Next-generation sequencing to investigate antimicrobial resistance

Fleres, Giuseppe

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Chapter 8

General Discussion & Future Perspectives

General Discussion

Antimicrobial resistance (AMR) is one of the biggest threats to global health. The ever-increasing human population and rising number of infections caused by antimicrobial resistant bacteria (ARB) require innovative approaches to investigate and monitor the dissemination and preserve effectiveness of antimicrobials for future generations. Implementing novel approaches such as next-generation sequencing (NGS) in public health microbiology is crucial to fight the AMR issue. This thesis aimed to apply the use of NGS technologies for the detection and characterization of microorganisms in a One-Health perspective. Short and long-read whole-genome sequencing (WGS) was used to investigate the presence of ARB in the nosocomial environment. Additionally, we applied shotgun metagenomics (SMg) approach to monitor the dissemination of antimicrobial resistance (AMR) in both, the hospital environment and animal farms.

Part I | Antimicrobial Resistance in the Hospital Environment

The hospital environment offers a unique opportunity for bacteria to interact, proliferate and colonize/infect a very susceptible population. Humid compartments, including tap water, sinks and shower drains, can act as reservoir for nosocomial pathogens, contributing to health-care associated infections (HAIs)[1,2,3]. **Chapter 2** and **3** showed the potential and discriminatory power of the WGS approach to detect and type ARB strains isolated from the hospital humid environment, including tap water and drains (sinks and showers).

Traditional microbiological methods, such as Pulse Field Gel Eletrophoresis (PFGE), have been widely used by public health authorities and food regulators for outbreak investigations, however, this approach is limited by a lower discriminatory power compared to WGS and does not provide information such as virulence potential or presence of ARGs[4]. The application of WGS for hospital surveillance and infection prevention has several benefits, allowing to investigate the environmental source of an outbreak, the transmission of a given pathogen between patients and track the spread of antimicrobial resistance determinants in the hospital-built environment[5,6,7].

In **Chapter 2**, by combining classical culture methods with WGS-based investigation, we revealed the presence of a unique *Legionella anisa* clone in the water of several dental chair units in the same hospital ward. Surveilling the hospital water environment for *Legionella* spp. is key to prevent its further spread, to identify contamination sources[2] and to map its occurrence in the hospital environment for future reference in nosocomial Legionnaires' Disease (LD) cases.

WGS using Illumina technology, limited by short-read length, results in incomplete genome assemblies and the investigation of the genetic environment of specific genes of interest, such as antimicrobial resistance genes (ARGs), virulence factors (VFs) and heavy-metal resistance genes (HMRGs) remain computationally challenging[8]. The MinION (Oxford Nanopore Technologies (ONT)) is a long-read sequencing (LRS) platform that produces complete bacterial genome assemblies, allowing to characterize mobile genetic elements (MGEs) and their genetic content[9]. By combining ONT long reads and short reads from Illumina, we succeeded in obtaining two complete and circular plasmid sequences in *Legionella* spp., improving the quality of ARGs and VFs analyses. Despite these advantages, Nanopore sequencing is less accurate and less suitable for high throughput sequencing at present.

Monitoring the hospital water environment for bacteria resistant to last-resort antibiotics, such as colistin and carbapenems, is an essential tool to identify reservoirs, prevent further spread and to design appropriate infection control measures. In a recent study, the implementation of WGS into infection prevention procedures, sequencing nosocomial pathogens even in absence of an outbreak, had significant impact in patients' mortality and hospital costs[10]. In this thesis (**Chapter 3** and **4**), using selective culture approach, we reported the presence of "ESKAPE" pathogens[11], including *Enterobacter spp.*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, in hospital wastewater drains (sinks and showers), exhibiting a resistance phenotype for last-resort antimicrobials such as colistin and carbapenems.

While most of the data available regarding colistin-resistant Gram-negative (ColR-GN) in the hospital is related to wastewater and environmental water-bodies surrounding the hospital, there is a lack of knowledge about ColR-GN in tap water and humid compartments from the hospital built-environment. In **Chapter 3** we

demonstrated the possibility of applying a culture-based approach in combination with WGS to investigate the presence of colistin-resistant Gram-negative bacteria in the wastewater drains of high-risk hospital units, throughout the 5 months of the sampling period. Performing WGS and core-genome SNP analysis we succeeded in typing the strains with a higher resolution than MLST, revealing persistence of MDR bacteria in the hospital environment and allowing to investigate genetic relationships with clinical isolates. In addition, WGS allowed to investigate molecular mechanisms (e.g. *mcr* genes, chromosomal mutations and gene alterations) related to colistin resistance, revealing for the first time the truncation of the *mgrB* gene by IS element in an *Enterobacter cloacae* complex isolate, *E. kobei*, which was associated with the highest levels of colistin resistance.

