Università degli studi di Cagliari

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INNOVATION SCIENCES AND TECHNOLOGIES

Cycle XXXI

TITLE OF THE PHD THESIS

***Pseudomonas aeruginosa* related to Nosocomial and Animal infections. New approaches in diagnosis and therapy**.

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Dedicated to my Father, my Mother

and Antonio

Chapter 1

General introduction

Alessandra Scano

1.1 General characteristics

Pseudomonas aeruginosa is a small bacillus gram negative, aerobic, asporogenous, monoflagellate, non-fermenting, characterized by a thick capsule coated with alginate, an exopolysaccharide consisting of D-mannuronic acid and glucuronic acid monomers.

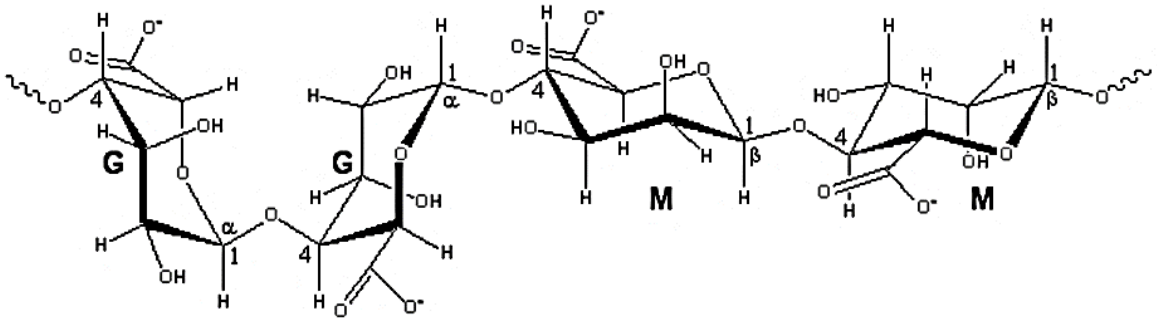

Fig. 1 *- Chemical structure of the alginate.*

P. aeruginosa infections often have a very severe course and are difficult to treat because of its to ability to invade the tissues and its aptitude to form biofilm. The sessile form and the outer structures in this bacterium represent a formidable tool for its pathogenicity. For example, the outer membrane of *P. aeruginosa* is characterized by different proteins which allows the entry of molecules and ions. These proteins (OprF) lower the permeability of the membrane, decreasing the possibility of entry of toxic substances, and this is one of the factors that makes *P. aeruginosa* so resistant to antibiotics [1]. This extracellular matrix makes these microorganisms resistant to antimicrobial agents (antibiotics) and leads to the emergence of multi-drug resistant clinical isolates (MDR) during therapy.

P. aeruginosa may develop resistance to an antimicrobial through several mechanisms: decreased uptake, development of efflux systems and inactivation or modification of the drug.

Among the mechanisms of resistance to antimicrobials we distinguish intrinsic or natural resistances and acquired resistances: the intrinsic resistance is due to characteristics related to bacterial physiology and includes the formation of biofilms, while the acquired resistance concerns mechanisms of resistance that the bacteria acquired later to different mechanisms of bacterial DNA mutation. Its versatility is determined by the ability to synthesize different enzymes that allow the bacterium to use a great diversity of substrates as moist nutrients [2]. *P. aeruginosa* is able to produce the elastase and zinc-protease enzymes that attack collagen and elastin proteins by destroying the cell structure; it also degrades human immunoglobulin, the alpha protein of the

serum and the plasma membrane of the eukaryotic cell through its exoenzyme ExoU, leading to the lysis of the cell.

The virulence mechanisms of *P. aeruginosa* are complex and only partially known; the pili, long homopolymeric strands of the pilin protein encoded by the *PilA* gene [3], which allows to adhere to the host tissue and the flagellum with motor function play a fundamental role. *Pseudomonas aeruginosa* uses, as the main virulence factor, the exotoxin A that inactivates via ADP-ribosylation, the elongation factor EF-2 of the protein synthesis in the host cell. Without the elongation factor the cell goes into necrosis [4] (Figure 2).

The production of exoenzymes and virulence factors is controlled by the quorum sensing, a mechanism of communication between bacteria, able to respond to the age of the population by modifying the gene expression [5]. During the process, the bacterium detects the presence of other bacteria, producing and responding with signal molecules known as auto-inducers; the quorum sensing in gram negative is based on the action of the LuxL and LuxR proteins that catalyze the formation of a specific self-inducer N-acyl-L-homoserine lactones (AHL), and detects its presence when the inductor concentration reaches a threshold level in the surrounding medium. The LuxR-AHL complexes activate the transcription of the target genes by recognizing specific binding sites on the promoters of the genes regulated by the quorum sensing detection. When the bacterial population reaches a critical threshold, a molecule will transmit the signal for the development of biofilm [5-6].

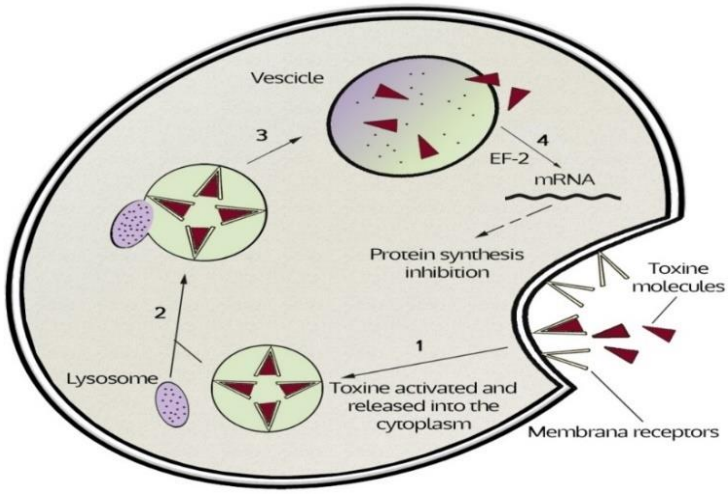

Fig. 2 *- Transport mechanism for exotoxin A and its activation in the cell. A) The toxin binds to the cell receptor and is transported inside the cytoplasm into a vesicle. B) The lysosome fuses with the vesicle containing the toxin and the lysosomal activity stimulates the toxin. C) Activated toxin is released into the cytoplasm where it inhibits cellular protein synthesis by interacting with the EF-2 elongation factor.*

1.2 Epidemiology

Epidemiological studies, performed using various genotyping methods, suggest that the transmission of *P. aeruginosa* can occur through direct contact between patients or through contaminated environmental sources. This ubiquitous bacterium able to live and multiply wherever they find sufficient soil for its growth. In veterinary field this bacterium represents an etiological agent of different infections able to allow serious sanitary and economic problems in breedings. In animal mastitis caused, for instance, by *Staphylococcus aureus*, the bacterium remains only temporarily in the external environment and it survives with difficulty. While in the mastitis of environmental germs the origin of the microorganisms involved is represented by the environment precisely: water, litter and milking system.

Eliminating all these elements is often impossible, and the contact of the udder with such microorganisms is continuous, in fact the penetration of the germs takes place through the canal of the nipple within 1-1.5 hours from the milking [7].

Pseudomonas aeruginosa is also one of the main causes of nosocomial infections, in hospitals where hygiene rules are not followed correctly, with real epidemics with serious consequences; in this context the incidence rate of *P. aeruginosa* infections may be alarming [8].

As before described, the species belonging to the genus *Pseudomonas* generally have a broad spectrum of resistance to antibiotics as they produce waterproofing coatings and enzymes capable of inactivating penicillins and aminoglycosides and are equipped with mechanisms for the expulsion of many antibiotics.

P. aeruginosa is inherently resistant to most of antimicrobial agents due to its selective ability to prevent various antibiotic molecules from penetrating into the external membrane. The antimicrobial groups that remain active include some fluoroquinolones (*e.g.* ciprofloxacin and levofloxacin), aminoglycosides (*e.g.* gentamicin, tobramycin and amikacin), some β-lactams and polymyxins. *Pseudomonas* turns out to be resistant to β-lactam, because the increase in Amp C β-lactamase production causes a reduced sensitivity to β-lactam. The resistance of *P. aeruginosa* to these agents can be acquired through one or more different mechanisms, including modified antimicrobial targets, efflux and permeability reduction [9]. The ubiquitous presence of *P. aeruginosa* and its prevalence and persistence in clinical settings, including intrinsic resistance to therapies are attributed to its extraordinary capacity for survival through responsive mechanisms [10].

The reasons why this bacterium is the subject of so much attention is its great ability to adapt to different environments: such as soil, water and plants. In this project we wanted to deepen the

aspects related to the nosocomial and the veterinary area in which *P. aeruginosa* can spread [11-12]. Currently the *P. aeruginosa* infections are characterized as a global problem in human and animal health because some recent works have demonstrated a transmission of drug-resistant *P. aeruginosa* strains from animals to humans and *vice versa* (zooanthroponosis) [13].

It has been decided to conduct studies in the veterinary field as *Pseudomonas aeruginosa* still causes considerable morbidity among dairy animals, in particular it is associated with severe clinical forms of diseases. It is also a causative infectious agent in both livestock and pet animals; it is associated with endometritis, haemorrhagic pneumonia and mastitis [14-15-16].

In the human field this species is involved in various infectious diseases such as cystic fibrosis, otitis, urinary tract infections and ulcers [17-18-19-20].

Due to a variety of mechanisms for adaptation and resistance to multiple classes of antibiotics, infections from *P. aeruginosa* strains can be life-threatening and are emerging worldwide as a public health threat.

In fact, recent studies using comparative genomics on several strains of *P. aeruginosa* isolated in the human and veterinary fields have identified some key genes involved in the state of the changing environment with respect to the pathogenic state of these bacteria. In particular, these studies have reported significant variations such as single nucleotide polymorphisms (SNP) and minor insertion / deletion variations in genes associated with biofilm formation and motility. A structured biofilm appears to be crucial in the pathogenicity of *P. aeruginosa* because the sessile condition leads to rather significant advantages compared to the planktonic counterpart [21].

The main benefits associated with the biofilm condition for *P. aeruginosa* are related to:

1) Bacterial cells enclosed in an extracellular polysaccharide matrix containing alginate extremely impenetrable to antibiotics. The antimicrobial concentration to eradicate the biofilm of *Pseudomonas* is 10-100 times higher than that used to eradicate the same cells in the planktonic state [22].

2) Recognition of the host immune system such as phagocytosis or surface interaction of antibody bacteria in *Pseudomonas* biofilms is strongly compromised [23].

3) Biofilm formation requires, in these bacteria, a complex change in the transcriptome profile with an increase in the virulence / pathogenicity profile [24].

For these reasons, the presence of a strong contamination of the *Pseudomonas* biofilm in the nosocomial areas could be a great risk for the patient and health professionals must implement all the hygiene procedures. Many studies have reported the Intensive Care Unit (ICU) as the focal point on patient risk for various microbial infections [25]. The high incidence of infections in ICU

patients is due to several concomitant conditions, use of broad-spectrum antibiotics, devices placed with interventional techniques, mechanical ventilation and cross-infection with multi-drug resistant *Pseudomonas* strains [26]. In hospitals the "*Pseudomonas* contamination hot-spots" were found mainly in the hydraulic lines of dental units [27-28]. Another field represents the infections that occur during surgical procedures (Figure 3) [29-39]. These data suggested that after intensive care units, ocular and cardiovascular surgery represent high-risk hospital departments often associated with Multi-Drugs Resistant strains (MDRs) of *Pseudomonas aeruginosa* [40].

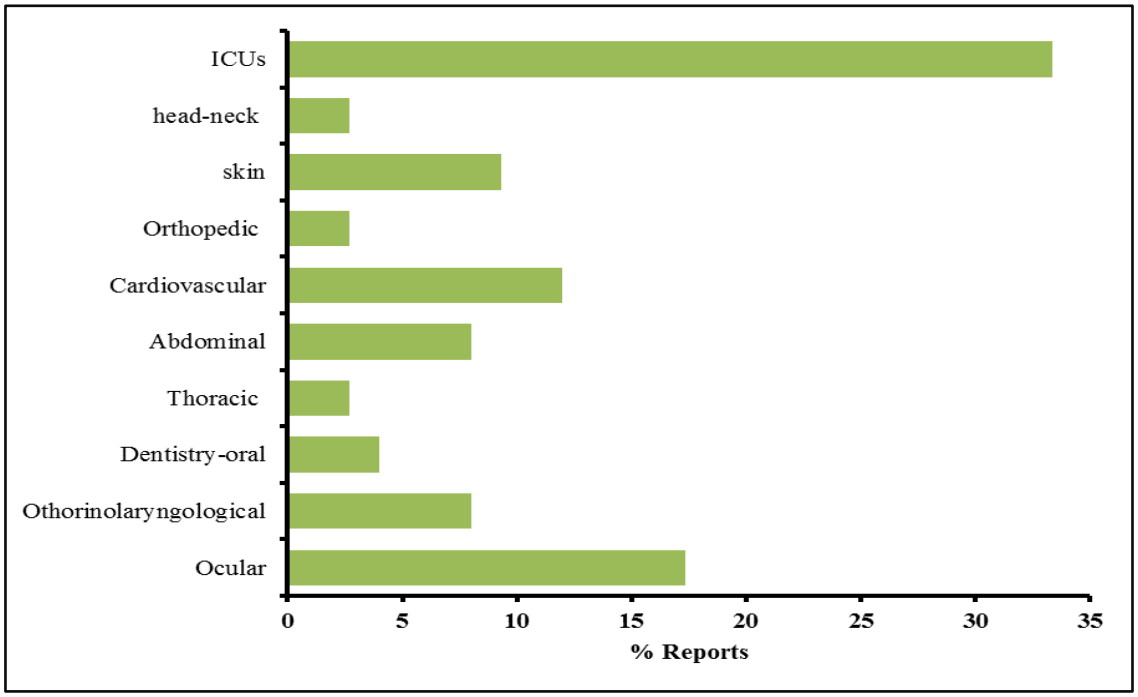

Fig. 3 *- Surgical-site and complications due to Pseudomonas infections reported on PubMed data.*

P. aeruginosa represents a microorganism capable of escaping antimicrobial therapies or prophylactic antisepsis procedures through genetic and epigenetic adaptive strategies [41]. A point of concern is the presence in the nosocomial area of Multi-Drug Resistant strains (MDR), *i.e.* resistant to more than one antimicrobial agent and Extensively Drug-Resistant strains (XDR).

Furthermore, the Pan-Drug Resistance phenotype (PDR) is defined as a bacterium resistant to all antimicrobial agents in all antimicrobial categories [41]. *Pseudomonas spp.* shows a serious therapeutic challenge for the treatment of nosocomial infections and the selection of the correct antibiotic therapy is essential to optimize the clinical outcome. Unfortunately, this selection could be complex due to the ability of these species, in particular *Pseudomonas spp.*, to develop resistance

to different classes of antibacterial agents, even during the treatment of an infection [42-50] (Figure 4). Considering various germicidal agents used in commercial liquid disinfectants such as Glutaraldehyde, Formaldehyde, Acetic acid, Hydrogen peroxide, Sodium hypochlorite, Phenol and Cupric ascorbate, these microorganisms have demonstrated an incredible ability to avoid their antibacterial activity.

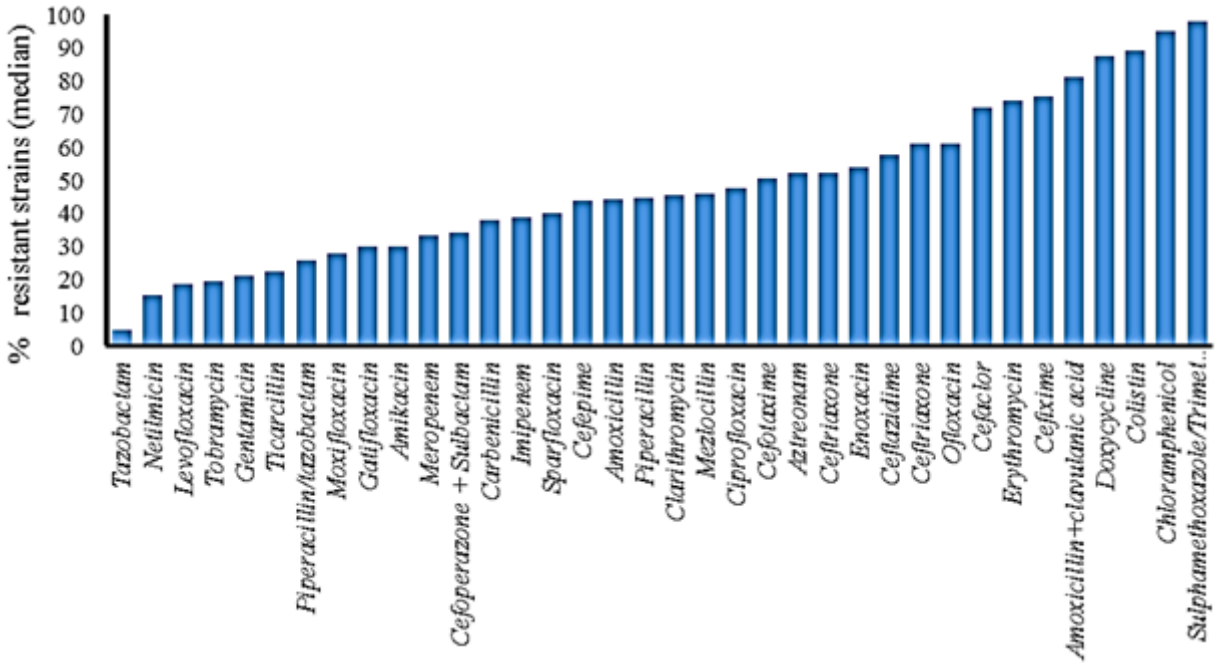

Fig. 4 *- Drug resistance rate% of Pseudomonas spp. Clinical isolates. 2015-2017 worldwide report.*

Several studies show the appearance of pharmacologically active *P. aeruginosa* strains following the use of inappropriate disinfectants, such as peroxides.

The risk of *P. aeruginosa* infection may be related to several factors such as inappropriate therapies, insufficient prophylaxis measures and primarily environmental monitoring systems, inadequate in detecting MDR strains. For example, the current use of Hydrogen peroxide can cause mutations in the muc operon with a consequent overproduction of alginate in the biofilm matrix, which causes impermeability to many disinfectants [51]. In the latest reports on *Pseudomonas spp*. infections in the nosocomial / surgical field have reported these bacteria as a "perfect war machine" and for this reason as proof of the concept, many researchers have focused on new therapeutic strategies for new antimicrobials and show interest in new bioactive molecules by chemical synthesis or plant extracts [52].

In fact, three different areas are involved in the study of new antimicrobials:

- Synthesis of new chemical compounds in particular gold-silver-platinum- complexes [53].
- New antibiotics.
- Biological active extracts, *i.e*. essential oils [52].

Many reports [54-55] recommend a precise and specific procedure within the nosocomial areas to minimize *Pseudomonas* infections, in particular:

- Measurement of *Pseudomonas* infection rates: it is important to know the types of *Pseudomonas* species and the profile of the genotypes involved.
- Infection control programs: prevent and control the onset of pathogens acquired in hospital, in this context the operational guidelines and training of health workers must be the core for efficient control.

This program must contain operational protocols for the sterilization and disinfection of invasive devices and medical instruments used for surgical interventions. Also, in the veterinary field it is very important to use disinfectants in dairy farms against bacterial contamination, especially in the pipes and tanks of the milking machine. It is also essential the microbiological and chemical control of water for zoo-technical use, especially if water is taken from a well; this is important to establish a proper and targeted potabilization treatment. The use of a non-compliant disinfection protocol and the use of an inactive disinfectant could lead to an increase in chemical-resistant strains with severe problems in infection eradication programs [56]. For these reasons international control bodies such as WHO (World Health Organizations [http://www.who.int]) and CDC (Centers for Disease Control and Prevention [http://www.cdc.gov/hai/organisms/*Pseudomonas*.html]) recommend monitoring environmental exposed to the risk of infections by *Pseudomonas aeruginosa*; in this way we are attempted to avoid the incidence of *Pseudomonas* related disease in veterinary and in human medicine.

