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Antimicrobial resistance, molecular epidemiology and virulence factors of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus aureus* in a third-level Pediatric hospital: new twists to old stories.

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Index of contents

Background……	Page 1
Staphylococcus aureus and antimicrobial resistance……..	p. 1
Community-acquired MRSA (CA-MRSA), hospital-acquired MRSA (HA-MRSA), Livestock-associated MRSA (LA-MRSA)……	p. 2
Molecular epidemiology of MRSA strains……	p. 4
Cystic Fibrosis and MRSA…….	p. 5
Intensive Care Units and MRSA…….	p. 7
SA, MRSA and virulence factors……	p. 8
Coagulase-negative *Staphylococci* as a possible *reservoir* of virulence factors……	p. 11
Aim of the study……	p. 12
Materials and methods……..	p. 13
Bacterial strains……	p. 13
Sample culturing and bacterial isolation……	p. 13
Biochemical identification……	p. 13
Antimicrobial susceptibility testing methods…….	p. 14
DNA extraction……..	p. 16
SCC*mec* typing…….	p. 16
spa typing……..	p. 19
MLST……..	p. 19
DNA sequencing…….	p. 20
ACME……..	p. 21
Toxins genes testing methods..……	p. 21
Results…….	p. 23
Strains collection……	p. 23
Antimicrobial susceptibility testing results…….	p. 26
Molecular characterization of MRSA isolates……...	p. 30
SCC*mec* typing results……	p. 30
spa typing results……..	p. 31
MLST analysis results……..	p.32
Virulence factors characterization……..	p.33
ACME typing results……	p.33
agr typing results……...	p.33
IEC typing results……..	p.34

PVL testing results…… p.35
Chronic MRSA infection in ICU…….. p.38
First MRSA infection in CF patients…… p. 38
Chronic MRSA infections in CF patients…… p.39
CF patients and lung transplantation…… p.40
Discussion…….. p.42

Index of Tables

Table 1……... Page 15
Table 2……... p. 16
Table 3……... p. 17
Table 4……... p. 18
Table 5……... p. 20
Table 6……..…… p. 22
Table 7……... p. 23
Table 8……... p. 24
Table 9……... p. 24
Table 10……. p. 25
Table 11……. p. 25
Table 12…… p. 26
Table 13……. p. 31
Table 14……. p. 34

Index of Figures

Figure 1…… Page 26
Figure 2…… p. 27
Figure 3…… p. 28
Figure 4…… p. 29
Figure 5…… p. 36
Figure 6…… p. 37
Figure 7…… p. 39
Figure 8…… p. 40
Figure 9…… p. 41

Background

Staphylococcus aureus and antimicrobial resistance

Staphylococcus aureus (SA) has been considered a potential pathogen since its discovery in 1880, and before the introduction of penicillin the mortality rate of patients with an infection caused by SA was about 80%. In 1942 the first penicillin-resistant strain emerged, and in 1960 almost 80% of all *S. aureus* (SA) strains resulted to be resistant to this antimicrobial agent. The introduction of methicillin dates from 1959, and two years later the first resistant strain (methicillin-resistant *Staphylococcus aureus*, MRSA) was isolated. During the next decades MRSA clones disseminated worldwide. This great ability to develop antimicrobial resistance led to the first isolation of vancomycin intermediate SA strain (VISA), as well as resistant strains (vancomycin resistant MRSA, VRSA), generated by the intensive selective pressure caused leading to intermediate phenotypes not inhibited *in vitro* at concentrations due to the more intensive vancomycin use[1]. To date, at least nine VRSA have been reported in USA[2]. No resistance to glycopeptides was detected in Italy during 2012; moreover, all MRSA isolates were susceptible to tigecycline and linezolid[3]. VISA strains isolated in bacteremia and endocarditis were described in Italy in 2012[3].

The last European Antimicrobial Resistance Surveillance (EARS) report highlighted a significant increasing trend of MRSA proportions in Italy (www.ecdc.europa.eu), whilst the countries showing a more evident and sustained decrease of MRSA proportion were Austria, Cyprus, Estonia, France, Ireland, Latvia and UK[3].

Differences in antimicrobial resistance patterns are described between community-acquired MRSA (CA-MRSA) and hospital-acquired MRSA (HA-MRSA), being the latter often multi-drug resistant (MDR). CA-MRSA isolates may present different sensitivity patterns to antimicrobial agents, being usually sensitive to clindamycin in

US, gentamicin in Australia, ciprofloxacin in England[2]. Italian data concerning cystic fibrosis (CF) patients reported similar resistance rates HA- and CA-MRSA, with a statistically significant difference in gentamycin sensitivity[4].

Community-acquired MRSA (CA-MRSA), Hospital-acquired MRSA (HA-MRSA), Livestock-associated MRSA (LA-MRSA)

In 2000, the Centers for Disease Control and Prevention (CDC) stated that a case of MRSA infection could be defined "community-acquired" if diagnosed in an out-patient, or within 48 hour of hospitalization when traditional risk factors for MRSA infection are lacking. On the other hand, an infection was considered "hospital acquired" in in-patients, or when diagnosed after the receipt of hemodialysis, surgery, residence in long-term facilities or hospitalization during the previous year, the presence of catheters or percutaneous devices, previous isolation of MRSA, as reported by David and Daum[2]. Recent studies demonstrated that defining CA-MRSA on the basis of the lack of exposure to risk factors allowed the misidentification of a large part of CA-MRSA infections; to date, CA-MRSA and HA-MRSA are distinguished by means of genetic determinants, but these definitions were starting to blur since 2003[2]. The Staphylococcal Chromosome Cassette *mec* (SCC*mec*) is the genetic determinant on the basis of which it is possible to correctly discriminate between CA-MRSA and HA-MRSA. SCC*mec* is a mobile genomic island containing genes encoding various determinants of resistance both to antimicrobial agents, and to other compounds. This genomic island contains the *mecA* gene, which encodes the penicillin-binding protein conferring resistance to methicillin as well as to β-lactams. The *mec* complex, *ccr* genes and junkyard regions represent the basic structure of the SCCmec element. The combination of *mec* and *ccr* genes and junkyard regions *plus* the above-mentioned mobile elements are able to confer different characteristics to the different SCC*mec* types.

To date SCC*mec* types from I to XI have been classified, as recently described by Hiramatsu and colleagues[5]. HA-MRSA are currently associated with SCC*mec* type I, II, III and VIII, and CA-MRSA are mainly represented by types IV, V, VI and VII. Each SCC*mec* type is characterized by different size and associated genetic determinants which confer different antimicrobial resistance profiles. Insertion sequences, transposons, plasmids are often encoded by SCC*mec* genomic islands. HA-MRSA are often multi-drug resistant germs, whilst CA-MRSA appear to be less resistant to antimicrobial agents than virulent. A correct discrimination between CA- and HA-MRSA is crucial to obtain a better understanding of epidemiology and strains diffusion since CA-MRSA started to spread in hospital settings, representing a further threat for hospitalized patients due to their virulence. Moreover, some community-onset infections are caused by HA-MRSA strains, probably related to the management at home of complex HA-MRSA infections [2].

There is an additional type of MRSA: livestock-associated (LA) MRSA were recently described. These strains were first associated with human disease in 2003, when a MRSA clone associated with *reservoir* in pigs was isolated from a human. This particular clone has a peculiar genetic background, and is characterized by the impossibility to obtain a pulsed field gel electrophoresis (PFGE) profile. To date, this clone was described in the Netherlands, Belgium, Europe, Asia and United States[6], constituting another challenge for physicians and microbiologists.

The additional step to properly characterize MRSA strains is to determine their sequence type (ST) using Multi Locus Sequence Typing (MLST) analysis because at the moment, the nomenclature of MRSA is based on the combination of ST and SCC*mec* type data[7]. MLST analysis allows to assign a clone basing on partial sequencing of seven different house-keeping genes, being each sequence assigned as a distinct allele. MLST allows also to determine clonal complexes (CCs), defined as groups of strains sharing five of the seven house-keeping genes used for MLST analysis[8].

