VRIJE UNIVERSITEIT BRUSSEL

Master thesis submitted to obtain the degree of Pharmacist and Master of Science in Drug Development

THE SINK AS SOURCE OF TRANSMISSION OF VIM METALLO-β-LACTAMASE-PRODUCING PSEUDOMONAS AERUGINOSA IN THE INTENSIVE CARE UNIT

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List of abbreviations

A

Analytical profile index (API)
Apotheker/Pharmacist (Apr.)

B

Basis local alignment (BLAST)

C

Chronic lung obstructive disease (COPD)
Colony forming units (CFU)
Clinical Laboratory Standards Institute (CLSI)

D

Deoxyribonucleic acid (DNA)
Doctor (Dr.)

E

European Congress of Clinical Microbiology & Infectious Diseases (ECCMID)
European Committee on Antimicrobial susceptibility testing (EUCAST)
Exempli gratia (e.g.)

F

Florence imipenemase (FIM)

G

Germany imipenemase (GIM)

H

Haemophilus agar (HEM)

I

Infectious dose 50 (ID50)
Imipenemase (IMP)
Intensieve zorgen (IZ)

L

Lethal dose 50 (LD50)
Lipopolysaccharides (LPS)

M

Mac Conkey agar (MAC)
Master in sciences (MSc.)
Matrix assisted laser desorption/ionization time-of-flight analyzer (MALDI-TOF)
Minimal inhibitory concentration (MIC)
Minimum spanning tree (MST)
Multilocus sequence typing (MLST)

N

New Delhi metallo-β-lactamase (NDM)
Next generation sequencing (NGS)

P

Pseudomonas aeruginosa (P. aeruginosa)
Chromogenic medium for direct identification of *P. aeruginosa* (PAID)
Polymerase chain reaction (PCR)
Professor (Prof.)

R

Ribonucleic acid (RNA)

S

Sao Paulo metallo-β-lactamase (SPM)
Sequence type (ST)

U

Ultraviolet (UV)
Universitair ziekenhuis Brussel (UZ Brussels)

V

Verona integron-encoded metallo-β-lactamase (VIM)
Viable but nonculturable (VBNC)

W

Whole genome sequencing (WGS)

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1. Aim of the study

Nosocomial infections are infections that are acquired during admission to the health institution or hospital that were not present at admission. They really are a major problem in healthcare, both in terms of health and finances. According to the World Health Organization, hundreds of millions of people are affected every year by such infections, mainly in developing countries, partly due to the circumstances in which people work there. However, the figure for Europe is also very high: about seven in 100 hospitalized patients are affected. It is noteworthy that this amount is much higher in the intensive care unit (ICU), where on average 30 per 100 hospitalized patients are afflicted. The reason why the number is higher here can be explained, among other things, by the weakened condition of the patient and the frequent use of invasive devices such as central catheters, urinary catheters and ventilators. Every year, no fewer than 37 000 patients in Europe die from the effects of such an infection which cost us all around seven billion euros (1).

Unfortunately, the UZ Brussels also had to deal with this problem, in particular with Verona integron-encoded metallo-β-lactamase (VIM) producing *P. aeruginosa* at the intensive care department. In 2015, following an outbreak of carbapenemase-producing *Enterobacteriaceae,* the department of Microbiolgy and infection control of the UZ Brussel demonstrated that the sinks in the hospital were the source of multi-resistant bacteria and leaded to transmission to patients. The goal of this study was to verify the previous findings in particular for *Pseudomonas aeruginosa* and to investigate whether patients are indeed colonized/infected by germs present in the sink drains with the help of next generation sequencing.

2. Theoretical background

2.1. General characteristics

P. aeruginosa is an encapsulated Gram-negative, facultative aerobic bacillus belonging to the bacterial family *Pseudomonaceae*, responsible for both acute and chronic infections. It is a free-living bacterium that normally inhabits the soil and surfaces in aqueous environments and can survive with very few nutrients. *P. aeruginosa* can occasionally cause opportunistic infections in a wide range of tissues such as the lungs. This bacterium has a flagellum for motility and pili that help with adhesion to other cells. Its adaptability, ability to form biofilms and high intrinsic antibiotic resistance enable it to survive in a wide range of natural and artificial settings, including surfaces in medical facilities. It uses oxygen for metabolizing through cellular respiration and never uses fermentation. It gains it energy by oxidizing glucose and transferring electrons to oxygen, but it can respire anaerobically if nitrates or other alternative electron acceptors are available. It does not ferment lactose and has not the ability to form spores. (2) (3) (4). The vast majority of the *P. aeruginosa* strains produce very characteristic water-soluble pigments which can diffuse through the medium. The best known are the blue-green and yellow green pigments (pyocyanin and pyoverdine), the red-brown pigment (pyorubin) is only produced by a small proportion of strains. Pyocyanin also causes a typical 'sweet' odor in vitro. These characteristics could be used as preliminary identification (5).

2.2. Pathogenicity and virulence factors

The pathogenicity is the potential to make someone sick. The term 'virulence' is also often used but actually refers to the degree of pathogenic potential. This virulence can be determined experimentally by measuring the infectious dose 50 (ID_{50}) or the lethal dose 50 (LD_{50}). This is the dose that is necessary to infect or kill 50% of the exposed test animals. A number of conditions must be met before *P. aeruginosa* can cause an infection, for example: the bacterium must be able to reach and penetrate the host, it must be able to multiply within the host and it must be able to escape from the defense mechanisms (6). The traits that are responsible for virulence are called 'virulence factors'. These often provide the ability to bypass and overcome the host defense mechanisms and subsequently cause disease. *P. aeruginosa* produces multiple virulence factors to help it invade epithelial cells and survive attacks from the immune system. The most important ones of *P. aeruginosa* are: endotoxins, exotoxins, biofilm-formation, type three secretion system and encapsulation. This list is not exhaustive.

Endotoxins are lipopolysaccharides (LPSs) found in the outer membrane of Gram-negative bacteria, such as *P. aeruginosa.* They are composed out of three parts: like the name says it is composed out of a 'lipid' and a core-'polysaccharide' joined by a covalent bond, and a O-antigen. Lipid A is the toxic part of the LPS. Because the O-antigen is embedded in the outer membrane, the toxic effect will only be expressed by lysis of the bacterium by, for example, phagocytosis. The toxicity of LPS is mainly due to the activation of the complement system and the stimulation of cytokines (7). LPSs play an important role in maintaining the integrity and functionality of the outer membrane of *P. aeruginosa* and in the pathogenesis because they can act as immunostimulators and can cause fever, disseminated intravascular coagulation, septic shock and organ failure. Septic shock can be induced by bacteremia when there is a massive release of LPS. It might be possible that cell wall-active antibiotics such as beta-lactams are also responsible for LPS release, but this will most likely depend on different parameters like the bacterial strain, concentration of bacteria, time that the bacteria are exposed to the antibiotic, type of antibiotic used and concentration of the antibiotic reached (8). But this is of course relative because in the treatment of septic shock, it is especially important to administer active antibiotics quickly, and often these are beta-lactams.

Other very important virulence factors are exotoxins. They can be secreted by *P. aeruginosa* but they could also be released during lysis of the cell, similar to endotoxins. Their effect could be local or systemic. *P. aeruginosa* can produce various exotoxins, some well-known examples are: phospholipase C, exotoxin A and pyocyanin. Phospholipase C is an enzyme that degrades the host cell membranes causing cell-lysis. Exotoxin A is taken up by host cells where it will target the endoplasmic reticulum and inactivates protein synthesis causing the cell to die, it is probably the most toxic toxin produced by *P. aeruginosa*. When pyocyanin is taken up by nearby host cells it will generate reactive oxygen species damaging deoxyribonucleic acid (DNA) and proteins leading to cell death. (4).

P. aeruginosa can form a biofilm. A biofilm is a multicellular community of bacteria held together by a self-produced extracellular matrix, they act like a kind of shield and protect the bacteria from immune cells and antibiotics. Most of the time biofilms occur on artificial surfaces like metals or plastics. But biofilms can also occur in the human body, on teeth, prostheses or even on soft tissues. A well-known example of a biofilm location of *P. aeruginosa* are the lungs: patients who suffer on cystic fibrosis have an excess mucus production and a lot of dead white blood cells in their lungs, which forms a good base to construct a biofilm. It is important to know that the pili on the surface of the bacterium are important to allow adherence to the surface the biofilm is formed. Biofilms can be very resistant to antibiotic treatment, which makes it very hard to treat (9) (10) (11).

Then there is the type three secretion system. The type three secretion system is a 'needle' shaped transport protein that is found in Gram-negative bacteria like *P. aeruginosa.* This protein can form a channel that crosses the bacterial envelope and the host cell membrane, making it possible to inject toxins into the host cell making it a very important virulence factor (12).

And last but not least, *P. aeruginosa* has the ability to form a capsule. This is a poorly structured network of polymers (usually polysaccharides, but sometimes also proteins) that covers the cell surface of a bacterium. This capsule protects the bacteria against the host's immune system by limiting activation of the complement system and thus reducing phagocytosis (13) (14).

2.3. The sink drains as source of *P. aeruginosa* infection

P. aeruginosa infections are not very common in the general population. But this bacterium frequently causes infections and colonization in high-risk individuals like those with cystic fibrosis, chronic granulomatous disease, chronic lung obstructive disease (COPD) and type 2 diabetes mellitus. Other peoples at risk are individuals with an immunodeficiency, intravenous drug abusers or those with deep wounds or severe burns. Hospitalized patients often have wounds or a reduced resistance through a primary pathology, and could therefore be a potential host for *P. aeruginosa* (4). *P. aeruginosa* can infect multiple parts of the body. Some important examples are: bacteremia, pneumonia, urinary tract- and skin infections (15).

P. aeruginosa is seen as a common cause of healthcare infections, and has been considered an important causative agent in hospital outbreaks over the last ten years (16). A large number of these outbreaks have been linked to sources out of the environment, especially the water systems (17). Sinks and all the associated components can function as reservoirs for *P. aeruginosa*, especially in the presence of a biofilm, which can harbor large microorganism communities, protecting them from environmental stresses and favoring their growth (18). But actually, *P. aeruginosa* can be found all over the environment: researchers at the hospital of the Provincial Hospital Center of Mohammedia (Morocco) found *P. aeruginosa* on the floors and walls, on the beds of patients, on door handles and

on various objects scattered in the hospital (19). There are studies that already report and describe outbreaks of multidrug-resistant *P. aeruginosa* that caused significant morbidity and mortality in immunocompromised patients in different hospitals (20) (21).

It is well known that sink drains beneath washbasins in hospitals contain huge amounts of bacteria. These sinks are especially prone to colonization by waterborne bacteria such as *Pseudomonas* species. When water runs into the sink drain, aerosols can be formed that can contaminate the environment and the hands of health care workers, particularly if tap water directly impacts the drain causing splashing. The survival times of various *P. aeruginosa* strains in aerosols was dependent on strain characteristics, light and humidity. The $t_{1/2}$ differed between three and 76 min (22). The sink is seen as an open, actively emitting pathogen reservoir. Free-swimming micro-organisms and a biofilm are found in the siphon of the sink, the seal water can contain easily up to 200 billion pathogens (23). Studies have already suggested that it might be possible that bacteria in the biofilm of the sink can get carried up into the air above the drain through aerosols when the tap water is running (24) (22). Döring et al. suggested already in 1991 that sinks could play a role in the transmission of *P. aeruginosa*. Upon entering the hospital, all personnel included in his study hand cultures were *P. aeruginosa-negative*. However, during duty, 42.5% of the personnel members carried different *P. aeruginosa* strains on their hands. They saw identical genotypes of the *P. aeruginosa* on the hands of the personnel and in the sink, suggesting a transmission route from sink to hands (22). Emilie Bédard et al. showed that failure to maintain good practices may act as promoting factors leading to increased concentrations and risk of patient exposure (25). On a large ICU in a hospital in the Netherlands, all sinks were removed from all the patient rooms and a water-free method of patient care was introduced. This intervention resulted in a significant reduction of colonization with multidrug-resistant Gram-negative bacteria (26). This also suggests that the sink indeed can play an important role in the transmission of multidrug-resistant bacteria.

Furthermore, it is very important to know is that *P. aeruginosa* can be in a state called viable but nonculturable (VBNC). This refers to a state of very low metabolic activity where the bacteria cannot grow on standard growth media, but are alive and have the ability to become culturable once resuscitated. They can enter this VBNC state through for example stress. This decrease of culturability without loss of viability might have an influence on human health due to an underestimation of the actual contamination in drinking water and drainage systems. Studies show that VBNC *P. aeruginosa strains*, for example found in the sink drains, can regain its viability and toxicity and therefore might influence the health of the patient (27).

Finally, E.M. Moloney et al. performed a study on the wastewater networks and washbasin U-bends. They used *P. aeruginosa* to investigate trafficking of bacteria between hospital washbasin U-bends. Highly related *P. aeruginosa* strains were identified in multiple U-bends in several Dublin Dental University Hospital locations, indicating trafficking via the wastewater network (28).

