Next-generation sequencing to investigate antimicrobial resistance

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Appendices

Summary

Antimicrobial resistance (AMR) is a leading cause of death around the world and rising numbers of infections caused by antimicrobial-resistant bacteria (ARB) represent a global threat to public health and agriculture. Given the occurrence and dissemination of ARB and ARGs in different ecological niches, such as human population, wild and farm animals and environment, it is necessary to look at it from a One-Health perspective. Therefore, new approaches that can be applied to human, animal, and environmental samples are essential to improve surveillance of AMR. Next-generation sequencing allows to determine the whole genome of a single pathogen (culture-dependent) but also to detect microorganisms and ARGs composition by sequencing the DNA/RNA content of a given sample without previous isolation (culture-independent). This thesis aimed to use NGS technologies for the surveillance of ARB and to track the dissemination of resistance determinants in human healthcare and animal farms.

In **Chapter 2** we demonstrate the possibility of applying short- and long-read whole-genome sequencing (WGS) to characterize environmental *Legionella* spp. strains and investigate bacterial molecular epidemiology in the nosocomial environment. Three dental-chair units were found positive for *Legionella anisa*, and four isolates were cultured. WGS and whole-genome multi-locus sequence typing (wgMLST), allowed the identification of a unique clone of *Legionella anisa* in several dental chair units in the same hospital dental ward. By combining ONT long reads and Illumina short reads, we succeeded in obtaining two contigs representing two complete and circular plasmid sequences, improving the quality of ARGs and VFs analyses. Our analysis revealed tap-water contamination in direct contact with patients and the usefulness of WGS to investigate bacterial molecular epidemiology.

I would add here what your study could contribute in the change of regular water analysis for risk assessment and prevention of infections in the nosocomial environment.

In **Chapter 3** we highlighted the possibility of applying a culture-based approach and WGS to investigate the presence of colistin-resistant Gram-negative (ColR-GN) bacteria in the wastewater drains of high-risk hospital units, throughout the 5 months

of the sampling period. We found ColR-GN in all investigated units with *Enterobacte*r spp. being the most abundant genus. WGS allowed to investigate molecular mechanisms of resistance, including chromosomal mutations and mobile colistin resistance (*mcr*) genes. In addition, the use of core-genome SNP analysis allowed to investigate persistence in the drains and reveal genetic relationships between clinical and environmental isolates with high discriminatory power. Colistin resistance surveillance in intensive care and hematology units for ColR allowed to identify a possible reservoir and could provide insights into transmission routes, helping design appropriate control measures and prevent further dissemination.

In **Chapter 4** we showed the potential of applying shotgun metagenomics (SMg) to monitor the bacterial community and resistome profiles of tap-water samples collected from high-risk hospital units. In addition, we use selective culture to investigate the presence of: ARB resistant to last-resort antibiotics, such as Carbapenem-resistant (CRE) and Colistin-Resistant Enterobacterales (ColRE), in tap-water and hospital wastewater drains (sinks and showers). Tap-water SMg analysis revealed the presence of opportunistic pathogens, such as *Pseudomonas aeruginosa*, and ARGs related to last resort antimicrobials, including gene conferring resistance to carbepenems such as bla_{VIM}. The hemotology unit had the highest rates of ARGs, with genes conferring resistance to beta-lactams, aminoglycosides, and macrolides, being the large majority. In addition, using selective culture we detected several opportunistic pathogens, mostly extended-spectrum beta-lactamases (ESBLs) producing *Enterobacter* spp. and carbapenem resistant *P. aeruginosa*. The presence of pathogenic bacteria and genes conferring resistance to "last-resort" antimicrobials pose a potential risk for patients and healthcare workers and highlights the importance of surveillance of the hospital water environment.

In **Chapter 5** we reported for the first time an *mcr-5* gene in an indoor and healthcare water environment. We characterized a novel *mcr-5.4* variant using SMg and, combining short- and long-reads metagenomic sequencing, assembled an accurate and long contig harbouring the *mcr-5.4* gene. Despite low coverage and sequence depth, we succeeded in characterizing the ARG genetic environment and assigned the bacterial host to the order of Pseudomonales using metagenomic binning analysis.

The findings of this study highlight the possible role of tap-water in dental units as reservoirs for clinically relevant ARGs and the potential of applying SMg in hospital environment surveillance.

In **Chapter 6** we evaluated the use of SMg in combination with oral fluid (OF) sampling to monitor the presence of pathogens and ARGs in pig farms. We discovered a diverse resistome with aminoglycosides, macrolides, tetracyclines, and beta-lactams resistance genes being the most abundant ARG classes. More than half of the ARGs (52.8%) were predicted to be located on plasmids. Viral enrichment enabled the detection of several clinically significant viruses such as influenza A virus and porcine hemagglutinating encephalomyelitis virus. Metagenomic sequencing of OF samples enabled the characterization of a wide range of pathogens, heavy metal genes, ARGs directly from the sample.