Molecular epidemiology of MRSA strains

An increased knowledge of MRSA clones distribution is crucial to avoid the spread of virulent lineage and to monitor global epidemiology in order to set up more efficacious infection control measures and therapies. MRSA epidemiology drastically has changed during the last years, due to the spreading of CA-MRSA lineages: since the early 1990s, many CA-MRSA clones disseminated worldwide. CA-MRSA has recently started to replace HA-MRSA in hospital settings, especially in areas where CA-MRSA prevalence is high[7]. A limited number of both HA- and CA-MRSA lineages has spread since 1961, when the first MRSA emerged[7]. Major HA-MRSA clones are represented by STs 250, 45, 239, 247, 8, 5, 228, 22, 36, important lineages belonging to CC 5, 8, 22, 36, 45. Five major CA-MRSA clones are widely worldwide diffused: European clone ST80-MRSA-IV; Soutwest Pacific clone ST30-MRSA-IV, USA 400 ST1-MRSA-IV; USA 300 ST8-MRSA-IV; USA1000 ST59-MRSA-IV or VII[7]. An accurate description of MRSA global epidemiology and CCs diffusion was published by Monecke and colleagues in 2011[9]. CC1 includes several strains of CA-MRSA, diffused in Europe, Australia, Egypt and US. CC5 and CC8 represent common and widespread clonal complexes, comprising a large number of both HA- and CA-MRSA strains. CC22 is common and largely diffused, including pandemic clones such as ST22-MRSA-IV. Several HA- and CA-MRSA originated from CC30, including the pandemic Soutwest Pacific clone ST30- MRSA-IV. CC45 strains cluster into two distinct groups, being this CC one of the major MRSA lineages. A recent European study[10] defined a high percentage of community-associated clones, both MRSA and MSSA. Most MRSA were related to USA 300 genetic background (ST8-MRSA-IV), followed by European clone (ST80-MRSA-IV) and Taiwan clone (ST59-MRSA-IV). A surprising high genetic diversity among MRSA was highlighted, describing also novel associations between genetic

background and SCC*mec*[10]. Different SA lineages appear to be evolved independently due to the presence of two different systems of restriction-modifications, avoiding uptake of DNA originated from different bacterial *species* and also from other SA lineages. Therefore, strains belonging to different lineages possess a unique but highly conserved combination of genes and their regulators[11].

Nowadays Italian data concerning molecular epidemiology of MRSA represent an important tool to improve infection control measures. A few reports describing the peculiar situation of outbreaks or particular regions are currently available[12,13,14], and it was recently demonstrated that in some European countries epidemic MRSA clones are in replacement. Recent work by Campanile and colleagues showed the changing epidemiology of HA-MRSA Italian isolates during a long period of time[15]. The majority of MRSA clones belonged to six major clones: ST8-MRSA-I, ST247-MRSA-I, ST239-MRSA-III, ST228-MRSA-I, ST22-MRSA-IV, and their prevalence varied from 1990s to 2007[15]. ST228-MRSA-I increased its presence during the period between 1990 and 2007[15], while to date Southern Germany clone ST228-MRSA-I was replaced by ST22- MRSA-IV due to its ability to produce bio-film and to inhibit ST228-MRSA I growth in co-culture[16]. The genetic background of CA-MRSA and HA-MRSA, their transmissibility, antibiotic susceptibility and virulence have been poorly investigated in pediatric patients in Italy and there is little data regarding CA-MRSA or HA-MRSA in pediatric intensive care units and high risk wards.

Cystic Fibrosis and MRSA

Cystic fibrosis (CF) is the most common autosomal inherited disorder in the Caucasian population. It is a multisystem disorder resulting from a disruption in chloride transport at the cellular level leading to abnormal dehydrated secretions in the lungs. This results in impaired mucociliary clearance leading to recurrent pulmonary infections, bronchiectasis and progressively deteriorating lung function, which is the main cause of morbility and mortality for CF patients[17]. The bacterial

species most commonly associated with respiratory tract infection in CF include common human pathogens such as SA and *Haemophilus influenzae* as well as several opportunistic pathogens, the most important of which is *Pseudomonas aeruginosa,* and nosocomial pathogens such as *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans.* Other species that are only occasionally associated with human infections apart from CF, are *Burkholderia cepacia* complex, *Burkholderia gladioli*, *Ralstonia* species, *Cupriavidus* Species, *Inquilinus limosus* and *Pandoraea* species[18]. Some of these species have been associated with poor outcomes in CF, the role of some others was not clearly determined[18].

The increase in prevalence of MRSA seems less relevant in CF than in other patient populations, data from the American CF foundation patient registry showed that the prevalence of MRSA infection in CF has also increased from 6.1% in 2001 to about 26.5% in 2011 (www.cff.org). Recently published studies demonstrated that the presence of MRSA in CF patients' airways may worsen their clinical conditions and increase the frequency of antibiotic treatment they must undergo[19,20,21] moreover, the drastic worsening of clinical conditions was demonstrated to lead to a reduced life expectancy[20]. Although MRSA chronic infections were recently recognized to be associated with poor survival and reduced lung function[19,20], to date a clear guidance on how to manage these infections is still needed. A more accurate knowledge of local epidemiology, global epidemiology and antimicrobial resistance patterns would be helpful to this purpose. Few studies regarding MRSA characterization have been published concerning CF patients[4,22]. A thorough knowledge of the dissemination and molecular evolution of MRSA is required to develop effective strategies to prevent the spread of MRSA and MSSA strains in hospital settings. Data concerning molecular epidemiology and diffusion of Italian MRSA strains in CF are scanty. These patients are traditionally considered at risk for hospital-acquired infections due to their frequent hospitalizations and medical invasive treatment, but a recent study described a CA-MRSA prevalence[4] higher

than US[22]. This Italian prospective study reported the presence and diffusion across the country of nine epidemic clones, whilst US data concerned particularly molecular typing of CA-MRSA, mainly represented by ST8-MRSA-IV. A well-delineated picture of MRSA global epidemiology would be helpful to set up therapeutic strategies and infection control measures. Data concerning molecular epidemiology of MRSA strains responsible for both first and chronic infections are still poor, so that the appropriateness of therapies remains unclear. To date lung transplantation is an established treatment option for patients with advanced CF, and is associated with improved quality of life and survival benefits. Severe pulmonary infections are characteristics of CF advanced disease stages, and the management of such infections is crucial for lung transplantation because the role that MRSA could play in these circumstances is still poorly investigated.

Intensive care units (ICUs) and MRSA

Recent studies demonstrated a continuing increase in MRSA hospital infections, probably reflecting the growing impact of medical interventions, devices, older age, and co-morbidities of patients[23]. The incidence of nosocomial infections in pediatric intensive care units (PICUs) due to use of mechanical ventilation, indwelling catheters and invasive monitoring is often higher than other wards. Rates of pneumonia are considerably higher among patients hospitalized in ICUs compared with those in hospital wards. Moreover, the risk of pneumonia is increased 3- to 10-fold for the intubated patients receiving mechanical ventilation, as described since 2002[24]. Ventilator-associated pneumonia (VAP) is defined as a pneumonia occurring more than 48 hours after endotracheal intubation and initiation of mechanical ventilation. VAP usually shows mortality percentages from 24 to 50%, reaching higher values in specific settings[24], and representing higher values comparing to urinary tract and skin infections. Appropriate antimicrobial treatment as well as rapid identification of infected patients represents important clinical goals. To this purpose,

clinical significance of MRSA colonization on hospital admission is still under debate. A recent US retrospective cohort study tracking patients for up to one year assessed that MRSA nasal colonization is a significant risk factor for future MRSA infection. The study showed that colonization with Panton-Valentine Leukocidin (PVL) positive MRSA is associated with a greater risk of developing MRSA infection[25]. Although MRSA screening at hospital admission seems to be promising, questions concerning the more effectiveness of universal *versus* targeted screening, as well as the more appropriate combination of body sites screening, are still waiting for an answer[26,27]. To date literature offers insufficient evidence to determine the consequences of universal screening or the effectiveness of other screening strategies[26]. A recent study comparing different body sites screening confirmed that testing at least three of them (groin, nose and throat) allow to reach a higher sensitivity in detecting MRSA[27]. Moreover, infection is often a cause of ICU admission, influencing both the clinical outcome of the patient and the microbiological environment of the ward[28]. Italian ICUs started in 2005 an observational, prospective, multicenter infection surveillance program. This project, named “Margherita”, allowed to collect data concerning ICU infections from all-over Italy. MDR microorganisms represented one of the most important problems concerning ICU-acquired infections, being MRSA on of the most represented MDR causing both VAPs and blood stream infections[28]. Molecular epidemiology of MRSA strains responsible for ICUs and PICUs infections is still a not really diffused tool, although a more delineate picture could represent a powerful tool to improve infection control measures and therapeutic schemes.

SA, MRSA and virulence factors.

SA strains may produce a large variety of toxins: cytolitic, including leukocidins, alpha-toxin and phenol-soluble modulins. Further important toxins are the SA superantigens, which include toxin shock syndrome toxin and lead to exacerbate

immune response by polyclonal T cell activation and massive cytokine release. SA also produces a wide range of toxins interfering with almost every mechanism of human innate host defense[29].