2.3.1. Prevention of nosocomial infections

Apart from the story of the sink, numerous measures can be taken to minimize nosocomial infections. It has been demonstrated that validated and standardized prevention strategies are indeed capable of reducing healthcare associated infections. With a good organization and some simple and inexpensive measures at least 30% of these infections could be prevented. In Belgium, the organization of hospital hygiene is entrusted to a team consisting of one or more physician-hospital hygienists and one of more nurse-hospital hygienists. This team takes care of the development, implementation and follow-up of a policy in the hospital (29).

As an example, a few prevention measures are briefly discussed here with regard to nosocomial infections that can be caused by *P. aeruginosa*.

- Only apply mechanical ventilation and intubation if really necessary
- Remove the catheter immediately when possible (50% of the nosocomial infections are catheter-related)
- A strict hand hygiene is always required
- The intubation of the nose should be limited to 48 hours to avoid sinusitis
- Limit the patient's pre- and post-operative stay in the hospital
- "Drop precautions" (it is assumed that drops of 5 microns or more are not transferred if the distance between patient and caregiver/sink is more than one meter)
- "Personal hygiene" (care providers should not wear jewelry on their hands, long hair must be tied, correct service clothing must be worn, …)
-…

2.4. Prevalence and colonization

It is important to know that not all patients who carry *P. aeruginosa* actually have an infection, the majority of patients carry this bacterium without being sick of it. This is called colonization. Colonization with *P. aeruginosa* can occur in the airways, the gastrointestinal tract and in moist locations like the armpits, perineum, ears, nose and throat. Colonization is more common in patients that undergo mechanical ventilation, in the gastrointestinal tract of patients receiving chemotherapy and on the skin in patients with burns. But especially patients with cystic fibrosis are sensitive for colonization with *P. aeruginosa*. (30)

Researchers in Maryland did a study to the prevalence, risk factors and clinical outcomes of *P. aeruginosa* colonization in an ICU of a tertiary hospital in Maryland (31). They noticed that 213 of 1840 patients (11,6%) were colonized with *P. aeruginosa* on admission in the ICU. They saw that age, anemia and neurological diseases are significant risk factors and that patients colonized were more likely to have a subsequent positive clinical culture than patients not colonized. Their data emphasized the need to identify patients colonized with *P. aeruginosa* on admission in the hospital.

Patients with multidrug-resistant *P. aeruginosa* cannot be decolonized routinely. In most of the cases there must be hoped that the normal flora of the patient will take over after some time. Patients who suffers from cystic fibrosis could be treated with antibiotics to eradicate *P. aeruginosa* in the lungs. In some cases, a fecal transplant is suggested in the literature: feces from a healthy donor are used to recolonize the patient's gut with 'good' bacteria. However, this treatment is currently only applied to a *Clostridium difficile* infection, but research is ongoing (32).

2.5. Therapy

In the case of serious infections such as pneumonia, antibiotics can be of crucial importance and should often be started as soon as possible. However, with some infections such as those of the upper respiratory tract, antibiotic treatment is not always useful (33). Like mentioned before, colonization of the lungs with *P. aeruginosa* is also possible, especially for patients that suffer from cystic fibrosis. The pneumologists at the UZ Brussels are screening very frequent for *P. aeruginosa* by cystic fibrosis patients to detect new colonization. When tested positive they'll try to eradicate this early colonization with a very aggressive antibiotherapy. In practice, the following schedule is often used: four weeks with inhaled tobramycin simultaneously with oral ciprofloxacin for two to three weeks (34).

It is important that sick patients with severe *P. aeruginosa* infections are treaded quickly and with an adequate antibiotic regimen. Other factors like optimal dosing, duration of therapy and the drug-administration interval are also very important factors influencing the clinical outcome (15). The infection guide (IGGI) of the Belgian association of infectiology and clinical microbiology (BVIKM/SBIMC) says that *P. aeruginosa* is intrinsically resistant to amoxicillin, amoxicillin-clavulate, first and second generation cephalosporins, cefotaxime, ceftriaxone, macrolides, trimethroprim/sulfamethoxazole, tetracyclines including tigecycline, temocillin, chloramphenicol, vancomycin and linezolid. The local epidemiology of the hospital must be taken into account when starting up and putting together the regime. The guide also says that although there is no clear evidence of the clinical benefit of combination therapy (e.g. aminoglycoside+ betalactam). However; such combination therapy can still be considered in patients with severe sepsis due to their potentially synergistically effect. Finally, it says that monotherapy with a carbapenem should be avoided due to the high risk of developing resistance (35). Choosing an empiric antibiotic therapy in the ICU setting is difficult: there is a need for a good balance between excessively broad coverage and too narrow coverage.

In the UZ Brussels, most of the time, piperacilline/tazobactam (Tazocin®) is used as a first-line empirical therapy for a *P. aeruginosa* infection. When the results of susceptibility are known, definitive therapy should be considered using the antibiotic to which the isolate is susceptible. Depending on multiple factors like the site of the infection, severity of the infection and physiological parameters such as kidney function and age, the most suitable antibiotic therapy is chosen. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendation of 2019, the following antibiotics are susceptible for wildtype *P. aeruginosa*: piperacillin/tazobactam, ticarcillin, cefepime, ceftazidime, azetreonam, ciprofloxacin, levofloxacin, gentamycin, amikacin, imipenem and meropenem (36). When all these options are not suitable, the highly toxic and old antibiotic colistin can sometimes be considered.

2.5.1. Alternative therapies

A new and promising approach to control antibiotic-resistant bacterial infections like those with VIM-producing *P. aeruginosa* is based on the use of laser light, in particular the blue wavelengths between 400 and 470 nm. The efficacy of blue laser light in eradicating *P. aeruginosa* was shown both in vitro and in vivo with minimal toxicity to mammalian cells and tissues. This approach looks very promising in treating skin wound infections and the inhibition of bacterial growth and biofilm formation. The mechanism is largely unknown but probably relies on a photochemical effect: blue light interacting with endogenous photosensitizing molecules, generating reactive oxygen species (37).

Another new and promising antibiotics-independent option to control some of these *P. aeruginosa* infections is with fecal transplantation. Mrazek et al. addressed that fecal microbiota transplantation could effectively combat multidrug-resistant *P. aeruginosa* carriage in the intestines, and thus prevent from future endogenous infections of patients at risk (38). Then there is 'phage therapy': combating bacteria with bacteriophages. This therapy has multiple advantages compared to antibiotics: they infect very specific bacterial cells avoiding damage to healthy commensal cells, they can self-control their dose increasing their number at the infection site only as long as the target bacteria are eliminated and they are able to kill MDR bacteria like the VIM-producing *P. aeruginosa* (39). Furthermore, there are studies describing honey as an inexpensive new approach for treating multidrug resistant *P. aeruginosa*. Honey has been successfully used in the treatment of surgical wounds and burns wounds. Mechanisms of antibacterial action of honey remain speculative but the osmotic effect, low pH and the presence of antibacterial substances such as inhibine most likely play a role. Vishnu *et al.* showed a bactericidal effect on *P. aeruginosa* with honey in concentrations of 20% or more. Orla *et al*. compared the activity of Ulmo90 and Manuka honey and reported similar activity against the *P. aeruginosa* at a concentration of 12.5%, better than the results from the study of Vishnu. These results suggest that honeys from different countries and regions may have wide variations in their antimicrobiological activity (40).

2.6. Resistance

As mentioned earlier, *P. aeruginosa* is known as a naturally highly resistant germ with many virulence factors outsmarting the host defense mechanisms and many antibiotics. Partly due to the massive and injudicious use of antibiotics in the past, there has been a worrying increase in the resistance of bacteria to existing antibiotics. Multidrug-resistant bacteria are without any doubt one of the most important and concerning threats to public health. The development and spread of these bacteria in the society is a crucial development that is associated with increased mortality, morbidity, healthcare costs and antibiotic use (41). The cumulative number of unique beta-lactamase enzymes identified is rising exponentially. While bacteria develop resistance to more and more drugs, fewer new drugs are being developed (42). The World Health Organization classifies *P. aeruginosa* as a "priority one pathogen": this is the most critical group of all and includes multidrug resistant bacteria that pose a particular public health threat (43).

The intrinsic or congenital resistance refers to the innate ability of a bacterial species to reduce the efficacy of a specific antibiotic through inherent functional or structural characteristics. It has been shown that *P. aeruginosa* possess a high level of intrinsic resistance to a wide variety of antibiotics through different mechanisms (44). A very important intrinsic antibiotic resistance mechanism is the extremely restricted outer membrane permeability. The outer membrane of *P. aeruginosa* is composed as an asymmetric bilayer of phospholipids and lipopolysaccharides embedded with porins that form protein channels. This membrane acts like a selective barrier preventing antibiotic penetration. Another important mechanism are the bacterial efflux pumps, they play a major role in expelling toxic compounds like antibiotics out of the cell. And last but not least, there are the antibiotic-inactivating enzymes. Commonly produced enzymes by *P. aeruginosa* are β-lactamases and aminoglycoside-modifying enzymes (44).

Antibiotic resistance can also be acquired when bacteria obtains the ability to resist the activity of an antimicrobial agent which it was previously susceptible to. Sometimes mutational changes are able to cause resistance by modifications of antibiotic targets, causing reduced antibiotic uptake and overexpression of antibiotic-inactivating enzymes and efflux pumps. Another option is through the acquisition of resistance genes carried on plasmids, transposons, integrons and prophages. Bacteria can acquire these genes via horizontal gene transfer from the same or different bacterial species. Horizontal gene transfer can go through different mechanisms: conjugation, transduction or transformation (44).

And last but not least, the ability of a bacterium to survive antibiotic attacks can increase due to transient alternations in gene and/or protein expression in response to an environmental stimulus. The best characterized and most important mechanisms of adaptive antibiotic resistance in *P. aeruginosa* are the formation of biofilms and persister cells, which can result in a persistent infection with poor prognosis in cystic fibrosis patients. Antibiotics can slowly penetrate the biofilm and some biofilm cells express an adaptive stress response that permit survival under harsh condition. An altered environment inside the biofilm induces slow growth of bacteria which reduces antibiotic uptake. Finally, multidrug-resistant persister cells are formed (44).

2.6.1. β-lactamases

Beta-lactamases are enzymes that provide multi-resistance. They are present in most of bacterial strains and confer intrinsic resistance against antibiotics. The expression of the antibiotic resistant gene occurs because of mutations or transfer of genetic material mediated by transposons, plasmids or integrons. Beta-lactamases inhibit the function of antibiotics by breaking down their structure. This takes place by a hydrolysis reaction in which amide bounds in beta-lactam antibiotics are cleaved. Consequently, antibiotics cannot act on penicillin binding protein (PBP) to any further extent and exert their function.

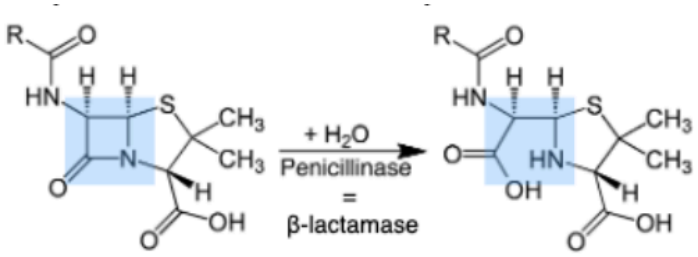


Figure 1: Beta-lactamase mechanism of action (Dimitri De Bundel, 2019)

There are different types of beta-lactamases reported in *P. aeruginosa*. The major groups are the penicillinases, the extended-spectrum beta-lactamases, the cephalosporinases and last but not least the carbapenemases. Beta-lactamase resistance, and in more particular carbapenem resistance in *P. aeruginosa* isolates, is an emerging problem worldwide. Carbapenemases are capable to hydrolyze carboxypenicillins, carboxypenicillins + beta-lactamase inhibitor, ureidopenicillins, ureidopenicillins + beta-lactamase inhibitor, ceftazidime, cefepime, azetronam and imipenem. Therefore, are carbapenemases known as the most powerful beta-lactamases. The beta-lactamases responsible for this powerful resistance are most of the time the metallo-β-lactamases, of which the metallo-β-lactamases are the most widespread. The most common ones found in Belgium are Verona integron-encoded metallo-β-lactamases (VIMs), VIM-2 is the most-reported metallo- β-lactamase in the world. Studies have shown that VIMs and resistance to carbapenems may be responsible for an increased mortality (45). VIMs have the ability to hydrolyze carbepenems, cephalosporins and penicillins and is resistance to the commercially available β-lactamase inhibitors (46).