In **Chapter 7**, the application of short- and long-reads SMg sequencing to an OF sample allowed us to characterize a small IncX4 plasmid harboring the mobile colistin resistance mcr-1.1 gene directly from the sample. The plasmid shares high homology with previously characterized plasmids in Enterobacterales from human, animal, food, and environmental specimens from several continents. However, it differed significantly in size due to the absence of part of the T4SS, which most likely resulted in the plasmid becoming non-self-transmissible. In this chapter, we showed the potential of culture-independent SMg approach to detect complete plasmids.

Samenvatting

Antimicrobiële resistentie (AMR) is wereldwijd een belangrijke doodsoorzaak. Het stijgend aantal infecties veroorzaakt door antimicrobieel resistente bacteriën (ARB) vormt een wereldwijde bedreiging voor de volksgezondheid en de landbouw. Gezien het voorkomen en de verspreiding van ARBs en antimicrobiële resistentie genen (ARGs) in verschillende ecologische niches, zoals de wereldbevolking, wilde-, landbouw, en huisdieren en het milieu, is het noodzakelijk om dit vanuit het "One Health" oogpunt te bekijken. Daarom zijn nieuwe benaderingen toepasbaar op menselijke, dierlijke en milieumonsters essentieel om de surveillance van AMR te verbeteren. Next-generation sequencing maakt het mogelijk om het volledige genoom van één pathogeen te bepalen (kweek-afhankelijk) maar ook om de micro-organismen en ARG-samenstelling vast te stellen door direct het DNA/RNA van een monster te sequencen zonder het eerst te kweken (kweek-onafhankelijk). Dit proefschrift heeft als doel om NGS technologieën te gebruiken voor de surveillance van ARB en om de verspreiding van resistentie determinanten in de gezondheidszorg en veehouderij te traceren.

In **Hoofdstuk 2** passen we short- en long-read whole-genome sequencing (WGS) toe om omgevingsstammen van *Legionella* spp. te karakteriseren en de bacteriële moleculaire epidemiologie in de nosocomiale omgeving te onderzoeken. Drie tandartsstoelen werden positief bevonden voor *Legionella anisa*, en vier isolaten werden gekweekt. WGS en whole-genome multi-locus sequence typing (wgMLST), maakten de identificatie mogelijk van een unieke kloon van *L. anisa* in verschillende tandartsstoelunits in dezelfde tandartsafdeling van het ziekenhuis. Door ONT long reads en short reads van Illumina te combineren, slaagden we erin twee contigs te verkrijgen die twee volledige en circulaire plasmide-sequenties vertegenwoordigen, hetgeen de kwaliteit van de ARGs- en VFs-analyses verbeterde. Onze analyse onthulde een besmetting van kraanwater waarmee patiënten in aanraking komen. Ook de bruikbaarheid van WGS om bacteriële moleculaire epidemiologie te onderzoeken werd aangetoond.

In **Hoofdstuk 3** hebben we de aanwezigheid van colistine-resistente Gram-negatieve (ColR-GN) bacteriën in de afvalwaterafvoeren van hoog-risico ziekenhuisafdelingen, gedurende 5 maanden gemonitord met behulp van kweek-gebaseerde. We vonden ColR-GN in alle onderzochte afdelingen, waarbij Enterobacter spp. het meest voorkomende genus was. WGS maakte het mogelijk moleculaire mechanismen van resistentie te onderzoeken, waaronder chromosomale mutaties en mobiele colistineresistentie (mcr)-genen. Bovendien maakte het gebruik van kern-genoom SNP-analyse het mogelijk om een mogelijke persistente aanwezigheid van bacteriën in de afvoerkanalen te onderzoeken en genetische relaties tussen klinische en omgevingsisolaten met een hoog discriminerend vermogen aan het licht te brengen. Colistine-resistentie surveillance op intensive care en hematologie afdelingen voor ColR maakte het mogelijk om een mogelijk reservoir te identificeren. Dit kan verder inzicht geven in transmissieroutes, wat kan helpen bij het ontwerpen van passende controlemaatregelen en het voorkomen van verdere verspreiding.