SA strains are known to cause three basic syndromes: skin abscesses and wound infections; deep-seated and systemic infections; toxemic syndromes such as toxic shock syndromes and staphylococcal scarlet fever, staphylococcal scalded-skin syndrome, and staphylococcal food poisoning[30]. Pathogenesis of SA infections is a process based on two different stages. During the first stage, corresponding to the exponential phase of growth of *in vitro* cultures, adhesins and surface proteins are produced. The second stage corresponds to the post-exponential and stationary phases of growth *in vitro*, and is characterized by increased toxin production and consequent tissue damage and bacterial spread. This process has multifactorial molecular basis, depending on the expression of accessory gene products. In particular, accessory gene regulator (*agr)* locus controls the expression of most virulence factors. The *agr* quorum-sensing system represses the expression of surface proteins, including protein A (*spa*), and up-regulates the production of toxins, such as α-haemolysin and protease. The *agr* system is comprised of two divergent transcripts, RNAII and RNAIII. RNAII carries four genes, *agrDBCA*, which are dedicated to the synthesis, sensing, and processing of the quorum-sensing autoinducing peptide (AIP) required for the activation of the RNAII promoter. The divergent transcript RNAIII, activated in response to RNAII via AgrA, is a regulatory RNA molecule expressed maximally during the post-exponential phase and controls virulence gene expression at the transcriptional and translational levels, the latter via an antisense mechanism. A polymorphism in the aminoacid sequence of the auto-inducing peptide and its receptor is the basis on which SA can be divided into four *agr* groups.

Recent studies made it possible to establish a relationship between *agr* group and clinical onsets: group I and II strains are able to cause SE-mediated disease (food

poisoning), group III strains are usually involved in toxic shock syndrome due to toxic shock syndrome toxin 1 (TSST-1), and group IV strains are generally involved in generalized exfoliative syndrome and bullous impetigo[30].

Immuno-evasion cluster (IEC) genes can be considered as virulence factors, since their presence appears to be related to escaping from immune system of the host. These genes are located on β-haemolysin-converting bacteriophages, and encode chemotaxis inhibitory protein of SA (CHIPS, encoded by *chp*), staphylococcal complement inhibitor (SCIN, encoded by *scn*), staphylokinase (SAK, encoded by *sak*) and enterotoxin A (SEA, encoded by *sea* gene), seven IEC variants have been identified basing on different combination of *chp, scn, sak* [31]. All IEC variants carry SCIN, which represent an efficient complement inhibitor of the lectin, preventing opsonophagocytosis, killing by neutrophils and neutrophils chemotaxis. The role of IEC in both colonization and infection processes is still unclear, as well as characterization of this important genetic determinant.

A further crucial element is PVL, a bipartite toxin with pore-forming activity against human polymorphonuclear cells, monocytes and macrophages. Both SA and MRSA are able to produce it, and PVL-positive strains can be associated with foruncles and carbuncles, and/or community pneumonia with lung abscesses. The clinical *sequelae* of infections caused by PVL-positive strains tend to be more severe than PVL-negative SA. For example, pneumonia associated with PVL-positive SA is more frequently associated with sepsis, high fever, leucopenia, hemoptysis, pleural effusion and death[32]. Due to the relevance of clinical manifestations caused by PVL, the importance of a correct isolation and identification of producer strains is clear. Soft skin infections are currently poorly investigated, causing the loss of strains responsible for a dramatic underestimation of PVL producer strains, leading to a weak knowledge of diffusion and epidemiology PVL-positive strains.

Coagulase-negative staphylococci as a possible *reservoir* of virulence factors

Coagulase-negative staphylococci (CoNS) comprise a multitude of species, many of which are opportunistic pathogens, being *Staphylococcus epidermidis* (SE) the most frequently encountered CoNS species on human skin and by far the most frequent source of infections among CoNS[29]. Pathogenicity of CoNS appears to stem from molecular determinants that evolved for commensal life on the skin, but may rise to additional use during infection[29]. Moreover, there are several recent findings indicating that SA acquired other factors that facilitate survival during infections from SE or other CoNS[29]. In some settings different staphylococcal species live in the same niches on human epithelial surfaces, being in close enough contact for genetic exchange to occur, probably due to horizontal gene transfer[29]. The arginine catabolic mobile element (ACME), a 31 kb mobile genetic element encoding an arginine deaminase (*arc*) and an oligopepetide permease (*opp3*), represents an important virulence factor for USA300 worldwide diffused CA-MRSA clone, and is commonly found in SE strains[33]. ACME seems to contribute to competitive fitness of USA300 with *arc* or *opp* gene clusters facilitating colonization or transmissibility capacity of USA300. To date, three different ACME types were described, diffusing among different clones. ACME type I, having both *arc* and *opp*, is associated with USA300. ACME type II, having *arc* only, was found in ST5 and ST59. ACME type III has the only *opp* gene[29]. Data concerning ACME presence in CoNS strains isolated in nosocomial settings are still scanty, limiting a correct epidemiology knowledge and avoiding real consideration of the importance of CoNS as a *reservoir* of virulence factors.

Aim of the study

The picture emerging from recently published studies demonstrates that both MRSA, SA and CoNS epidemiology is rapidly changing: the prevalence of MRSA is increasing worldwide, and SE has become one of the major causes of indwelling devices infections. Moreover, MRSA epidemiology and genetic background are poorly described and characterized in high risk wards such as ICUs and CF patients, as well as the presence and characterization of genes encoding for virulence factors has also been poorly investigated, especially in pediatric patients. The aim of this study is to analyze molecular epidemiology and antimicrobial resistance of clinical MRSA strains, belonging to patients attending CF as well as PICU wards. Moreover, the presence of CoNS and their virulence factors would be investigated in PICU, to clarify the diffusion of such determinants. Molecular analysis of PVL-positive SA and MRSA strains represents another goal of this study, as well as their molecular characterization. This study should provide a detailed picture of molecular epidemiology and genetic background characterization of MRSA, SA and CoNS isolated in a third-level pediatric hospital, a useful tool to obtain a different point of view on infection control policies and therapeutic approaches.

Material and methods:

Bacterial strains

Putative MSSA, MRSA, CoNS isolates will be obtained from Department of Pediatric Medicine wards (DPMW), PICUs and CF patients. Each sample collected during the hospitalization period was cultured for detection of MRSA isolates.

Sample culturing and bacterial isolation.

Samples were cultured inoculating plates of selective agar, the specific selective medium for SA (Mannitol Salt 2 Agar, bioMérieux, Marcy L'Etoile, France). Respiratory samples were mainly bronchial aspirates (BA) in the case of PICU patients, or sputum and swabs in case of CF patients. BA samples were treated after a quality control based on microscopic observation. Then, samples were processed following recommendations: 100 µl of samples were cultured on selective media plates, focusing attention in this specific case on the specific selective medium for SA (Mannitol Salt 2 Agar, bioMérieux, Marcy L'Etoile, France). *Sputum* samples from CF patients were usually cultured after incubation with dithiothreitol (1:1, volume to volume). 20 µl of samples were plated on seven different media, as recommended by consensus guidelines (www.SIFC.it). After 48 hours of incubation in aerobic atmosphere at 37°C, plates were read in order to detect the presence of SA or CoNS.

Biochemical identification

Identification of putative SA isolates is based on typical colony morphology on selective culture media (Mannitol Salt 2 Agar, bioMérieux, Marcy L'Etoile, France). In order to confirm species-level identification, the Slidex Staph Plus (Bio-Rad) test was performed. This test is based on latex agglutination principle; latex particles have been sensitized with fibrinogen and IgG in order to detect clumping factor and

protein A, biochemical characteristics of SA. A positive reaction is evidenced by the formation of aggregates with the reagent test only, visible to the naked eye. CoNS are identified on the basis of the growth on Mannitol Salt Agar, negative results of Slidex Staph Plus test, and Vitek GP cards (bioMérieux, Marcy L'Etoile, France). Methicillin resistance was initially tested using Oxa Screen Test Agar (Becton and Dickinson). Methicillin resistant strains are individuated until 2011 following CLSI instruction, and since 2012 EUCAST (www.eucast.org). All MRSA, SA, CoNS strains were stored at -80°C.

Antimicrobial susceptibility testing methods

Antibiotic susceptibility was evaluated assessing the activity of a large panel of drugs by means of the disk diffusion test on Mueller-Hinton agar (Kirby Bauer) until 2011. This method is based on the diffusion of antibiotic into agar inoculated with a liquid culture of the isolate of interest. As soon as the antibiotic-impregnated disk comes into contact with the moist agar surface, water is absorbed into the filter paper and the antibiotic diffuses into the surrounding medium. The concentration immediately adjacent to the disk may exceed that in the disk itself. As the distance from the disk increases there is a logarithmic reduction in the antibiotic concentration. Simultaneous growth of bacteria occurs on the surface of the agar. When a critical cell mass of bacteria is reached, the inhibitory activity of the antibiotic is overcome and bacterial growth occurs. The interpretation criteria of the Kirby-Bauer test have been offered by different international committees, such as the NCLSI or ESCMID's EUCAST since 2011. The interpretative standards are derived from a correlation between zone sizes and minimum inhibitory concentrations (MICs) of those species that can be tested by disk diffusion method. The E-test (AB-Biodisc) were used in case of intermediate phenotypes to delineate glycopeptides resistance. A plastic strip calibrated with a MIC scale in µg/ml is laid in an agar plate streaked with the bacterial culture of interest. A predefined

concentration gradient of antibiotic agent, across 15 twofold dilutions of a conventional MIC method, is immobilized on the other surface of the carrier. After overnight incubation, the point at which the elliptical zone of growth inhibition intersects a quantitative scale on the strip determines the MIC.