	WT	PENI		ESBL		CEPH	CARBA
	WT	TEM PSE CARB	OXA	PER VEB TEM SHV CTX-M	OXA	AmpC	IMP VIM NDM KPC
Carboxypenicillins	S	R	R	R	R	R	R
Carboxypenicillins +BLI	S	S/I	I/R	S/I	I/R	R	R
Ureidopenicillins	S	I/R	R	I/R	R	I/R	R
Ureidopenicillins +BLI	S	S/I	I/R	S/I	I/R	I/R	R
Ceftazidime	S	S	S	R	I/R	I/R	R
Cefepime	S	S	I/R	R	I/R	I/R	R
Aztreonam	S	S	S	R	I/R	I/R	S
Imipenem	S	S	S	S	S	S	R

Figure 2: Beta-lactamase activity summary. BLI, β-lactamase inhibitor; CARBA, carbapenemase; CEPH, cephalosporinase AmpC; ESBL, extended-spectrum β-lactamase; I, intermediate resistance; PENI, penicillinase; R, resistance; S, susceptible; WT, wild type

The presence of genes responsible for the degradation of antibiotics (like the VIM-gene) can be confirmed by different methods such as polymerase chain reaction (PCR) or whole-genome sequencing (WGS). These methods allow to study the molecular resistance mechanisms responsible for the acquired resistance. PCR can only screen a limited amount of resistance genes which are selected because of their resistance to key antibiotics like the carbapenems. WGS in contrast, allows screening of the whole genome's isolate and collects information about all the present resistance genes (47).

There are different tools available for the genotypic identification and detection of resistance genes like VIM. A well-known is the Basis local alignment search tool (BLAST), which is a program that compares the nucleotides sequence of isolates to sequence databases and calculate if the matches are statistical significant. In this thesis ResFinder, a web tool from the Center for Genomic Epidemiology was used. This tool allows to identify acquired antimicrobial resistance genes and/or chromosomal mutations in partial or totally sequenced isolates based on BLASTing (47). Samples can test phenotypical positive for VIM with an immunochromatography.

2.7. Identification of *P. aeruginosa*

As a first-line option for the identification of germs, it is possible to look at the growth on species-specific plates. Furthermore, one can also look at the morphology of the colonies. However, these 2 techniques offer no certainty and additional biochemical tests like MALDI-TOF (matrix-assisted laser-desorption/ionization time of flight) or API (analytical profile index) are required to identify the germ. After inoculating and incubating flat and smooth oxidase-positive colonies with regular margin and a 'grape like smell' are formed when *P. aeruginosa* is present. When a disc of amoxicillin is added to the agar plate before incubating, growth should appear around it in case of *P. aeruginosa* because of its intrinsic resistance against amoxicillin.

The oxidase test is used to identify bacteria that produce cytochrome c oxidase: an enzyme of the bacterial electron transport chain. All bacteria that are oxidase positive are aerobic. Bacteria that are oxidase negative may be anaerobic or aerobic. It is possible to test if the bacterium is 'oxidase positive' with strips that contain tetramethyl-p-phenylenediamine. When cytochrome c oxidase is present, it will oxidize the reagent to indophenols and a deep purple color will appear within 10 seconds. When the enzyme is not present, the reagent remains reduced and colorless. This test is helpful in screening colonies suspected of being *Pseudomonas aeruginosa* because they all have to be positive. Other screening-tests where *Pseudomonas* should give a positive result are the citrate and catalase tests (48).

Then there is the matrix assisted laser desorption/ionisation time-of-flight mass spectroscopy analyzer (MALDI-TOF MS). A laser beam will cause ionizing irradiation of the bacterial proteins and will create characteristic peaks or a spectrum. In a first step the analyte is crystallized with small organic compounds or a matrix to protect against direct contact with the laser beam and to prevent its degradation. The organic compounds will transfer the energy to the proteins that will be positive ionized when exposed to the radiation of the laser. These positive charged molecules are then accelerated in an electric field. After these ions passed through the electric field, the ions will go into a tube that is not subjected to an electric field where they are separated according to their mass/load ratio. Ultimately, associated software that is based on a database with spectra will search for similarity with the species of the bacterium. However, MALDI-TOF MS does not provide information about pathogenicity or the serotype of the species (50).

Identification of different clones of *P. aeruginosa* can be done with multilocus sequence typing (MLST). MLST is a technique for characterizing isolates of microbial species using the DNA sequences of 'housekeeping genes'. Those housekeeping genes are typically constitutive genes required for maintaining the essential cellular functions (51). To determine the sequence type (ST) of *P. aeruginosa*, following seven genes are analyzed: acsA (product is acetyl-coenzyme A synthetase), aroE (product is shikimate dehydrogenase), guaA (product is GMP synthase), mutL (product is DNA mismatch repair protein), nuoD (product is NADH dehydrogenase), ppsA (product is phosphoenolpyruvate synthase) and trpE (product is anthranilate synthase). There are many variations of house-keeping genes possible per locus, allowing billions of allelic profiles to be distinguished using these seven house-keeping loci. Often these MLSTs are visually presented in a minimum spanning tree (MST). A MST is a subset of the edges of a connected, edge-weighted undirected graph that connects all the vertices together, without any cycles and with the minimum possible total edge weight (52).

2.8. Antibiotic susceptibility tests

2.8.1. Disk diffusion test

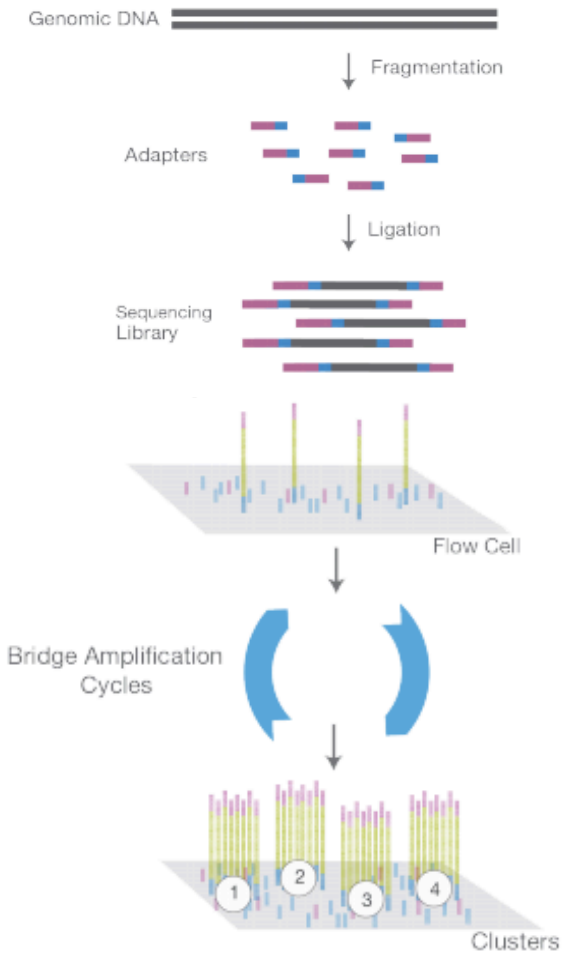
The disk diffusion test is the most used antibiotic susceptibility test in routine laboratories, it is used to determine the sensitivity of a strain for different antibiotics. The results can be interpreted with clinical breakpoints established by different expert committees like the EUCAST (European Committee on Antimicrobial susceptibility testing) and the CLSI (Clinical Laboratory Standards Institute). With these breakpoints, it is possible to determine if a bacterium is susceptible to an antibiotic agent by comparing it to the inhibition zone. These breakpoints and inhibition zones can guide the physician in choosing the right antibiotic therapy. To perform a correct disk diffusion test following steps should be followed: selection of colonies on an agar plate, preparation of the inoculum suspension, standardization of the inoculum suspension, grafting of the Mueller-Hilton agar plate, addition of the antimicrobial disks, incubation, measurement of the inhibition zones and the interpretation of the results.

2.8.2. Minimal inhibitory concentration susceptibility test

The minimum inhibitory concentration (MIC) of a certain bacterium relative to a certain antibiotic is the lowest concentration of that antibiotic that will inhibit the growth of the bacterium. Broth or agar media is used for the preparation of the microdilution, broth microdilution is used most of the time. To perform a correct MIC test the following steps should be followed: selection of colonies on an agar plate, preparation of the inoculum suspension, mixing the inoculum suspension with the broth microdilution, adding microdilution to the 96 well plate containing serial dilutions of antibiotics, checking the purity of the inoculum, incubating for 16-20 hours, MIC reading and the interpretation of the results. These results can be interpreted with the clinical breakpoints and based on these MIC values, a classification can be given for each antibiotic in sensitivity: S for sensible, I for intermediate and R for resistant.

2.9. Next generation sequencing

Next generation sequencing (NGS) or massive parallel sequencing is a technology that makes it possible to obtain millions of nucleic acid sequences or reads from a DNA/RNA source in a few hours to days. The concept is very similar to Sanger Sequencing: in both techniques DNA polymerase adds fluorescent nucleotides one by one onto a growing DNA template strand. Every incorporated nucleotide is identified by its fluorescent tag. The sequencing volume is one of the main differences: while the Sanger method can only sequence a single DNA fragment at a time, NGS can sequence millions of fragments simultaneously per run. This translates into sequencing hundreds to thousands of genes at one time (53).



Before preforming the actual NGS, DNA-extraction and purification should be done. This is necessary to isolate only bacterial DNA without other particles or impurities like proteins and membranes. Then the DNA-library needs to be prepared: the genome is mechanically cleaved in fragments through ultrasonic fragmentation. Then the five' and three' adapters were added to the five'- and three'-ends of the DNA fragments using DNA-ligase.

The next step in the process is the cluster generation. A flow cell is used to make clonal clusters. The surface of this flow cell is coated with oligo pairs or lanes, each one contains an adaptor on the surface which is compatible with the adaptors added at the ends of the DNA-fragments. DNA-fragments can be absorbed into the flow cell, meaning that each adaptor on the DNA-fragment hybridized with their compatible adaptor on the lane. Finally, DNA-polymerase will add homologous nucleotides to each lane resulting in the formation of DNA-strands that are completely compatible with the fragments of the DNA-library. When cluster generation is complete, the templates are ready for sequencing.

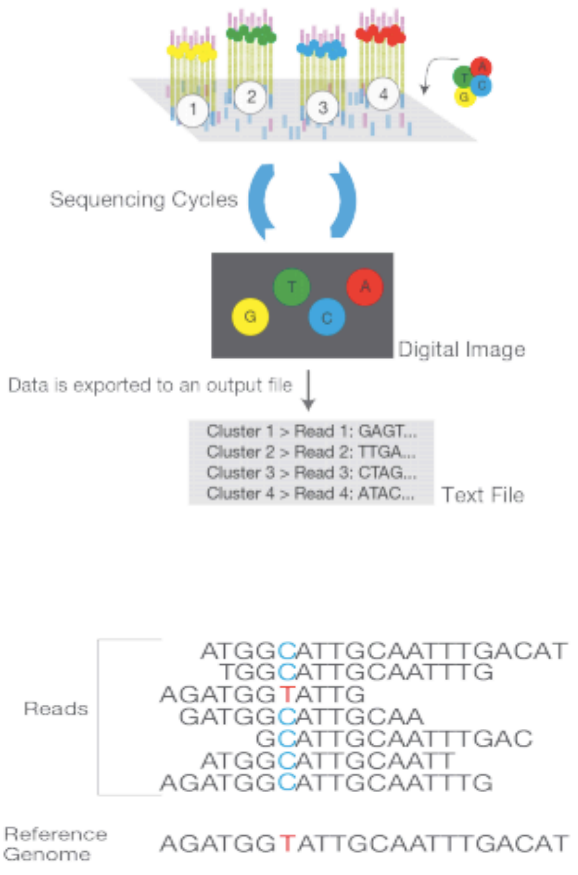


Figure 3: Summary of NGS (Illumina, 2019)

The next step is sequencing that is performed by the sequencing-by-synthesis method. Primers, DNA-polymerase and fluorescently labeled nucleotides (ATP, TTP, GTP, CTP) are added. Per cycle, the differently labeled nucleotides are offered for chain extension at the same time, but only one nucleotide can integrate per cluster. Non-incorporated nucleotides are washed away and the nucleotide integration is determined per cluster via fluorescence imaging. The fluorophore is cleaved away so that the next cycle can start. Finally, the signal of each base is then excited by a laser and measured by optical equipment. This cycle is repeated until all the bases are sequenced.

The sequenced reads can then be aligned to a reference sequence using software. After alignment, it is possible to identify differences between the reads and the reference genome (54).

3. Materials and methods

3.1 Patients

Isolates from clinical samples from patients with *P. aeruginosa* were stored between January and October of 2019 and were retrospectively selected for the study based on the presence of the blaVIM-enzyme, duration and place of hospitalization (eg ICU). Although the presence of the blaVIM-enzyme was preferred, also some blaVIM-negative samples were included. In total, we included 41 samples (four blood samples, nine bronchial aspirate samples, six endotracheal aspirates, four sputum samples, five anus surveillance samples, seven urine samples and 6 wound samples) obtained from 34 patients. Twenty-eight of these patients were hospitalized on the ICU.