1.3 The purpose and nature of this PhD thesis

This work aims to study *P. aeruginosa* infections and its environmental contaminations as a global health problem. In fact, this "multi-task" pathogen can contaminate different areas in human, veterinary and agricultural field. The fields studied in this work have been schematized in figure 5.

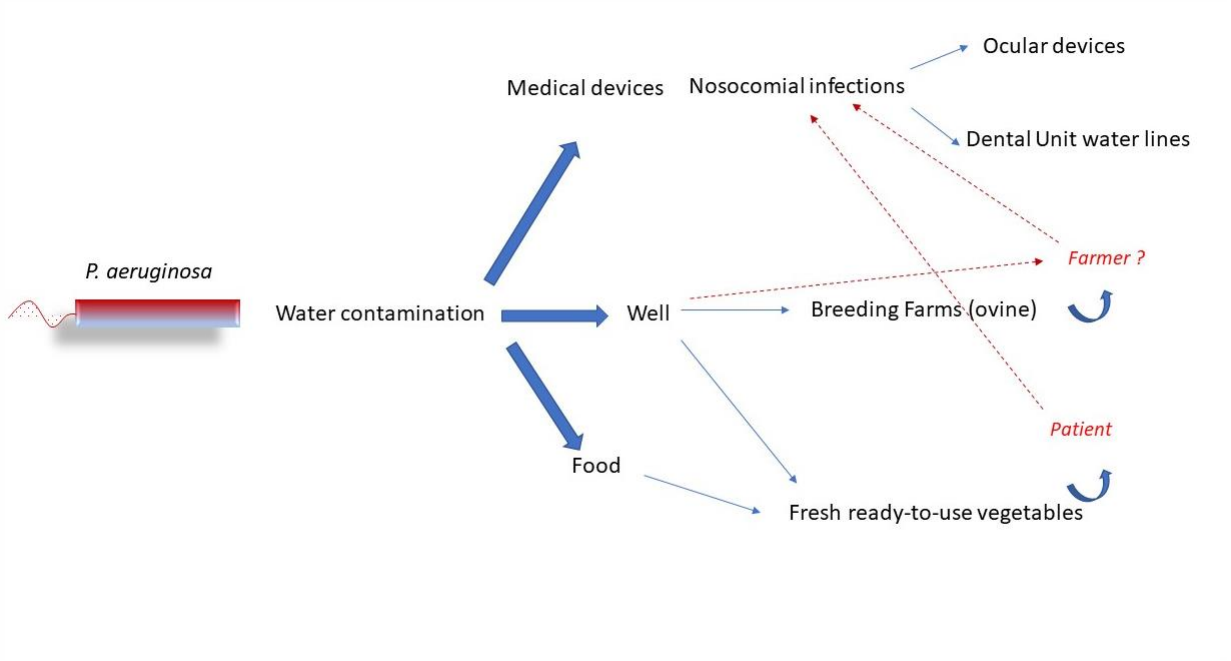

Fig. 5 *- Schematic representation of different P. aeruginosa modes of contamination and relative infections (Pseudomonas infective cycle) in human and animal field studied in the present work.*

The scheme represented in figure 5 suggest also that same *Pseudomonas* strain could contaminate different human activity areas. For this reason, a comprehensive work must be performed by different strategies described in these points:

1. Pathogen detection: development of a fast and easy laboratory method to determine the *P. aeruginosa* presence and its concentration in the biological sample.
2. *mucA*/alginate profile: in *P. aeruginosa*, *mucA* genotype resulted essential to reveal high drug-resistant strains due to alginate hyperproduction in the biofilm. For this reason, in this work, we have designed an integrated procedure for sequencing the *mucA* gene in environmental and clinical isolates of *P. aeruginosa*.

3. Evaluation of potential new antimicrobials: a critical point in anti - *P. aeruginosa* prophylaxis is represented by the absence of "highly performant disinfectant". The disinfection resulted in fact the first step for pathogen control.
4. New cultural systems: design/use of bioreactors able to reproduce, in standard controlled conditions, the initial parameters in the primary contamination area, for example the cold storage implants in the food production.
5. Future strategies: following the recent new molecular procedures obtained in biological field could be possible to design new clinical and laboratory strategies against *P. aeruginosa*, *i.e.* the study of antisense oligonucleotides or miRNAs.

Ovine mastitis due to *P. aeruginosa* are a great nosocomial infection model, having the same pathogenic mechanisms observed in humans. This model has been useful to perform/validate the original molecular methods used in this work. In fact, molecular biology techniques are indispensable for the characterization of pathogenic strains, evaluated through real time PCR technology and DNA Sequencing.

In human field, another relevant data is the presence of different microorganisms inside the hydraulic lines of the dental unit able to induce infections in humans in contact with water contaminated by *Pseudomonas* during dental care. Currently several clinical reports and scientific studies show an incidence of *P. aeruginosa* in the ducts of the dental unit, in some cases with prevalence in sampling near turbines and micromotors. Conversely other authors have found a low prevalence or total absence, this is due to differences in the sampling unit. The characterization of the biofilm in dental units will allow to define points and materials at risk, and whether low doses of disinfectant can induce more virulent and resistant bacterial phenotypes. It is important to evaluate which points of the dental unit are most sensitive to biofilm concentration. In fact, the biofilms in the ducts, mainly in the valves and in the joints, release the bacteria only when stimulated by the flow of water and if excessively extended or weakened.

The laboratory procedures used in this study could be divided in these following phases:

1) The genotypic analysis of *mucA* will be compared with the phenotypic profile to verify if there is a molecular epidemiology of SNP in *mucA* useful as a prognostic factor of the state of infection.

2) Molecular probes will be designed specifically for fast detection of *P. aeruginosa*.

3) Sequencing will be performed for research of mutations that characterize the status of "resistant drug" in *Pseudomonas*.

4) The *mucA* gene integrated in the drug resistance will be analyzed through bioinformatics software.

5) Identification in compliance with sanitary hygiene standards of specific disinfectants would prove useful for the control of biofilm supported by *Pseudomonas aeruginosa*.

6) New synthesis substances shall be tried for inhibition of *Pseudomonas* growth.

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*Part of this chapter has been published in the following article:

Germano Orrù, **Alessandra Scano**, Vincenzo Piras, Gloria Denotti, Manuele Liciardi, Giuseppe Speziale, Claudio Napoleone, Andrea Mascolo (2017).

Pseudomonas Infections in Surgery Practice Needs for Innovative Antimicrobial Procedures?

OPEN ACCESS JOURNAL OF SURGERY, p. 1-4, ISSN: 2476-1346, doi: 10.19080/OAJS.2017.03.555619

Chapter 2

A comparative evaluation on clinical *Pseudomonas aeruginosa* strains in nosocomial/veterinary areas suggests a global health problem

Alessandra Scano, Ferdinando Coghe and Germano Orrù

2.1 Incidence of *P. aeruginosa* infections in nosocomial area in Sardinia

Recently the Centers for Disease Control and prevention (CDC) indicating *P. aeruginosa* as an important pathogen in nosocomial area. These reports suggesting a strictly surveillance and outreach activities to help to prevent infections (CDC 27/7: saving lives, protecting people). These studies have estimated in USA 51.000 healthcare-associated infections and more than 6.000 (13%) of these were allowed from multi-drug resistant strains, with 4.000 deaths per year attributed at these infections. Up the present, no existing recent data in Italy regarding the epidemiology of *P. aeruginosa* infection in nosocomial field as well as the rate of MDR strains. In this chapter we describe a preliminary study performed in Cagliari University Hospital (AOU).

2.2 Materials and methods

We have conducted a microbiological study in this regard in the University Hospital of Cagliari.
In 2017 we analyzed the presence of microorganisms in 152 sample of blood present in the following departments: Thoracic Surgery, Emergency Medicine 1, Emergency Medicine 2, Medicine 1 and Medicine 2 (Table I).

Tab. I *- Distribution of invasive isolates 2017 (blood) by bacterial species (sentinel bacteria).*

Microorganisms	**%**
Escherichia coli	36.8
Enterococcus faecalis	7.2
Enterococcus faecium	2.6
Klebsiella oxytoca	2.6
Klebsiella pneumoniae	21.1
Pseudomonas aeruginosa	**3.3**
Staphylococcus aureus	21.7
Streptococcus pneumoniae	4.6

All *Pseudomonas aeruginosa* isolates were submitted to drug-resistance analysis for several antibiotics commonly used in clinical practice. The Antimicrobial Susceptibility Tests has been performed by The Vitek 2 Compact (Biomerieux, France) following the manufacture instruction.
The *P. aeruginosa* strains have been tested from colonies isolated in Trypticase Soy Agar with 5% sheep blood (Microbiol, Uta, Italy) after 24 hours of incubation at 37°C.

2.3 Results

Here we report the data obtained. All samples belonging to Emergency Medicine 1 and Emergency Medicine 2 were analyzed (Table II).

Tab. II *- Antibiotic resistance in Pseudomonas aeruginosa strains isolated inside University Hospital of Cagliari and referred to nosocomial infections.*

Antibiotics	**%R**	**%I**	**%S**
Amikacin			100.0
Amoxicillin/Clavulanic acid	**100.0**		
Ampicillin	**100.0**		
Cefepime			100.0
Cefotaxime	**100.0**		
Ceftazidime			100.0
Cefoxitin	5.3	7.9	86.8
Ciprofloxacin	28.6	1.8	69.6
Colistin			100.0
Gentamicin			100.0
Imipenem	40.0		60.0
Meropenem	40.0		60.0
Piperacillin			100.0
Trimethoprim	**100.0**		

The results showed a high resistance profile for some β-lactam antibiotics and pyrimidine inhibitors, in fact 100% of isolates were resistant to Amoxicillin, Ampicillin, Cefotaxime and Trimethoprim in according with previous published data [57]. While a complete sensitivity has been observed for:

- Large spectrum 3th - 4th generation cephalosporin, resistant to β-lactamases such as Ceftazidime and Cefepime (Maxipime) respectively;
- Colistin, also known as polymyxin E;
- Aminoglycosides *i.e.* Gentamicin and Amikacin;
- Ureidopenicillin class β-lactam antibiotics such as Piperacillin.

2.4 A comparative study in veterinary/nosocomial field.

At present, *P. aeruginosa* infections are characterized as a global problem in human and animal health because some recent works have demonstrated a zooanthroponotic-transmission of drug-resistant *P. aeruginosa* strains from animals to humans and *vice versa* [58]. Its adaptability makes it a frequent contaminant of water pipes in several nosocomial areas as well as on animal breeding farms. In fact, acute infection by this opportunistic pathogen is the leading cause of significant morbidity and mortality in infected subjects [59]. In patients with impaired immune systems, this bacterium causes severe infections such as: cystic fibrosis, infections of the cornea, skin burns, catheter-related infections and lung infections in patients with emphysema and obstructive lung disease [60-63]. It is also a causative infectious agent in both livestock and companion animals, linked to endometritis, hemorrhagic pneumoniae and mastitis [64-65]. In all these situations, one of the main difficulties in clinical management is due to the extensive resistance of *Pseudomonas* to antimicrobial agents, associated with a particular recognized biofilm *status* in sanitary device contamination, as well as in persistent or chronic infections [66-67].

The biofilm is characterized by the following phases:

1) The bacteria that are in the planktonic form start to decelerate and adhere to a surface through adhesi

2) The anchorage to the surface starts a cascade of reactions that activate the genes responsible for the phenotype "biofilm".

3) The increase of the cell density determines the activation of quorum-sensing systems, able to regulate the density of the bacterial population of the biofilm and promoting the expression of virulence genes.

4) During the maturation of the biofilm, the cellular elements breed to form bacterial towers.

5) Finally, the bacteria in planktonic form are released into the environment in order to colonize other districts.

These different sessile states of *P. aeruginosa* in comparison with planktonic liquid culture make these bacteria highly resistant to antimicrobial agents, including the disinfectants used in hospitals or in the veterinary field against bacterial contamination, especially in hospital water lines [68-69]. This process of biofilm formation and subsequent antimicrobial resistance indicates the most probable reason for the difficulty in the eradication of this etiological agent and calls for the enforced application of cleaning and sanitation protocols. Disinfectants play an important role in antibacterial prophylaxis and in the disinfection of different sanitary devices, *i.e.* protheses, catheters, dental unit water lines or for example, the milking machines used in the veterinary field. However, the use of a non-compliant disinfection at protocol and/or the use of an inactive disinfectant could produce an increase in chemical resistant strains with severe problems in eradication of infection programs [70]. At the moment, bibliographic data report the presence of multi-drug resistant (MDR) strains of *P. aeruginosa* in hospitals as well as on ovine farms worldwide, especially against chlorhexidine and benzalkonium chloride [71-72]. In this context, we believe that a disinfectant susceptibility profile linked to *P. aeruginosa* growth inhibition could be useful in the medical field in controller protocols against this pathogen. For this reason, the aim of this study is to reveal the antimicrobial susceptibility pattern of *P. aeruginosa* strains isolated from a large scale of biological samples, against the common disinfectants used in Sardinia region.

2.5 Materials and methods

Nosocomial samples

Ninety swabs recruited in 30 different dental units water lines (DUWL) were analyzed. These medical devices were located at Cagliari University's Odontoiatric Institute and in different private practices in the town of Cagliari. Sampling was carried out in three different positions for each DUWL:

- in water pipes related to the handpiece zone,
- disinfectant's tank,
- pipes related to pre-disinfection zone in private practice water pipelines derived from public water line [73].

Sampling procedure for veterinary specimens

The analyzed breeding farms contained around 4,000 Sardinian ewes distributed among 11 different sheep farms located in different areas in southern Sardinia. The flocks were milked with an automated milking machine and monitored throughout the lactation period by means of the California Mastitis Test (CMT) and routine bacteriology due to persistent cases of mastitis [74]. The period of sampling reported in this work was at the end of the lactation period. Animals selected for sampling included cases of clinically acute mono-lateral mastitis and cases of persistent acute infection. Before samples were taken, the teats have been carefully cleaned and the first three streams of milk were discarded. Ten milliliters were collected from each udder and separated into two different falcon tubes with 1 ml glycerol. After milking, teat swabs were taken. The swabs were put into test tubes with PBS and 10% glycerol. Four swabs have been taken from the teat cups of the milking machine reserved for the infected animals.
All samples were immediately placed in dry ice for transport to the lab where they were stored at -20°C. 30 strains were isolated in infected milk while 13 were isolated from different points of the milking machine and 1 was isolated from farm well water 23278.

Laboratory diagnosis

Pigmented colonies showing a typical morphology and positive for the oxidase test (Bactident Oxidase, Merck) were considered as belonging to the *P. aeruginosa* species. The identification response was performed using a biochemical system (API20NE BioMerieux). Further confirmation was carrying out by sequencing the 16S rRNA using already indicated protocols. The *P. aeruginosa* colonies were extracted by using the CTAB modified method [75]. Primer OG33 (5'-GACTACCAGGGTATCTAATC-3') and OG123 (5'- AGCAGCCGCGGTAATA - 3') [76] were used for PCR and sequencing reaction performed with Sanger's procedure. The isolates were stored before the experiments in Mueller Hinton (MH) Broth with glycerol at 15% at -80 ° C.

Strains used in this experiment

P. aeruginosa ATCC 15442 (American Type Culture Collection), recommended for disinfectant testing by official methods [77-78] was used as the high biocide-resistant strain for the comparison of clinical isolates behavior with disinfectants, while *P. aeruginosa* ATCC 2783 was used as the susceptible strain [79]. A total of 61 different clinical isolates of *P. aeruginosa* were studied, 17 from Dental unit water lines and 44 from veterinary samples. The presence or absence of no-mucoid or mucoid colonies was recorded for each strain to distinguish muc^-/muc^+ phenotypes respectively [80].

Disinfectants

Four different chemical compounds currently used in the Sardinian region for sanitary antimicrobial prophylaxis were tested:

- **Chlorhexidine,** CHX (Sigma C9394), in solution to 20% has been diluted to obtain a solution to 10%, which is equivalent to 100 mg/ml.
- **Benzalkonium chloride powder,** BZC (SIGMA B6295), has been prepared with a concentration of 100 mg/l in water.
- **Sodium hypochlorite** NaClO (SIGMA 425044) in solution at 13% of free chlorine, equivalent to 126000 mg/l, has been brought to a final concentration 1064 mg/l.
- **Hydrogen peroxide** H_2O_2 (Farve, Italy) in solution at 3%.

The various aliquots were stored in a freezer at -20°C. Following the literature data and commercial data sheets, we used the following concentration ranges for the tested antimicrobials:

- BZC and CHX from 128 to 0.25 μg/ml,
- NaClO from 8192 to 16 μg/ml and H_2O_2 from 10000 to 1.95 ppm [81].

All the consulted sheep-farmers reported using the products with the analyzed active compounds in the following order of frequency: BZC, CHX, NaClO and H_2O_2.

Broth Dilution Tests, MIC and MBC

Before any experiment, 50 μl of the frozen bacterial suspension was inoculated onto a Mueller Hinton plate and a single colony was inoculated into Mueller Hinton Broth (Microbiol, Cagliari, Italy) and incubated at 37°C for 8 hours, until the growth middle-logarithmic phase. 10^6 CFU/ml bacterial cell suspensions were used as inoculum for all subsequent experiments.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were performed according to National Committee for Clinical Laboratory Standards (NCCLS) protocols using the micro-broth dilution method with ½ serial dilutions of each tested disinfectant [82-83]. The main MIC point was positioned at the values detected for the high resistance, strain ATCC 15442.

Briefly, each antibacterial combination was suspended in a 96-well microplate where each well contained 100 μl of the tested compound and 100 μl of microbial inoculum suspended in the appropriate liquid medium described previously. Each strain was inoculated in the exponential (log) phase with a concentration corresponding to $1*10^6$ CFU/ml; the experiment was performed in triplicate and after 24 hours of growth at 37°C, the turbidity at λ = 550 nm of each set of combinations was measured. The minimum inhibitory concentration MIC for each microorganism was the lowest concentration of an antimicrobial that inhibited visible growth (absence of turbidity) [82-83].

Once determined the MIC it is possible to derive the MBC through sowing in solid medium (MH). Starting from the dilution corresponding to the least inhibiting concentration are seeded the suspensions that don't present bacterial growth. The first concentration which underlines absence of growth corresponds to the minimum bactericidal concentration (MBC).

Statistical analysis

Statistical analysis was done using Stat-VIEW for Windows (SAS packs data management, USA). To describe a correlation between the susceptibility pattern to one disinfectant *versus* others tested antimicrobials, the Pearson's chi-squared test was used (all P values < 0.05 were considered significant).

2.6 Results

A total of 21 sampling points (Dental unit and Breedings) suspected of *P. aeruginosa* contamination were analyzed: 10 different Dental water lines and 11 animal breeding farms. Of the 90 swabs recruited from dental surgeries, 17 (18,8%) resulted *P. aeruginosa* positives and 10/30 DUWLs resulted *P. aeruginosa* contaminated (33%). The results obtained from strains recruited in different places in Sardinia suggest the presence of different disinfectant susceptibility areas in this Region. In particular, if we order the mode of MIC values for each sampling zone from the most frequently used disinfectant to the least-used, we can observe a non-homogeneous distribution of the susceptibility patterns. Table III shows the situation at studied sampling points. In this context, a non-statistical association, calculated with Pearson's $\chi 2$ test was observed between BZC resistance and susceptibility to other studied disinfectants ($P > 0.05$), except when hydrogen peroxide MICs are considered as a main variable. The results exhibited also shown a non - homogeneous distribution of the disinfectant susceptibility patterns among the various sampling places. In fact, clinical isolates could be assigned to three different sensitivity groups by comparing the MIC values between high resistant ATCC 15442 and low resistant ATCC 2783 reference strains. The sampling points studied showed this situation [Table III]:

- Benzalkonium chloride (BZC) represents the compound with minor activity on all strains, with a high range of MIC values, from 128-to 64 μg/ml. This observation is in accordance with previous experimental works on drug susceptibility values for *P. aeruginosa*, where BZC represented a non-compliant disinfectant [84-85]. The experimental results found in the literature suggest that in resistant *P. aeruginosa*, the decrease in the amount of adsorbed BZC is likely to be the result of increases in the contents of phospholipids (PL) and fatty and neutral lipids in the bacterial membrane [86]. This bacterial adaptation to BZC could show resistance to other membrane-active agents, but not to clinically relevant antibiotics [87].