Since 2011, antimicrobial susceptibility tests were performed by means of Vitek (Vitek 2, bioMérieux, Marcy L'Etoile, France) using P580 cards and then P619; results were interpreted following CLSI and, since 2012, EUCAST criteria.

Table 1 shows a comparison of antimicrobial agents used for Kirby-Bauer and then Vitek 2 method, interpretation rules changed as well.

Table 1. Comparison of antimicrobial agents used for Kirby-Bauer and Vitek 2 testing.

Antimicrobial agent	abbreviation	Kirby-Bauer	Vitek II (P580)
Amoxicillin/ clavulanate	AMC	Yes	NO
Oxacillin	OXA	Yes	Yes
Cefoxitin	FOX	Yes	Yes
Cefaclor	CEC	Yes	NO
Ceftazidim	CAZ	Yes	NO
Cefuroxime	CXM	Yes	NO
Tobramycin	NN	Yes	Yes
Gentamicin	GM	Yes	Yes
Erythromycin	E	Yes	Yes
Ciprofloxacin	CIP	Yes	NO
Levofloxacin	LVX	Yes	Yes
Trimethoprim / Sulfamethoxazole	SXT	Yes	Yes
Rifampicin	RA	Yes	Yes
Vancomycin	VA	Yes	Yes
Teicoplanin	TEC	Yes	Yes
Doxycycline	DO	Yes	NO
Minocycline	MNO	Yes	NO
Clindamycin	CLI	Yes	Yes
Linezolid	LZD	Yes	Yes
Phosphomycin	PHO	NO	Yes
Fusidic Acid	FUS	NO	Yes
Moxifloxacin	MOX	NO	Yes
Mupirocin	MUP	NO	Yes
Tigecycline	TIG	NO	Yes
G Penicillin	PEN	NO	Yes
Tetracycline	TET	NO	Yes
Nitrofurantoin	NIT	NO	Yes

DNA extraction:

DNA extraction was performed according to manufacturer's instructions using Nucleo Spin Tissue kit (Macherey-Nagel GmbH&Co. KG, Düren, Germany), with lysostaphin at 0.5 mg/ml for the lysis step.

SCC*mec* typing:

SCC*mec* typing of MRSA strains were done following the protocol published by Oliveira and de Lancastre in 2002[34], primers are listed in Table 2. A multiplex PCR mix was set up in a 50-µl volume, containing 1x PCR buffer, 200 µM (each) deoxynucleotide triphosphate (Roche Italia), 50 mM $MgCl_2$; 400 nM concentrations of primers CIF2 F2, CIF2 R2, MECI P2, MECI P3, RIF5 F10, RIF5 R13; 800 nM concentrations of primers DCS F2, DCS R2, MECA P4, MECA P7; 200 nM concentrations of primers KDP F1, KDP R1, RIF4 F3, RIF4 R9; 1.25 U of AB Taq (AB Analitica, Italia) and 4 µl of template DNA. PCR amplifications were performed with the following parameters: 4 min at 94°C; 30 cycles of 94°C for 30 seconds, 53°C fors 30 seconds and 72°C for 1 minute; 4 min at 72°C. PCR products were resolved in a 2% agarose gel in 1X TBE buffer at 100V and visualized after staining with GelRed Nucleic Acid Gel Stainer (Biotium, California).

Table 2: list of primers for SCC*mec* typing[34].

Primer	Oligonucleotide sequence(5'–3')	Amplicon size (bp)	SCC*mec* type
CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	495	I
CIF2 R2	ATTTACCACAAGGACTACCAGC		
KDP F1	AATCATCTGCCATTGGTGATGC	284	II
KDP R1	CGAATGAAGTGAAAGAAAGTGG		
MECI P2	ATCAAGACTTGCATTCAGGC	209	II, III
MECI P3	GCGGTTTCAATTCACTTGTC		
DCS F2	CATCCTATGATAGCTTGGTC	342	I, II, IV
DCS R1	CTAAATCATAGCCATGACCG		
RIF4 F3	GTGATTGTTCGAGATATGTGG	243	III
RIF4 R9	CGCTTTATCTGTATCTATCGC		
RIF5 F10	TTCTTAAGTACACGCTGAATCG	414	III
RIF5 R13	GTCACAGTAATTCCATCAATGC		
MECA P4	TCCAGATTACAACTTCACCAGG	162	Internal control
MECA P7	CCACTTCATATCTTGTAACG		

The protocol published by Oliveira and colleagues in 2002 does not allow to detect SCC*mec* type V. To this purpose, primer list of another protocol proposed by Zhang and collaborators[35] is shown in Table 3.

Table 3. Primer list used in Zhang's protocol [35].

Primer	Oligonucleotide sequence(5'–3')	Amplicon size (bp)	SCC*mec* type
I-F	GCTTTAAAGAGTGTCGTTACAGG	613	I
I-R	GTTCTCTCATAGTATGACGTCC		
II-F	CGTTGAAGATGATGAAGCG	398	II
II-R	CGAAATCAATGGTTAATGGACC		
III-F	CCATATTGTGTACGATGCG	280	III
III-R	CCTTAGTTGTCGTAACAGATCG		
IV a-F	GCCTTATTCGAAGAAACCG	776	IV a
IV a-R	CTACTCTTCTGAAAAGCGTCG		
IV b-F	TCTGGAATTACTTCAGCTGC	493	IV b
IV b-R	AAACAATATTGCTCTCCCTC		
IV c-F	ACAATATTTGTATTATCGGAGAGC	200	IV c
IV c-R	TTGGTATGAGGTATTGCTGG		
IV d-F	CTCAAAATACGGACCCCAATACA	881	IV d
IV d-R	TGCTCCAGTAATTGCTAAAG		
V-F	GAACATTGTTACTTAAATGAGCG	325	V
V-R	TGAAAGTTGTACCCTTGACACC		
MecA147-F	GTGAAGATATACCAAGTGATT	147	Internal control
MecA147-R	ATGCGCTAT AGA TTG AAA GGA T		

Single target amplifications were performed in a 25 µl volume, containing 1x PCR buffer, 200 mM (each) deoxynulceotide (Roche, Italia), 50mM $MgCl_2$, 1 U AB Taq (AB Analitica,Italia), and 0,2 µM of each primer. PCR conditions were as follow: 94°C for five minutes, 30 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, 72° for 10 minutes. PCR products were resolved in a 2% agarose gel in 1X TBE buffer at 100V and visualized after staining with GelRed Nucleic Acid Gel Stainer (Biotium, California).

A further method was applied in SCC*mec* typing[36], in order to detect different types basing on different region of the SCC*mec* mobile element. Table 4 shows the primer list, used at different concentrations in a master mix containing 1X PCR buffer; 1.5 mM $MgCl_2$; 40 µM (each) deoxynucleotide triphosphate (Roche, Italia); 0.2 µM

primers KDP F1, KDP R1; 0.4 µM primers CIF2 F2, CIF2 R2, RIF5 F10, RIF5 R13, SCC*mec* III J1F, SCC*mec* III J1R, SCC*mec* V J1F, SCC*mec* V J1R; 0.8 µM primers mecI P2, mecI P3, DCS F2, DCS R1, mecA P4, mecA P7, ccrB2 F2, ccrB2 R2, ccrC F2, ccrC R2; 1.25 U of AB Taq (AB Analitica, Italia) and 2 µl of DNA. The optimal cycling conditions were the following: 94°C for 4 minutes, 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 minute and a final extension at 72°C for 4 minutes.

Table 4. Primer used following the protocol by Mileiriço and colleagues[36].