Using culture-based methods, several studies have shown that the hospital water environment, including tap-water, sink surfaces and wastewater drainage systems, serve as reservoirs for nosocomial pathogens that can cause outbreaks and healthcare associated infections[1,2,12]. However, culture methods are laborious, time-consuming and may underestimate the level of microbial pathogens, especially fastidious or non-culturable microorganisms.

A culture-independent approaches can also be applied for AMR surveillance in the hospital environment (**Chapter 4** and **5**). The use SMg allows to profile the overall microbial community, characterize individual microbes without previous culture-based isolation, and represents a scalable, high-throughput method for surveying hospital environments[13,14,15].

Currently, our understanding of the microbiome in drinking water is primarily obtained by culture-based methods and 16S amplicon sequencing. However, this approach cannot always discriminate between closely related species and does not allow the analysis of ARGs, VFs and HMRGs[16]. In **Chapter 4** we showed the potential of applying SMg sequencing to investigate and monitor AMR dissemination in tap-water samples collected from high-risk units, intensive care and hematology. We reported the presence of opportunistic pathogen, such as *P. aeruginosa*, and last-resort ARGs, including bla_{VIM}, in tap water, highlighting the advantages of using SMg to gather unbiased information of the bacterial and ARGs composition. Genes encoding

carbapenem-hydrolyzing beta-lactamases, such as *bla*$_{KPC}$ and *bla*$_{VIM}$, were previously observed in low abundances in water samples from environmental and clinical settings13. Antimicrobial resistant *P. aeruginosa* strains are often observed in contaminated hospital water and can cause outbreaks as previously described[17,18].

In addition, we employed selective culture approach and revealed the presence of carbapenem resistant and ESBL-producing Gram-negative bacteria in wastewater drains. Compared to culture-based approach and WGS, SMg provides a more comprehensive view, but several limitations make its use still challenging including the low level of biomass present in environmental samples (tap water, surfaces etc.) and presence of multiple pathogens at low abundances[15,19]. In addition, the SMg short-read sequence outputs do not provide enough resolution to reconstruct mobile genetic elements and investigate ARGs genetic background, making the analysis computationally challenging. In **Chapter 5** we highlighted the advantages of combining short- and long-read sequencing SMg data to detect and characterize a novel *mcr* gene in hospital tap water. Long-read sequencing technologies can be used to gain precious information regarding the localization of relevant ARGs in plasmid and other mobile genetic elements[20,21], yet the lower accuracy of this approach compered to Illumina short-read sequencing has limited its implementation in surveillance investigations.

Part II | Antimicrobial Resistance in Livestock

The One-Health approach highlights the importance of monitoring AMR in all microbial habitats, including humans, animals and the environment. The use of antimicrobials in food animals, including the drug classes relevant to human medicine, contributes to the selection and emergence of resistant microorganism, which can be transmitted in the community and ultimately affect the hospital environment[22,23,24].

In fact, despite the human and livestock populations are two separate ecological niches, they can occasionally overlap, cresting interfaces where clonal transfer of ARB and/or horizontal transmission of ARGs could occur[25,26]. Transmission of AMR

between humans and livestock take place through direct or indirect contact, with the latter involving either an environmental component (soil, animal manure, sewage and surface water) or an intermediate vector (wild animals, food-borne infections)[27].

Pathogens with zoonotic potential, including globally emerging porcine viruses, are considered a significant threat in human healthcare and animal sector. Many globally emerging bacteria and viruses, such as *Salmonella enterica*[28] or African swine fever virus[29], pose a threat to porcine health and production. Additionally, most emerging infectious diseases have zoonotic origins[30,31]. For example, the outbreak of swine-origin H1N1 influenza in 2009[32] and the detection of Eurasian avian-like H1N1 influenza strains with pandemic potential in China[33], highlighting the importance of viral monitoring of farm animals.

Intensified livestock farming in support of increasing food demand due to the ever-growing human population, facilitates disease transmissions within herds and between livestock and humans[30]. The implementation of novel approaches is crucial to monitor the dissemination of potential pathogens, as well as antimicrobial resistance determinants, and prevent transmission between livestock and humans. The application of NGS technologies has the potential to be a powerful tool to help identifying reservoirs of AMR and investigating the pathways of transmission.