- 9 out of 21 sampling points (42,8%) showed the max MIC value for at least two disinfectants. BZC and H_2O_2 were the most representative in this group.
- Max MIC value for one disinfectant (BZC) was 5 sampling points out of 6 (83,3%). The remaining sampling points showed intermediate MIC mode values: 28,5%,
- 42,8 % of the *P. aeruginosa* strains in this study reported a multi-drug resistant profile (MDR) with 7 derived from dental surgeries (33,3%).

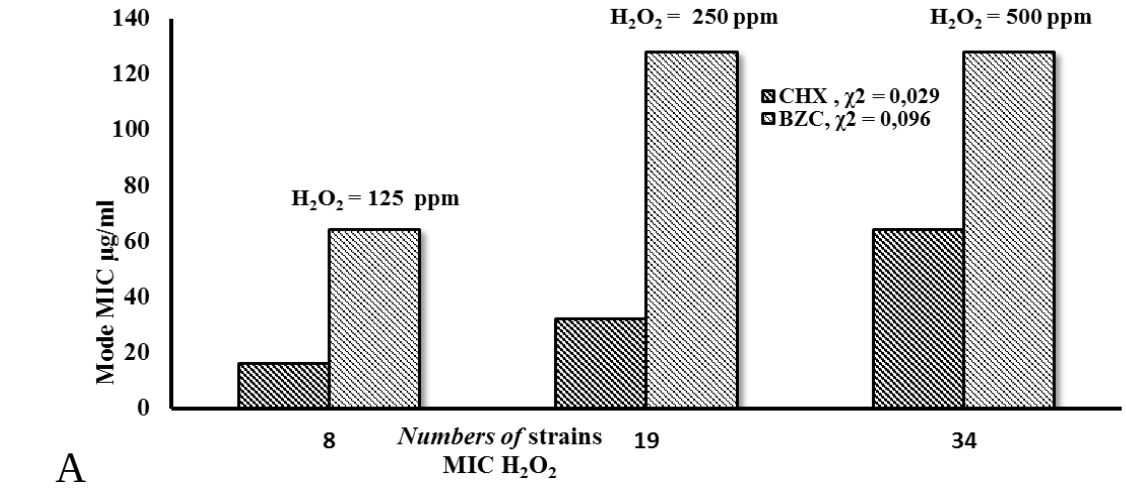
Tab. III *- Mode of MIC values of 61 clinical P. aeruginosa strains related to percentage of mucoid strains and the co-respective susceptibility group. The samples with the letter "**D**" are strains isolated from Dental Unit Water Lines while the samples with the letter "**B**" are strain isolated from sheep breeding.*

Sampling point	**Strains #**	**BZC**	**CHX**	**NaClO**	**H_2O_2**	
		μg/ml		ppm		% muc$^+$
ATCC 15442	1	128	128	4096	500	
Pabillonis^{B1}	6	128	64	4096	250	
Cagliari^{D1}	2	128	64	4096	250	
San Gavino ^{B2}	3	128	64	2048	500	
Cagliari^{D2}	1	128	64	2048	500	72% (I)
Bauladu ^{B3}	3	128	64	2048	500	
Cagliari^{D3}	2	128	64	2048	500	
Ortacesus ^{B4}	3	128	64	2048	250	
Cagliari^{D4}	2	128	64	2048	250	
Cagliari^{D5}	1	128	64	2048	250	
Cagliari^{D6}	2	128	32	2048	250	
Villaputzu ^{B5}	3	128	32	2048	500	
Musei ^{B6}	3	128	16	4096	250	65% (II)
Cagliari^{D7}	2	128	16	2048	250	
Villasor ^{B7}	3	64	64	4096	250	
Serdiana ^{B8}	4	128	32	2048	250	
Cagliari^{D8}	1	64	64	2048	250	
Furtei ^{B9}	7	64	64	2048	250	
Cagliari^{D9}	2	64	32	1024	250	
Sanluri ^{B10}	5	64	32	1024	125	48% (III)
Cagliari^{D10}	2	64	16	1024	250	
Codrongianos ^{B11}	3	64	16	2048	250	
Strain 23278	1	64	8	2048	250	
ATCC 2783	1	64	8	2048	250	

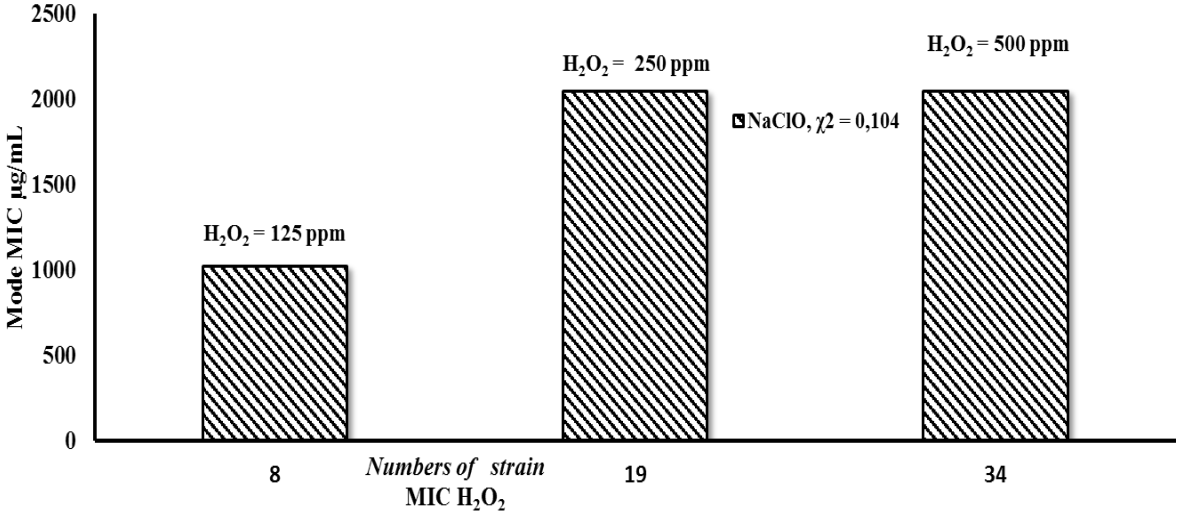
These results underline the emergence of antimicrobial resistance in *P. aeruginosa.*

In this paragraph we focalized our attention on another field where erroneous antisepsis practices could be the reason for the appearance of new drug resistant strains [88-89]. We have investigated on the role of Hydrogen Peroxide as a disinfectant related to drug resistance in this bacterium.

If we observe figures 6a and 6b, the mode MIC values are correlated with the MIC values for the H_2O_2 measured in all the analyzed strains. Normally raised concentrations of H_2O_2 MIC values corresponded to high modes for all the compounds used in this study (Figures 6a and 6b), and a significant correlation was observed with H_2O_2 and CHX, (χ^2=0.029).



A



B

Fig. 6 - A) *Mode of MIC distribution for Chlorhexidine (CHX) and Benzalkonium Chloride (BZC) in a series of P. aeruginosa strains ordered by H_2O_2 increased MICs.* ***B)*** *Behavior observed with Sodium Hypochlorite NaClO.*

This behavior could be explained by citing the results obtained by Mathee *et al.* in *P. aeruginosa* strains isolated from cystic fibrosis patients [90]. Mucoid phenotypes (muc^+) among the strains infecting cystic fibrosis patients have indicated overproduction of the alginate, a linear polysaccharide situated in the bacterial capsule. These muc^+ strains are the cause of mortality in patients with cystic fibrosis. However, these authors demonstrated that after treating non- mucoid strains with low sub-inhibitory levels of H_2O_2, the formation of mucoid variants was observed. All muc^+ variants showed the same mutations in the *mucA* gene that encodes an anti-sigma factor, leading to the deregulation of an alternative sigma factor, required for expression of the alginate biosynthetic operon. The positive correlation between Hydrogen peroxide MIC and susceptibility to other disinfectants could suggest the role of H_2O_2 and the susceptibility of the other compounds used in this work. As reported in Table III, the mode MIC of the analyzed sampling points was also related to the percentage of muc^+ strains. These results could be used to suggest the role of non-performant use of H_2O_2 in disinfection protocols with a possible use of sub-inhibitory concentrations of this disinfectant and the appearance of muc^+ strains. The extensive alginate capsule in these bacteria is then responsible for host tissue adherence and low cell diffusion for various antimicrobial [91].

2.7 Is the dynamism of the biofilm influenced by the disinfectants in minimal concentrations?

A study on Hydrogen peroxide it has been conducted to evaluate if the biofilm dynamism is influenced by minimal concentrations of this disinfectant. In fact, low concentration of peroxide induces conversion to the mucoid form of *P. aeruginosa*, a physiological state more virulent and resistant (Mathee *et al.*).

2.8 Materials and methods

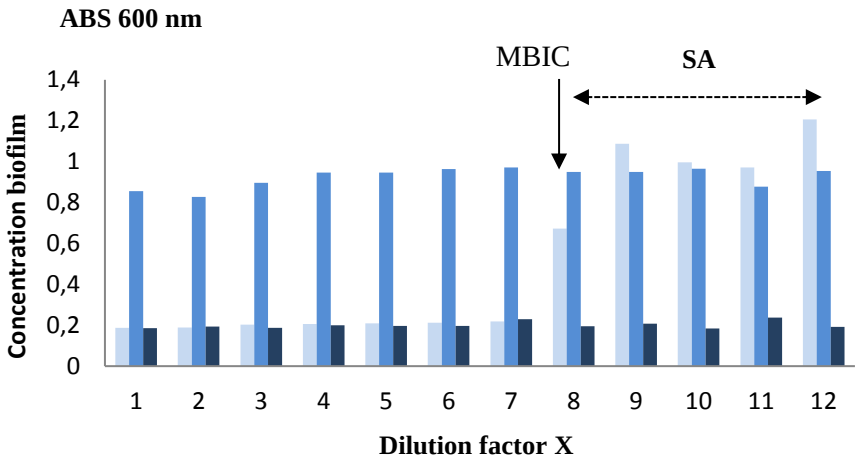
Reference strains

The experiments were performed on *P. aeruginosa* ATCC 2783 used as a biocide-susceptible, no-mucoid strain.

Biofilm measurement with different concentrations of Hydrogen Peroxide.

In a multiwell plate containing $1*10^6$ CFU/ml of *P. aeruginosa* cells, a serial concentration from 10000 to 1.95 ppm of H_2O_2 has been performed. This culture was maintained for 7 days at 37°C. The biofilm was measured following the colorimetric assay published in the "Center for biofilm engineering of Montana State University". Briefly: after a week of incubation, the plate has been washed 3 times with Phosphate-Buffered Saline (PBS Gibco, USA), thus the biofilm present in the bottom of the plate was stained with a crystal-violet 0.4% solution for 2 minutes. Following 2 washes with PBS, to each well has been then added 100 microliters of acetic acid at 30%. After this last step, the biofilm amount was evaluated at 620 nm by Microplate Spectrophotometer (SLT-Spectra II, SLT Instrument, Germany) (Figure 7).

2.9 Results



H_2O_2 concentration = $1*10^4/2^X$

Fig. 7 - *Final biofilm amount of a muc- P. aeruginosa strains after 7 days of incubation. An increase of biofilm has been observed, in comparison with positive control, in sub inhibitory area of H_2O_2 (SA).*

The biofilm behavior due to muc- *P. aeruginosa* strain, is different between a bacterial growth without hydrogen peroxide or in presence of low sub inhibitory concentration of this compound. As represented in figure 7, an increase of biofilm is evidenced after 39 ppm ($10000/2^8$) and this value represented the minimum biofilm inhibitory concentration (MBIC). Thanks to the results obtained it has been decided to study the speed of biofilm formation of two different *P. aeruginosa*

isolates: (1) highly resistant muc+ strain, isolated from animal with mastitis and (2) an environmental strain, muc - sensitive to disinfectants (Figure 8). We have used the cultural conditions already described and by using a sub-inhibitory concentration of H_2O_2 (12,5 ppm). Every hour we have measured the biofilm though the procedure described above.

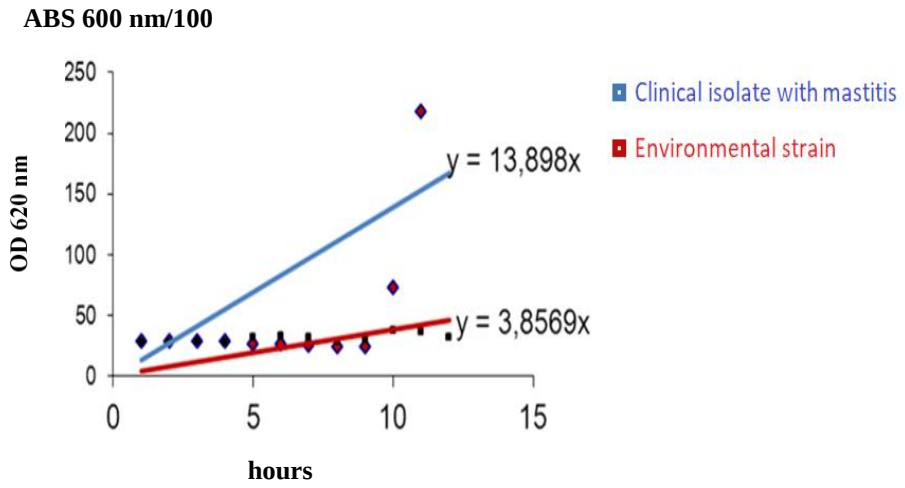


Fig. 8 *- Linear tendency curves and respective slope expressed in [biofilm]/hour between two different P. aeruginosa strains, muc+/muc-.*

The results show how the behavior in the form biofilms at sub-inhibitory doses of disinfectant is completely different between two strains muc+/muc-. In fact, the environmental strain showed a lowest 3,6 - fold biofilm formation rate, in comparison with clinical muc+ isolate (13,8/3,8).

2.10 Conclusions

In this chapter we have investigated the antimicrobial profile of *P. aeruginosa* strains recruited from human/animal related contaminated areas. We observed a correlation between the hydrogen peroxide resistance profile, the presence of mucoid strains and the susceptibility patterns to 3 disinfectants, CHX, BZC and NaClO. These results suggest a possible role of H_2O_2 in the disinfection procedure in the Sardinian region as a "*P. aeruginosa* mucoid-converting compound" and a subsequent increase in the spread of high virulent strains in the environment. However, more studies are necessary to confirm this hypothesis, such as: *mucA* gene sequencing for these strains (Chapter 3).

Links:

- CDC Pseudomonas report. https://www.cdc.gov/hai/organisms/pseudomonas.html
- Center for biofilm engineering of Montana State University. http://www.biofilm.montana.edu/biofilm-basics/index.html

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*A part of this paragraph has been submitted in Large animal review Journal.

Alessandra Scano, Giuseppe Serafi, Sara Fais, Silvia Bomboi, Marcella Peri, Antonella Ibba, Carolina Girometta, Germano Orrù, Paola Rossi, Manuele Liciardi.

Activity of 4 Antimicrobials Against Clinical *Pseudomonas aeruginosa* Strains Isolated from Different Ovine Breeding Farms in Sardinia.

Large Animal Review. 7-2018.

Chapter 3

mucA Mutations in Clinical Isolates of *Pseudomonas aeruginosa*

Alessandra Scano, Maurizio Fossarello, Vincenzo Piras and Germano Orrù

3.1 *Pseudomonas aeruginosa* and Biofilm related diseases

Pseudomonas aeruginosa is at the attention in health care by its ability to form biofilms, a complex aggregation of microorganisms characterized by the secretion of an adhesive and protective matrix that makes the microorganisms impermeable and resistant to antibiotics and disinfectants. The extreme capability of this bacillus to form biofilm is very interesting not only for purely biological aspects but also from a clinical point of view.

The dynamism of the biofilm is influenced by the disinfectants in minimal concentrations, under the Minimum Bactericidal Concentration (MBC); in this regard, Mathee *et al*., report a phenomenon of induction by Hydrogen peroxide at low concentrations [92]. In experiments performed in vitro in flow chambers, low concentrations of H_2O_2 induce the conversion to the mucoid phase of *P. aeruginosa*, a physiological condition characterized by an extremely virulent and antimicrobial resistant biofilm. This phase is characterized by the appearance of mutations present in a key gene for the production of alginate, *mucA.*

The analysis of the biofilm of *P. aeruginosa* allows to characterize new antimicrobials and to ascertain what are the useful ranges of the disinfectant that do not induce the mucoid phenotype.

Allelic and gene expression profiles will be able to provide information on critical system levels of biofilm entities. Mutations in the *mucA* gene encode a protein involved in the production of Alginate. Mutations present in the promoter of the gene or along the amino terminal part of the protein modulate an alginate hyper-expression giving the biofilm a barrier almost impermeable to the antimicrobials; this aspect must be considered during the use of oxidizing microbicides such as Hydrogen peroxides, in order to determine mutations in the *mucA* gene. H_2O_2 or other organic peroxides are the disinfectants of choice for water systems in dental units, which makes these devices at high risk for contamination of resistant *P. aeruginosa* strains [93]. *P. aeruginosa* resistances are due to different genetic mechanisms, plasmid acquisition or mutations in the chromosome. The phenotypic analysis of *Pseudomonas aeruginosa* is useful to evaluate a correlation between the reduced susceptibility to antimicrobials and the ability to form biofilm and alginate.

In the literature, some studies suggest that the biofilms of the mucoid phenotype of *P. aeruginosa* are more resistant to antibiotics than the biofilms of the non-mucoid phenotype (Figure 9) [94].

Phenotypically non-alginate strains genotypically show a perfectly in-match sequence with the reference sequence of the non-mucoid PAO1 strain (NCBI Reference sequence: NC_002516).

The mucoid strains, on the other hand, produce the alginate that has a protective function in a relatively hostile environment, in which the bacteria are continually subjected to oxidative stress and attack by the immune system.

Fig. 9 *- On the left a plate containing mucoid colonies of P. aeruginosa is exposed; on the right a plate containing non-mucoid colonies. M = Mucoid strain, NM = no mucoid isolate.*

3.2 Alginate

Alginate is the main component of the extracellular matrix of the biofilm; as a consequence, the alginate lysis facilitates the spread of antimicrobial substances. The synthesis of alginate involves 12 genes belonging to the operon *algA-algD*. The expression of alginate operon is regulated and controlled by the promoter *PalgD* located upstream of the gene *algD* regulated by the anti-sigma factor *mucA* [95]. The overproduction of alginate is linked to mutations in a gene cluster called *mucABCD* that inhibit the activity of the alternative factor σ *AlgU*.

AlgU acts on *algD* gene, that codes the guanosine of phospho-D-mannose dehydrogenase, a key process in the biosynthesis of alginate and on *algR*, a regulator gene that increases the production of alginate.

Mutations of the negative regulators *mucA, mucB and mucD* lead to an overproduction of alginate and to conversion to a stable mucoid phenotype in *P. aeruginosa*.

The products of the *algA*, *algC* and *algD* genes are necessary for the formation of the alginate precursor, the PIL-mannuronate. The product of the alginate gene is instead involved in the export of the polymer on the surface of the bacterial cell. Enzyme-coding genes in alginate biosynthesis are found in an 18kb operon on the circular chromosome of *P. aeruginosa*. A gene is the promoter for the operon of the biosynthetic alginate pathway, called *PalgD*. This promoter is silenced in non-mucoid *P. aeruginosa* strains but is instead very active in hyper productive strains (Alg+). The activation of genes for alginate biosynthesis seems to occur thanks to the deregulation of the *algT* gene product, the factor σ^{22}, a 22 kDa protein that is an alternative sigma factor (initiation factor implicated in prokaryotic transcription that involves the attack of the specific RNA polymerase sequence to the promoter). The factor σ^{22}, product of the *AlgU* gene, is called ECF (extracellular function) [96].

3.3 *mucA* gene

The *mucA* genes, *mucB, mucC and mucD* control the gene expression of the alginate operon [97].