3.1. Sampling and incubating

On different moments, samples were prospectively collected from all sinks in the ICU of the UZ Brussels. There are four units for adults: 13, 14, 15 and 16. In each unit, there are six beds, each with their exclusive sink right next to the bed. Often the distance between the bed and the sink was no more than one meter. There are also three other sinks present in each unit, which means that there are nine sinks per unit. Unit 13 and 14 were sampled on 27/08/2019, unit 15 and 16 were sampled on 28/08/2019. All the units were sampled again on 07/10/2019. In total, 36 environmental samples were taken both in August and October.

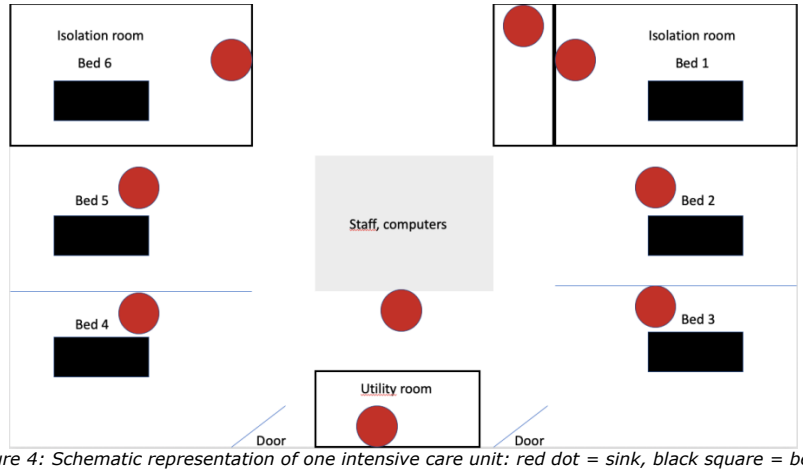


Figure 4: Schematic representation of one intensive care unit: red dot = sink, black square = bed

ESwabs® (Copan, Brescia, Italy), flocked swabs with liquid Amies medium *(a transport medium containing charcoal to prolong the viability of the bacteria)* were used to take and preserve the samples during transport to the laboratory. After that, 1 ml fastidious broth was added to the ESwabs® before incubating 48 hours at 37°C in an aerobic environment. PAID-, MAC- and HEM-plates were used to grow and isolate *P. aeruginosa* colonies.

Composition of PAID agar (Chromogenic medium for direct identification of *P. aeruginosa)*

15,0g agar, 8,0g peptone and yeast extract, 8,0g salts, 2,2g chromegenic mix, 1L demineralized water

Composition of HEM agar (Haemophilus agar)

2ml haemine, 40,0g trypticase soy agar, 2ml nicotinamide adenine dinucleotide, 50ml horse blood, 1L demineralized water

Composition of MAC agar plate (Mac Conkey agar)

17,0g bacto peptone, 3,0g proteose peptone, 10,0g lactose, 1,5g, sodium chloride 5,0g, agar 13,5g, neutral red 0,03g, crystal violet 0,001g, 1L demineralized water

Composition of Amies medium

10,0g charcoal, 3,0g sodium chloride, 1,15g sodium hydrogen phosphate, 0,2 potassium dihydrogen phosphate, 0,2g potassium chloride, 1,0g sodium thioglycollate, 0,1g calcium chloride, 0,1g magnesium chloride, 4,0g agar, 1L demineralized water

3.2. Isolation and identification of *P. aeruginosa*

After inoculating and incubating the PAID-plates for 48 hours (37°C, aerobic), violet-colored colonies should appear in case of *P. aeruginosa*. However, these plates were difficult to interpret because many different color intensities and colony-types appeared. The purple colonies also gave unclear results on the MALDI-TOF. After inoculating and incubating the MAC-plates for 48 hours (37°C, aerobic), flat and smooth oxidase-positive colonies with regular margin and a 'grape like smell' are formed when *P. aeruginosa* is present. When a disc of amoxicillin was added to the MAC-plate before incubating, growth appeared around it in case of *P. aeruginosa* because of its intrinsic resistance against amoxicillin. The positive colonies weren't purple, because *P. aeruginosa* is a non-lactose fermenter and lactose fermenters colors purple on these plates. MAC-plates are not *P. aeruginosa* specific, so further identification with the help of biochemical tests was necessary.

Oxidase test

An oxidase test (Microbiology Bactident ®, Merck KGA, Darmstadt, Germany) was used to screen colonies before analyzing them with MALDI-TOF.

MALDI-TOF MS

MALDI-TOF MS was performed with Microflex LT mass spectrometer with MALDI Biotyper 3.0 software and reference library (Bruker DaltonikGmBH, Bremen, Germany).

3.3. Disk diffusion test

A colony was selected on the agar plate. A (part of) that colony was suspended in 1 ml of sterile physiological water with a glass pasteur pipette until a suspension of 0,5 McFarland was made. A sterile cotton-tipped swab was used to inoculate with the suspension the entire surface of the Mueller-Hilton agar (MHA) plate. Then antibiotic disks were applied on the inoculated MHA plate using a multichannel disk dispenser. The MHA plates were incubated with the agar side up during +-24 hours at +- 37°C. After incubation, the zone of inhibition was automated measured by SIRscan® (I2A, Montpellier, France). The disk diffusion diameters were interpreted by using the EUCAST clinical breakpoints.

In the first sampling round, disk diffusion tests were performed to study the resistance patterns of the strains. However, this was a very time-intensive activity and not very relevant to the thesis' research question, so it was decided not to do this again for the second round. It was also decided not to include the results of the disk diffusion tests of the first round.

Composition of the disk diffusion test

The follow antibiotics (I2a, Montpellier, France) were used for the disk diffusion-test; Ampicillin (10 µg), piperacillin/tazobactam (30/6 µg), colistin (10 µg), aztreonam (30 µg), gentamicine (10 µg), ceftriaxone (30 µg), meropenem (10 µg), amikacin (30 µg), ceftazidime (10 µg), amoxicillin/clavulanic acid (20/10 µg), cefuroxime (30 µg), moxifloxacin (5 µg), temocillin (30 µg), cefepime (30 µg), ciprofloxacin (5 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg).

Other materials that were used: demineralized sterile water, nephelometer (DEN-1B, Biosan, Riga, Latvia), 0.5 McFarland polymer turbidity standard, Mueller-Hinton agar plates (I2a, Montpellier, France) and an aerobe incubator (37°C).

This is the standard disk diffusion test for all Gram-negative bacilli and not specific for *P. aeruginosa. P. aeruginosa* is naturally resistant to amoxicillin/clavulanic acid, cefuroxime, moxifloxacin and temocillin. Therefore, these antibiotics should not further be analyzed. Another problem is that collistin shouldn't be tested with disk diffusion, because there is no good diffusion and therefore these results are not reliable. Therefore, this analyze should be seen as a screening. After screening, very resistant samples were tested with an immunochromatography test (RESIST-4 O.K.N.V., Coris BioConcept, Gembloux, Belgium) for the presence of the VIM-gene.

3.4. MIC susceptibility test

A colony was selected on the agar plate and a broth microdilution was prepared by suspending the colony in 5ml demineralized water until a turbidity of 0,5 McFarland was achieved. 10 µl of the suspension was added to 11 ml Mueller-Hilton inoculum broth. The purity of the inoculum was checked before MIC reading by inoculating some of the broth suspension on a blood agar plate after which the agar was incubated and checked for contamination. Thereafter, 50 µl of the broth solution was added to each well of the 96 well plate with the help of the Sensititre® AIM. The 96 well plate was then incubated for +- 24 hours at +- 37°C. A plastic seal was placed over the panel to prevent dehydration. Before the MIC could be read, the positive control well was checked for growth and the blood agar plates were checked for contamination. The 96 well plate was then placed in the Sensititre® Vizion to read the results and to determine the MICs.

Composition of the MIC test

Amoxicillin (1-32 µg/ml), amoxicillin/clavulanic acid 2:1 ratio (1/0.5 – 32/16 µg/ml), amoxicillin/clavulanic acid with fixed dose clavulanic acid (1/2 – 64/2 µg/ml), aztreonam (0.5 – 32 µg/ml), cefepime (0.5 – 32 µg/ml), cefotaxime (0.5 – 8 µg/ml), ceftazidime (0.5 – 32 µg/ml), ciprofloxacin (0.06 – 8 µg/ml), colistin (0.25 – 32 µg/ml), gentamicine (0.5 – 8 µg/ml), meropenem (0.12 – 32 µg/ml), piperacillin (2 – 32 µg/ml), piperacillin/tazobactam (1/4 – 64/4 µg/ml), temocillin (1 – 64 µg/ml), tigecycline (0.25– 16 µg/ml), tobramycin (1 – 16 µg/ml), trimethoprim/sulfamethoxazole (0.5/9.5 – 32/608 µg/ml)

Other materials that were used: Sensititre AIM™ Automated Inoculation Delivery System, Sensititre® Vizion, Sensititre® demineralized water, Sensititre® cation adjusted Mueller-Hinton broth (broth is suspended to divalent cation level of 12,5 mg Mg^{2+}/L and 25 mg Ca^{2+}/L), nephelometer, 0.5 McFarland polymer turbidity standard and HEM plates.

Schematic summary

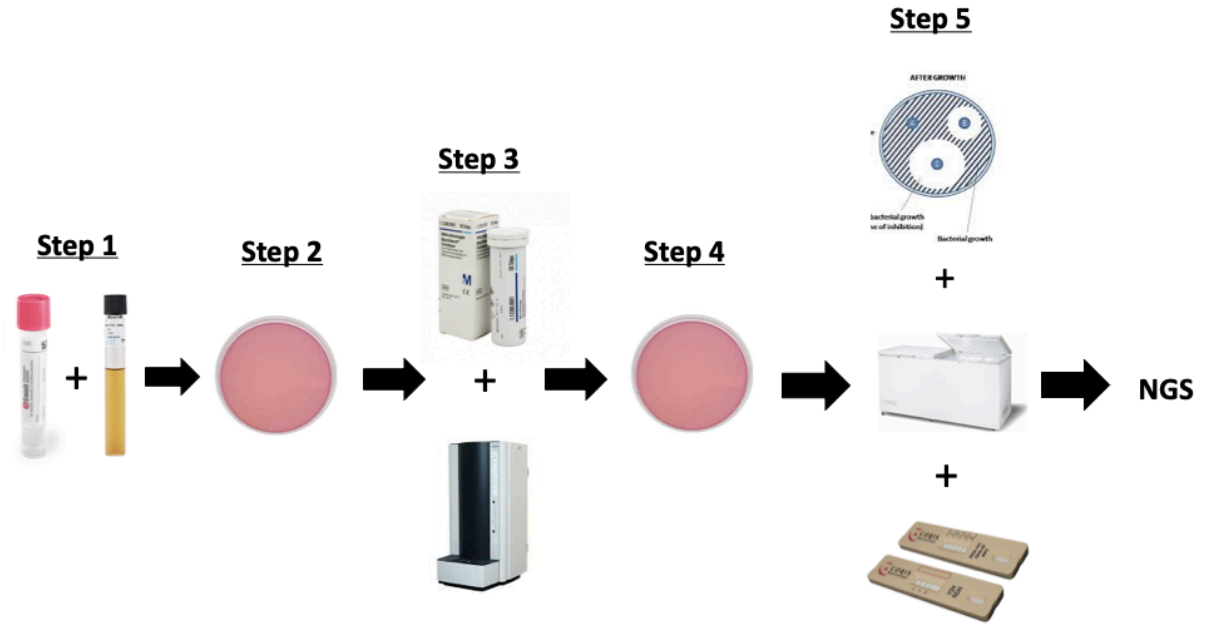


Figure 5: Schematic representation of the steps before NGS and antibiotic susceptibility tests: (1) sampling, adding fastidious broth and incubating, (2) grafting the MAC-plates and incubating, (3) oxidase-test and MALDI-TOF MS, (4) isolating P. aeruginosa and incubating on HEM-plates, (5) isolating pure P. aeruginosa sample for NGS, disk-diffusion, MIC and VIM-test (disk diffusion and MIC only performed on the first sampling round)

3.5. DNA purification/extraction

Before performing the actual NGS, DNA-extraction and purification should be done. This is necessary to isolate only bacterial DNA without other particles or impurities like proteins and membranes. The Maxwell® RSC Cell DNA Purification Kit was used on the Maxwell® RSC Instrument for DNA extraction. The extraction and purification of the DNA is based on the use of paramagnetic magnetic beads.