In **Hoofdstuk 4** laten we zien dat het mogelijk is om shotgun metagenomics (SMg) toe te passen om de bacteriegemeenschap en resistentieprofielen van kraanwatermonsters van hoog-risico ziekenhuisafdelingen te monitoren. Daarnaast gebruiken we selectieve kweek om de aanwezigheid te onderzoeken van: ARB resistent tegen laatste-resort antibiotica, zoals Carbapenem-resistente (CRE) en Colistine-resistente Enterobacterales (ColRE), in kraanwater en ziekenhuis afvalwater afvoeren (gootstenen en douches). Kraanwater SMg-analyse onthulde de aanwezigheid van opportunistische pathogenen, zoals *Pseudomonas aeruginosa*, en ARGs gerelateerd aan resistentie tegen laatste redmiddel antimicrobiële middelen, waaronder genen die resistentie veroorzaken tegen carbepenems, zoals blaVIM. De hemotologieafdeling had de hoogste ARG-percentages, waarbij genen die resistentie verlenen tegen beta-lactams, aminoglycosiden en macroliden de grote meerderheid vormden. Bovendien ontdekten we met behulp van selectieve kweek verschillende opportunistische pathogenen, voornamelijk Enterobacter spp. en carbapenem-resistente *P. aeruginosa*, die uitgebreide-spectrum bèta-lactamases (ESBLs) produceerden. De aanwezigheid van pathogene bacteriën en genen die resistentie verlenen tegen "laatste redmiddel" antimicrobiële stoffen vormt een potentieel risico voor patiënten en gezondheidswerkers en benadrukt het belang van toezicht op het ziekenhuiswatermilieu.

In **Hoofdstuk 5** rapporteerden we voor het eerst een mcr-5 gen in een wateromgeving binnenshuis en in de gezondheidszorg. We karakteriseerden een nieuwe mcr-5.4 variant met behulp van SMg en, door het combineren van short- en long-read sequencing, stelden we een nauwkeurige en lange contig samen. Ondanks een niet volledige dekking van het genoom en een lage sequentiediepte slaagden we erin de genetische omgeving van ARG te karakteriseren en de bacteriële gastheer aan de orde van de Pseudomonales toe te wijzen met behulp van metagenomische binning-analyse. De bevindingen van deze studie benadrukken de mogelijke rol van kraanwater in tandheelkundige afdelingen als reservoirs voor klinisch relevante ARGs en het potentieel van toepassing van SMg in de bewaking van het ziekenhuismilieu.
In **Hoofdstuk 6** evalueerden we het gebruik van SMg in combinatie met orale vloeistof (OF) monsterafname om de aanwezigheid van pathogenen en ARGs in varkensbedrijven te monitoren. We ontdekten een divers resistoom met aminoglycosiden, macroliden, tetracyclinen en beta-lactam resistentiegenen als de meest voorkomende ARG klassen. Van meer dan de helft van de ARGs (52,8%) werd voorspeld dat ze zich op plasmiden bevonden. Virale verrijking maakte de detectie mogelijk van verscheidene klinisch significante virussen zoals influenza A-virus en porcine hemagglutinating encephalomyelitis virus. Metagenomische sequencing van OF monsters maakte de karakterisering mogelijk van een breed scala aan pathogenen, zware metalen genen, en ARGs direct uit het monster.
In **Hoofdstuk 7**, heeft de toepassing van short- en long-reads SMg sequencing op een OF monster ons in staat gesteld om een klein IncX4 plasmide, dat het mobiele colistine resistentie mcr-1.1 gen bevat, direct uit het monster te karakteriseren. Het plasmide vertoont een hoge homologie met eerder gekarakteriseerde plasmiden in Enterobacterales aanwezig in menselijke, dierlijke, voedsel- en milieu monsters uit verschillende continenten. Het verschilt echter aanzienlijk in grootte door de afwezigheid van een deel van de T4SS, wat er waarschijnlijk toe geleid heeft dat het plasmide niet-zelfoverdraagbaar was. In dit hoofdstuk toonden we het potentieel van de kweek-onafhankelijke SMg benadering aan om complete plasmiden te detecteren.

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About the author

Giuseppe Fleres was born on the 23rd of December in 1989 in Augusta (SR), Italy. In 2013, he obtained his Bachelor of Science in Biology at the University of Catania (Italy) with a thesis entitled "Identification and molecular characterization of coagulase-positive and mannitol non-fermenting *Staphylococci* spp.". He then pursued his Master's degree in Cellular and Molecular Biology with a thesis entitled "Skin and Soft Tissue Infections caused by *Staphylococcus aureus*: three case reports". In 2017, he started his new position at the University Medical Center Groningen, The Netherlands, as a PhD candidate in the "Pronkjewail" doctoral training program funded by the Marie Sklodowska-Curie Actions, European Union's Horizon 2020. His PhD research focused on the application of next-generation sequencing (NGS) technologies to investigate antimicrobial resistance dissemination in a one-health perspective under the supervision of Prof. Alex Friedrich, Prof. John W.A Rossen and Dr. Silvia Garcia-Cobos. In 2022, he obtained a PhD (COFUND – Marie Sklodowska-Curie Actions - European Union) in Medical Microbiology at Department of Medical Microbiology and Infection Prevention, University Groningen, the Netherlands.

He explored different NGS and sequence data analysis approaches for identification, characterization and typing of bacterial pathogens isolated from the nosocomial environment and animal farms. His PhD research projects also involved the collaboration of international research groups in Denmark and France. In 2022, while finalizing his thesis, he started working as Postdoctoral Associate in Microbial Genomics at the Division of Infectious Diseases, Department of Medicine, University of Pittsburgh, Pittsburgh (PA), USA.

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