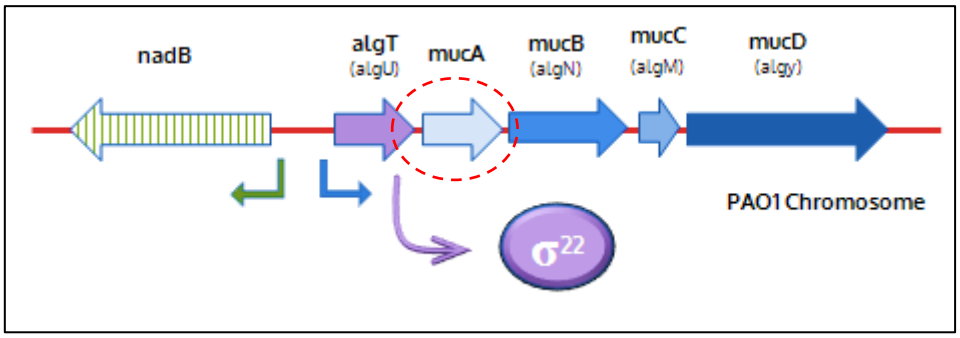

Fig. 10 *- Segment of the chromosome region of the P. aeruginosa wild type PAO1 strain containing algT and its control elements mucA, mucB, mucC and mucD.*

The inactivation in vitro of *mucA* in *Pseudomonas aeruginosa* PAO1 (non-mucoid) produces Alg+ strains; this seems to indicate, therefore, that *mucA* acts as a negative regulator of the production of alginate because it can bind and sequester the factor σ^{22} through the N-terminal cytoplasmic domain [98].

The promoter is silenced in non-mucoid strains of *P. aeruginosa* but is very active in hyperproducers strains (Alg+). The activation of the genes for the biosynthesis of alginate seems to

occur thanks to the deregulation of *algT* of the gene product, the σ^{22} factor. *MucA* may also act as a regulator of *algU* transcription by forming a complex with *mucB* and changing the signal transduction pathway that maintains *algU* transcriptional activity low [99]. Several studies have shown that *mucA* is an anti-sigma factor, due to its affinity with the σ^{22} factor produced by *algT* and its ability to inhibit transcriptional activity in vitro [100]. The *mucB* and *mucD* genes have been identified as negative regulators of alginate synthesis, while the *mucC* product seems to act as a positive regulator.

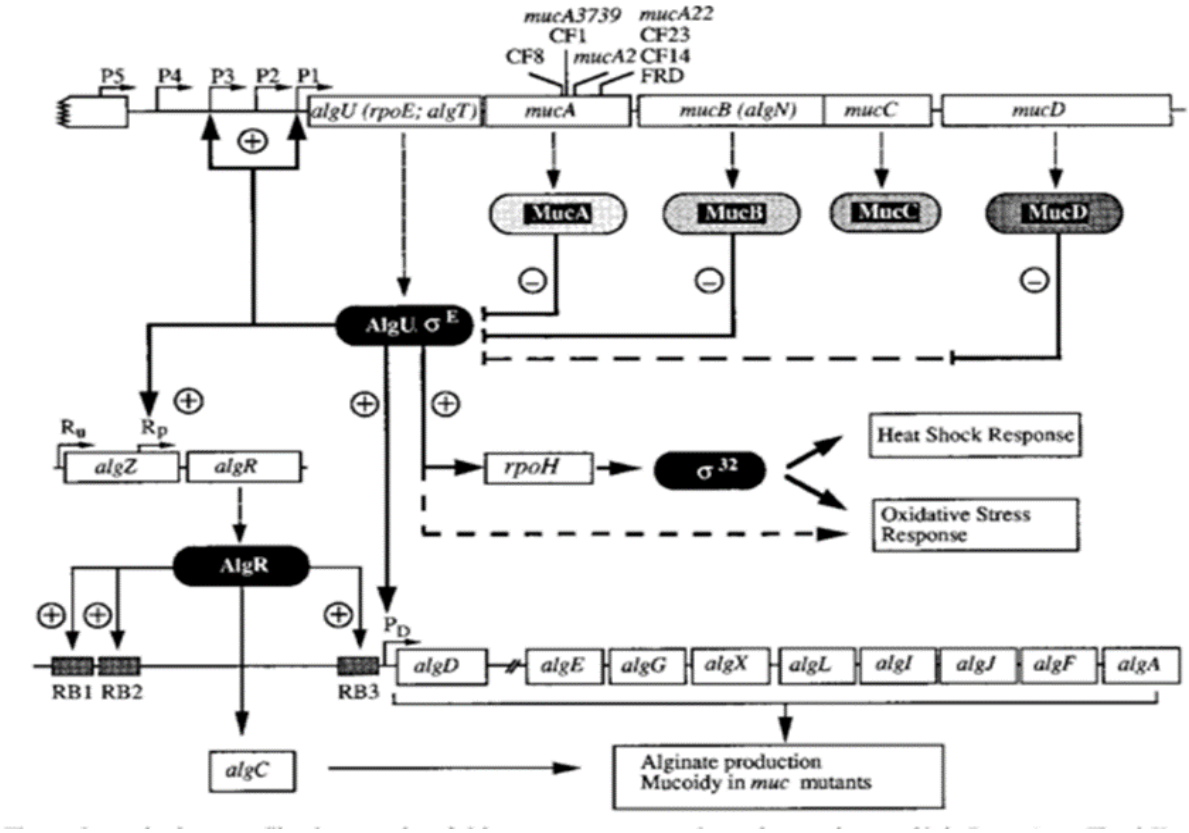

Fig. 11 *- Activation cascade of alginate [101].*

The *mucA* gene has a length of 585 base pair (bp) and encodes a 194 amino acid protein. The *mucA* protein has a molecular weight of 20.999 kDa, a N-terminal portion of 83 amino acids, located in the cytoplasm, a trans membrane segment and a C-terminal portion of 30 amino acids located in the periplasmic space (*Pseudomonas* Genome Database http://www.*pseudomonas*.com). A model has been proposed in which *mucA* - *mucB* - σ^{22} interact through a complex on the internal membrane of the bacterium to control the stability of the protein σ^{22}, in order to regulate the alginate biosynthesis [102].

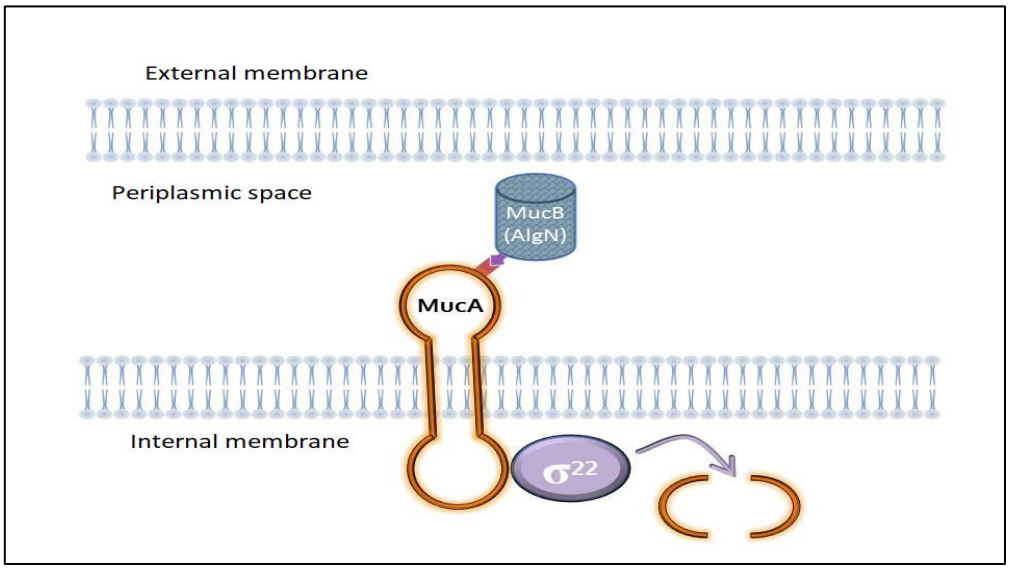

Fig. 12 – *Posttranslational control model of* σ^{22} *by mucA*

The *mucA* gene has been completely sequenced [103] and recent studies [104] have revealed that the majority of mutations involved are frame shift or nonsense mutations, which cause the premature end of protein synthesis (stop mutations).

P. aeruginosa presents a non-mucoid phenotype with high sensitivity to antimicrobials and a mucoid phenotype resistant to antimicrobials [105]. In some cases, the conversion of non-mucoid phenotype to mucoid phenotype is caused by mutations present in two distinct chromosomal loci denominated MUC. These mutations are induced by antibiotics treatments.

The mutations in the gene codify for MucA a protein involved in the production of alginate.

In particular the mutations in the promoter of the gene or along the amino-terminal part of the protein modulate an alginate overexpression by giving the biofilm a nearly impermeable barrier to antimicrobials. In this context, it is strictly necessary to use in our experiments two reference strains: ATCC 2783 non-mucoid strain, and ATCC 15442 multi-drug resistant strain. For example, we can predict the alginate production profile by *mucA* gene, aligning the sequence obtained with the ATCC 2783 (Figure 13).

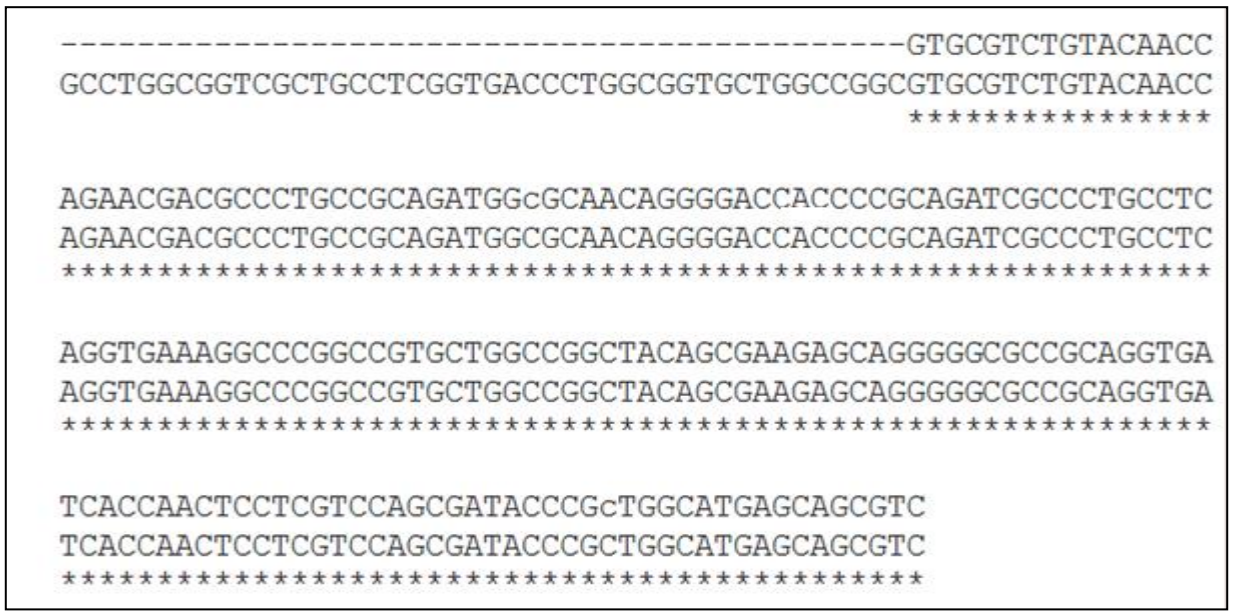

Fig. 13 - *The drug sensitive reference strain (ATCC 2783) and a clinical isolated showed a perfect match (no mutation detected). In this case we can predict a low alginate production for the analyzed strain.*

3.4 Materials and methods

Sampling

During the period October 2015 to June 2018 we collected 103 clinical isolates of *Pseudomonas aeruginosa* coming from:

- 11 ovine flocks in different areas of Sardinia, of which 24 isolates from milk levies of sheep infected by mastitis and one from the washing water drawn out of the milking machine.
- 76 *P. aeruginosa* strains recruited from Odontoiatric Institute of University of Cagliari, 75 from Dental unit water lines coming from different locations as cannula, spray, filter, seat of the filter, bowl, under bowl (Figure 14) and 1 from oral swab.
- 2 *P. aeruginosa* strains isolated from corneal swabs recruited in Eye Clinic of University of Cagliari.

For each patient, approval was obtained, and informed consent was given to patients for the collection of biological samples. The study has been approved for Odontoiatric patient by the Independent Ethic Committee (Prot. PG/2017/16799).

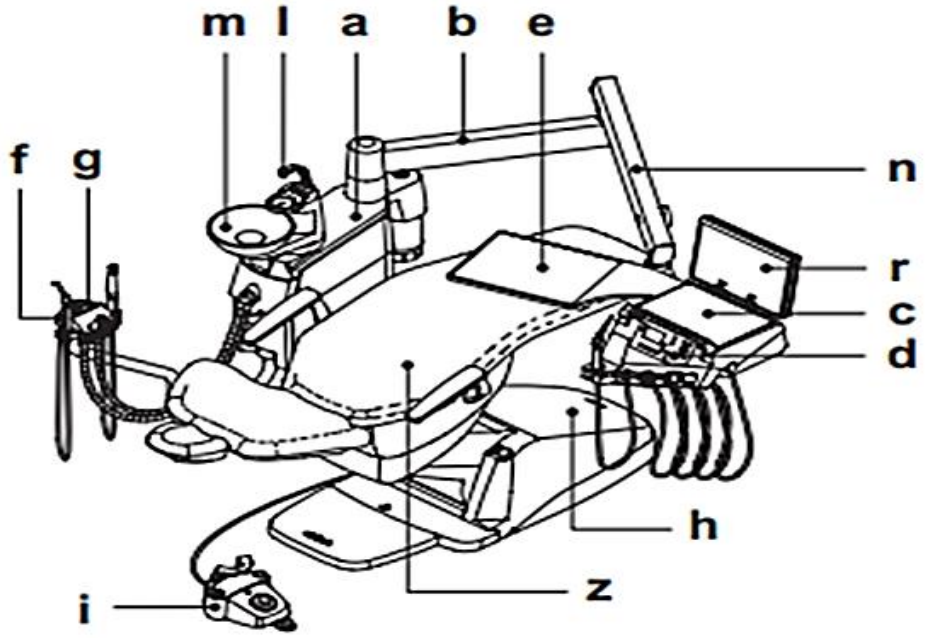

Fig. 14 - ANTHOS A3 PLUS *a) Hydrogen group b) Adjustable arm c) Instrument board d) Doctor's console e) Tray holder (optional) f) Assistant's board g) Assistant's control console h) Utility service center i) Multifunction foot control l) Water to cup m) Bowl n) Auto balancing arm q) Instrument tray on assistant's board (optional) r) X-ray film viewer for panoramic x-rays (optional) z) ANTHOS A3 PLUS dental chair.*

Laboratory diagnosis

All samples were immediately placed in dry ice for transport to the lab where they were stored at -20°C. Pigmented colonies, showing a typical morphology and positive for the oxidase test (Bactident Oxidase, Merck) were considered as belonging to the *P. aeruginosa* species. The identification response was performed by mean of a biochemical system (API20NE BioMerieux). In addition, further confirmation was performed by 16S rRNA sequencing. Briefly, the *P. aeruginosa* colonies were extracted by using the CTAB modified method. Primer OG33 (5'- GACTACCAGGGTATCTAATC - 3') and OG123 (5'- AGCAGCCGCGGTAATA - 3') were used for PCR and for sequencing reaction with sanger's method.

The isolates were stored before the experiments in Mueller Hinton (MH) broth with glycerol at 15% at -80 °C.

Reference strains used in this work

The *Pseudomonas aeruginosa* strains were tested in an exponential growth phase, calculated using a growth curve with the reference strain mucoid and non-mucoid. The growth curve data indicated that the isolated strains reached the exponential phase after 8 hours of growth in liquid medium MH at 37 °C. The 'inoculum' experiment (starter) was prepared by titrating the bacteria through reading in a spectrophotometer at 550 nm, using the McFarland indexes. Our reference standard was 0.5, which includes $1{,}5x10^8$ bacteria/ml corresponding to 0,125 Optical Density (OD) units. In practice, the bacterial suspensions were prepared by collecting and dissolving the colonies in MH broth.

Primers design

Primers for real time PCR and for capillary sequencing were designed by using the entire *mucA* gene extracted from the NCBI database on complete genome of PAO1 strain (GenBank with NC_002516 accession numbers). Possible oligonucleotide dimer formation, self-complementarity and the annealing temperatures of the real time PCR were calculated using the Oligo program version 4 (Med-Probe, Oslo, Norway). A fragment containing 491 bp from *mucA* gene was obtained by real time PCR by using these primers (Pa*mucA*F-seqf:5'-GGATAACGAAGCGGATGAA-3' and Pa*mucA*R-seqr:5'-CACTGACGGCGGATTGTT-3'), (Figure 15).

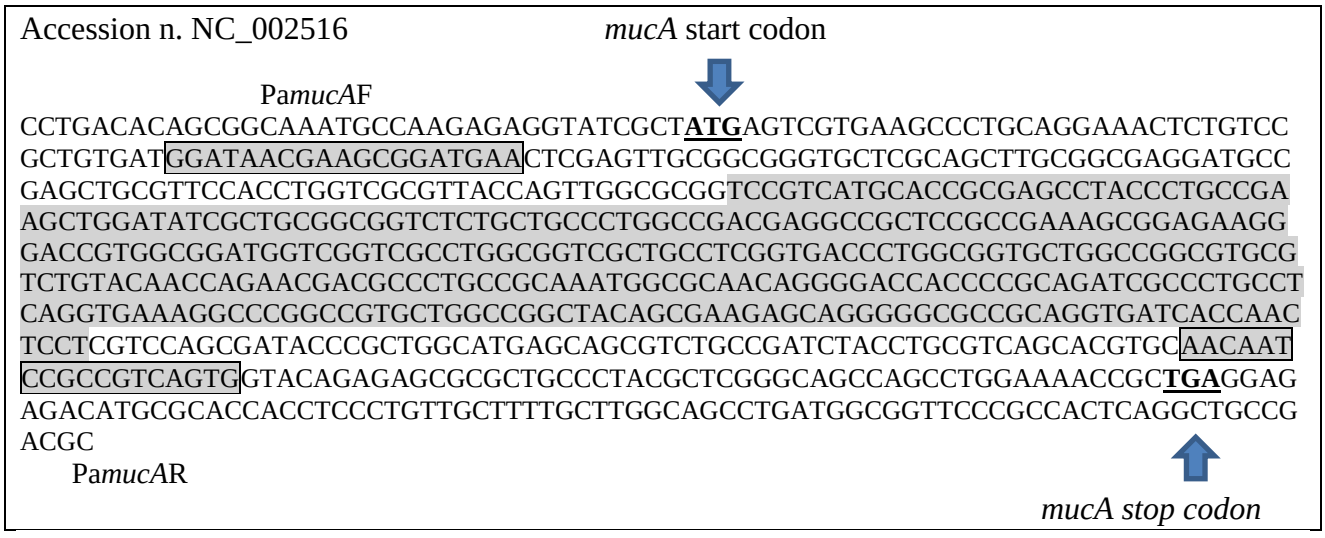

Fig. 15 *- The primer sequence and its position in the mucA gene. Position of oligonucleotide primers used in this work to amplify through real time PCR and for sequencing of mucA gene; in evidence the hot-spot region with a high frequency for gene mutations.*

Real time PCR conditions

Real time PCR was performed with a LightCycler instrument and a LightCycler DNA Master SYBR Green I kit (Roche Diagnostics Mannheim, Germany), according to the manufacturer's instructions. The 20 µl final volume contained 4 mM $MgCl_2$, 1 µM of each primer (Pa*mucA*R-seqf:5'-GGATAACGAAGCGGATGAA-3' and Pa*mucA*R-seqr:5'-CACTGACGGCGGATTGTT-3') and 2 µl of DNA extract. The thermal cycles were set as follows:

95° for 30", 40 cycles of 95° for 1', 52° for 10' and 72° for 20' and 3" at 74.3 °C. The melting curve was performed for 0 seconds at 95 °C, 45 °C, 95 °C. Transition rates were: 5 °C/s in 72 °C segment, 0.1 °C/s in 45 °C segment and 20 °C/s for other steps. Fluorescence was detected at the end of the 74.3 °C segment in the PCR step (single mode), and at 45 °C segment in the melting step (continuous mode) in the F1 channel.

During the initial optimization of the real time reaction, products were analyzed using agarose gel to ensure a correct sample product size (Figure 16). Positive samples showed a melting curve with 86°C *Tm*. After real time PCR, samples were recovered from capillaries by reverse centrifugation into microcentrifuge tubes (Eppendorf 0,2 µl).