Primer	Oligonucleotide sequence(5'–3')	Amplicon size (bp)	SCC*mec* type
CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	495	I, J1 region
CIF2 R2	ATTTACCACAAGGACTACCAGC		
ccrC F2	GTACTCGTTACAATGTTTGG	449	V, *ccr* complex
ccrC R2	ATAATGGCTTCATGCTTACC		
RIF5 F10	TTCTTAAGTACACGCTGAATCG	414	III, J3 region
RIF5 R13	ATGGAGATGAATTACAAGGG		
SCCmec V J1 F	TTCTCCATTCTTGTTCATCC	377	V, J1 region
SCCmec V J1 R	AGAGACTACTGACTTAAGTGG		
dcs F2	CATCCTATGATAGCTTGGTC	342	I, II, IV, VI, J3 region
dcs R1	CTAAATCATAGCCATGACCG		
ccrB2 F2	AGTTTCTCAGAATTCGAACG	311	II, IV, *ccr* complex
ccrB2 R2	CCGATATAGAAWGGGTTAGC		
kdp F1	AATCATCTGCCATTGGTGATGC	284	II, J1 region
kdp R1	CGAATGAAGTGAAAGAAAGTGG		
SCCmec III J1 F	CATTTGTGAAACACAGTACG	243	III, J1 region
SCCmec III J1 R	GTTATTGAGACTCCTAAAGC		
mecI P2	ATCAAGACTTGCATTCAGGC	209	II, III *mec* complex
mecI P3	GCGGTTTCAATTCACTTGTC		
mecA P4	TCCAGATTACAACTTCACCAGG	162	Internal positive control
mecA P7	CCACTTCATATCTTGTAACG		

PCR products were resolved in a 3% agarose gel in 1XTris-Borate-EDTA buffer, at 100V and were visualized after staining with GelRed Nucleic Acid Gel Stainer (Biotium, California).

spa typing:

The sequence of the polymorphic region X of the SA protein A (*spa*) locus has been used to developed a single-locus sequence typing technique for MRSA[37]. The *spa* locus consists of a number of mainly 24-bp repeats, and its diversity is attributed to deletions and duplications of the repeats, and due to point mutations. PCR amplification of a portion of *spa* gene was performed in a 25 µl volume, containing 1X PCR Buffer, 50 mM $MgCl_2$,350 mM total deoxynucleotide (Roche, Italia), 0.5 mM PA 1095F forward primer (5'-AGACGATCCTTCGGTGAGC-3') and PA 1517R reverse primer (5'-GCTTTTGCAATGTCATTTACTG-3'), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 2 µl of chromosomal DNA. Thermal cycling parameters included an initial 10 minutes at 95°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 60°C, 45 seconds at 72°C; 72°C for 10 minutes. PCR products were resolved in a 1% agarose gel in 1X TBE buffer at 100V and visualized after staining with GelRed Nucleic Acid Gel Stainer (Biotium, California). Spa types were assigned using a free web-application named spa typer finder (http://spatyper.fortinbras.us/).

MLST

PCR partial amplification of the seven house-keeping genes was performed following protocol by Enright and colleagues[38]. Table 5 presents the primer list.

Table 5. Primer list for MLST analysis.

Primer	Oligonucleotide sequence(5'–3')
arcC UP	TTGATTCACCAGCGCGTATTGTC
arc DN	AGGTATCTGCTTCAATCAGCG
aroE UP	ATCGGAAATCCTATTTCACATTC
aroE DN	GGTGTTGTATTAATAACGATATC
glpF UP	CTAGGAACTGCAATCTTAATCC
glpF DN	TGGTAAAATCGCATGTCCAATTC
gmk UP	ATCGTTTTATCGGGACCATC
gmk DN	TCATTAACTACAACGTAATCGTA
ptA UP	GTTAAAATCGTATTACCTGAAGG
ptA DN	GACCCTTTTGTTGAAAAGCTTAA
tpI UP	TCGTTCATTCTGAACGTCGTGAA
tpI DN	TTTGCACCTTCTAACAATTGTAC
yqiL UP	CAGCATACAGGACACCTATTGGC
yqiL DN	CGTTGAGGAATCGATACTGGAAC

PCRs were carried out in a 25-µl reaction volumes containing 2 µl of chromosomal DNA, 1X PCR buffer, 50 mM $MgCl_2$, 0.10 µM of each primer, 200 mM (each) deoxynucleotide (Roche, Italia), 1 U of AB Taq DNA polymerase (AB Analitica, Italia). Thermal cycling parameters included an initial 5-minutes denaturation at 95°C, followed by 30 cycles of annealing at 55°C for 1 min, extension at 72°C for 1 min, followed by a final extension time of 5 minutes at 72°C. PCR products were resolved in a 1% agarose gel in 1X TBE buffer at 100V and visualized after staining with GelRed Nucleic Acid Gel Stainer (Biotium, California). Sequence types (STs) were attributed submitting the DNA sequences obtained to the online MLST database (http://www.mlst.net/).

DNA sequencing

PCR products were purified by means of ExoSAP-IT (Affymetrics USB, Santa Clara, CA), following manufacturer's recommendations. 5 µl of PCR product were mixed with 2 µl of ExoSAP-IT, and then incubated at 37°C for 15 minutes. The enzyme combination was inactivated incubating it at 80°C for 15 minutes.

Nucleotide sequencing reactions were set up using Big Dye Terminator v. 3.1 Cycle Sequencing chemistry (Applied Biosystems, Life Technologies) on 310 Genetic Analyzer (Applied Biosystems, Life Technologies). Sequencing products were

analyzed using the free software BioEdit, Biological sequence alignment editor (copyright by Tom Hall, Ibis Bioscience, CA).

Phylogenetic trees were obtained using the free software MEGA6 [39], following recommendations by Hall[40].

ACME

The molecular characterization of ACME was performed according to previously described methods (Ellington MJ, 2008). Reactions were set up in a 25-µl volume using 1X PCR Buffer, 0.1 µM of each primer, 3 mM $MgCl_2$, 200 µM of each deoxynucleotide (Roche, Italia), 1 U of AB *Taq* (AB Analitica), and 2 µl of chromosomal DNA.

Optimal cycling conditions were the following: 5 minutes at 95°C, 30 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes, followed by 72°C for 5 minutes. PCR products were resolved in a 1% agarose gel in 1X TBE buffer at 100V and visualized after staining with GelRed Nucleic Acid Gel Stainer (Biotium, California).

Toxin genes testing

PCR to detect PVL genes was set up as previously described by Lina and colleagues [41], using primers *luk*-PV1 (5' - ATCATTAGGTAAAATGTCTGGACATGATCCA-3') and *luk*-PV2 (5'-GCATCAASTGTATTGGATAGCAAAAGC-3'). Thermal cycling conditions were the following: 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute. PCR products were resolved by electrophoresis through 1.5% agarose gel, and analyzed after GelRed Nucleic Acid Gel Stainer (Biotium, California) staining. Nucleotide sequences of PCR products were determined to confirm PCR specificity, and to set up diagrams.

The *agr* typing was performed as previously described by Shopsin and colleagues in 2003[42]. Table 6 shows the primer list: one forward primer named pan-*agr* was used in combination with four different reverse primers to detect *agr* types basing on different sizes of PCR products.

Table 6. List of primers used for *agr* typing.

Primer	Oligonucleotide sequence(5'–3')	Amplicon size (bp)
pan-*agr*	ATGCACATGGTGCACATGC	-
agr I	GTC ACAAGTACTATAAGCTGCGAT	440
agr II	GTATTACTAATTGAAAAGTGCCATAGC	572
agr III	CTGTTGAAAAAGTCAACTAAAAG CTC	406
agr IV	CGATAATGCCG TAATAC CCG	588

PCR was performed in a 25-µl volume mixture, containing 2 µl of chromosomal DNA, 2 mM MgCl2, 0.1 µM of each primer, 350 µM (total) deoxynucleoside (Roche, Italia), 2.5 U of AB Taq (AB Analitica). Thermal cycling was performed following these conditions: 25 cycles consisting of 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C. PCR products were resolved on a 1% agarose gel, and detected after GelRed Nucleic Acid Gel Stainer (Biotium, California) staining.

Results:

Strains collection:

Clinical strains were collected until the last day of 2012, with the exception of DPMW samples sent to search PVL-producers isolates, collected until the last day of 2013. Table 7 resumes sample collection and MRSA and MRCoNS obtained sampling PICU and CF patients.

Table 7. MRSA and MRCoNS collected during the study period in PICU and CF wards.

Ward	Year	Sample or patients	MRSA (%)	MRCoNS (%)
PICU	2010	202	25 (11.4%)	15 (7.5%)
	2011	210	29 (12.8%)	31 (14.7%)
	2012	180	10 (5.5%)	16 (9%)
CF	2010	283	28 (9.9%)*	-
	2011	301	26 (8.6%)*	-
	2012	285	33 (11.5%)*	-

* MRSA percentage referred to patients' number instead of samples.
MRSA: meticillin-resistant *Staphylococcus aureus*;
MRCoNS: meticillin-resistant coagulase-negative *staphylococci*.

During routine clinical sampling, MRSA and MRCoNS were isolated from PICU samples. Due to the lack of data concerning MRSA and MRCoNS molecular epidemiology of Italian PICU patients, more than one strain/patient were collected and analyzed in order to obtain a more delineated epidemiological PICU picture and more information concerning long-term patients. Considering that CF patients are microbiologically monitored every three months, one strain/year/patient was collected and analyzed. Forty-nine patients supplied all the analyzed strains.

Clinical suspects of both soft tissue and invasive infections led to collect several samples, as described in Table 8.

Table 8. Collected samples and PVL-positive ones.

Year	Total number of samples	PVL- positive samples
2011	10	4
2012	50	9
2013	52	17

During this period of time, it was possible to collect PVL-positive staphylococcal isolates belonging to Pediatric patients as well as their sibling or parents, and all these strains were analyzed.

Each antimicrobial susceptibility testing result was considered to build up a more accurate picture, but molecular analysis were performed on a selected number of strains, as shown in Table 9.

