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Exploring next-generation sequencing in clinical microbiology

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Summary

Rapid and accurate detection of pathogens is a fundamental step in infection management and ensuring an optimum antimicrobial therapy is provided to the patient, ultimately reducing the antimicrobial resistance selection and medical costs of healthcare. Next-generation sequencing (NGS) technologies have transformed diagnostics in clinical microbiology by overcoming some of the challenges of conventional methods: culturing, relying on the growth of the pathogens, and molecular assays, limited by a restricted panel of pathogen targets at a time. NGS can provide detailed sequence information for specific genetic regions or the whole genome in a single assay. However, the interpretation of the sequence data requires sophisticated and robust bioinformatics pipelines to be standardized. In this thesis, we aimed at studying state-of-the-art molecular diagnostic techniques that strive for an accelerated microbial diagnosis. More specifically, we studied the application of several NGS approaches for different clinical applications and evaluated bioinformatic data analysis tools/approaches for pathogen detection, characterization, and typing.

In **Chapter 2**, a literature search was conducted on the available molecular methods used in diagnosing bloodstream infections (BSIs). We discussed the implications of molecular methods in microbiological diagnostics of BSIs. We concluded that **the molecular tests reviewed, integrated as part of the diagnostic workflow complementary to conventional blood culture diagnostics and antimicrobial stewardship programs, may optimize infection management.**

Chapter 3 focused on evaluating currently in use bioinformatics tools employed for WGS of *Mycobacterium tuberculosis* provided by both short- and long-read sequencing technologies, aiming at guiding investigators to choose the appropriate tools for different clinical diagnostic applications. We assessed the significance of Illumina short-read and ONT long-read sequencing for i) anti-TB drug resistance prediction using the TBProfiler and Mykrobe tools, ii) the fraction of genome recovery and assembly accuracies, and iii) the robustness of cgMLST and cgSNP typing of *M. tuberculosis* based on either reads or assemblies. In our evaluation, **anti-TB drug resistance prediction, particularly with long-read sequence data, was challenging, especially for first-line drugs. Short-read assemblies were more accurate than long-read assemblies**. **Moreover, the cgSNP approach, which resulted in reproducible phylogenies, was more robust than the cgMLST approach, especially for long-read sequence data**.

Chapter 4 described the use of an amplicon-based NGS method in a retrospective study of a CTX-M-15-producing *Escherichia coli* outbreak. In

total, 149 fecal samples and rectal eSwabs were analyzed. ESBL-producing bacteria and resistance genes were detected directly from samples without prior enrichment cultures using an amplicon-based NGS method. The same samples were cultured for comparison purposes. While the sensitivity for CTX-M detection was 96.3% and 85.2% for the phenotypic and NGS methods, respectively, the specificity was 100% for both methods. This resulted in a PPV of 100% for both methods and an NPV of 99.2% and 96.8% for the phenotypic and NGS methods, respectively. Given the 14 hours and 4 days' time-to-results for the NGS method and the phenotypic method, respectively, we concluded that **an amplicon-based NGS method could be advantageous for outbreak management to identify positive samples rapidly**.

In **Chapter 5**, we aimed at assessing the optimum data analysis approach providing the highest discriminatory power for NGS of the 16S-23S rRNA encoding region to identify bacterial species. 16S-23S rRNA NGS was performed for 28 clinical tissue and body fluid samples. We tested three different NGS data analysis approaches using an in-house compiled 16S–23S rRNA encoding region database for the correct assignment of bacterial species directly from clinical samples: i) *de novo* assembly followed by BLASTN, ii) mapping, and iii) OTU clustering. The speed and diagnostic accuracy of these approaches compared to the gold standard of culture and 16S rRNA gene Sanger sequencing was evaluated. ***De novo*** **assembly followed by BLAST using the in-house database was the optimal data analysis approach** with the shortest turnaround time, approximately 2 h less than OTU clustering and 4.5 h less than mapping, and had a sensitivity of 80%.