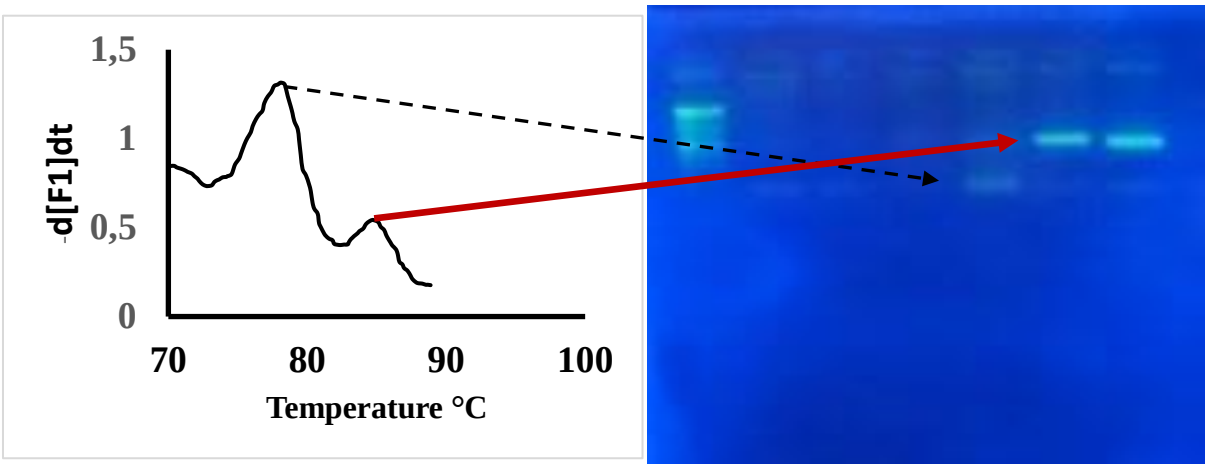

Fig. 16 *- Melting curve of Real time PCR and agarose gel electrophoresis results. Melting peaks at 82°C correspond to mucA amplicon. Melting peak at 77°C in negative control and the small band in the control samples are primer dimer products.*

DNA Sequencing

The amplified products have been purified and subjected at sequence reaction, characterized by dideoxynucleotides (ddNTPs), called nucleotide terminators. DdNTPs are marked with four fluorochromes, one for each base. The four fluorochromes will give a different emission when will

be read by the sequencer machine. The products of the sequencing reaction were purified according to the method of precipitation: the magnesium chloride and ethanol have been added to nucleic acids solution and finally the DNA was denatured before being subjected to sequencing. After a centrifugation the ethanol was eliminated, and the pellet suspended in Formamide. For research of possible mutations has been used capillary sequencing (ABI PRISM 310). The analysis of the results of sequences has been realized using software like:

- Cromas Pro (http://www.technelysium.com.au) that shows the electropherogram of the whole sequence.
- Blast - Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST) that research the similarity between the obtained sequence and a sequence deposited in a data base.
- Subsequently Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) has proven useful for search similarities between the reference sequence and the non-mucoid sequence PAO1 (sequence available online at the National Center for Biotechnology Information - NCBI).

3.5 Results

mucA mutation and allelic profile in clinical isolates of P. aeruginosa

In this study on different 103 clinical strains of *P. aeruginosa* from south and central Sardinia we could detect the presence of a 2 mutated *mucA* alleles, on 12 different mutations (Figure 17).

ATGAGTCGTGAAGCCCTGCAG**G**AAACTCTGTCCGCTGTGATGGATAACGAAGCGGATGAACTCGAGTTG
CGGCGGGTGCTCGCAGCTTGCGGCGAGGATGCCGAGCTGCGTTCCACCTGGTCG**C**G**T**TACCAG**T**TG**G**C**G**C
G**G**TCCGTCATGCAC**C**G**C**GAGCCTACCCTGCCGAAGCTGGATATCGCTG**C**GGCGGTC**T**C**T**GCTGCCCTGGC
CGACGAGGCCGCTCCGCCGAAAGCGGAGAAGG**GAC**CGTGGCGGATGGTCGGTCGC**C**T**G**GCGGTCGCTGC
C**T**C**G**GTGACCCTGGCGGTGCTGGCCGGCGTGCGTCTGTACAACCAGAACGACGCCCTGCCG**C**AA**A**TGGC
GCAACAGGGG**A**C**C**ACCCCGCAGATCGCCCTG**C**C**T**CAGGTGAAAGGCCCGGCCGTGCTGGCCGGCTACAG
CGAAGAGCAGGGGGCGCCGCAGGTGATCACCAACTCCTCGTCCAGCGATACCCGCTGGCATGAGCAGCG
TCTGCCGATCTACCTGCGTCAGCACGTGCAACAATCCGCCGTCAGTGGTACAGAGAGCGCGCTGCCCTAC
GCTCGGGCAGCCAGCCTGGAAAACCGC**T**GA

***Fig. 17** - Mutations found in mucA gene, in comparison with wild type sequence of antimicrobial susceptible P. aeruginosa strains (PAO1, sequence accession. NC _002516). In red the codons supporting missense mutations.*

By comparing our data with literature and already published *mucA* sequences we observed that two missense mutations occurring most frequently in these Sardinian strains (65G**G**C and 63G**G**G) have different relative frequencies in strains isolated from different medical or veterinary area. While GAC65G**G**C is absolutely the most common mutation isolated in nosocomial area (9% of strains), GCG63G**G**G appears to be in only veterinary field (4.1% of analyzed strains) (Table IV and Table V).

Tab. IV *- mucA mutation observed in P. aeruginosa strains isolated in sheep's milk samples, in comparison with PA01 mucA sequence.*

Samples #	Nucleotide	Mutation	Type
1	126	CGT42CGC	*Silent*
3	133	TTG65CTG	*Silent*
3	156	CGC52CGT	*Silent*
1	188	GCG63G**G**G	*Missense:Gly-Ala*
1	194	GAC65G**G**C	*Missense:Asp-Gly*
1	198	TCT66TCG	*Silent*
13	342	CAA114CAG	*Silent*
1	381	CCT127CCG	*Silent*

Tab. V *- mucA mutation observed in P. aeruginosa strains isolated in nosocomial-human samples, in comparison with PA01 sequence.*

Samples #	Origin	Nucleotide	Mutation	Type
6	Dental unit-Eye session (2)	126	CGT42CGC	silent
9	Dental unit	133	TTG65CTG	silent
1	Eye session	141	CGG47CG**C**	silent
7	Dental unit	156	CGC52CGT	silent
7	Dental unit	194	GAC65GGC	missense: Asp-Gly
8	Dental unit-oral swab (1)-Eye session (2)	198	TCT66TCG	silent
4	Dental unit	267	CTG89CTA	silent
2	Dental unit	282	TCG94TCT	silent
12	Dental unit-oral swab (1)-Eye session (2)	342	CAA114CAG	silent
12	Dental unit	360	ACC120ACA	silent
10	Dental unit-Eye session (2)	381	CCT127CCG	silent

One possible explanation of different rate of the same representative missense mutation 65G**G**C between veterinary and nosocomial samples (4.1% and 9% respectively) could be the different

disinfections protocols used in *P. aeruginosa* prophylaxis. In fact, as suggested by Mathee *et al.*, the use of hydrogen peroxide-based antimicrobials could increase the mutation frequency of this gene. These results are in accordance with the considerations and comments described in the previous chapter, in fact the frequency of antimicrobial susceptibility patterns, as well as the percentage of mucoid strains were geographically related and linked at possible different use of disinfectants. Of course, genetic exchange mechanisms responsible for the spread of antimicrobial resistant alleles in *P. aeruginosa* cannot be completely ruled out, even if, due to the current understanding of genetics of *Pseudomonas*, they would be more difficult to postulate.

Genbank

This work has allowed to deposit sequences obtained in a database Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Below are reported the accession numbers of these sequences of *Pseudomonas aeruginosa*:

- MG710801
- KU726585
- KU877946
- KU877945
- KU877944
- KU744948
- KU687333

3.6 Correlation between production of alginate and mutations in *mucA* in veterinary field

Some clinical isolates are represented by a total of 36 strains of *Pseudomonas aeruginosa* coming from 11 ovine flocks in different areas of Sardinia, of which 35 isolates from milk levies of sheep infected by mastitis and one from the washing water drawn out of the milking machine.

3.7 Materials and Methods

Alginate quantification test

The alginate production has been valued using a carbazole methodology through the spectrophotometric method. An alginate solution at a concentration equal to 16.000 µg /ml has been used as mother solution; the curve points were distributed in a range of concentrations between 8.000 and 3.8 micrograms/ml.

To 100 µl of this solution has been added 200 µl of H3BO3 - H2SO4 and immediately after 10 µl of carbazole to 2%. After 10 minutes at 55º C has been read in a spectrophotometer at 530 nm. The OD value at 530 nm obtained was compared to the standard curve to extrapolate the concentration of alginate.

3.8 Results

The samples were sequenced with the Sanger method and submitted to the Alginate test (Figure18).

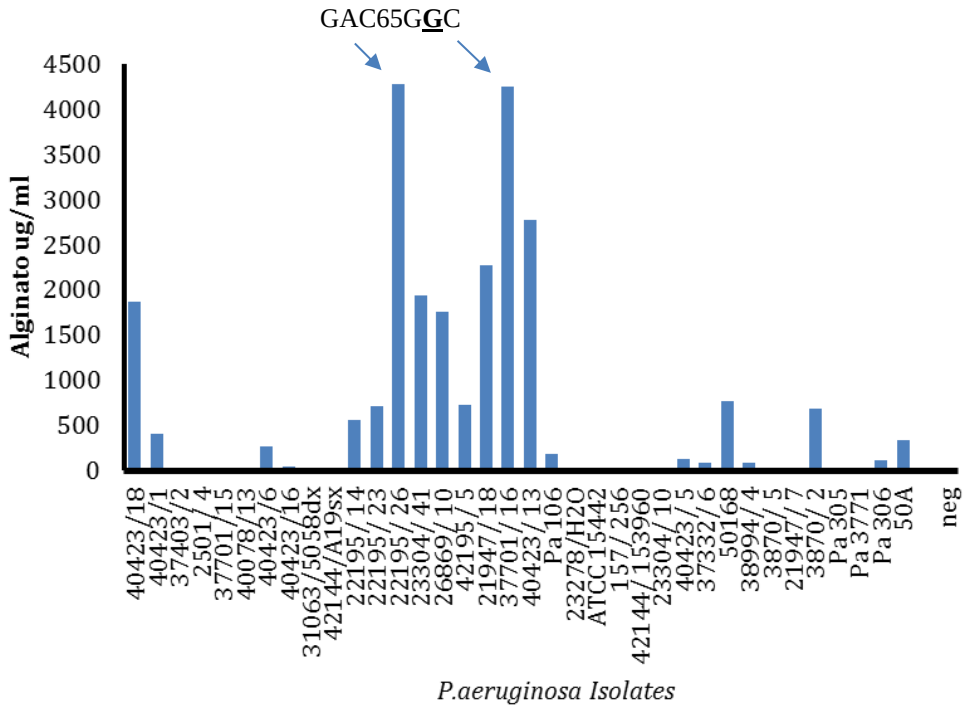

Fig. 18 - *Correlation between production of alginate and mutations in mucA.*

The results indicate the presence of mutations in the gene *mucA,* which could lead to overproduction of extracellular matrix (alginate) with consequent resistance to biocides.

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*A part of this chapter has been published:

A. Scano, G. Serafi, S. Fais, V. Piras, G. Orrù.
Ruolo del laboratorio di biologia molecolare nelle contaminazioni in ambito ospedaliero di *Pseudomonas aeruginosa*.
Biochimica clinica, 2016, vol. 40 SS S47. Abstrac 48° Congresso SIBIOC.

A. Scano, G.Orrù, G.Serafi, F.Puggioni, S. Bomboi, L. Pateri, S.Fais, M.Liciardi
Phenotypic/genotypic characters for the production of Alginate in the clinical isolates of *Pseudomonas aeruginosa* present in sheep breeding.
Abstract, 2018, XXIII Congresso NAZIONALE S.I.P.A.O.C

Chapter 4

Study of new antimicrobials against *P. aeruginosa*

Alessandra Scano, Massimiliano Arca, Antonio Zucca, Agostina Cinellu, Enrica Tuveri and Germano Orrù

4.1 In vitro study

As previously described, a session of this thesis work aimed the identification of new antibacterial substances against *P. aeruginosa* to use in anti-Pseudomonas prophylaxis, such as for example in the disinfection environmental procedure.

4.2 Materials and methods

Reference strains used in this work

P. aeruginosa mucoid strain ATCC 15442 was used, in these antimicrobial assays, as the high biocide-resistant reference strain. The bacterial *inoculum* was standardized by using a *P. aeruginosa* suspension after 8 hours of incubation at 37 °C in Muller Hinton broth. This growth time corresponded to middle exponential phase of its growth curve (ABS 550 nm = 0.3, about 2.5 $*10^8$ CFU/ml), (Figure 19). From here, a work suspension of $1*10^7$ CFU/mL has been obtained by dilution with Mueller Hinton (MH) Broth (Microbiol, Uta, Cagliari).

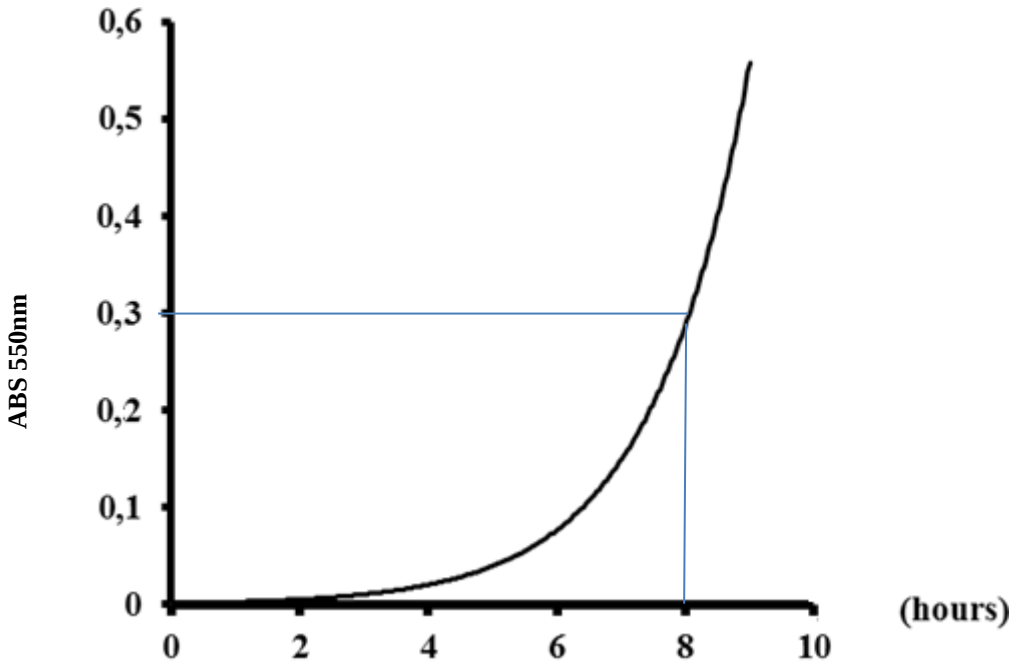


Fig. 19 *- Growth curve of P. aeruginosa ATCC 15442 strain*

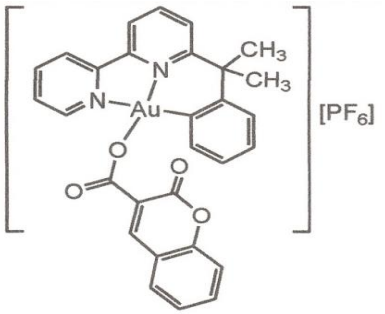
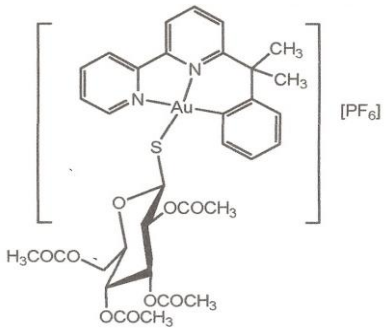
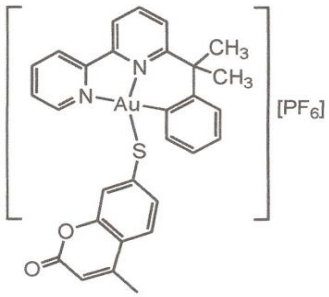
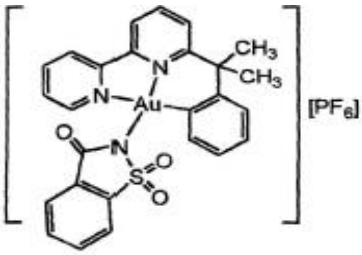
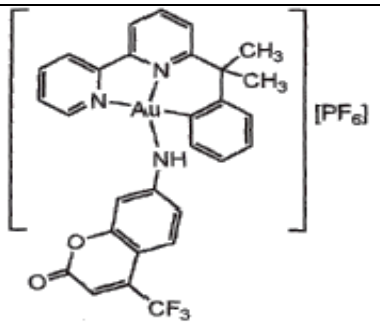
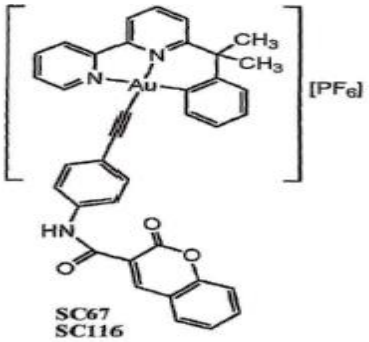
Agar diffusion test (Kirby-Bauer)

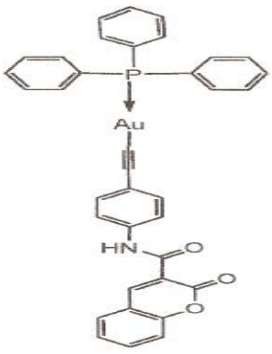
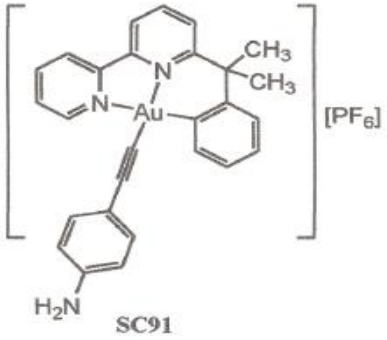
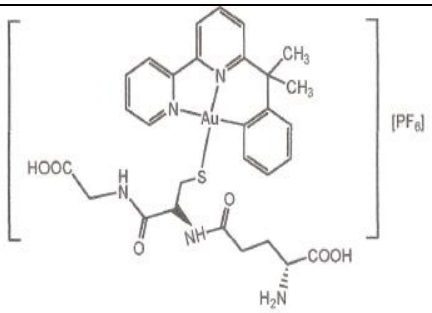
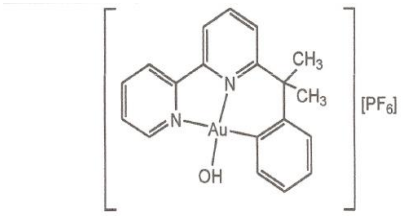
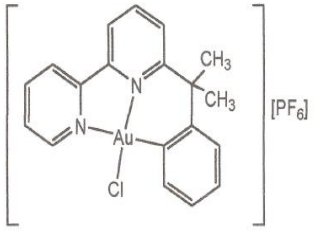
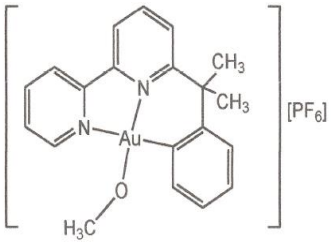
In this work we have used the modified procedure recently described from Orrù *et al.* For each strain, 15 ml of agarized Muller Hinton medium (Microbiol, Uta, Cagliari) at 55°C was added to a 90 mm Petri dish and, prior to agar solidification, four sterile iron rivets, Ø 10 mm diameter and 2 mm thick, were put into the agar mixture and then removed from the medium, leaving in the medium's thickness a well contained 50 µl in volume. The strain was inoculated onto the plate surface using a sterile swab with the bacterial $5*10^7$ (CFU) standardized inoculum. Two wells were used for the antibacterial substance testing and two for the negative control. The Petri dishes were incubated at 37°C for 24 hours. After incubation, the diameter of the possible inhibition alone was measured, and the experiment was performed in triplicate. For each compound 50 µl derived from a solution of 1.000 µg/ml in MH broth with 10 % DMSO has been used.