Table 9. Strains belonging to PICU and CF patients undergoing to molecular characterization.

Ward	**Year**	**Number of MRSA strains**	**Selected MRSA strains**
PICU	2010	25	17
	2011	29	11
	2012	10	6
CF	2010	28	27
	2011	27	27
	2012	33	29

PICU patients were admitted with different diagnosis, 15 out of 23 (65%) underwent a tracheotomy before the admittance. Five out of 23 patients (21%) were progressively monitored during the study period. Table 10 shows characteristics of PICU patients.

Table 10. Main characteristics of PICU patients.

Ward	Year	MRSA positive patients	Median age (range, years)	Number of Tracheotomy positive
PICU	2010	12	2.44 years (0.8-17)	6
	2011	12	1.88 years (0.08-7)	8
	2012	6	2.83 years (1-5)	5

CF patients showed different characteristics, as shown in Table 11, in particular adult patients also attend to CF Center of Florence. MRSA strains belonging to adult patients were included since they attend to the Center, being subject to follow-up in a Pediatric hospital.

Table 11. Description of CF patients.

Ward	Year	Patients	Median age (range)	Number of First infections	Number of Chronic infections
CF	2010	28	19.5 (0.4-49)	6	22
	2011	27	20.3 (5-40)	6	21
	2012	33	17.6 (0.8-48)	14	19

A chronic infection is defined as “When more than 50% of months, when samples had been taken, were MRSA culture positive”. Patients having no growth of MRSA during the previous twelve months, but having previously been MRSA culture positive were defined free of infection. These definitions were chosen according to Leeds criteria used to define patients chronically infected by *Pseudomonas aeruginosa* [43] due to the lack of an universally accepted definition of chronic MRSA infection.

As shown in Table 12, PVL-positive strains collection started in 2011 since this problem emerged in that period, being necessary to set-up molecular approaches to determine the presence of such isolates in selected cases.

Table 12. Characteristics of patients infected by PVL-positive strains.

Year	PVL-positive strains	MRSA strains	SA strains
2011	4	0	4
2012	10	6	4
2013	13	2	11

Antimicrobial susceptibility testing results

Technological progresses, such as automated systems utilization, had a strong impact on collected data, leading to use extreme carefulness in interpretation. The number of comparable antimicrobial agents became smaller, as shown in Table 1 of Materials and Methods Section, and some change occurred during the study period. Figure 1 compares data concerning antimicrobial resistance during this three-year study.

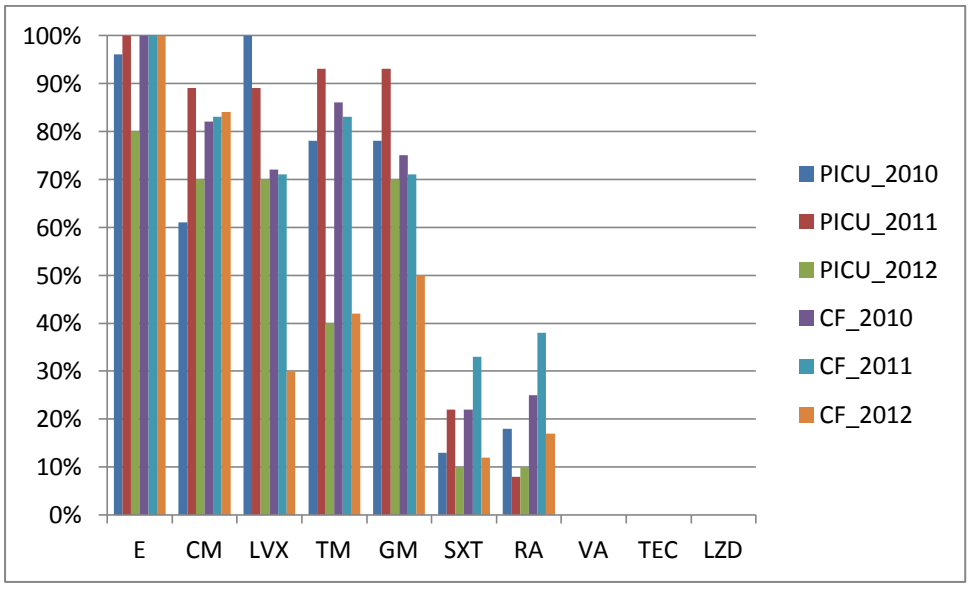

Figure 1. Comparison of antimicrobial resistance patterns of CF and PICU MRSA isolates. **E**: Erythromycin; CM: Clindamycin; LVX: Levofloxacin; TM: Tobramycin; GM: Gentamicin; SXT: Trimethoprim / Sulfamethoxazole; RA:Rifampicin;Va: Vancomycin; TEC: teicoplanin; LZD: linezolid.

Statistical comparisons are unfortunately not suitable due to different methods and interpretation rules, progressively changed during the project time, as reported in Materials and Methods section.

Resistance to glycopeptide and linezolid was not detected, whereas other antimicrobial agents showed high percentages of resistance. Resistance level appear to be comparable in PICU and CF patients, although differences are detectable. A higher percentage of CF strains showed resistance to thimetoprim-sulfomethoxazole and rifampin, whilst comparable values of resistance against erythromycin, tobramycin, gentamicin were showed with difference during the years. PICU strains appeared to be more resistant against levofloxacin, and showed higher resistance percentages during 2011, suggesting the diffusion of particularly resistant strains.

If we get a look on singular wards, we can see how resistance modified through these three years. Figure 2 shows CF wards.

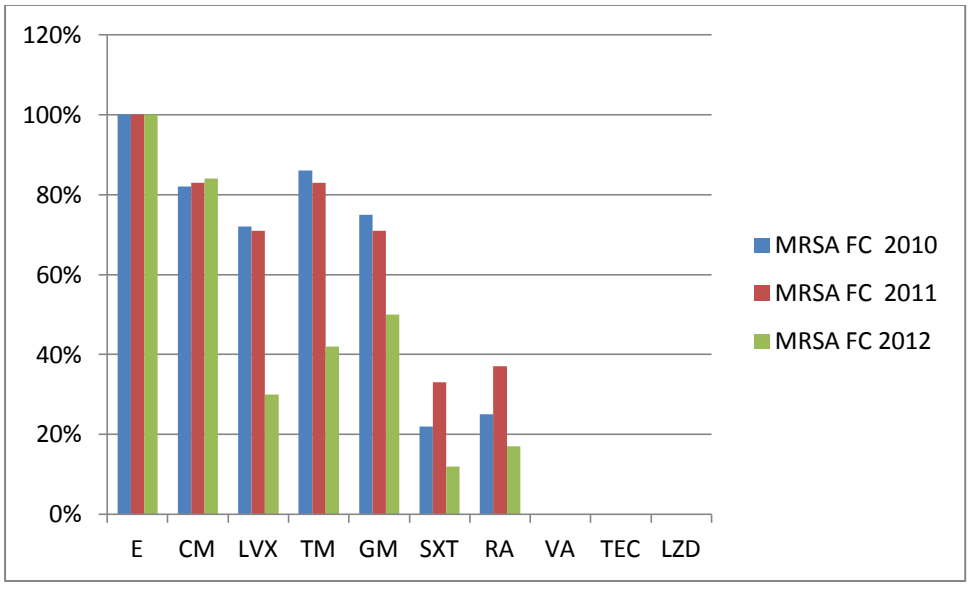

Figure 2. Changing MRSA antimicrobial resistance in CF ward, from 2010 to 2012.

E: Erythromycin; CM: Clindamycin; LVX: Levofloxacin; TM: Tobramycin; GM: Gentamicin; SXT: Trimethoprim / Sulfamethoxazole; RA:Rifampicin;Va: Vancomycin; TEC: teicoplanin; LZD: linezolid.

It is interesting to note that percentage of resistance against levofloxacin, tobramycin, gentamycin, trimethoprim-sulfomethoxazole and rifampin changed in 2012. The statistical valence of such differences are unfortunately not definable, and may be referred to the use of automated methods and changes in expert rules and interpretation standards.

In Figure 3 are showed PICU percentage of resistance against antimicrobial agents.