Chapter 6 presents our opinion on the future potential of clinical metagenomics next-generation sequencing (mNGS) as a diagnostic tool in clinical microbiology laboratories. We discussed how mNGS meets the expectations of clinical microbiology laboratories by considering the challenges associated with costs, turnaround time, sensitivity/specificity, validation, and reproducibility. We furthermore described a diagnostic algorithm of potential workflow for the intended use of mNGS, which is currently performed on a case-by-case basis. **mNGS has the potential to become a cost-competitive option for routine diagnostics as it could be used as a direct "rule in" or "rule out" test to confirm the presence or absence of an infectious aetiology especially for specific patient populations.**

In **Chapter 7**, we investigated the application of shotgun metagenomics (SMg) to diagnose BSIs using the fast MinION (ONT) long-read sequencing technology. In this study, we experimented on whole blood spiked with a single common BSIs causing bacterium, a mock microbial community at different concentrations, and plasma obtained from spiked whole blood. We evaluated

DNA extraction with the Blood Pathogen Kit (Molzym, GmbH Bremen, Germany) and the MinION sequencing protocols for their efficiency in recovering/identification of bacteria from both whole blood and plasma samples. Overall, **the number of bacterial reads recovered from spiked whole blood was higher than from plasma samples**. However, **more than >95% and >50% of the sequencing reads were classified as human in single bacteria and mock microbial community spiked whole blood samples, respectively**. Further optimization of the DNA extraction protocol is needed to increase the bacterial DNA recovery combined with a low level of human DNA.

In **Chapter 8**, our goal was to develop an SMg method for rapid diagnosis of different mycobacterial infections, most importantly infections caused by slow-growing mycobacteria like *M. tuberculosis*. We first evaluated the DNA extraction efficiency of three nucleic acid extraction kits for mycobacteria using *Mycobacterium abscessus* as a model species. Then, we assessed the microbial detection sensitivity of the selected kit (AllPrep PowerFecal DNA/RNA kit [Qiagen]) and a host depletion technique of saponin pre-treatment on *M. abscessus* spiked sputum at three different dilutions. Using **Illumina sequencing, up to an 89% increase was observed in the percentage of reads mapped to *M. abscessus* in saponin treated samples compared to the untreated ones**. This methodology could also be efficiently applied to the direct detection of *M. tuberculosis* and other mycobacteria from sputum.

The findings of this thesis present the wide-ranging applications of different NGS and associated data analysis approaches to make clinical microbiology diagnostics better and faster.

Nederlandse Samenvatting

Snelle en nauwkeurige detectie van pathogenen is essentieel voor infectiebestrijding en -preventie en het voorschrijven van een optimale antimicrobiële therapie aan de patiënt. Het draagt bij aan het voorkomen van antimicrobiële resistentie en het verminderen van de kosten in de gezondheidszorg. Next-generation sequentie (NGS) technologieën hebben het diagnostisch landschap getransformeerd, doordat ze in tegenstelling tot de kweek niet afhankelijk zijn van de groei van de pathogenen en, in tegenstelling tot op amplificatie-gebaseerde testen, niet beperkt zijn tot een panel van pathogenen per test. NGS kan gedetailleerde informatie geven over specifieke genetische regio's of het volledige genoom. De interpretatie van de sequentiegegevens vereist echter verfijnde en gestandaardiseerde bioinformatica pijplijnen. In dit proefschrift hebben we state-of-the-art moleculaire diagnostische technieken bestudeerd die een versnelde microbiële diagnose beogen. Meer specifiek, we bestudeerden de toepassing van verschillende NGS-benaderingen voor verschillende klinische toepassingen en evalueerden bioinformatica data-analyse tools/benaderingen voor pathogeen detectie, karakterisering en typering.

In **Hoofdstuk 2** werd een literatuuronderzoek uitgevoerd naar de beschikbare moleculaire methoden die worden gebruikt bij de diagnose van bloedstroom infecties (BSIs) en hun betekenis voor de diagnose hiervan. We concludeerden dat **de moleculaire testen die we hebben onderzocht, geïntegreerd als onderdeel van de diagnostische workflow in aanvulling op de conventionele bloedkweekdiagnostiek en antimicrobiële stewardship programma's, infectiebestrijding en -preventie optimaliseren.**

