downstream analysis. Then the adapter trimming takes place to remove adapters and primers introduced during library preparation. Subsequently, taxonomic classification based on reads can be done for SMg sequencing, but for WGS, usually assembly, i.e., building contigs (contiguous sequences) out of shorter sequences, is performed first. The assembled genome can then be used for i) taxonomic assignment/confirmation, ii) AMR and virulence detection, and/or iii) pathogen typing through cgMLST, cgSNP analysis. Besides the assembly, bioinformatics tools enable pathogen characterization (AMR/virulence detection, typing) directly from the sequencing reads.

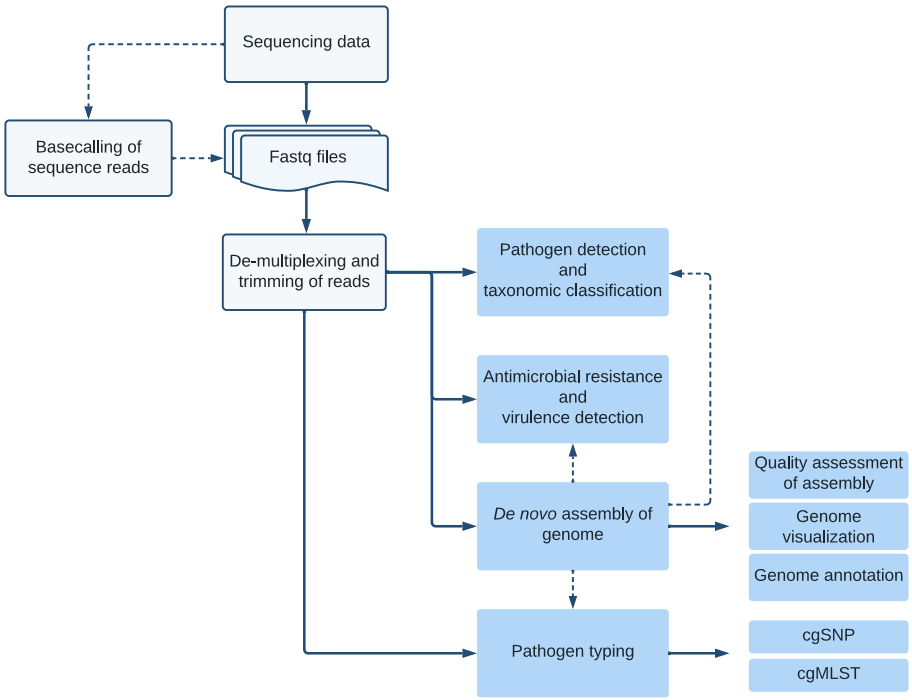

Fig. 2: NGS data analysis workflow. First, raw signals of sequencing data (e.g., bcl files of Illumina sequencing, fast5 files of ONT sequencing) are converted to fastq files, which can be already performed by the sequencing device (e.g., MiSeq). Then the reads are adapter and quality trimmed and demultiplexed. After pre-processing the sequence data, further analysis ranging from pathogen identification to pathogen characterization can be performed.

Implementation of NGS to diagnose infectious diseases needs standardized, validated bioinformatics tools/pipelines. The most commonly used bioinformatics tools, including the ones used in this thesis, and their purpose are summarized in Table 2.

Table 2. Non-exhaustive list of current, most commonly used bioinformatics tools and their purposes are summarized.

Application	Tool	Interface	Link
Basecalling	Guppy	command-line	https://github.com/nanoporetech
Sequence read quality check	FastQC	desktop application	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
	NanoPlot	command-line	https://github.com/wdecoster/NanoPlot
Trimming reads	trimmomatic	command-line	http://www.usadellab.org/cms/index.php?page=trimmomatic
	qcat	command-line	https://github.com/nanoporetech/qcat
FASTA/Q file manipulation	SeqKit	command-line	https://github.com/shenwei356/seqkit
Filtering long reads	Filtlong	command-line	https://github.com/rrwick/Filtlong
De novo assembly	SPAdes	command-line	https://github.com/ablab/spades
	Velvetoptimiser	command-line	https://github.com/tseemann/VelvetOptimiser
	ABySS	command-line	https://github.com/bcgsc/abyss
	Shovill	command-line	https://github.com/tseemann/shovill
	Canu	command-line	https://github.com/marbl/canu
	Unicycler	command-line	https://github.com/rrwick/Unicycler
	Flye	command-line	https://github.com/fenderglass/Flye
Assembly polishing	Racon	command-line	https://github.com/lbcb-sci/racon
	Medaka	command-line	https://github.com/nanoporetech/medaka
Quality assessment tool for genome assemblies	QUAST	command-line	http://quast.sourceforge.net/index.html
Sequence alignment	Minimap2	command-line	https://github.com/lh3/minimap2
	bwa-mem2	command-line	https://github.com/bwa-mem2/bwa-mem2
Reading/writing/editing/indexing/viewing	samtools	command-line	https://github.com/samtools/samtools
Calling/filtering/summarising SNP and short indel sequence variants	bcftools	command-line	https://github.com/samtools/bcftools
SNP calling pipeline	Snippy	command-line	https://github.com/tseemann/snippy
SNP distance matrix	snp-dists	command-line	https://github.com/tseemann/snp-dists
maximum-likelihood phylogenetic trees	FastTree	command-line	http://www.microbesonline.org/fasttree/