In our study, the protocol for Gram-negative bacterial cells was followed. The whole procedure was performed in the laboratory of clinical microbiology of the UZ Brussels. Up to $2x10^9$ cells (=one loop) from the pure *P. aeruginosa* colonies on the HEM-plates were added to 400µl of culture medium, no preprocessing steps were required. The cartridges were placed into the holder and the seals from each cartridge was removed. These cartridges consist of 8 wells filled with lysis and wash buffers to purify the DNA. The cultured sample was transferred to well #1 of each cartridge and thoroughly mixed by pipetting at least 10 times. After that, a plunger was placed in each cartridge in well #8. Finally, an empty elution tube was placed in the cartridge and 200 µl of elution buffer (Tris-HCl, pH 8,0, 1ml /100ml MilliQ water). The platform will now automatically purify the sample, this procedure will take 43 minutes for 16 samples. When the automatic procedure was done, the magnetic beads in the elution tube were removed: the tube was centrifuged at 12 000 rounds per minute for 2 minutes and placed on a magnetic rack to concentrate the beads on the bottom of the tube.

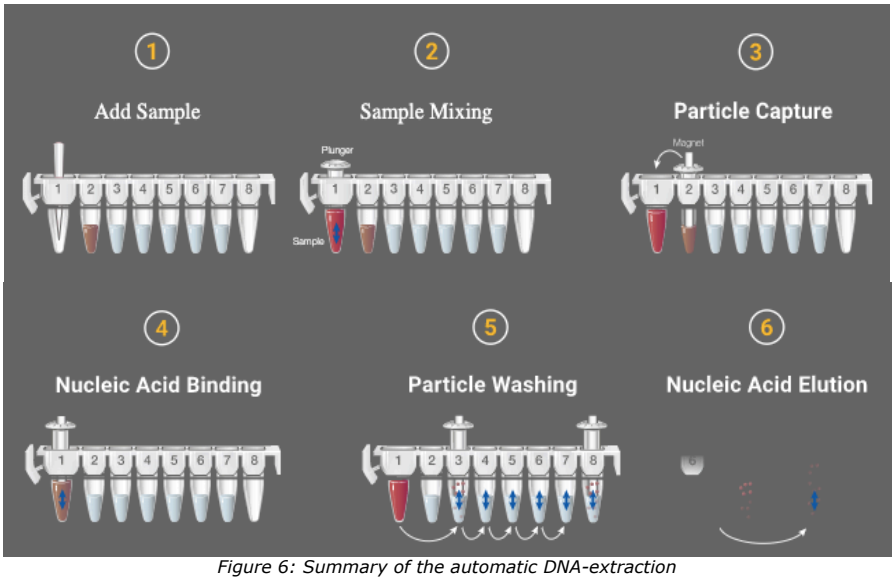


Figure 6: Summary of the automatic DNA-extraction

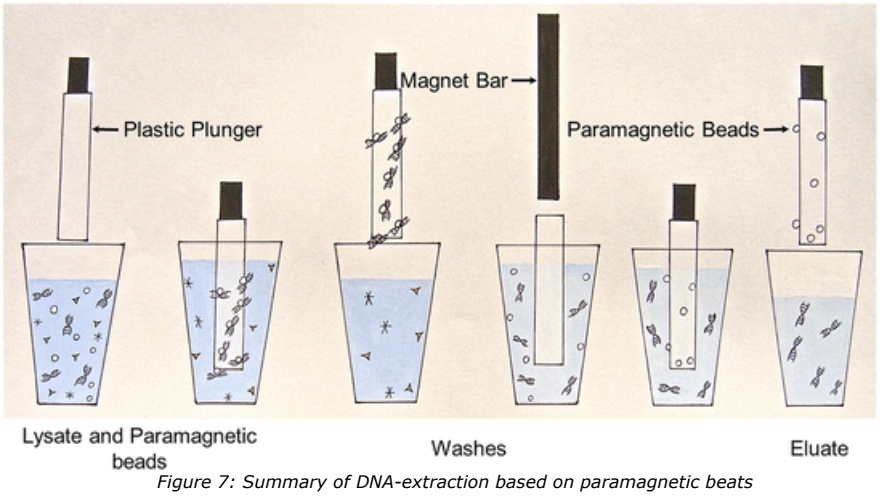


Figure 7: Summary of DNA-extraction based on paramagnetic beats

3.5.1. Quality control on the extracted DNA

The quality of the extracted DNA was determined with a Qubit® Fluorometer and Nanodrop® Spectrophotometer. The Qubit® Fluorometer was used to quantify the amount of extracted DNA. The Nanodrop® was used to determine the presence of protein contamination.

For the Qubit® assay/analysis, the Qubit dsDNA BR Assay kit from Invitrogen (ThermoFisher, Waltham, U.S.) was used, which is based on target-selective dyes that emit fluorescence once bound to DNA. Each sample requires a minimal concentration of 28,6 ng/µl for the NGS library preparation. Before analyzing, the reagents need to be at room temperature. The Qubit working solution was prepared by dilutingthe Qubit dsDNA BR reagent 1:200 in theQubit dsDNA BR buffer. One hundred and ninety-eight µl was further added into each Eppendorf Two µl extract of each sample was brought into an Eppendorf's, in order to obtain a total volume of 200µl. The mixture was vortexed shortly and incubated for two minutes at room temperature. Standard 1 and 2 were used to calibrate the fluorometer. Each sample was analyzed twice and the average concentration (ng/µl) was calculated. For the Nanodrop®analysis, the NanoDrop2000c spectrophotometer from Thermoscientific (Waltham, U.S.) was used. The ratio of nucleic acid/protein is measured by measuring the absorbance at 260/280 nm: nucleic acids have a peak absorbance at 260 nm, proteins have a peak absorbance at 280 nm. The ratio nucleic acid/protein should be between 1,8 and 2,0 (+-0,05).

3.6. Whole genome sequencing

The principle of NGS is illustrated in point 2.9. NGS was performed on purified and extracted DNA from *P. aeruginosa* isoaltes by the Brussels Interuniversity Genomics High Throughput core (BRIGHTCore). Fragmentation of genomic DNA was carried out using the NEBNext® Ultra™ II FS module. DNA libraries with an insert size of on average 550 bp were prepared via the KAPA Hyper Plus kit (Kapa Biosystems, Netherlands) and a Pippin prep size selection. In order to avoid PCR bias, the PCR amplifications step was excluded and a 500 ng imput of genomic DNA was used. After equimolar pooling libraries were sequenced on a Novaseq 6000 instrument (Illumina, U.S.) using an SP flowcell with 500 cycli. For this the library was denatured and diluted according to manufacturer's instructions. A 1% PhiX control library was included in each sequencing run. Sequence quality was assessed with FastQC (version 0.11.4) software.

3.7. Genome assembly and analysis

SPAdes genome assembler was used to perform de novo assembly. BRIGHTcore delivered us fastq files and de novo assemblies (SPAdes). ResFinder, a web tool from the Center for Genomic Epidemiology, was used to identify acquired antimicrobial resistance genes in totally sequenced isolates. In addition, the sequencing data was analyzed using the whole genome multi-locus sequence typing (*wg*MLST) scheme for *P. aeruginosa* of the BioNumerics software v.7.6 3 (Applied Maths, Biomérieux, Belgium). Minimal spanning trees (MSTs) were generated using BioNumerics.

4. Results

After sampling the sinks drains on 28/08/2019, they were incubated and identification of strains was performed. In the first sampling-round, 20 of the 36 samples tested positive for *P. aeruginosa*. This means that at least 56% of the sinks were contaminated at that moment. For some samples, no growth was observed. After a second sampling-round on 07/10/2019, 20 of the 36 sink drains were again tested positive. A complete summary of the environment-samples that tested positive for *P. aeruginosa* and blaVIM, go to appendix one.

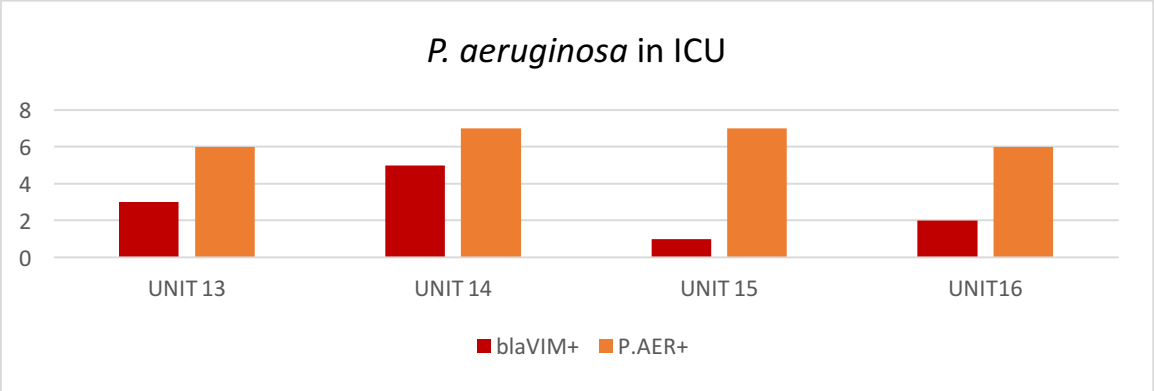


Figure 8: Distribution of the (blaVIM producing) P. aeruginosa found in sink drains between 28/09'19 and 07/10'19

Disk diffusion summary

Disk diffusion was performed as a screening for MDR *P. aeruginosa* before the first NGS run. This was not repeated the second run.

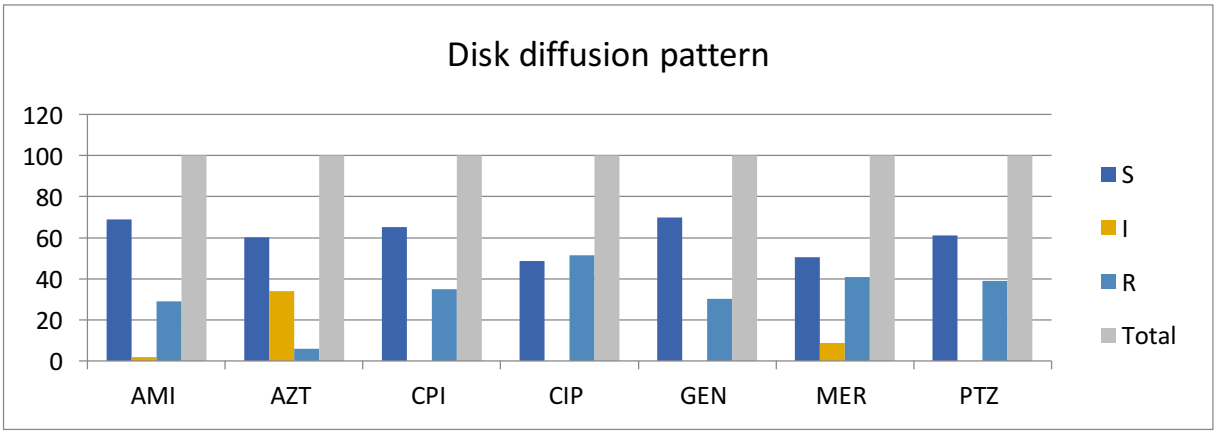


Figure 9: Summary of the disk diffusion results for AMI (amikacin), AZT (aztreonam), CPI (cefepime), CIP (ciprofloxacin), GEN (gentamicin), MER (meropenem) and PTZ (piperacilline-tazobactam) in %

The standard disk diffusion test for all Gram-negative bacilli and not specific for *P. aeruginosa* was performed. *P. aeruginosa* is naturally resistant to amoxicillin/clavulanic acid, cefuroxime, moxifloxacin and temocillin. Therefore, these antibiotics should not further be analyzed. Another problem is that colistin shouldn't be tested with disk diffusion, because there is no good diffusion and therefore these results are not reliable. Therefore, this analyze should be seen as a screening. Only some important antibiotics against *P. aeruginosa* are shown in this graph: amikacin (69% susceptible), aztreonam (60% susceptible), cefepime (65% susceptible), ciprofloxacin (49% susceptible), gentamicin (70% susceptible), meropenem (50% susceptible) and piperacilline – tazobactam (61% susceptible).

MIC-testing summary for blaVIM positive samples

MIC-testing was performed on five samples that were tested positive for blaVIM to get an idea of the resistance patterns. As suggested in figure 2 on page 13, resistance was seen in all the tested beta-lactams except aztreonam. There can also be noted that colistin could be used in some cases to treat serious infections with blaVIM-producing *P. aeruginosa*.