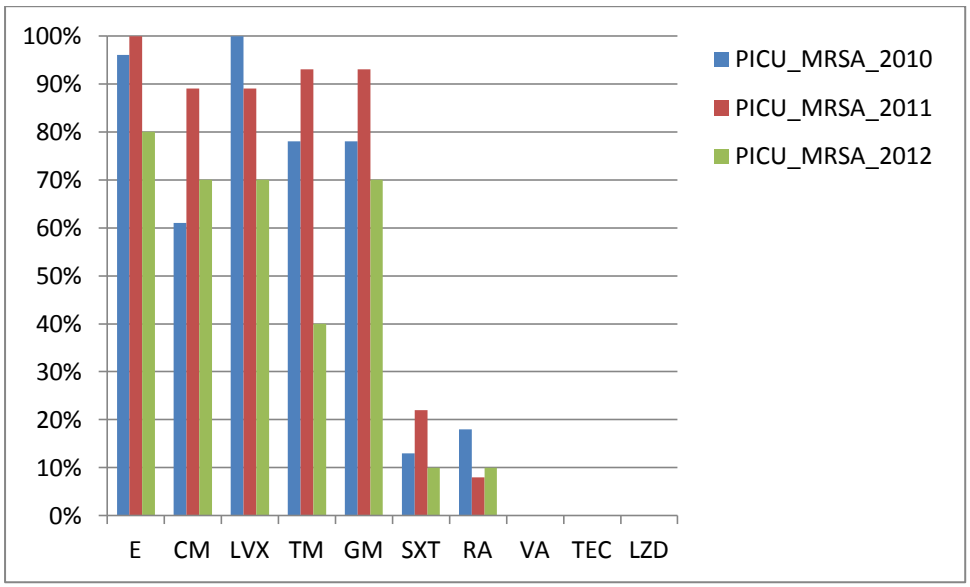

Figure 3. Antimicrobial resistance percentages of MRSA isolates in PICU.

E: Erythromycin; CM: Clindamycin; LVX: Levofloxacin; TM: Tobramycin; GM: Gentamicin; SXT: Trimethoprim / Sulfamethoxazole; RA:Rifampicin;Va: Vancomycin; TEC: teicoplanin; LZD: linezolid.

There is no resistant strain against glycopeptides, but differences comparing each year are notable concerning other antimicrobial agents. During 2010 23 MRSA strains were collected from specimens belonging to 13 patients; in 2011 29 strains came from 16 different patients; during 2012 10 strains were collected from 9 patients. A total of 31 patients supplied 62 strains from 2010 to 2012. Five out of 31 patients supplied strains for more than one year, three out of five showed infection in two different years, and the remaining two patients covered the entire study period.

These circumstances may have given a contribution to differences, the statistical valence of which could not be determined.

Methicillin-resistant ConS (MRCoNS) were detected and collected from specimens belonging to PICU patients. Figure 4 shows the comparison of antimicrobial resistance percentage of MRSA and MRCoNS.

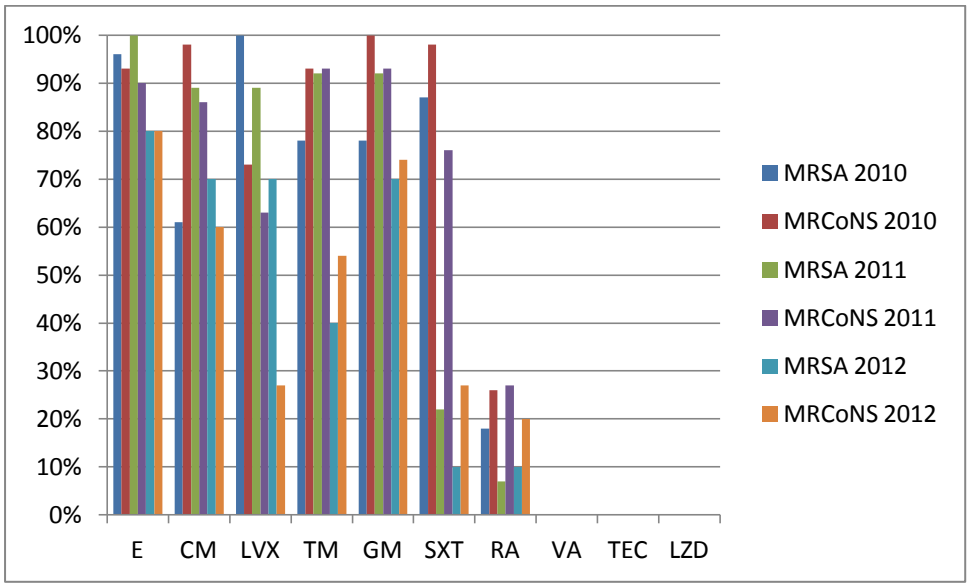

Figure 4: Comparison of antimicrobial resistance between MRSA and MRCoNS from PICU patients.

E: Erythromycin; CM: Clindamycin; LVX: Levofloxacin; TM: Tobramycin; GM: Gentamicin; SXT: Trimethoprim / Sulfamethoxazole; RA:Rifampicin;Va: Vancomycin; TEC: teicoplanin; LZD: linezolid.

MRCoNS represent an important *reservoir* of resistance genetic determinants as well as SCC*mec* elements. MRCoNS show an higher percentage of resistant strains, but it should be taken into account that more than one strain/year/patients was chosen, determining a possible influence on data. Statistical analysis can't be performed due to the different antimicrobial susceptibility testing methods as well as changing in interpretation rules.

Molecular characterization of collected isolates

A thorough knowledge of the dissemination and molecular evolution of MRSA are required to develop effective strategies to prevent the spread of such strains. Therefore, various molecular typing techniques have been developed. In general, there are “band-based” and “sequence-based” methods to determine the genetic background of SA and MRSA, but “sequence-based” methods are preferred because the data are exchangeable. The most commonly used typing methods are pulsed-field gel electrophoresis (PFGE), MLST, *spa* typing, and SCC*mec* typing. Although PFGE is still regarded by many authors as a gold standard for benchmarking new typing methods, sequence-based typing methods generate unambiguous and portable data, easily comparable in different laboratories of different countries. Moreover, due to their important role in virulence and pathogenicity of MRSA strains, molecular characterization of toxins such as PVL is definitely a relevant issue.

SCC*mec* typing

All the available methods for SCC*mec* typing are based on different structural properties of this genetic element, but a single universal method for its classification is still needed. A number of methods have been developed to investigate the structure of SCC*mec*[34,35,36]. Multiplex PCR assays are available to discriminate among SCC*mec* type I to IV[34], in which *mecA* and different loci on SCC*mec* are detected, as well as type I to V[35].

Three different methods were applied to properly discriminate among SCC*mec* types from I to VI[36]; since others SCC*mec* types occurred rarely, further methods are going to be set up in the next future. Table 13 illustrated the distribution of different SCC*mec* types in PICU and CF wards during the study period.

Table 13. SCC*mec* typing of collected strains.

Ward	SCC*mec* type I	SCC*mec* type II	SCC*mec* type III	SCC*mec* type IV	SCC*mec* type V	Not determined
PICU_2010	7	0	0	5	5	0
PICU_2011	7	0	1	2	1	0
PICU_2012	6	0	0	0	0	0
CF_2010	14	1	3	6	2	1
CF_2011	13	1	3	4	3	1
CF_2012	11	2	4	4	5	3

Only two strains did not result to belong to a known SCC*mec* type, being necessary to test the less frequently noted chromosomal cassettes *mec*.

SCC*mec* typing of CoNS strains showed the presence of 8 out of 27 (30%) SCC*mec* type I, 1 (4%) SCC*mec* type II, 6 (22%) SCC*mec* type III, as well as type IV and not determined.

Nine out of 30 (30%) collected PVL-producer strains were MRSA, being the remaining 21 SA. All MRSA strains belonged to SCC*mec* type IV, and SA strains did not harbor SCC*mec* element.

Spa typing results

This analysis is a single-locus sequence typing based on the polymorphic region X of the *S. aureus* protein A (*spa*)[37]. This *locus* consist of repeats of 24 base-pair and its diversity is attributed to deletions and duplications of the repeat due to point mutations. This method can be used successfully to investigate both molecular evolution and hospital outbreaks of MRSA.

This analysis was carried out on all the 147 collected strains, and results were obtained for 30 isolates due to the use of a public database. One of the most represented type was t2167, followed by t127 as well as t002 and t008.

Performing *spa* typing was possible to assess strain sharing as well as the presence of a particular type indicating a LA-MRSA. This *spa* type, t127, was shared

by three CF patients: two siblings and another patient. MLST analysis was the necessary further step to properly characterize collected strains.

MLST analysis results

Basing on the nucleotide sequences of seven different housekeeping genes, it is possible to assign a sequence type (ST): isolates are defined by the alleles present at the seven loci (the allelic profile), and each unique allelic profile is assigned an ST[38]. Isolates with the same ST, therefore, have identical sequences at all seven MLST loci and are considered to be members of a single clone. Data analysis allows to provide an evolutionary picture of isolates collection compared to already known strains. MLST is an excellent tool to investigate the evolution of MRSA: a nucleotide sequence-based approach for the unambiguous characterization of bacterial isolates and other organisms. The aim of MLST is to provide a portable, accurate, and highly discriminating typing system. This approach allows a comparison of results with global epidemiology data, assessing worldwide strain diffusion.

MLST profiles of 102 MRSA and SA strains were obtained. PICU predominant clone appeared to be ST128-MRSA-I, and epidemic clone named Southern Germany clone. This particular clone was shared by four patients in 2010, 2011, and 2012. The other representative clone emerged during this study was ST22-MRSA-IV, an already described CA-MRSA clone.