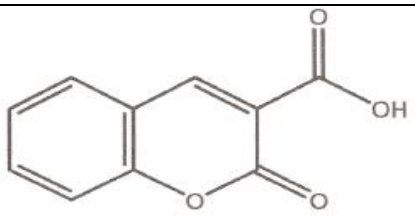
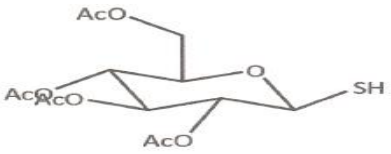
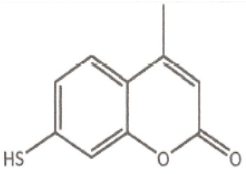
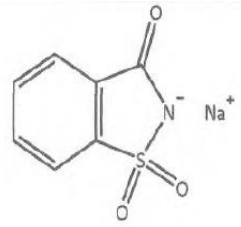
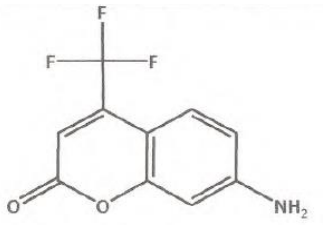
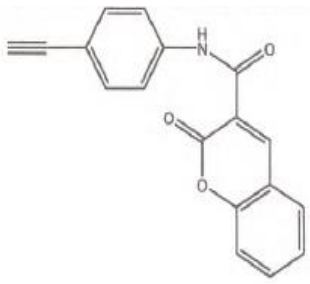
4.3 Evaluation of the antibacterial activity of new synthesis substances

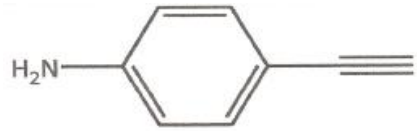
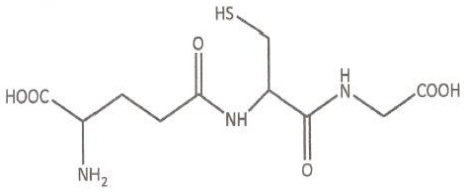
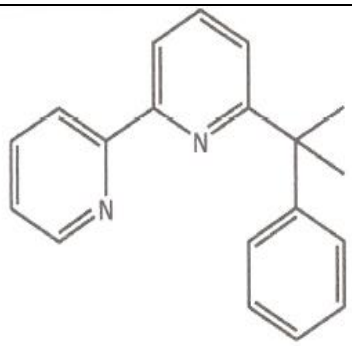
A library of coordination compounds, featuring a central gold ion, has been analyzed as potential antimicrobic agents against a set of bacteria and yields. Both gold (I) and gold (III) have been examined. Most of the complexes display nitrogen donors (variously functionalized phenanthroline, bipyridine), with a variety of ancillary ligands (such as OH-, Cl-, saccharinate, 1,3,5-triaza-7-phosphaadamantane (TPA), 4-ethylaniline, reduced glutathione (GSH), coumarin derivatives). All the compounds were provided by the research group of Prof. M. Agostina Cinellu and Prof. Antonio Zucca from the University of Sassari, and Prof. Massimiliano Arca from the University of Cagliari (Table VI). The compounds were fully characterized by micro analytical and spectroscopic (NMR, IR, UV-Vis) means. Where possible, electrochemical measurements (CV, DPV) and theoretical calculations were also carried out in the spectroelectrochemical and computational laboratories of the Department of Chemical and Geological Science at the University of Cagliari.

Tab.VI *- The table lists the chemical formula of the examined gold complexes and ligands.*

Compound	Chimical formule	Compound	Chimical formule
1	 **SC6 SC101** Chemical Formula: $C_{29}H_{22}AuF_6N_2O_4P$ Exact Mass: 804,09 Molecular Weight: 804,43	2	 **SC7 SC94** Chemical Formula: $C_{33}H_{36}AuF_6N_2O_9PS$ Exact Mass: 978,14 Molecular Weight: 978,64
3	 **SC21** Chemical Formula: $C_{29}H_{24}AuF_6N_2O_2PS$ Exact Mass: 806,09 Molecular Weight: 806,51	4	 **SC48 SC111** Chemical Formula: $C_{26}H_{21}AuF_6N_3O_3PS$ Exact Mass: 797,06 Molecular Weight: 797,46
5	 **SC35A SC115** Chemical Formula: $C_{29}H_{22}AuF_9N_3O_2P$ Exact Mass: 843,10 Molecular Weight: 843,43	6	 **SC67 SC116** Chemical Formula: $C_{37}H_{27}AuF_6N_3O_3P$ Exact Mass: 903,14 Molecular Weight: 903,56

7	 SC74 Chemical Formula: $C_{36}H_{25}AuNO_3P$ Exact Mass: 747,12 Molecular Weight: 747,53	8	 SC91 Chemical Formula: $C_{27}H_{23}AuF_6N_3P$ Exact Mass: 731,12 Molecular Weight: 731,42
9	 SC97 Chemical Formula: $C_{29}H_{33}AuF_6N_5O_6PS$ Exact Mass: 921,15 Molecular Weight: 921,60	10	 $Au(bipy^{dmb}\text{-}H)OH][PF_6]$ $C_{19}H_{18}AuF_6N_2OP$ Exact Mass: 632,07 Mol. Wt.: 632,29 C, 36.09; H, 2.87; Au, 31.15; F, 18.03; N, 4.43; O, 2.53; P, 4.90
11	 $Au(bipy^{dmb}\text{-}H)Cl][PF_6]$ $C_{19}H_{17}AuClF_6N_2P$ Exact Mass: 650,04 Mol. Wt.: 650,74 C, 35.07; H, 2.63; Au, 30.27; Cl, 5.45; F, 17.52; N, 4.30; P, 4.76	12	 $Au(bipy^{dmb}\text{-}H)OMe][PF_6]$ $C_{20}H_{20}AuF_6N_2OP$ Exact Mass: 646,09 Mol. Wt.: 646,32 C, 37.17; H, 3.12; Au, 30.48; F, 17.64; N, 4.33; O, 2.48; P, 4.79

13	 Coumarin-3-carboxylic acid $C_{10}H_6O_4$ Exact Mass: 190,03 Mol. Wt.: 190,15 C, 63.16; H, 3.18; O, 33.66	14	 GluSH 1-thio-ß-D-glucosio tetraacetato $C_{14}H_{20}O_9S$ Exact Mass: 364,08 Mol. Wt.: 364,37 C, 46.15; H, 5.53; O, 39.52; S, 8.80
15	 7-mercapto-4-methyl-coumarin 7-mercapto-4-methyl-2*H*-chromen-2-one Chemical Formula: $C_{10}H_8O_2S$ Exact Mass: 192,02 Molecular Weight: 192,23 Elemental Analysis: C, 62.48; H, 4.19; O, 16.65; S, 16.68	16	 Saccarinato di Sodio $C_7H_4NNaO_3S$ Exact Mass: 204,98 Mol. Wt.: 205,17 C, 40.98; H, 1.97; N, 6.83; Na, 11.21; O, 23.39; S, 15.63
17	 7-amino-4-(trifluoromethyl)-2*H*-chromen-2-one 7-Amino-4-(trifluoromethyl)coumarin $C_{10}H_6F_3NO_2$ Exact Mass: 229,04 Mol. Wt.: 229,16 C, 52.41; H, 2.64; F, 24.87; N, 6.11; O, 13.96	18	 3-((4-ethynylphenylamino)(hydroxy)methyl)-2*H*-chromen-2-one $C_{18}H_{11}NO_3$ Exact Mass: 289,07 Mol. Wt.: 289,28 C, 74.73; H, 3.83; N, 4.84; O, 16.59

19	 4-etinilanilina C_8H_7N Exact Mass: 117,06 Mol. Wt.: 117,15 C, 82.02; H, 6.02; N, 11.96	20	 GSH : Glutatione ridotto $C_{10}H_{17}N_3O_6S$ Exact Mass: 307,08 Mol. Wt.: 307,32 C, 39.08; H, 5.58; N, 13.67; O, 31.24; S, 10.43
21	 $bipy^{dmb}$ 6-(1,1-dimetilbenzil)-2-2'dipiribile $C_{19}H_{18}N_2$ Exact Mass: 274,15 Mol. Wt.: 274,36 C, 83.18; H, 6.61; N, 10.21		

4.4 Results

Figure 20 summarizes the biological activity of the complexes in terms of alone inhibition in *Pseudomonas aeruginosa*.

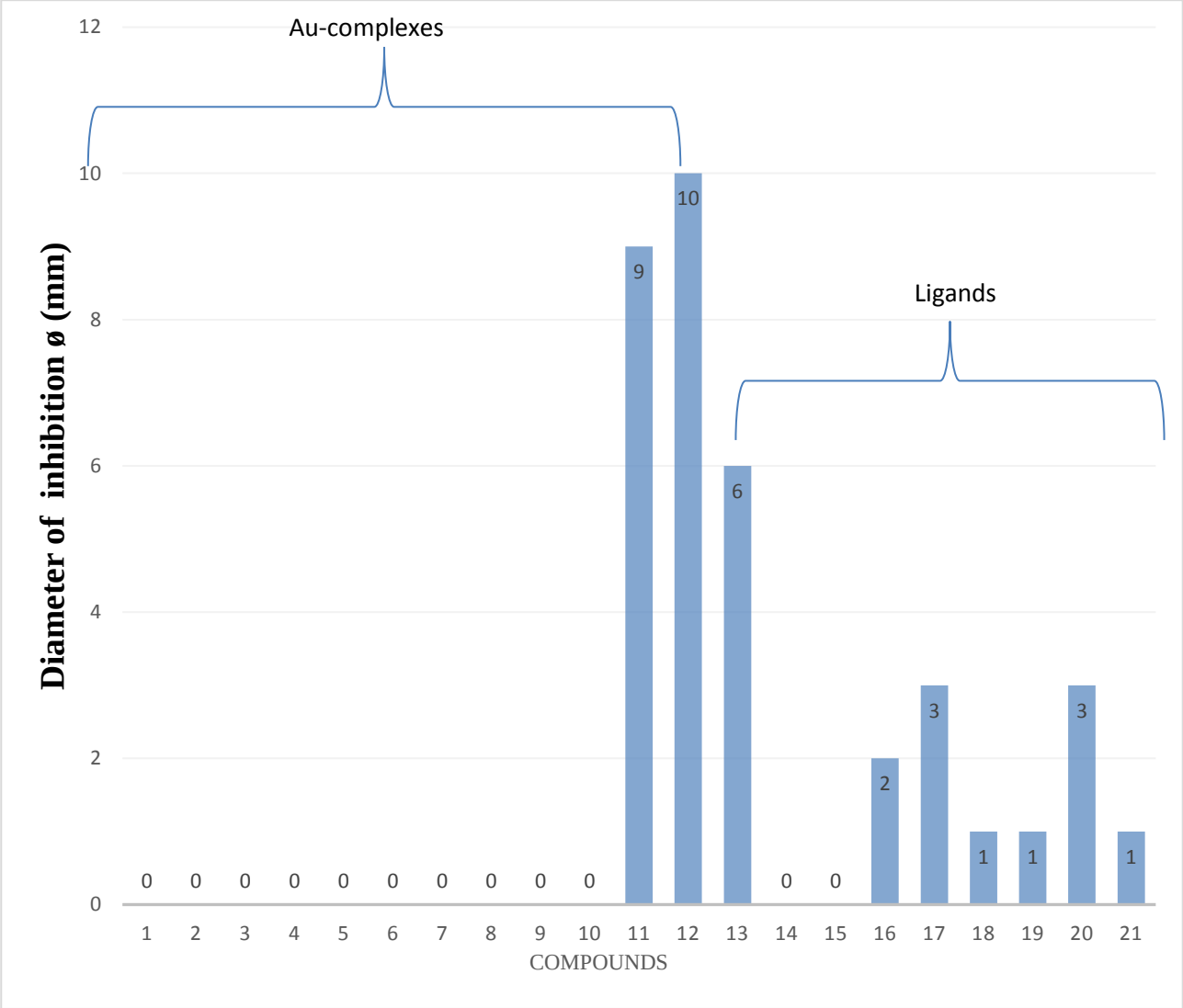


Fig. 20 *- Gold -based substances and respective ligands used on P. aeruginosa ATCC 1544. Compounds 11 and 12 showed the better activity on Kirby Bauer antibacterial test.*

In order to establish sound structure-activity relationships, the microbiological essays have been carried out not only on the gold complexes, but also on the isolated ligands. Generally, the complexes were found to be more active than the relevant ligands (compound 21) (Figure 21).

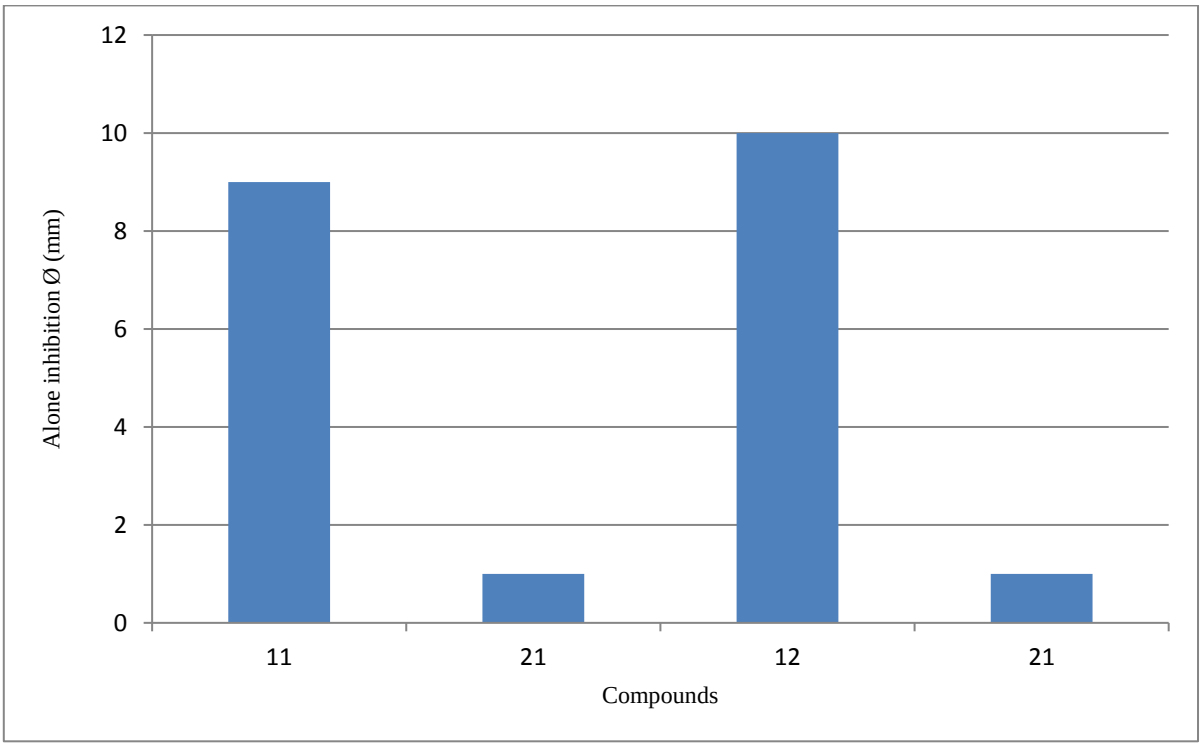


Fig. 21 *- Compounds 11 and 12 showed the better activity respect at ligands.*

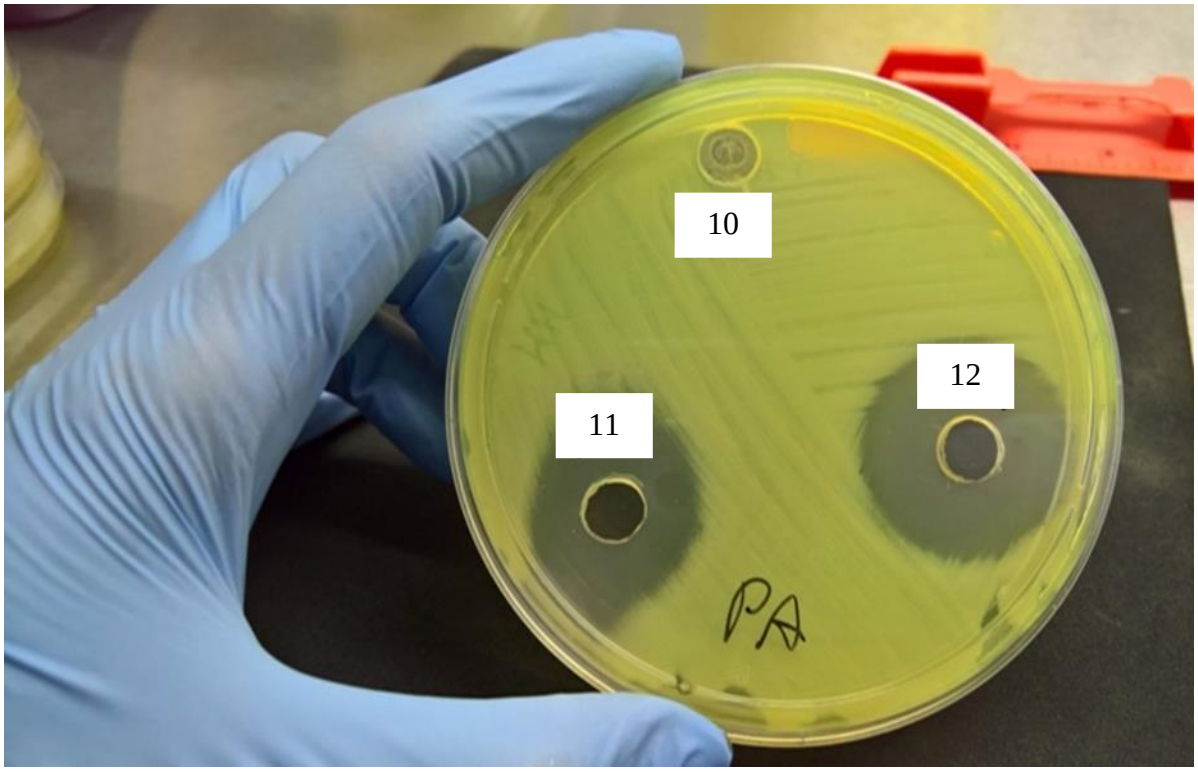


Fig. 22 *- Kirby Bauer results by using Au complexes 11 and 12.*

The search for new synthetic antimicrobial agents is a topic of growing interest, mainly because of the continuous and serious problem of antimicrobial resistance.

The compounds containing transition metal ions (*i.e.* Au or Pt) represent a promising class of drugs [107]. The aim of this study was to characterize novel Au complexes as antimicrobial agents.

A comparison of their antimicrobial properties revealed important structure-activity relationships and showed that small modifications on the main ligand can dramatically affect the pattern of activity. Au ^{III} complexes are widely studied in the medical field as potential anticancer drugs. There are few literature data for these compounds that report a significant antimicrobial activity, especially against *Pseudomonas spp*. and these are mainly cyclo-metal complexes, able to strongly stabilize the metal.

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*A part of this chapter results or methologies has been published in:

Pilloni A, Carere M, Orrù G, **Scano A**, Trezza C, Rojas M A., Zeza B (2017).
Adjunctive use of an ethyl lauroyl arginate-(lae-)-containing mouthwash in the nonsurgical therapy of periodontitis: a randomized clinical trial.
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Chapter 5

Development of a molecular method to detect *Pseudomonas spp.* in the biological samples

Alessandra Scano and Germano Orrù

5.1 Dual-Fret probes for a fast *P. aeruginosa* detection

Normally *P. aeruginosa* is ubiquitous in soil and water, and on surfaces in contact with soil or water. For this reason, a diagnostic tool must calculate the titre of this pathogen in the complete microbiome in the biological samples, for example foods, in order to avoid false negative results due to casual environmental contamination with very low concentrations of this bacterium. Another problem is that we can observe a contamination of different *Pseudomonas* species such as *P. fluorescens and P. putida* that can determine critical microbiological status in this product, due to their pathogenicity.