Antimicrobial agent	**Breakpoint for resistance (μg/ml)**	**% Resistance (*N=5*)**
Amikacin	≥64	100%
Aztreonam	≥32	40%
Cefepime	≥32	100%
Ceftazidime	≥32	100%
Ciprofloxacin	≥4	100%
Colistin	≥8	40%
Meropenem	≥8	100%
Piperacillin/tazobactam	≥8	100%

Figure 10: Summary of the MIC results for the blaVIM-producing samples in %

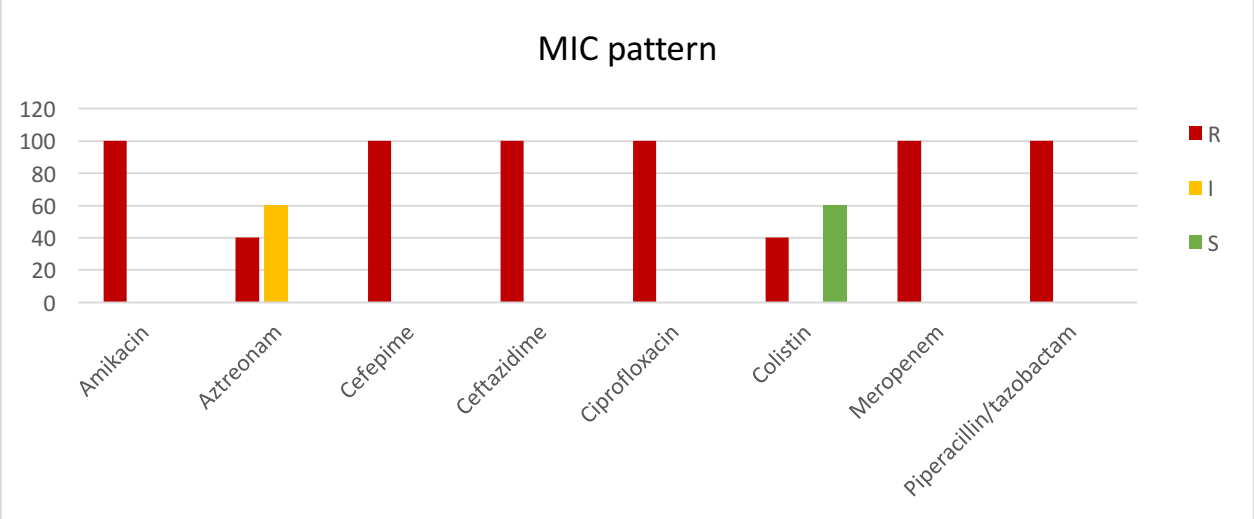


Figure 11: Summary of the MIC results for the blaVIM-producing samples in % (N=5)

Minimum spanning tree of multilocus sequence typing

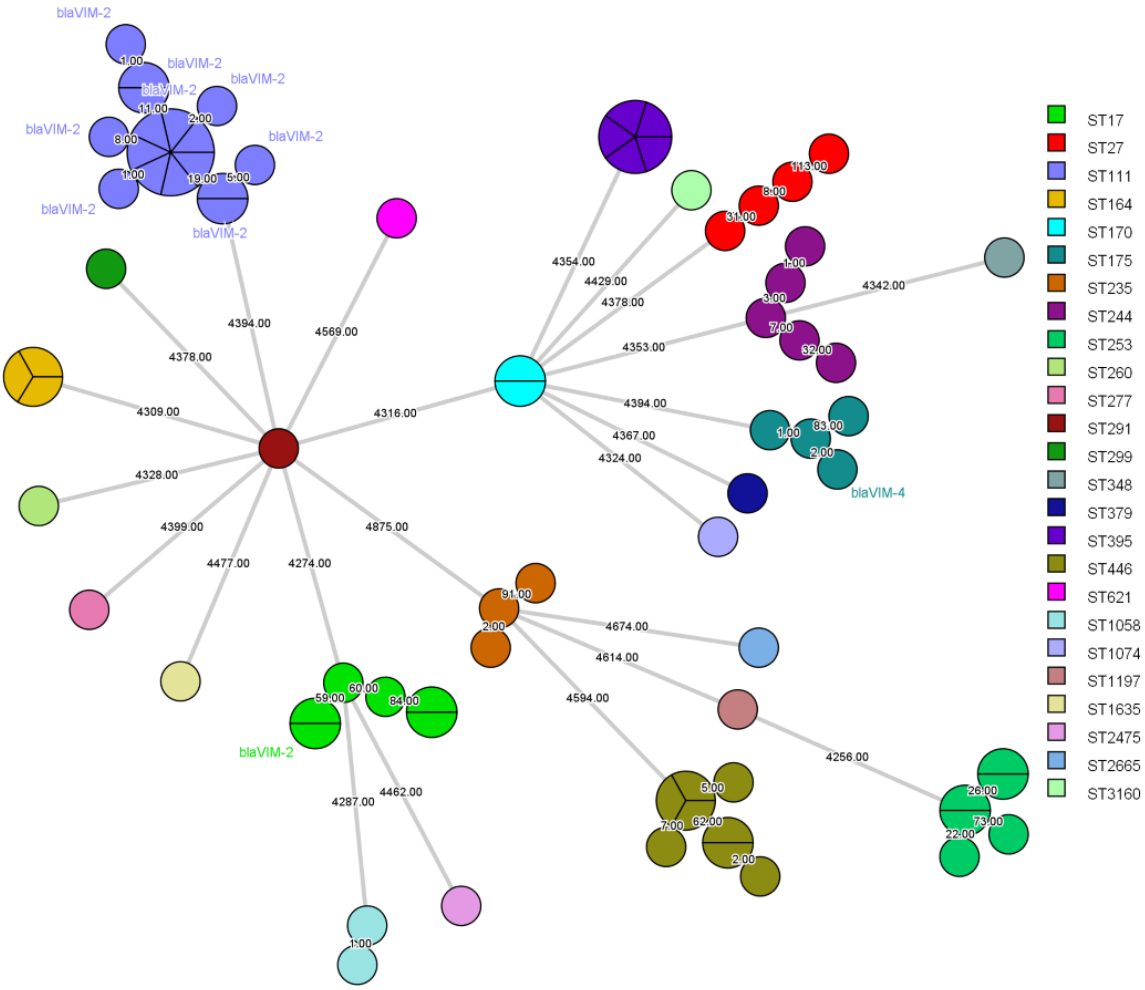


Figure 12: Minimum spanning tree based on the wgMLST allelic profiles of 70 P. aeruginosa isolates built from wgMLST analysis.

In the above figure the samples are grouped according to their MLST in a MST. As can be seen in the image above, there are 25 different MLSTs identified among the 70 samples. Colors highlight isolates based on MLST, numbers of allelic differences are indicated on the lines connecting various STs + blaVIM-2/-4 resistance genes represented.

ST111 (*N=16*) is overall the most found type in this study. With the exception of one environmental sample and two patients, all the blaVIM positive samples belong to this group. The other blaVIM positive samples belong to the ST17 (*N=6, but only N=2 carrying the blaVIM-2 gene*) and ST175 (*N=4, but only N=1 carrying the blaVIM-4 gene*) genotype. ST111 and ST17 were both found in clinical samples and environmental samples, ST175 was only found in clinical samples. ST111 and ST175 are worldwide well known as high-risk clones, responsible for epidemics of nosocomial infections. ST111 in particular is known as a very dangerous strain and according to the *P. aeruginosa* PubMLST database (55), ST111 is mainly reported from Europe, Asia and North and South America. Multiple studies describe a hospital outbreak with the ST111 strain.

In the sink drains following STs were found: ST111 (*N=13*), ST17 (*N=1*), ST164 (*N=3*), ST395 (*N=5*), ST253 *(N=2*).

Minimum spanning tree of population structure

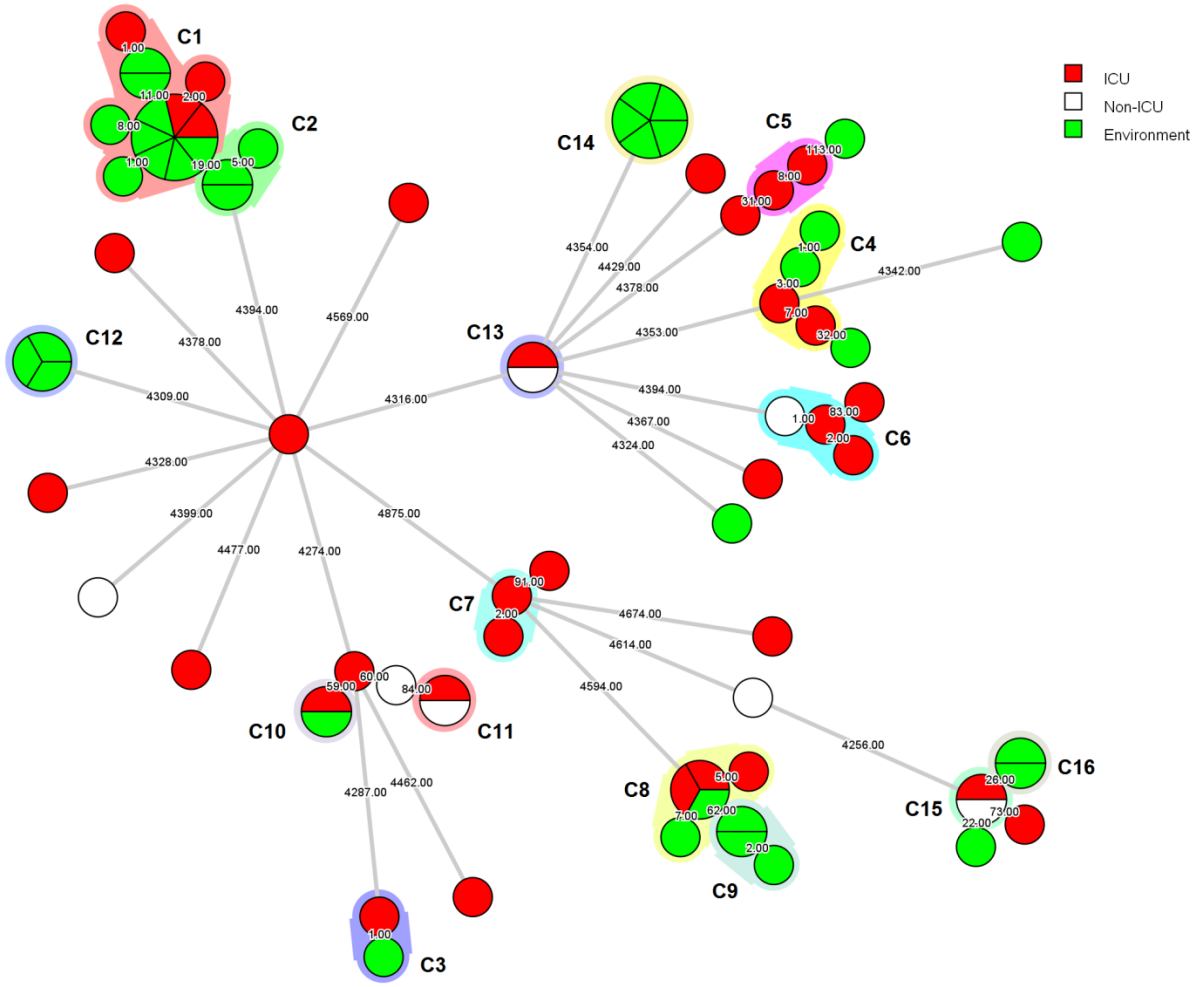


Figure 13: Minimum spanning tree based on the wgMLST allelic profiles of 70 P. aeruginosa isolates built from wgMLST analysis

In the above figure, the 36 environmental strains are highlighted in green; the clinical isolates from 28 patients residing at the ICU are highlighted in red and the seven clinical isolates from six patients that were not admitted at the ICU are shown in white. The numbers of allelic differences are indicated on the lines connecting the different strains. A fixed threshold of 14 allelic differences was used for clustering of isolates by wgMLST analysis (28). With this threshold, 16 different clusters can be distinguished.

The first, and most important cluster, is the cluster (C1) containing isolates (*N=13*) from the blaVIM-producing ST111-group. Seven of the 13 isolates had an allelic difference of 0, suggesting these isolates were exactly the same. These seven isolates include samples from two different patients and five different sink drains from ICU subunit 14, 15 and 16. The other six isolates had an allelic difference of no more than 12, suggesting that these isolates were very closely related. In this cluster, a clear link is proven between four different patients and the sink drains all carrying the blaVIM-2 gene. Also, it is proven that at least five different sink drains carried the exact same blaVIM-producing isolate. A second important cluster is cluster four (C4). This cluster contains isolates (*N=4*) from the ST244-group, which doesn't produce blaVIM. Two of these isolates are clinical isolates, the other two are environmental isolates. Both environmental isolates were from ICU subunit 14. All the four isolates had no more than an allelic difference of 12, suggesting that these isolates were very closely related. The third important cluster is cluster eight (C8). This cluster contains isolates (*N=5*) from the ST446-group, which doesn't produce blaVIM. Two of these

isolates are clinical isolates, the other three are environmental isolates. The environmental isolates were from ICU subunit 14 and 15. All five isolates had no more than an allelic difference of 12, suggesting that these isolates were very closely related. Furthermore, three of the five isolates had no allelic difference at all. These three isolates include samples from two different patients and one sink drain. Finally, in cluster then (C10) and cluster three (C3) links between patient and sink drain is proven. In total, 12 of the 28 clinical samples from the ICU can be linked to isolates found in the sink drains.