Hoofdstuk 3 richtte zich op de evaluatie van de bioinformatica tools die momenteel worden gebruikt voor WGS van *Mycobacterium tuberculose* middels zowel short- als long-read sequentie technologieën, met als doel onderzoekers te adviseren bij het kiezen van de juiste tools voor verschillende klinisch diagnostische toepassingen. We onderzochten het belang van Illumina short-read en ONT long-read sequensen voor i) het voorspellen van antibioticaresistente in *M. tuberculose* met behulp van de TBProfiler en Mykrobe tools, ii) het percentage van het genoom dat wordt verkregen en de nauwkeurigheid van de assemblage, en iii) de robuustheid van cgMLST en cgSNP typering van *M. tuberculose* gebaseerd op afzonderlijke of geassembleerde reads. **Het voorspellen van resistentie van *M. tuberculose* tegen met name eerstelijns geneesmiddelen, vooral met long-read sequentiegegevens, bleek een uitdaging. Short-read assemblages waren nauwkeuriger dan long-read assemblages. Bovendien was de cgSNP benadering, die resulteerde in reproduceerbare fylogenie, robuuster**

dan de cgMLST benadering, wederom met name voor long-read sequentie data.

Hoofdstuk 4 beschrijft het gebruik van een op amplicon-gebaseerde NGS-methode in een retrospectieve studie van een CTX-M-15-producerende *Escherichia coli* uitbraak. In totaal werden 149 fecale monsters en rectale eSwabs geanalyseerd. ESBL-producerende bacteriën en resistentiegenen werden rechtstreeks uit monsters gedetecteerd zonder voorafgaande verrijkingskweken met behulp van een op amplicon-gebaseerde NGS-methode. Dezelfde monsters werden ter vergelijking gekweekt. De gevoeligheid voor CTX-M-detectie bedroeg 96,3% en 85,2% voor respectievelijk de fenotypische en de NGS-methode, terwijl de specificiteit 100% was voor beide methoden. Dit resulteerde in een PPV van 100% voor beide methoden en een NPV van 99,2% en 96,8% voor respectievelijk de fenotypische en de NGS-methode. Gezien de 14 uur en 4 dagen tijd-tot-resultaat voor respectievelijk de NGS-methode en de fenotypische methode, concludeerden we dat **een op amplicon-gebaseerde NGS-methode kan helpen tijdens een uitbraak om snel positieve monsters te identificeren.**

In **Hoofdstuk 5** hebben we ons gericht op het beoordelen van de optimale data-analyse aanpak die het hoogste discriminerende vermogen levert voor NGS van de 16S-23S rRNA coderende regio voor het identificeren van bacteriesoorten. 16S-23S rRNA NGS werd uitgevoerd voor 28 klinische weefsel- en lichaamsvloeistofmonsters. We testten drie verschillende NGS data-analyse benaderingen met behulp van een in-house samengestelde 16S-23S rRNA coderende regio database voor de juiste toewijzing van bacteriële soorten direct uit klinische monsters: i) *de novo* assemblage gevolgd door BLASTN, ii) mappen tegen referentie-sequenties, en iii) OTU-clustering. De snelheid en diagnostische nauwkeurigheid van deze benaderingen in vergelijking met de gouden standaard van kweek en 16S rRNA gen Sanger sequensen werd geëvalueerd. ***De novo* assemblage gevolgd door BLAST met behulp van de in-house database bleek de optimale data-analyse aanpak met de kortste doorlooptijd (ongeveer 2 uur minder dan OTU-clustering en 4,5 uur minder dan mappen), en had een gevoeligheid van 80%.**

Hoofdstuk 6 geeft onze mening over het toekomstige potentieel van klinische metagenomics next-generation sequensen (mNGS) als diagnostisch hulpmiddel in klinische microbiologische laboratoria. We bespraken de verwachtingen van klinische microbiologische laboratoria van mNGS en de uitdagingen hiervan die samenhangen met kosten, doorlooptijd, gevoeligheid/specificiteit, validatie, en reproduceerbaarheid. Verder beschrijven we een diagnostisch algoritme voor het gebruik van mNGS, dat van geval tot geval varieert. **mNGS heeft het potentieel om een kosteneffectieve techniek binnen de routinediagnostiek te worden,**

omdat het gebruikt kan worden als een directe "rule in" of "rule out" test om de aan- of afwezigheid van een infectieuze etiologie te bevestigen, vooral voor specifieke patiëntenpopulaties.