In this context, the laboratory analysis for infection or contamination due to *Pseudomonas spp*. it is not easy. In nosocomial area it must be based on an accurate assessment of the hygienic-sanitary state of the hospital ward, as well as in food is necessary to assess the hygienic status of the farms. In human's infections the refractory nature of the Pseudomonadaceae to the antimicrobials, often make the only inadequate clinical diagnosis criteria, as the latter tend to highlight the effects rather than the cause and the current state of the disease. This often involves a delay or a reduced treatment which cannot therefore be considered correct. Therefore, the need for new diagnostic procedures is first considered to consider *Pseudomonas* infections as a "biofilm disease" with long pre-clinical conditions. In this scenario, the evaluation of the critical mass of the pathogen, to be evaluated with the method described here, could represent a winning system for prevention.

In this work, we describe a fast-molecular approach for detect the initial pathogen biofilm samples contaminated with *Pseudomonas spp.* (*P. aeruginosa, P. fluorescens and P. putida*). This strategy is based on early detection of the subsequent rate: *Pseudomonas vs* total bacteria in biofilm specimens. This procedure is based on particular bi-functional FRET oligonucleotide probes named DUAL-FRET.

5.2 Materials and methods

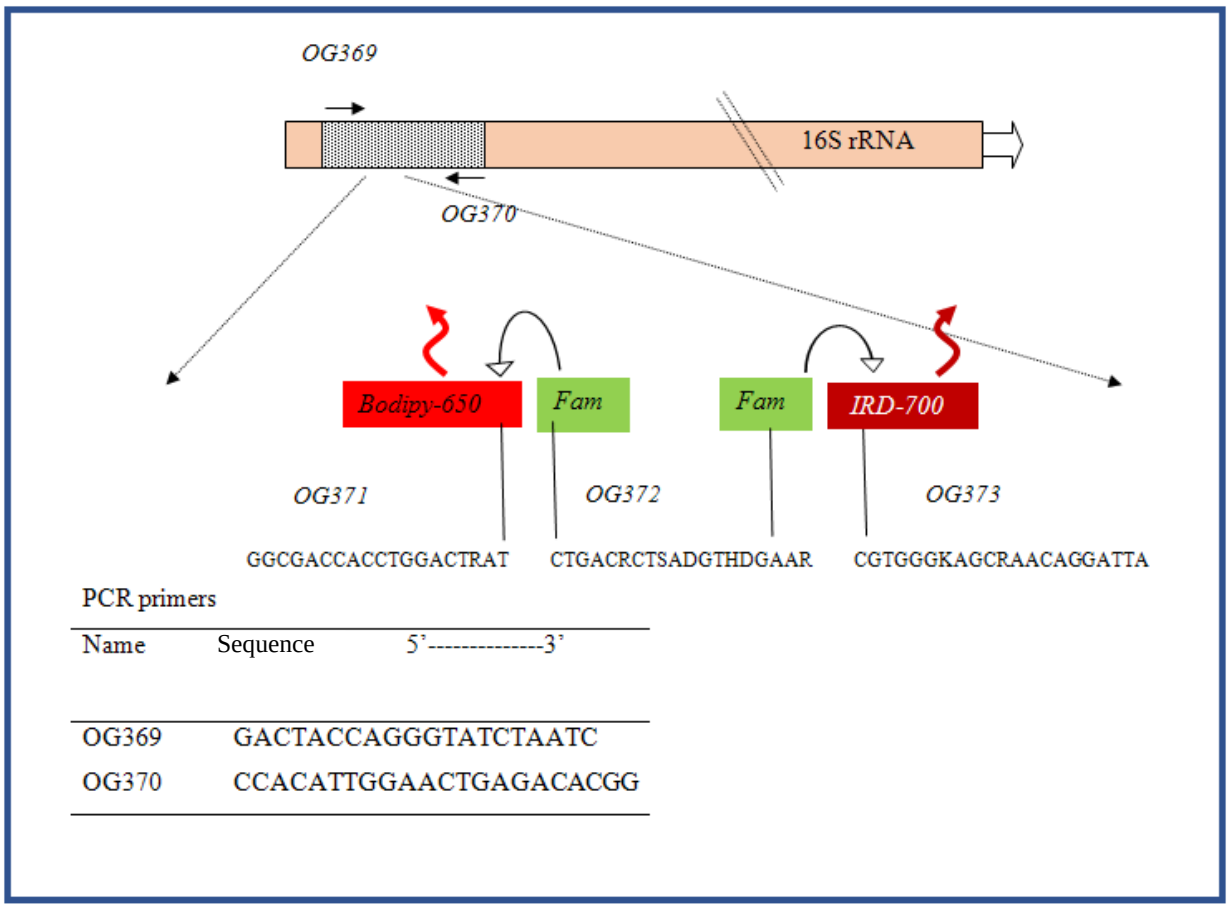
Reference strains

The sensitivity and specify procedures have been evaluated by different reference strains: *P. aeruginosa* mucoid ATCC 15442, *P. aeruginosa* ATCC 27583 not mucoid, clinical isolate of *P. fluorescens* (LM2) and clinical isolate of *P. putida* (LM3).

The specificity tests are concerned, they were made with microorganisms belonging to the genera *Bacillus, Escherichia, Staphylococcus* and *Streptococcus* (Table VII). For each strain a suspension was performed in 1 ml of H_2O RNase DNase free, equivalent to 3 McFarland (10^8 cfu / ml). From this "mother suspension" serial dilutions were performed from 10^8 to 10^3 CFU / ml, corresponding from 10^5 to 10^1 CFU / PCR. Subsequently these control suspensions were used for DNA extraction by using the CTAB method, already described in this thesis work; this procedure was executed in triplicate.

Molecular Probes designed to detect Pseudomonas spp.

Figure 23 represents the position and the structure of the bi-functional probe (Dual-Fret).
The central oligo bi-labeled at 5' and at 3' with fluorescein (OG372) and the one positioned on the right marked at 3' with IRD 700® (OG373) are placed in a nucleotide residue of 30 bp inside of a highly conserved 16S rRNA gene domain. In contrast, the oligo labeled at 5' with Bodipy 630 / 650® (OG371) is positioned in a highly variable domain of the gene (region V8) and it contains a complementary sequence to *P. aeruginosa, P. fluorescens* and *P. putida.*
In these conditions, a 710 nm (infrared) emission is relative to the total bacteria, while the 650 nm (red) one indicates the presence of the *Pseudomonas spp.* described above. In these conditions it is possible to measure simultaneously the respective threshold cycles *Pseudomonas spp*. (Ctp) and those of total bacteria (Ctt) to evaluate the *Pseudomonas spp*. in the biofilm. The primers for the PCR reaction and the bi-functional probe were developed using the Oligo program version 4.0 (Medprobe, Oslo, Norway). The universal primers for the 16S rRNA gene were designed using the 16S rRNA sequence (GenBank accession NC_X73965), in a fragment common to all Schizomycetes.



PCR primers

Name	Sequence	5'----------3'
OG369	GACTACCAGGGTATCTAATC	
OG370	CCACATTGGAACTGAGACACGG	

Fig. 23 *- Schematic representation of the DUAL-FRET system used in this work.*

Real Time PCR Conditions

The quantification of the bacteria present in the samples was made using LightCycler (Roche Diagnostics, Indianapolis, Ind). The reaction mix was composed as follows: 2 µl of DNA Master Hp (Roche Molecular Biochemicals, Mannheim, Germany), 3 µl of $MgCl_2$ (final concentration 5 mM), 10 pmol of each primer, and 0.25 pmol of each oligo constituting the DUAL-Fret probe. Finally, 2 µl of DNA to obtain a final volume of 20 µl. The thermal cycles were set as follow: (i) initial denaturation for 30 seconds at 95 ° C, (ii) 35 cycles consisting of: denaturation at 95 ° C for 0 seconds, alignment 53 ° C for 10 seconds, extension at 72 ° C for 12 seconds. The fluorescence reading was performed in the 53 ° C phase in the F2 channel (650 nm) for the genus *Pseudomonas* and F3 (710 nm) for the total bacteria. Once the PCR was completed, a melting cycle was carried out with a progressive heating from 45 ° C to 95 ° C at a constant rate of 0.1 ° C / s. To avoid interference, between the visible and infrared signals, the "compensation color" procedure was activated by following the instructions with the relative kit (Roche Molecular Biochemicals, Mannheim, Germany).

5.3 Results

With samples containing different bacterial species the fluorescence signal was positive both in the F2 and F3 channels, while reading in F2 (650 nm) the only positive signal is the one emitted by *Pseudomonas spp*. (Table VII, Figure 24). The results showed a maximal specificity able to easily discriminate Pseudomonas from total bacteria that can be detected in the sample. By implementing serial dilutions in *P. aeruginosa* and *E. coli*, quantification standards have been set up, both lines show an acceptable correlation coefficient R^2 (from 0.95 to 0.98).

Tab. VII - *Fluorescence signal obtained with different bacteria species.*

Bacteria strain	**Real Time PCR results**	
	650 nm	710 nm
P. fluorescens	+	+
P. putida	+	+
P. aeruginosa	+	+
A. actinomycetemcomitans	-	+
E. coli	-	+
S. mitis	-	+
S. *oralis*	-	+
S. gordonii	-	+
S. mutans	-	+
B. cereus	-	+
B. subtilis	-	+
S. aureus	-	+
M. micros	-	+

The sensitivity limit was around 50 CFU / PCR for *Pseudomonas spp*. and 150 CFU / PCR for *E. coli* (on 2 µl DNA extract). Similar results were obtained using as representative of the total *P. aeruginosa*, *S. aureus* and *B. subtilis* bacteria, where the sensitivity range oscillated between 300 and 50 CFU / PCR.

During *Pseudomonas* laboratory contamination level assessment, for example in foods or in environmental samples, biological and technical problems are encountered. In fact, the presence of Pseudomonadaceae in a sample can be considered as a necessary, but not sufficient, condition for the onset and progression of the infection/contamination.

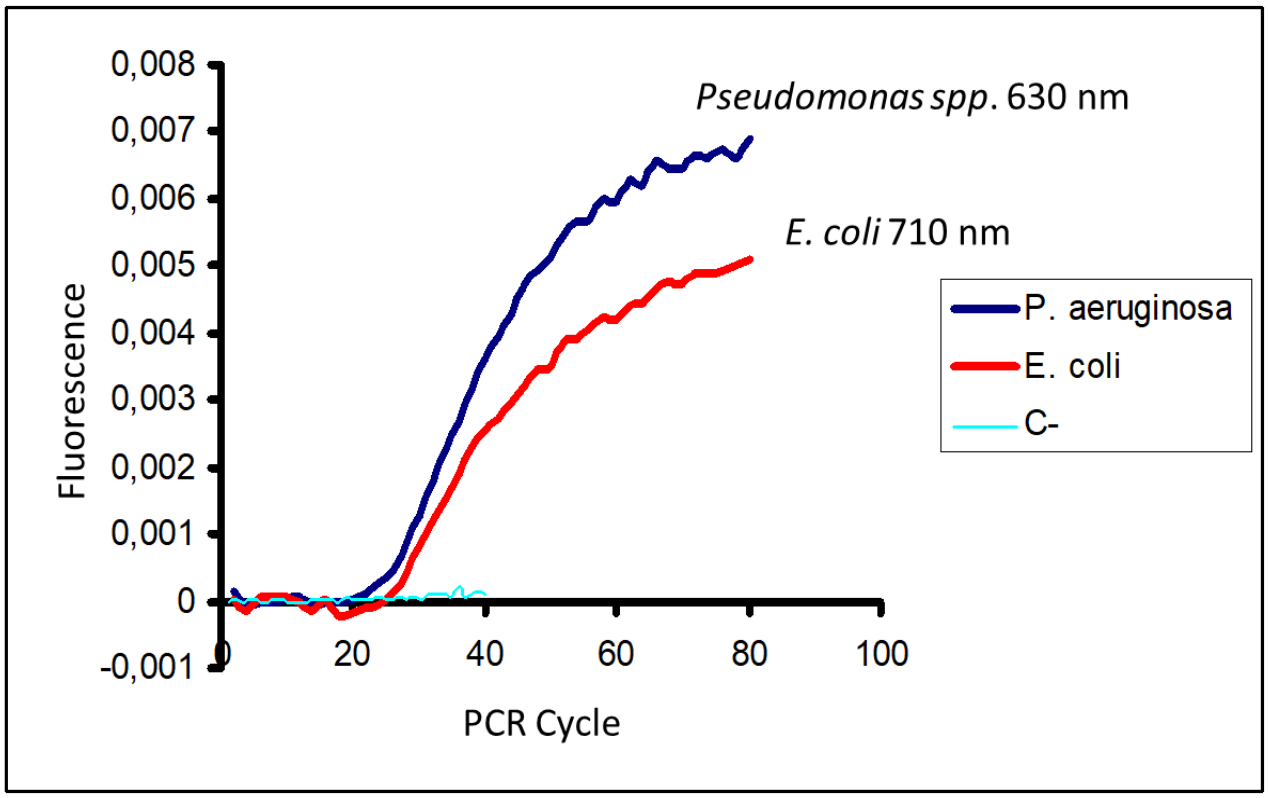


Fig. 24 *- PCR real time fluorescence curve (using F2 and F3 channels) obtained with different Pseudomonas spp. and E. coli. Data suggest a good specificity level, in fact the system was able to discriminate these different bacteria genera.*

In fact, the ubiquity of these microorganisms therefore imposes a precise evaluation of the mass of the bacterium in the sample, *i.e.* the verification if the pathogen has reached excessive titers, close to the infective dose. The difficulty of a technical nature lies in the fact that breeding farms are not always close to the laboratory of analysis, hence the need, particularly in Mediterranean regions with a temperate climate, to control the sample temperature during transport, this to avoid artifacts due to unwanted bacterial growth. The methodology shown in this article can represent in the laboratory diagnosis a helpful tool for clinician as well as to the laboratory technician, presenting some peculiarities with respect to the current microbiological diagnostics: fast procedure (average 30 minutes for 30 samples), consequently rapidity in prophylaxis responses and a smoother job, compared to the culture examination, when a large number of samples must be analyzed. The data shown in this thesis are to be considered preliminary and purely "technical", as the evaluation of the critical mass of *Pseudomonas* and therefore the degree of relative risk for example in nosocomial samples. It is required the longitudinal study of a large number of pathological and normal situations in order to evaluate the threshold values useful to the Clinicians and Biologists.

Chapter 6

Ready-to-eat vegetables as new sources of contamination-infection of *Pseudomonas spp.*

Alessandra Scano, Antonio Barberis, Guy D'hallewin and Germano Orrù

6.1 RTE as optimal basis for *P. aeruginosa* growth

Ready-to-eat vegetables (RTE) are fresh products with limited days of shelf life that must be stored in refrigeration conditions. These products belong in our days to the convenience foods category, in fact they offer commodity of use. For this food products, data of 2016, show an increase in the value of sales, the volume sold, and the individual packs sold, compared to the same period of 2015. Nevertheless, as the raw material is characterized by a high enzymatic content and water activity, they are a good substrate for microorganisms, in particular those derived from water or human contamination [108]. In this context *Pseudomonas spp.* represent one the main isolated bacteria in this product, followed by *Erwinia spp., E. coli, Salmonella enterica* etc. [109-110]. In Italy RTE showed these commercial data for these consumable forms: to be cooked (-1.5%) and crudité (-6.7%) is down. On the other hand, purchases of RTE salads (+ 3.5%) and flavorings (+ 6.1%) have increased, while there is a boom in snacks and appetizers (+ 86.9%) which up until now had represented a share minimum in the field of RTE. Therefore, the stabilities of these products, *e.g.* salads, depend on the handling procedures, the packaging modality, the storage conditions, all the respect of cold chain during distribution, but first for all the qualities of raw materials. The aim of this study was to determine the kinetic of contamination/growth of different strains of *P. aeruginosa*, non-mucoid and mucoid, with a floating bioreactor. This approach would represent an innovative aspect to study the problem of contamination of RTE products with *P. aeruginosa*. As described before, despite the nutritional and practical characteristics, several international reports highlight many cases of human infections associated with the consumption of RTE fruits and vegetables [111-115]. Recent epidemiological data show an increase of infections and this can be attributed to several concomitant factors: the increase in demand, difficulty in production chain controlling in a short time. The time factor in this process is crucial, as the times of laboratory analysis can greatly exceed collection and delivery times, making the analytical result ineffective in preventing risks for the consumer. In RTE production chain the "hot points" for food contamination are: feces, uncontrolled water coming from cisterns or wells, human manipulation during the pre- and post-harvest phases, and partial transformation procedures such as peeling or cutting. On the one hand they make the product practical for the consumer, on the other hand they deprive it of the natural protective barriers from pathogenic microorganisms and make it more susceptible to contamination.

Recent reports from the Atlanta Centers for Disease Control and Prevention (CDC) as well as different literature report highlight the growing problem of human infections due to the ingestion of minimally processed products contaminated by bacteria, among which *Pseudomonas spp*. [116-

117]. In this chapter we have simulated a contamination between a highly mucoid strain ATCC 15442 (*mucA* gene GAC65G**G**C mutated) and a non-mucoid ATCC 2783 (*mucA* gene wild type) one with a bioreactor able to simulate the physical-storage conditions normally used for RTE. *Lactuca sativa L.* has been used as test RTE product.

6.2 Materials and method

Bioreactor structure

In this thesis work, we developed a growth system to normalize the contact area between *P. aeruginosa* cells and vegetable surfaces by means of a floating bioreactor. The system contained 2 cm^2 of *Lactuca sativa* leaf suspended in 100 ml of sterile water (Figure 25).
The system reproduced some physical characteristics used for RTE storage inside the production chain: the temperature was maintained at 4 °C and the air contained 30 % of H_2O [118].

P. aeruginosa strains used in this work

Our strains of reference are: phenotypically mucoid highly biocide-resistant strain (muc+) ATCC 15442 and phenotypically non-mucoid highly susceptible strain (muc-) ATCC 2783. These isolates were stored at -80 °C with15% glycerol stock on tryptic soy agar (TSA, Microbiol Uta, Italy) until use. Before any experiment, 50 μl of the frozen bacterial culture was inoculated onto a TSA plate and a single colony was inoculated into Mueller Hinton Broth (Microbiol, Cagliari, Italy) and incubated at 37 °C for 8 hours, until the growth middle logarithmic phase. A final concentration of 10^6 CFU/ml bacterial cell was used for all *P. aeruginosa* strains.