Furthermore, there are cluster (C5, C7, C11, C13 and C15) including multiple clinical samples with no or no significant allelic difference between themselves. No environmental isolates can be linked to these clusters, making this links between different patients unexplainable. This can perhaps be explained by the fact that many positive environmental samples have been missed like explained on *page 22*, or the fact that there are likely to be other sources of *P. aeruginosa* whether or not in the ICU department. Finally, there is also the chance that certain health care providers are carriers of these specific isolates without being sick themselves, which could cause them to spread from patient to patient.

Unfortunately, there are also many 'missing links'. For example, by cluster 12 (C12) and cluster 14 (C14). C12 contains three samples from three different sink drains from ICU subunit 16 and 15. Those three samples belong to the ST164-group. There is no allelic difference between those three samples, concluding these are exact the same. But no link was found by clinical samples. C14 contains five samples from four different sink drains from three different ICU subunits. No allelic difference is seen between those isolates, concluding these are exact the same. Here again, no link was found with clinical samples. A possible explanation can be found in the fact that it is possible that there are biofilms in the drains 'connected' between those sinks, so that no more human vector is required for the spread from sink to sink. It has been shown that *E. coli* biofilm could grow up to one inch/day along the waste water pipework (28).

Summary of resistance

In appendix two an overview of the identified resistance-mechanisms presents in the 33 analyzed blaVIM-positive *P. aeruginosa* isolates is shown. Three different types of genes responsible for acquired aminoglycoside modifying enzymes, three types of genes responsible for beta-lactamases and four other acquired types of resistance genes were found in those blaVIM-positive samples. In total, 31 acquired resistance genes were found in the 77 samples analyzed by ResFinder, 11 genes were found in the samples containing the blaVIM-2 gene. In the underlying figures those resistances are shown in graphs. Notice that the appearance of some genes (aac(6')-29a, aac(6')-29b, blaOXA395, crpP and sul1) are more frequent than in the mean population. This is not a coincidence, studies show that these genes can also be found in the same integron where blaVIM-2 positions itself. Most of the integrons containing blaVIM-2 has following structure: intI1-aacA29a-blaVIM2-aacA29b-qacE-sul1-orf5, explaining why these genes are found together in this study.

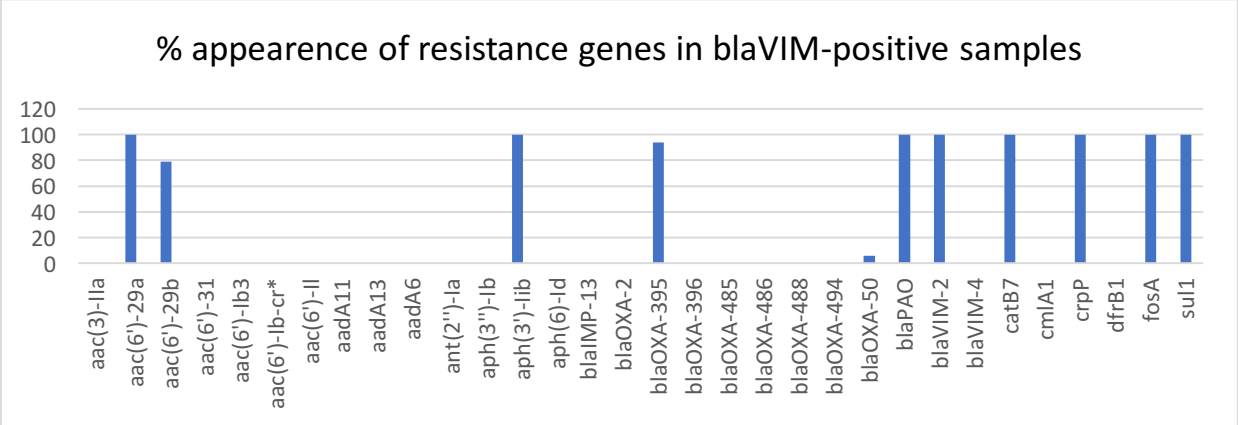


Figure 14: Summary of all the detected genes by ResFinder by all the analyzed samplespositive for blaVIM in %

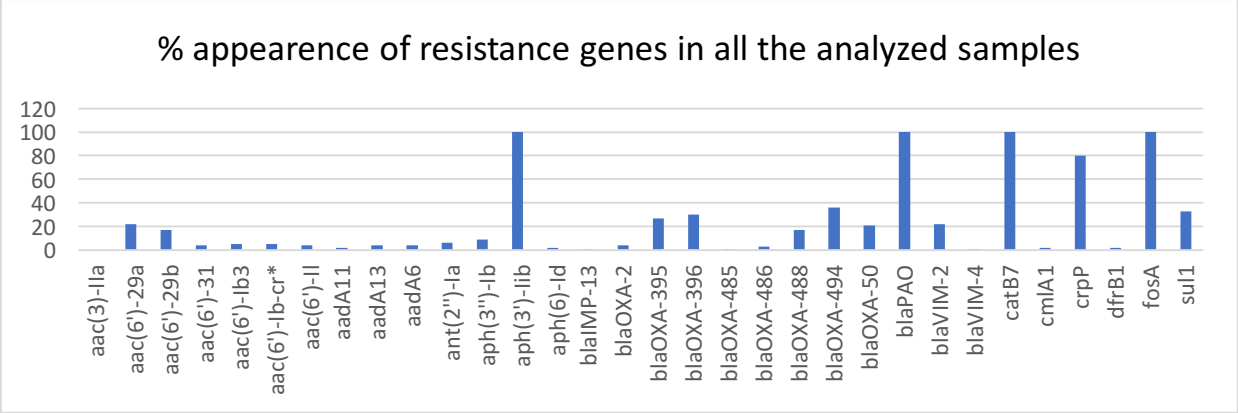


Figure 15: Summary of all the detected genes by ResFinder by all the analyzed samples in %

Cases

In this part, some clinical samples from the same patients are discussed as 'cases'. In particular, the MLST and wgMLST results are used to investigate interesting concerns. Since only retrospective samples with *P. aeruginosa* were kept for this study, only these are shown.

Casus 1

Sample	**Sampling date**	**ST-type**	**Sample type**	**VIM**
ROB121	21/06/19	ST299	Endotracheal aspirate	/
ROB201	22/07/19	ST111	Bronchus aspirate	blaVIM-2
ROB269	15/08/19	ST111	Endotracheal aspirate	blaVIM-2
ROB315	29/08/19	ST111	Bronchus aspirate	blaVIM-2

When first sampled on the ICU, this patient carried a *P. aeruginosa* from the ST299-group that did not produce blaVIM. One month later, the patient tested positive for a blaVIM-producing *P. aeruginosa* from ST111. This ST was found in multiple sink drains on the ICU, inclusive the sink drains on subunit 14 and 15 were the patient was hospitalized. After whole genome sequencing, it is clear that the blaVIM-producing isolates found in this patient are very close related to those found in the sink drains (less than 14 loci of 15143 differed). This strongly suggests that the patient has been infected in the ICU by a bacterium that was present in the sink drain.

Casus 2

Sample	**Sampling date**	**ST-type**	**Sample type**	**VIM**
ROB108	16/06/19	ST27	Urine	/
ROB113	17/06/19	ST27	Blood	/
ROB170	10/07/19	ST17	Bronchus aspirate	blaVIM-2

When first sampled on the ICU, this patient didn't carry a *P. aeruginosa* producing the blaVIM-enzyme. The patient carried a ST27 isolate. One month later, the patient tested positive for a VIM-producing *P. aeruginosa* from ST17. This ST-type was also in a sink drain in the ICU on subunit 13, but this was not the subunit where the patient was hospitalized. After whole genome sequencing, it is clear that the blaVIM-producing strain found in the patient is very close related to the one found in the sink drain (less than 14 loci of 15143 differed). This suggests that this patient isn't infected directly through aerosols formed in the sink, but possibly through a healthcare worker functioning as a vector from sink to patient. Or that we missed to find and identify this isolate in the sinks on the subunit where the patient was hospitalized.

Casus 3

Sample	Sampling date	ST-type	Sample type	VIM
ROB254	29/05/19	ST17	Urine	/
ROB065	30/05/19	ST170	Urine	/
ROB067	14/08/19	ST17	Urine	/

This case suggests that it is possible to be infected with two different STs of the same species at a time. Here the patient tested positive for the ST17 and ST170 type simultaneously. The ST17 type was also found in one sink drain in unit 13, but the patient was carrying this strain before hospitalized in the ICU. Furthermore, wgMLST analysis clears out that the strain in the sink drain wasn't closely related to the strain found in the patient (more than 14 loci of 15143 differed). ST170 was found in one other patient, and ST17 was found in three other patients.

An interesting thing to see is that exactly the same strain (0 loci of 15143 differed) was found in another patient (ROB021) hospitalized on another part of the hospital at a different time. This is an inexplicable link and suggests that there are for example other sink drains in the hospital also carrying *P. aeruginosa.* Further research is needed.

Casus 4

Sample	Sampling date	ST-type	Sample type	VIM
ROB127	24/06/19	ST175	Anus	/
ROB169	08/07/19	ST175	Blood	blaVIM-4
ROB171	11/07/19	ST175	Anus	blaVIM-4

In this case, no link was seen between patient and sink: ST175 was found in the patient but in no single sink drain on the ICU. After wgMLST it becomes clear that these three strains aren't the same: the last two have the blaVIM-4 gene, the first one didn't. Eighty-two of the 15143 loci differed, suggesting that the patient has probably contracted this strain somewhere in the hospital. However, it is not clear where or how since this ST-type or blaVIM-4 producing *P. aeruginosa* are not found anywhere in the hospital. Further research is needed. Another possible explanation is that some genes, like the blaVIM-4 gene, can be carried on plasmids, transposons, integrons and prophages. Bacteria can then acquire these genes via horizontal gene transfer from the same or different bacterial species *(See page 13).*

5. Conclusion

A previous study conducted in our hospital revealed that sinks drains of the ICU were a possible source of various multi-drug resistant pathogenic bacteria and suggested that transmission from these drains could indeed play a role in nosocomial infections colonized/infected by bacteria present in the sink drains. The results of this study point out that sink drains are indeed a possible source of blaVIM-producing *P. aeruginosa* strains after contamination with patients' materials, and that patients indeed could get infected from sink drains containing these blaVIM-producing *P. aeruginosa*.

In both sampling rounds together, at least 72% (*N=26*) of the sampled sink drains (*N=36*) tested positive for *P. aeruginosa*. Of these positive samples, 31% (*N=11*) tested positive for the blaVIM-2 gene between 8/'19 and 10/'19. An allelic threshold of 14 was used and 16 clusters were distinguished among all the analyzed isolates (*N=60*). In five of these 16 clusters, a connection is visible between clinical isolates and environmental isolates. At least 12 of the 28 (42%) clinical samples of patients from the ICU can be linked to isolates found in the sink drains. Five of the five (100%) of the patients included in this study that carried a *P. aeruginosa* with a blaVIM-2 gene were linked to environmental samples, highly suggesting that the sink indeed plays a role in harboring and transmission of blaVIM-producing *P. aeruginosa*. No single link was seen between environmental isolates and non-ICU clinical samples (*N=6*). However, it is very likely that the real percentage of positive samples is even greater. This is because MAC-plates are not specific for *P. aeruginosa* and positive colonies can easily be overlooked or overgrown by other Gram-negative species like *Escherichia coli.* Also, there are studies that mention that the cultivation of samples from a *P. aeruginosa* biofilm can take a very long time to become positive: they saw that some samples only become positive after 48-240 hours of incubating (56). It is possible that a lot of positive results have been missed in this way.

Furthermore, it may also be that some sinks were just cleaned because a few times no bacterial growth at all was observed on the MAC-plates. In this study, there are unfortunately some 'missing parts' and inexplicable links. There are for example multiple strains found in different sink drains very closely related to each other, without one single link to a patient. There are also links between patient themselves that can't be explained with this study, suggesting that the problem isn't strictly limited to the sink drains in ICU. This can perhaps be explained by the fact that many positive environmental samples have been missed like explained on *page 22*, or the fact that there are likely to be other sources of *P. aeruginosa* whether or not in the ICU department. Finally, there is also the chance that certain health care providers are asymptomatic carriers of these specific isolates that can cause spread to patients, which could cause them to spread from patient to patient.

General discussion and conclusion

The prevalence of infections with multidrug resistant Gram-negative bacteria is increasing worldwide. Recently, the World Health Organization has classified *P. aeruginosa* as a "priority 1 pathogen": this is the most critical group of all and includes multidrug resistant bacteria that pose a particular public health threat (43). Apart from the danger to public health, these infections contribute to an enormous cost for the healthcare institutions and can be associated with increased mortality (57).