In **Hoofdstuk 7** onderzochten we de toepassing van shotgun metagenomics (SMg) voor de diagnose van BSIs met behulp van de MinION (ONT) long-read sequentie technologie. In deze studie hebben we geëxperimenteerd met volbloed gespiket met een veel voorkomende bacterie die BSIs veroorzaakt, een mock microbiële gemeenschap in verschillende concentraties, en plasma verkregen uit gespiket volbloed. We evalueerden DNA-extractie met de Blood Pathogen Kit (Molzym, GmbH Bremen, Duitsland) gevolgd door MinION sequensen voor het identificeren van bacteriën uit zowel volbloed- als plasmamonsters. In het algemeen was **het aantal teruggevonden bacteriële reads in gespikete volbloedmonsters hoger dan in plasmamonsters. Echter, meer dan >95% en >50% van de gesequenste reads werden geclassificeerd als menselijk in volbloed monsters gespiket met één bacterie en de mock microbiële gemeenschap, respectievelijk.** Verdere optimalisatie van het DNA-extractie protocol is nodig om het percentage bacterieel DNA te verhogen en menselijk DNA te verwijderen.

In **Hoofdstuk 8** was ons doel om een SMg methode te ontwikkelen voor snelle diagnose van verschillende mycobacteriële infecties, met name infecties veroorzaakt door langzaam groeiende mycobacteriën zoals *M. tuberculose*. Eerst evalueerden we de DNA-extractie-efficiëntie van drie verschillende nucleïnezuurextractiekits voor mycobacteriën, met *Mycobacterium abscessus* als modelsoort. Vervolgens evalueerden we de microbiële detectiegevoeligheid van de geselecteerde kit (AllPrep PowerFecal DNA/RNA kit [Qiagen]) en een gastheer DNA-depletie techniek met behulp van een saponine voorbehandeling op met *M. abscessus* gespiket sputum bij drie verschillende verdunningen. **In de daaropvolgende Illumina sequentie-analyse, werd in saponine behandelde monsters een tot 89% toename waargenomen in het percentage *M. abscessus* reads in vergelijking tot de onbehandelde monsters.** Deze methodologie kan ook efficiënt worden toegepast voor de directe detectie van *M. tuberculose* en andere mycobacteriën uit sputum.

De bevindingen van dit proefschrift laten de veelzijdige toepassingen zien van verschillende NGS en bijbehorende data-analyse benaderingen om klinisch microbiologische diagnostiek beter en sneller te maken.

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About the author

Nilay Peker was born on the 18th of March in 1987 in Gazipasa, Turkey. She obtained her undergraduate degree in Molecular Biology and Genetics at Izmir Institute of Technology (Izmir, Turkey) in 2011. She then pursued her Master's degree in Medical Biotechnology at the Royal Institute of Technology (KTH) in Stockholm, Sweden. Her Master's thesis topic was about the development of microdevices for digital ELISA to be used for diagnostic purposes in clinics. After her graduation in 2014, she worked as a visiting researcher in the Clinical Microfluidics group of the Nanobiotechnology Department at KTH. She then worked as a molecular biologist at Biyo-Gen Genetics Diagnostics Laboratory in Antalya, Turkey. In 2017, she started her new position at the University Medical Center Groningen, The Netherlands, as a PhD candidate in the Pronkjewail doctoral training program funded by the Marie Skłodowska-Curie Actions, European Union's Horizon 2020. Her PhD research focused on the development of methods for rapid identification of micro-organisms and resistance determinants by next-generation sequencing (NGS) technologies under the supervision of Prof. Bhanu Sinha, Prof. John W.A Rossen and Dr. Natacha Monge Gomes do Couto at the microbiology department. She explored different NGS and sequence data analysis approaches for identification, characterization and typing of pathogens in clinical microbiology. Her PhD research projects also involved the collaboration of international research groups in Germany, Switzerland and Sweden. During writing her PhD thesis, she started working as a molecular biologist and bioinformatician at Örebro University Hospital, Sweden in 2021.

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