Anti-TB drug resistance prediction	TBProfiler	command-line	https://github.com/jodyphelan/TBProfiler
	Mykrobe	command-line	https://github.com/Mykrobe-tools/mykrobe
Antimicrobial resistance and virulence gene detection	ABRicate	command-line	https://github.com/tseemann/abricate
	ResFinder	web-interface	https://cge.cbs.dtu.dk/services/ResFinder/
	ARIBA	command-line	https://github.com/sanger-pathogens/ariba
	AMRFinderPlus	command-line	https://github.com/ncbi/amr#ncbi-antimicrobial-resistance-gene-finder-amrfinderplus
Taxonomic classification	Kraken2	command-line	https://github.com/DerrickWood/kraken2
Taxonomic classification/mapping/*de novo* assembly/alignment	CLC Genomics Workbench	desktop application	https://digitalinsights.qiagen.com/
Pathogen Typing	Ridom SeqSphere+	desktop application	https://www.ridom.de/seqsphere/

A. Scope of the thesis

Clinical microbiology seeks to improve patient care by rapidly and reliably identifying and characterizing pathogens to ensure optimal treatment and infection control. Gold standard culture-based methods are relatively slow, and current rapid molecular tests only cover a few resistance determinants. Therefore, this PhD study aims at developing or optimizing (novel) approaches to speed up the diagnostic process in clinical microbiology for timely administration of appropriate therapy and prevention of the spread of infections. The main objectives are exploring innovative ways of using NGS as a diagnostic tool, looking into different sequence data analysis approaches, and evaluating them accordingly for different diagnostics purposes in clinical microbiology: pathogen identification, AMR determination, and pathogen typing.

Part I of this thesis presents the recent developments in molecular diagnostic approaches, mainly focusing on diagnosing bloodstream infections (BSIs). **Chapter 2** provides an update on recent developments in molecular-based diagnostic platforms used for identifying BSIs, focusing on assays performed on positive blood cultures and blood samples.

Part II of this thesis addresses whole-genome sequencing in clinical diagnostics. In **Chapter 3**, we assess the relevance of recent advances in WGS of *Mycobacterium tuberculosis* (MTB), provided by both short-read (SR) and long-read (LR) sequencing technologies for anti-TB drug resistance prediction and MTB typing. Overall, the study provides a comparison of the currently in use bioinformatics tools employed for both SR and LR sequencing of MTB,

aiming at guiding investigators how to choose the appropriate tools for different clinical diagnostic applications.

Part III of this thesis presents the application of amplicon sequencing (a form of targeted metagenomics) to identify and type pathogens and detect AMR genes. **Chapter 4** describes an amplicon-based NGS method, based on a commercial kit, used for screening during a CTX-M-15-producing *Escherichia coli* outbreak in a Dutch nursing home. This study presents the value of amplicon-based NGS for screening during outbreaks to rapidly distinguish negative from positive patients using primary samples.

Chapter 5 depicts various data analysis approaches proposed to identify pathogens directly from patient samples using an amplicon-based NGS method targeting the 16S-23S rRNA encoding region. This study compares the speed and diagnostic accuracy of different data analysis approaches, i.e., *de novo* assembly and Basic Local Alignment Search Tool (BLAST), Operational Taxonomic Unit (OTU) clustering, or mapping using an in-house developed 16S-23S rRNA encoding region database for the identification of bacterial species.

Part IV of the thesis presents the application of shotgun metagenomics in clinical microbiology. **Chapter 6** describes the future potential of metagenomic next-generation sequencing (mNGS) in clinical laboratories. We discuss i) the expectations of integrating clinical mNGS in routine diagnostics; ii) how this can be achieved by explaining the main hurdles, e.g., cost, turnaround time, different sensitivity/specificity, validation and reproducibility to overcome; and iii) how mNGS could be implemented in clinical laboratories. **Chapter 7** evaluates whole blood and plasma protocols for shotgun metagenomics-based diagnosis of BSIs using the a long-read sequencing platform. **Chapter 8** studies the development of an SMg protocol to improve the microbial detection sensitivity using a saponin pre-treatment directly on patient material. We developed a method for NGS-based diagnosis of *Mycobacterium* spp., using *Mycobacterium abscessus* as a model species for spiking sputum samples.

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Part I.
Molecular Diagnostics in Clinical Microbiology