As studies show, blaVIM-producing *P. aeruginosa* can occur in a wide range of environmental reservoirs and objects in the hospital like floors and walls, on the beds of patients, on door handles and on various other objects. However; it appears that the sink drain is probably the most important reservoir with a huge amount of CFU's. The strains present in these sink drains could be spread to other sinks and patients through the formation of aerosols and contaminating in this way the air, environment and hands of healthcare-workers. Another potential and important transmission route is via the sewers

of the sinks: Moloney et al. performed a study to investigate trafficking of bacteria between hospital washbasin U-bends and indicated trafficking via the wastewater network (28). Finally, *P. aeruginosa* can also be transmitted through mechanical ventilation of the lungs. The relatively high amount of sink drains containing blaVIM-producing *P. aeruginosa* is probably partly due to the fact that fluids, whether or not from patients and which often contain antibiotics, have been and are being flushed down the sink. These antibiotics could promote the selection of resistant bacterial strains, like the blaVIM-producing *P. aeruginosa*. For example: beta-lactams are excreted in the urine and reach very high concentrations there. When this urine enters the sink, the bacteria there are exposed to very high concentrations of antibiotics, thus promoting the selection of resistance. In the next years, a new ICU will be built in the UZ Brussels. Certainly, the problem of the sink drains should be taken into account. There are multiple options, but the most obvious and probably best solution would be to no longer place sinks next to the patient's beds and even ban them in the hole room. In this way, aerosols formed next to the patient are no longer a treat. However, in practice this is currently difficult to achieve since the service's doctors and nurses have not supported this idea so far. Another (probably less effective) method is to ban all the sinks next to the patient, but to keep one or two 'central' sinks in the middle of each unit. Finally, various special designed sinks can be used so that hands can be washed without touching the taps or the risk of splashback and creating aerosols. There are even sinks on the market that can disinfect themselves through heating. Fusch C. *et al*. showed that these sinks were superior to other sinks in preventing emissions from aerosols pathogens (58). However, providing every bed with one of these sinks seems like a very expensive option. If the UZ Brussels want to install them in the 28 ICUs rooms, it would cost at least 66.000 Euro. De Geyter D. *et al*. installed and tested in 2015 a self-desinfecting siphon in the ICU of the UZ Brussels. Unfortunately, they had too few results to make conclusions about the effectiveness, but concluded that the installation and maintenance were a crusade (24). In addition to the architectural aspect, various other measures can prevent the spread of blaVIM-producing *P. aeruginosa*. It is very important that the staff stops flushing organic material that may contain bacteria and antibiotics in the sinks. These materials could contain bacteria that contaminate the sink drains. The antibiotics on the other hand could promote selection and proliferation of resistant strains. Furthermore, a good hand hygiene is also important: the "elbow-way" was shown to be the best method to wash hands under a sink, providing no evidence for post-contamination when performed correct (59).

And last but not least: consistently disinfecting the sink drains can be a cheap and temporarily option. The most effective substance to treat the sink drains against *P. aeruginosa* and their biofilm is acetic acid. Despite the fact that bacterial biofilms are often difficult to eradicate, data shows that acetic acid has an exceptional effect against *P. aeruginosa* biofilm *in vitro*. However, acetic acid will not completely disinfect the growth in the sink and drains, but weekly treatment will significantly reduce the bacterial burden. It is important to maintain a good adherence to the acetic acid routine, enabling the bacterium to re-emerge. A potential limitation could be the strong and irritating smell of the acid (60).

APPENDIX ONE: SUMMARY OF THE ENVIRONMENT-SAMPLES FOR *P. AERUGINOSA*

'+' stands for *P. aeruginosa* present in sample, 'VIM' stands for VIM-phenotype positive. All the samples phenotypical positive for VIM were also tested genotypic positive by NGS. Between 28/09/2019 and 07/10/2019, at least 11 of the 36 (31%) sink drains carried blaVIM-producing *P. aeruginosa.*

Sample	***P.aeruginosa* 28/08**	***P.aeruginosa* 07/10**	**Sample**	***P. aeruginosa* 28/08**	***P.aeruginosa* 07/10**
VP 13.1	-	-	VP 15.1	+	+
VP 13.2	-	+ VIM	VP 15.2	+	+ VIM
VP 13.3	+	-	VP 15.3	+	-
VP 13.4	-	+	VP 15.4	+	-
VP 13.5	+ VIM	+ VIM	VP 15.5	+*	-
VP 13.6	+ VIM	+*	VP 15.6	-	-
VP 13.midden	+	-	VP 15.midden	+	+
VP 13.UR	-	-	VP 15.UR	-	-
VP 13 sas	-	-	VP 15 sas	+	+
VP 14.1	-	+ VIM	VP 16.1	-	-
VP 14.2	+	+ VIM	VP 16.2	+	-
VP 14.3	+ VIM	+	VP 16.3	+ VIM	+ VIM*
VP 14.4	+	+	VP 16.4	-	+
VP 14.5	+ VIM	+ VIM	VP 16.5	+	+*
VP 14.6	+	+ VIM	VP 16.6	-	+ VIM
VP 14.midden	-	-	VP 16.midden	+	+
VP 14.UR	-	-	VP 16.UR	-	-
VP 14 sas	-	+	VP 16 sas	-	-

Summary of the environment-samples for P. aeruginosa
** contaminated, no NGS results available*

APPENDIX TWO: SUMMARY OF THE DETECTED GENES BY NGS RESPONSIBLE FOR RESISTANCE

		Acquired aminoglycoside													Acquired Beta-lactam												Other					
Staal		aac(3)-IIa	aac(6')-29a	aac(6')-29b	aac(6')-31	aac(6')-Ib3	aac(6')-Ib-cr*	aac(6')-Il	aadA11	aadA13	aadA6	ant(2'')-Ia	aph(3'')-Ib	aph(3')-IIb	aph(6)-Id	blaIMP-13	blaOXA-2	blaOXA-395	blaOXA-396	blaOXA-485	blaOXA-486	blaOXA-488	blaOXA-494	blaOXA-50	blaPAO	blaVIM-2	blaVIM-4	catB7	cmlA1	crpP	dfrB1	fosA	sul1
19/1903																		1								1						
ATL19/112																		1								1						
H.ANITA																		1								1						
ROB129																		1								1						
ROB162																		1								1						
ROB170																								1	1	1						
ATL19/282-ROB192	92																	1								1						
ATL19/290-ROB201	01																	1								1						
ROB269																		1								1						
ROB269																		1								1						
ROB314																		1								1						
ROB315																		1								1						
ROB377																		1								1						
ROB470		1	1										1					1								1				1		
ROB491																		1								1						
ROB493																		1								1						
ROB513																		1								1						
ROB516																		1								1						
ROB641																		1														
ROB677																		1														
VP13.2																		1								1						
VP13.5																		1								1						
VP 13.5																		1								1						
VP13.6																								1		1						
VP14.1																		1								1						
VP14.2																		1								1						
VP14.3																		1								1						
VP14.5																		1								1						
VP14.5																		1								1						
VP14.6																		1								1						
VP15.2																		1								1						
VP 16.3																		1								1						
VP16.6																		1								1						

An overview of the identified resistance-mechanisms presents in the 33 analyzed blaVIM-positive P. aeruginosa isolates.

ABSTRACT

The sink as source of transmission of VIM metallo-beta-lactamase-producing *Pseudomonas aeruginosa* in the intensive care unit.

Robin Vanstokstraeten, Deborah De Geyter, Ingrid Wybo, Florence Crombé, Denis Piérard,

Background: Hospital-acquired infections, caused by multi-drug resistant, Verona Integron-encoded Metallo-β-lactamase (VIM)-producing *P. aeruginosa,* are a major problem in intensive care units (ICU). A previous study conducted in our hospital revealed that sinks drains of the ICU were a possible source of various multi-drug resistant pathogenic bacteria and suggested that transmission from these drains could indeed play a role in nosocomial infections colonized/infected by bacteria present in the sink drains. This study investigates whether the sinks at the ICU are effectively carriers of VIM-producing *P. aeruginosa*. This study examines whether there is a genetic link between sink isolates and patient isolates, in order to demonstrate whether the sinks play a role in the transmission of these multidrug-resistant bacteria

Materials/methods: Thirty-six sinks located in the ICU of the UZ Brussel were sampled twice with Eswabs® (Copan, Italy). Samples were inoculated on MacConkey agar (Biomérieux, France) and identification of suspicious colonies was performed by MALDI-TOF (Bruker, US). Twenty-six sinks were found to be positive and isolates were analyzed by whole-genome sequencing (WGS) together with *P. aeruginosa* strains from 34 patients in the hospital, positive for *P. aeruginosa* between 01/2019 and 09/2019, in order to investigate the relationship between those strains. DNA-purification was done with a Maxwell® RSC Instrument (Promega, US) and WGS was performed on a NovaSeq® 6000 (Illumina, US) by Brussels Interuniversity Genomics High Throughput core (BRIGHTcore). The sequencing data was analysed using the *wg*MLST schema for *P. aeruginosa* of the BioNumerics software v.7.6 3 (Applied Maths, Biomérieux, Belgium).

Results: Thirty-one % of the *P. aeruginosa* strains found in the sink drains carried the VIM2-gene. Five patient samples carried the VIM2-gene and one carried the VIM4-gene. Twenty-five different *P. aeruginosa* sequences types (ST) were identified among 77 isolates, of which 28 belong to ICU-patients. Twelve of these were also found in strains recovered from the sink samples. *Wg*MLST- analysis of all VIM2-positive isolates showed that isolates within each ST were very closely related. Transmission in both directions is suspected: patients who contaminated the sink drains with *P. aeruginosa* strains, but also patients who became infected over time by VIM-producing *P. aeruginosa* strains present in the sink drains.

Conclusions: This study points out that sink drains are a possible source of VIM-producing *P. aeruginosa* strains after contamination with patients' materials. We also showed that patients could get infected from sink drains containing VIM-producing *P. aeruginosa*.

ABSTRACT

De gootsteen als bron van transmissie voor VIM metallo-beta-lactamase-producerende *Pseudomonas aeruginosa* op de dienst intensieve zorgen.

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Achtergrond: Het Universitair Ziekenhuis Brussel wordt op de dienst intensieve zorgen (IZ) regelmatig geconfronteerd met nosocomiale infecties, in het bijzonder met multi-drug resistente Verona Integron-encoded Metallo-β-lactamase (VIM)-producerende *P. aeruginosa.* Een eerdere studie in ons ziekenhuis toonde reeds aan dat de gootstenen op IZ drager kunnen zijn van verschillende multidrug-resistente kiemen. In deze studie wordt onderzocht of de gootsteen op de dienst IZ effectief drager zijn van blaVIM-producerende *P. aeruginosa*. Daarna wordt er nagegaan of er een genetische link is tussen isolaten uit de gootsteen en isolaten afkomstig van de patiënt, om op deze manier aan te tonen of de gootstenen een rol spelen bij de transmissie van deze multiresistente kiemen.

Materialen/methoden: Zesendertig gootstenen op de dienst IZ van het UZ Brussel werden tweemaal bemonsterd met Eswabs® (Copan, Italië). Monsters werden geïnoculeerd op MacConkey-agar (Biomérieux, Frankrijk) en identificatie van verdachte kolonies werd uitgevoerd door MALDI-TOF (Bruker, VS). Zesentwintig gootstenen bleken positief te zijn en isolaten werden geanalyseerd door middel van sequentiebepaling van het gehele genoom (WGS) samen met *P. aeruginosa-stammen* van 34 patiënten in het ziekenhuis, positief voor *P. aeruginosa* tussen 01/2019 en 09/2019 om zo de relatie tussen die stammen te onderzoeken. DNA-extractie werd gedaan met een Maxwell® RSC Instrument (Promega, US) en WGS werd uitgevoerd op een NovaSeq® 6000 (Illumina, US) door Brussels Interuniversity Genomics High Throughput core (BRIGHTcore). De sequentiegegevens werden geanalyseerd met behulp van het wgMLST-schema voor *P. aeruginosa* afkomstig van de BioNumerics-software v.7.6 3 (Applied Maths, Biomérieux, België).

Resultaten: Eenendertig% van de *P. aeruginosa*-stammen in de gootstenen droegen het VIM2-gen. Vijf patiëntmonsters droegen het VIM2-gen en één het VIM4-gen. Vijfentwintig verschillende *P. aeruginosa*-sequentietypen (ST) werden geïdentificeerd onder 77 isolaten, waarvan 28 behoren tot IZ-patiënten. Twaalf hiervan werden ook aangetroffen in stammen die werden gewonnen uit de gootsteenmonsters. WgMLST-analyse van alle VIM2-positieve isolaten toonde aan dat isolaten binnen elke ST zeer nauw verwant waren. Transmissie in beide richtingen wordt vermoed: patiënten die de gootsteenafvoeren hebben verontreinigd met *P. aeruginosa*-stammen, maar ook patiënten die na verloop van tijd zijn geïnfecteerd door VIM-producerende *P. aeruginosa*-stammen die aanwezig zijn in de gootsteenafvoeren.

Conclusies: Deze studie wijst erop dat gootsteenafvoeren een mogelijke bron zijn van VIM-producerende *P. aeruginosa*-stammen na besmetting met materiaal van patiënten. We laten ook zien dat patiënten geïnfecteerd kunnen raken door gootsteenafvoeren die VIM-producerende *P. aeruginosa* bevatten.

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