Current conventional porcine sampling methods can be invasive, laborious, and cover a limited microbial spectrum. In **Chapter 6** we used a culture independent NGS approach in combination with oral fluid sampling to characterize the porcine microbiome (bacterial and viral composition) and the resistome profile of pigs in animal farms. SMg was applied to these oral fluid samples and detected several bacteria, ARGs, heavy metal resistance genes, and clinically significant viruses. Some of the detected ARGs are known to be clinically significant in humans e.g., the mobile colistin resistance (*mcr*) gene, conferring resistance to the last-resort antibiotic colistin. In **Chapter 7** we reported the characterization of a small IncX4 plasmid carrying the mcr-1.1 gene, found in a pig oral fluid (OF) sample, using short- (SRS) and long-read sequencing (LRS). Oral Fluid (OF) sampling is a versatile, non-invasive approach that could provide, in combination with SMg, a powerful tool of ARGs and pathogens surveillance at the herd level.

Several publications in the literature showed that worldwide, humans and animals (particularly livestock) are colonized by commensal and potentially pathogenic colistin-resistant bacteria carrying mcr genes[34,35,36].

Colistin (polymyxin B) has been used in both humans and animals for decades and the dissemination of colistin resistance and *mcr* genes has almost certainly been intensified by the use of colistin in Chinese and Southeast Asia animal farms[37]. In recent years, *mcr* variants have been reported in more than 40 countries from all continents excluding Antarctica[36]. The plasmid-borne *mcr* gene has been observed in several host, including enterobacteria, such as *Escherichia coli*, *Salmonella spp.*, *Aeromonas spp.*, *Enterobacter cloacae*, *Klebsiella pneumoniae*[38]. Poultry and livestock, including chickens, pigs, and cattle, have been described as reservoir hosts for bacteria harbouring *mcr* genes[38].

Future Perspectives

To combat AMR is it crucial to support a One-Health approach and to implement new technologies to investigate different ecological niches and prevent transmission between humans, animals and environment at their interfaces. Next-Generation Sequencing technology applied in a One-Health context is a promising tool to investigate AMR dissemination and the emergence of resistant pathogens. Despite Illumina is the most widely sequencing technology currently in use, ONT sequencing is developing rapidly and its latest chemistry enables >99% raw read accuracy at high data yields. In addition, ONT has the potential for many new innovative sequencing approaches, such as real-time analysis, and sequencing in the field. However, in January 2022, Illumina introduced its new long-read technology named "Infinity" which merged the accuracy of the Illumina sequencing with the benefits of the long-read approach[39].

AMR Surveillance in the Hospital Environment. Environmental sampling in hospitals usually only takes place during an outbreak investigation and only if the infection control team has the interest and resources for such analysis. Deep sampling and WGS could be used to map pathogen occurrence in the hospital environment for

future reference in outbreaks and HAIs. Recently, the implementation of WGS into infection prevention procedures, sequencing nosocomial pathogens even in absence of an outbreak, had significant impact in patients' mortality and hospital costs[10].

Although tap-water may be classified as safe according to regulations and monitoring, mainly targeting fecal contamination and common water-borne pathogens, other risks such as the occurrence of last-resort ARGs may be neglected. In this thesis we provide evidence of last-resort ARGs and ARB occurring in hospital humid compartments and tap-water in high-risk unit. In the future, water AMR surveillance should be part of infection prevention measures to monitor the dissemination of relevant ARGs in the hospital built-environment. As next-generation sequencing costs continue to decline (http://genome.gov/sequencingcosts), a shotgun metagenomics approach could be implemented, providing unbiased information of the microbial community and ARGs composition. Pooling samples from different waters sources belonging to the same hospital environment (e.g. hospital wards and rooms) (Chapter 4) may be already used to decrease costs and obtain information about overall resistome profiles for risk assessment. In addition, standardization of NGS workflow and their integration in One-Health AMR surveillance will enable fast and accurate user-friendly bioinformatic analysis.

Surveillance of AMR in Livestock. In this thesis, we demonstrated that both wet lab and dry lab methodologies used for hospital AMR surveillance can be also applied to animal samples. Oral Fluid sampling, combined with SMg analysis, could be a very efficient and animal-friendly way to monitor pathogens and AMR dissemination in pig farms in the future. However, in order to introduce this technic on a wide scale, further studies are needed for a direct comparison of the use of oral fluid samples with gold standard sample matrices (e.g., nasal swabs, blood serum, and faecal samples from animals of the same farm, to detect pathogens and AMR determinants.

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