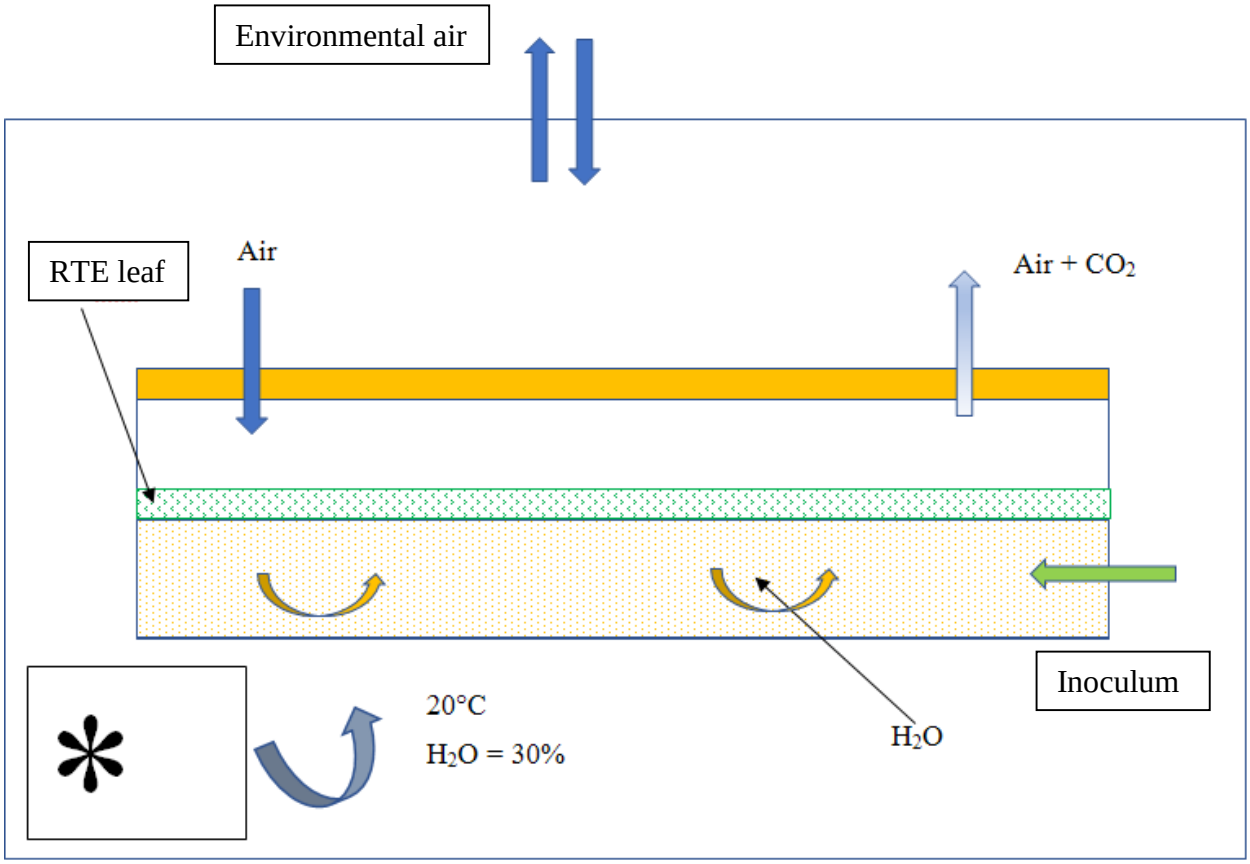


Fig. 25 *- Schematic diagram of the floating bioreactor used in this work, containing an RTE leaf floating in a liquid medium inoculated in two different experiments, with two different P. aeruginosa strains, muc- and muc+ respectively.*

DNA extraction procedure

After 48 hours of growth, 1 cm^2 of leaf has been cut with a precise aluminum ring. This sample has been used for DNA extraction by CTAB procedure. The leaf has been suspended in 400 µl of ultrapure DNAse free water (GIBCO) and grinded by strong vortexing with sterile glass balls. Subsequently 70 µl of 10% sodium dodecyl sulphate (SDS) and 5 µl of proteinase K at 10 mg/ml concentration (SIGMA-Aldrich, ST. Louis, Missouri, USA) have been added; after vigorous vortexing, this mixture was incubated for 10 minutes at 65°C. Next, 100 µl of NaCl [5 M] and 100 µl of CTAB/NaCl (0.274 M CTAB, Hexadecyl trimetylammonium bromide and 0.877 M NaCl, Sigma-Aldrich) were added to the tube, which was vortexed briefly and incubated at 65°C for 10 minutes. 750 µl of SEVAG (Chloroform: Isoamyl Alcohol 24:1, Sigma-Aldrich) were added and the mixture was vortexed for 10 sec. After centrifuging for 5 min (at 12000 rpm) 0.6 volumes of isopropanol (Sigma-Aldrich) were added to the supernatant. After 30 min at -20°C and after being centrifuged for 30 min at 12.000 rpm, the pellet was dried at room temperature for 20 min and

suspended in 20 µl of molecular biology-grade distilled water (Gibco, Invitrogen Paisley, Scotland, UK). 2 µl of this were used as DNA suspension for real time PCR reaction.

Real time PCR conditions

The total mass of these muc- and muc+ *Pseudomonas* strains on RTE surfaces was evaluated through the method described by Denotti *et al.* by using a Real time PCR [119]. Briefly, the Real time PCR reaction was performed by using the LightCycler instrument and LightCycler DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). PCR reaction has been considered on a region of the 16S rRNA gene. The primers for the PCR (OG347 and OG348) were designed to a flanking sequence of 177 bp (GenBank accession NC_AF104671). All experiments were performed in triplicate and the mean of *Pseudomonas* titers was used for the subsequent analysis. To describe a correlation between muc+ and muc-, the Fisher's test was used, all P values <0.05 were considered significant.

6.3 Results

Figure 26 shows the final biofilm amount obtained with *P. aeruginosa* muc+ and muc- phenotypes after 48 hours of growth in the *Lactuca sativa L.* leaf, used in this work as RTE product. The difference between muc+ and muc- strains in biofilm amount was assessed approximately to 1 log ($P < 0.05$).

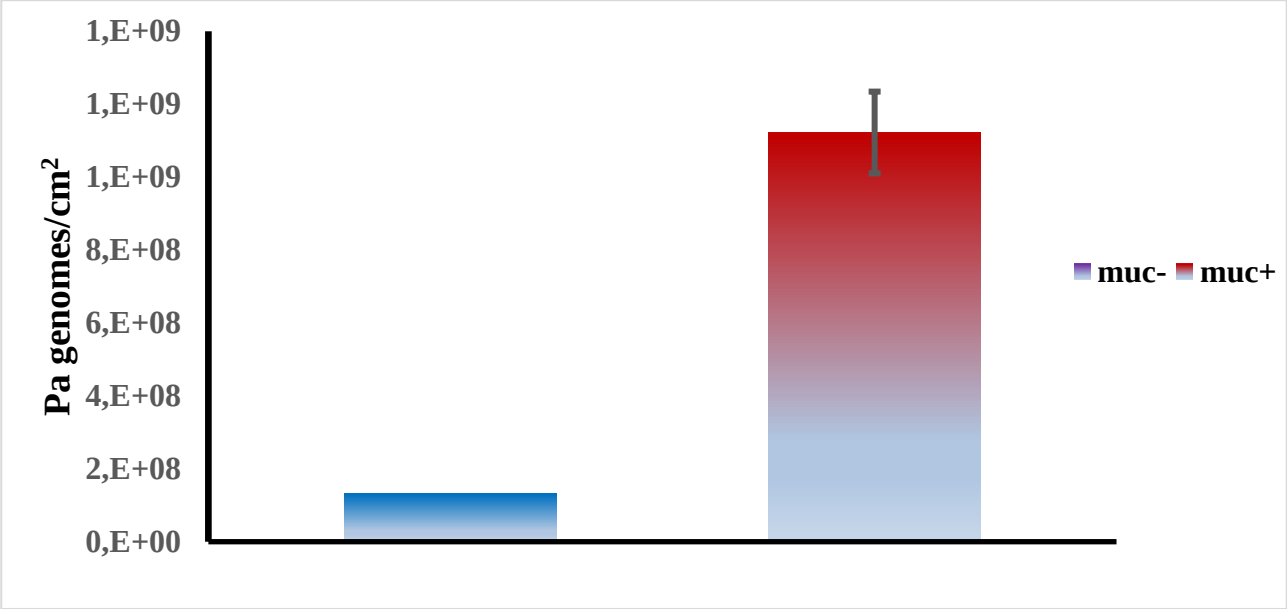


Fig. 26 *- Different biofilm amount in a cm^2 of L. sativa leaf after 48 of growth.*

The data obtained in this work show a significative difference between two reference strains used in the experiment. In particular *muc*+ ATCC 15442 strain resulted most able in biofilm increase in this vegetable. This result suggests also the risk of water contamination with muc+ *P. aeruginosa* strains and in the same point the importance of typing sequencing method to evaluate the *mucA* gene mutations responsible for alginate production in this bacteria genus, in fact this muc+ strain resulted *mucA* mutated (missense GAC65G**G**C Asp-**Gly)**. The role of alginate in *P. aeruginosa'* s biofilm formation has been explained from Lim J. *et al.*, by using atomic force microscopy (AFM) and confocal laser scanning microscopy [120]. The authors suggest that the alginate overproduction greatly affects the physical properties (topography and stickiness) of *P. aeruginosa* biofilms as well as the physiological properties (cell death and growth) of the bacterial cells inside the biofilms. The preliminary work explained in this thesis's chapter, allows different considerations for biologist or bio-technologist that could be useful for future experimental projects based on environmental *Pseudomonas* food contamination problem. In particular, these points could be synthetized as follows:

- ✓ the mutated *mucA* strains, characterized for alginate hyperproduction, can be contaminating for vegetables used in RTE products and its could be most effectiveness in the biofilm formation in comparison with *mucA* wild type strain. The mutated *mucA* strains can show main pathogenicity profile for humans and animals and consequently the risk of these contaminations could be evaluated in future RTE production chain;

- ✓ this work is strictly preliminary and further investigations are necessaries, for example, to study the role of different vegetal species to contain the bacteria biofilm formation through its natural components, such as polyphenols, terpenes etc.

Chapter 7

Pseudomonas aeruginosa in human infections, future perspectives for diagnosis and therapy

Alessandra Scano and Germano Orrù

7.1 The role of cell RNAs as possible target for diagnosis and therapy against Pseudomonas infections

In humans *P. aeruginosa* represent the most frequent and important pathogen responsible for chronic infections especially in patients with cystic fibrosis or in organ transplantation [121-122]. As already reported in this thesis work, there are many genotypically different strains of *P. aeruginosa* and these may behave differently during patient infection. A critical point during the infection history is that we can isolate in the same biological samples a set of different genotypes often multi-drug resistant and this represents a worldwide health care problem because of the difficulty in treating these infections [122-123]. The presence of multi-drug resistant strains is not the unique problem during the clinical government with illnesses due to *P. aeruginosa*. The initial problem is to evaluate a good prognostic factor that can be used to estimate the change of patient recovery or the change of infection recurring. During this work we have evaluated the news described in literature that can be used for future considerations for *Pseudomonas* infection and treatment.

Within the biomolecules currently used for laboratory diagnosis in human disease such as infection and cancer, many of these are shown not be sufficient to predict the prognosis in the critical stages of the diseases. This in spite of the significant achievement made in early diagnosis and therapeutic strategies, *e.g.* with extensive SNPs analysis for oncogenes in cancer, or during a bacterial infection the presence of different genotypes in the same patient. In this last decade for example the micro-RNA functions (miRNAs) are changing our understanding of cells and diseases with a particular emphasis on the eukaryotic cell cancer biology. For this reason, several miRNAs are actually and intensively studied and proposed as promising candidates in therapy and/or as biomarker to reveal the disease progression and prognosis. The use of miRNAs in diagnostic tools could represent several advantages, first among all owing to non-invasive nature of miRNA-based assays and their sensitivity, selectivity and specificity in detecting tissue cell changes. Furthermore, the possibility to use easy and less expensive tools to detect their expression level, such as confocal microscopy or Real time quantitative PCR, is another positive point for suggest these biomolecules as interesting molecular targets. In this contest the article based on cellular RNAs presents interesting peculiarities if speculate a use for *Pseudomonas* infections in humans. The most articles that describe miRNAs are focalized on colon cancer (CRC) but ignoring some data on long non-coding RNAs that could be transferred in *Pseudomonas* biology and consequently these could be a basis for further studies.

7.2 Long non-coding RNAs in *Pseudomonas aeruginosa* infection

The biological function of long non-coding RNAs (lncRNA) has often been described in the literature in these last years and represents a very interesting field of research in translational medicine [124-125]. In fact, non-coding RNA oligos (>200 bp) are now thought to have regulatory roles in different fundamental biological pathways and provide cells with an additional layer of response to different environmental stimuli. The regulation of many non-coding RNAs is thought to occur in a variety of human diseases, including cancer progression, bacterial infections and microbial drug resistance. Here we discuss recent research on the molecular functions of long non-coding RNAs in cellular pathways mediating colon cancer, which was recently described by Gu *et al.* [126], and bacterial infections caused by *P. aeruginosa* [127]. Although the clinical features, etiological aspect and general biological pathways are different for these disease, we hypothesize that they share a common mechanism in terms of pathological features, namely the interaction between the lncRNA and the E-cadherin pathway during cancer development as well as during bacterial infections [127-130].

7.3 Biological regulation by lncRNAs, a common point in cancer and in bacterial infections

lncRNAs in eukaryotic cells belong to long intergenic RNAs with transcripts that are not translated into proteins. An interesting aspect is their high abundance within the cell's cytoplasmic area with around more than 30,000 lncRNAs per cell, which suggests that these RNAs play a crucial role in the eukaryotic biological network [131]. These RNA oligos represent a class of non-coding RNAs transcribed by RNA polymerase II and most of these transcripts are adenylated and spliced. As a result, most of them are able to regulate gene expression at the level of transcription or translation, but somewhere lncRNA expression is restricted to precise biological stages or is located in a particular tissue. Traditionally, the study of these lncRNAs falls within cell developmental studies or in the field of tumorigenesis, but very recent publications have shown that lncRNAs are also involved in the response against pathogenic bacteria. In the field of human infections, most of the roles of lncRNAs are still unknown, but the recent scientific results obtained in oncological research could be used to suggest new biological mechanisms in microbial diseases such as *P. aeruginosa* infections *i.e.* in cystic fibrosis patients.

In this context, the article by Gu *et al*. focalizes on the role of lncRNAs (URHC) in the proliferation and invasion of colorectal cancer cells *in vitro*. The authors suggested that the down-regulation of this molecule in colorectal cancer cells could enhance the expression of a cell junction protein E-cadherin with a subsequent decrease in tumor proliferation and invasion rate. Other authors have reported that an alteration in phosphorylation, as well as the transcription status of E-cadherin, or other junction proteins, are involved in the changes in cell junction associations and in the enhanced paracellular permeability to *P. aeruginosa* infection of the aerial tissues. Our research focused on this crucial role of the intercellular bridge mediated by lncRNA E-cadherin and any subsequent clinical and diagnostic tools in *P. aeruginosa* infections.

P. aeruginosa supports different emerging human/animal infective illnesses and is considered a "superbug". In fact, it is a leading cause of dramatic nosocomial infection, often associated with high drug resistance, especially in surgical, geriatric and oncological hospital divisions. In addition, these bacteria cause high morbidity in individuals afflicted with cystic fibrosis. This ubiquitous Gram-negative bacillus has a non-clonal epidemic population structure, but several genotypes (ST111, ST175, ST235, ST244 and ST395) are distributed worldwide and frequently associated with severe outbreaks [132]. These clinical isolates identified as "invasive types" often invade epithelial cells, a process that includes the deactivation of E-cadherin – catenin bridges by phosphorylation with consequent cell-cell junctional gate failure. Figure 28 represents a probable schematic LPS mediated biological pathway described by other authors for Gram negative bacteria.

E-cadherins represent a type of cell-cell adhesion molecules belonging to the cadherin group and are involved in the formation of adherent junctions to bind cells with each other. This class -1 of transmembrane proteins is strictly associated in the cytoplasmic domain with catenin proteins. This E-cadherin–catenin complex plays a key role in cellular adhesion and the loss of this function has been associated with greater tumor metastasis, as well as bacterial tissue invasion [132].

Tissue cell junctional gate failure has been shown to be essential in tumorigenesis, especially for tumor and metastasis progression, and a comprehensive study of this mechanism could be very useful to improve new therapies in cancer research. For example, according to the latest publications, colorectal cancer is the second and third main cause of cancer deaths in women and men. The disease is also characterized by a low survival rate after 5-years and this is why new therapeutic strategies are much needed in this field.

7.4 Usefulness of a translational study for lncRNA and E-cadherin

Disruption of the intercellular junctions is a strategy that several microorganisms and neoplastic cells use to their advantage and intervention in these mechanisms by new drugs or new genetic engineering strategies could be useful in several fields of Medicine. Although only a fragmentary study presently exists on the role of lncRNA in severe bacterial infections, such as *P. aeruginosa* in cystic fibrosis, the results obtained in another field by Gu *et al*, promise new light in the research field of microbiology. We speculate a similar mechanism in lncRNA and E-cadherin expression pattern also in the lung-aerial tissues of cystic fibrosis patients, infected with *P. aeruginosa*. In this context, lncRNA could be down-regulated by the expression of the E-cadherin pathway in colon cancer, as suggested by Gu *et al*., or activate the phosphorylation mechanism in *P. aeruginosa*, as described by Vikström *et al.*, (Figure 27).

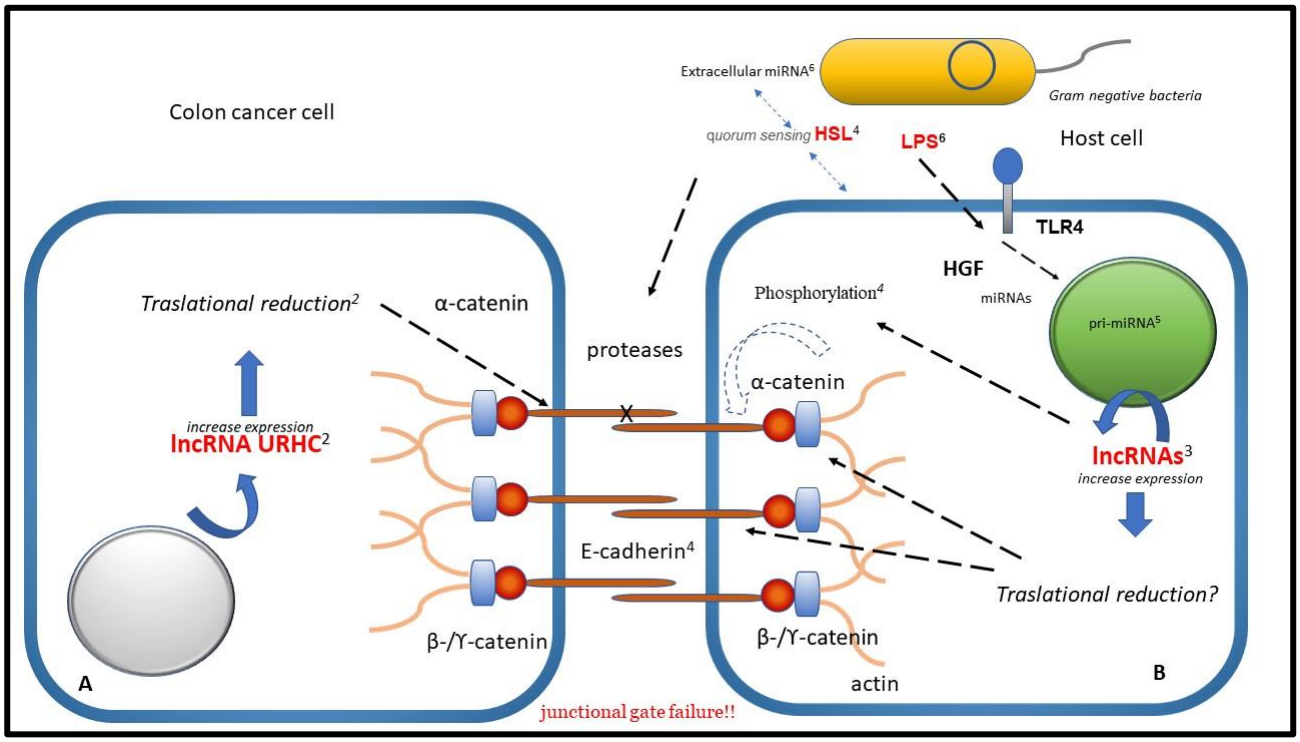


Fig. 27 *- Schematic representation of the role of lncRNA in colon cancer, as described by Gu et al., (A) and a mechanism for P. aeruginosa host infection (B).*

7.5 Conclusions

As regards its therapeutic application, in both these ways, lncRNA on E-cadherin could be an interesting candidate for the new evolving concept of host-directed therapies to treat bacterial and cancer infections. In particular this cross-talk study could prove interesting for the development of an *in vitro* model cell-bacteria to study new therapeutic tools by using, for example, antisense oligonucleotides (ASOs) against specific lncRNA targets. Another interesting field could be use of lncRNAs as predictive targets during early infection stage in fibrosis cystic patients. It could be possible to use a specific oligonucleotide fluorescent probe such as molecular beacon [133] designed for each lncRNA sequence showed a predictive value for disease *status*. These probes set (molecular beacon array) could be used efficiently in the laboratory diagnosis for *P. aeruginosa* infection.

Links/footnotes

Genecard: http://www.genecards.org/cgi-bin/carddisp.pl?gene=MIR552
miRbase: http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0003557

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*This paragraph has been published:

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