CF patients showed the presence of different clones. Eight out of 49 patients were infected by strains belonging to Southern Germany clone, 4 to ST5-MRSA-I, 3 to ST8-MRSA-IV, one to ST45-MRSA-IV or Berlin clone. One patient harbored ST239-MRSA-III or Brazilian-Hungarian clone, two patients ST1-MRSA-III. Two patients shared ST152-MRSA with undetermined SCC*mec*, and the USA300 variant represented by ST8-MRSA-V. Moreover, ST398 (traditionally a LA-MRSA) was found in two different patient, one of which harbored SCC*mec* type V.

15 out of 30 PVL-positive strains were genotyped by means of MLST, showing the presence of epidemic lineages such as USA300, ST5-MRSA-IV or Pediatric clone, and ST30-MRSA-IV or Southwest Pacific clone (SWP).

Virulence factors characterization

Several virulence factors were tested, to obtain a more accurate characterization of collected strains.

ACME typing results

ACME is a novel genetic element able to confer an enhanced ability to grow and survive within the host[29]. This genomic island appears to have been transferred into USA300 from *Staphylococcus epidermidis*. Strains able to acquire this genetic element belong to USA300. ACME contains two gene clusters (*arc* encoding a secondary arginine deiminase system and *opp*-3 encoding an ABC transporter) that are homologous of virulence determinants in other bacterial species, highlighting their importance.

Two MRSA-IV strains belonging to PICU and 10 belonging to CF patients were tested for ACME presence. Nineteen CoNS isolates were tested. None of the PICU tested strain was positive for ACME. Only two CF strains belonging to the same patient chronically infected tested positive for ACME type I. CoNS showed a higher grade of variety: 7 out of 27 strains belonged to type I, 3 to type II and the last one was a type III; the remaining strains tested negative for ACME.

agr typing results

This *locus* controls the expression of most virulence factors. The *agr* quorum-sensing system represses the expression of surface proteins, including *spa* and upregulates the production of toxins, such as α-hemolysin and protease. The *agr* system is comprised of two divergent transcripts, RNAII and RNAIII. RNAII carries

four genes, *agrDBCA*, which are dedicated to the synthesis, sensing, and processing of the quorum-sensing autoinducing peptide (AIP) required for the activation of the RNAII promoter. The divergent transcript RNAIII, activated in response to RNAII *via* AgrA, is a regulatory RNA molecule expressed maximally during the post-exponential phase and controls virulence gene expression at the transcriptional and translational levels, the latter via an antisense mechanism. A polymorphism in the aminoacid sequence of an auto-inducing peptide and its receptor is the basis on which SA can be divided into four groups.

All collected strains were tested for *agr* grouping; Table 14 illustrates the distribution of types among the collected strains.

Table 14. Distribution of *agr* types among the collected isolates.

Ward	Number of *agr* type I	Number of *agr* type II	Number of *agr* type III	Number of *agr* type IV
PICU_2010	6	2	1	6
PICU_2011	2	1	0	3
PICU_2012	4	1	1	0
CF_2010	11	0	2	6
CF_2011	7	0	4	8
CF_2012	6	0	5	6

Association between *agr* and clinical manifestations were demonstrated[30], as well as with nasal colonization[44]. Data concerning both chronic and first infections are still scanty, so that it's difficult to highlight eventual correlation between infection and *agr* type.

IEC typing results

Eleven PICU and 41 CF MRSA strains were tested for IEC typing. PICU strains were type A (4 out of 11), and B (one strain). Five strains tested positive for some IEC genes: *chp, sak, sea, hlb*. 40 CF strains were tested for IEC presence, 5 of which belonged to type A, 2 to type B, 10 to type D, 3 to type E, 3 to type F, and 10

tested positive for only some genes. At this stage, it is impossible to highlight a correlation between chronic or first infection in CF patients and IEC, or to compare IEC types of MRSA belonging to patients attending to these two wards.

PVL testing results

PVL is one of the major virulence factors characterizing SA and MRSA. Due to the importance of the clinical manifestations caused by PVL, a correct isolation and identification of producer strains appear to be crucial. Soft skin infections are currently poorly investigated, causing the loss of strains responsible for a dramatic underestimation of PVL producer strains, leading to a weak knowledge of diffusion and epidemiology PVL-positive strains. Basing on clinical symptoms, 30 PVL-positive SA and MRSA strains were collected from DPMW during the study period. Moreover, PVL presence was tested on 25 out of 34 PICU strains, and 38 out of 83 CF strains. All PICU strains tested negative, whilst one CF strain tested positive. PVL gene partial sequencing was performed, and this 500 bp portion allowed to set-up a diagram describing differences between DPMW and the CF strain, as shown in Figure 5.

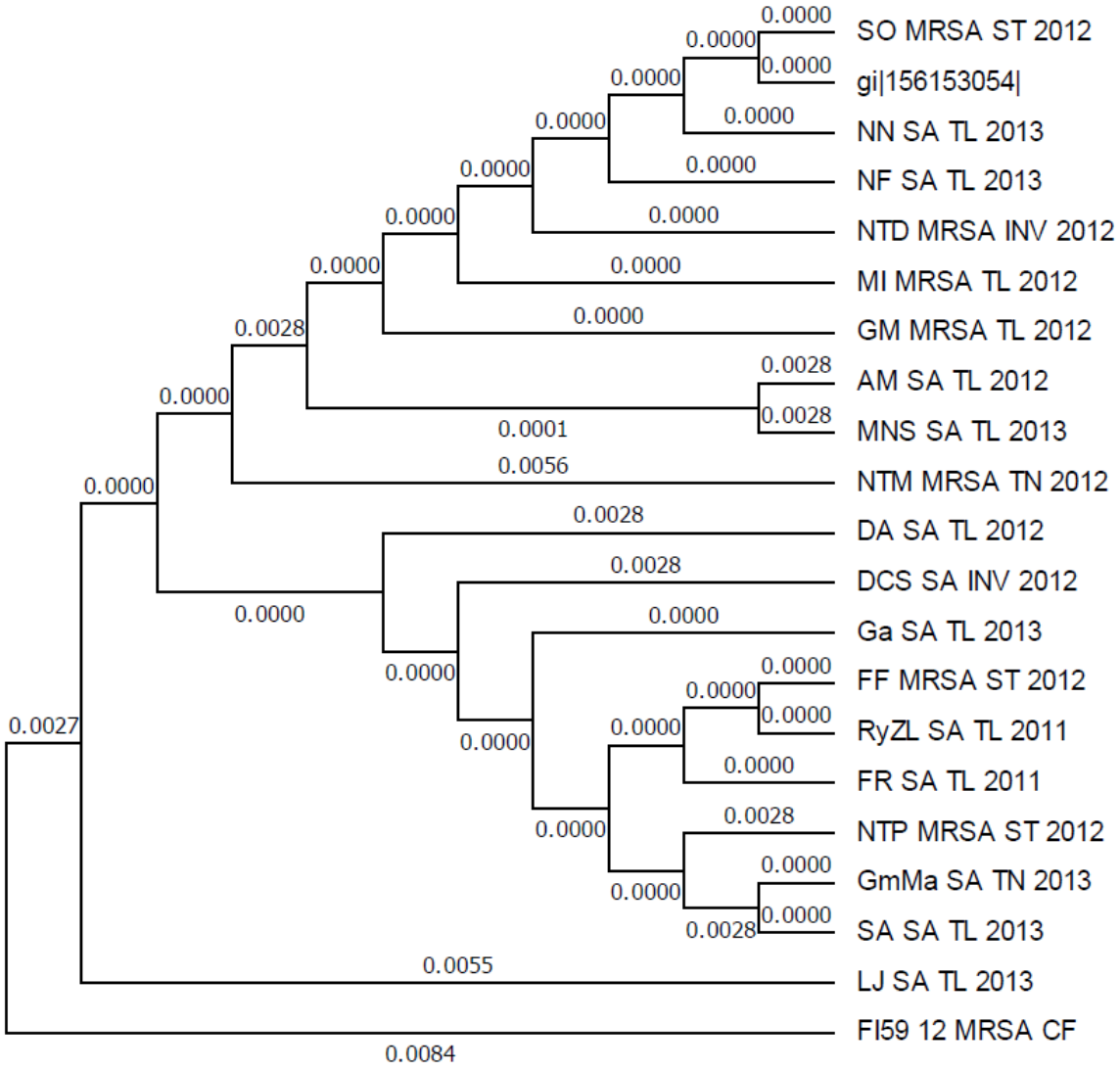

Figure 5. Diagram of partial PVL sequences, and a reference sequence (gi|156153054|.).
ST: soft tissue infection; INV: invasive infection; TL: sample from lesion; TN: nasal swab.

FI59_12 represents the isolate belonging to CF patient, chronically infected by MRSA since 2006. One MRSA strain/year was tested for PVL, highlighting the presence of PVL genes in all these isolates. PVL partial sequences were used to built another diagram to examine if some changes occurred, as shown in Figure 6.

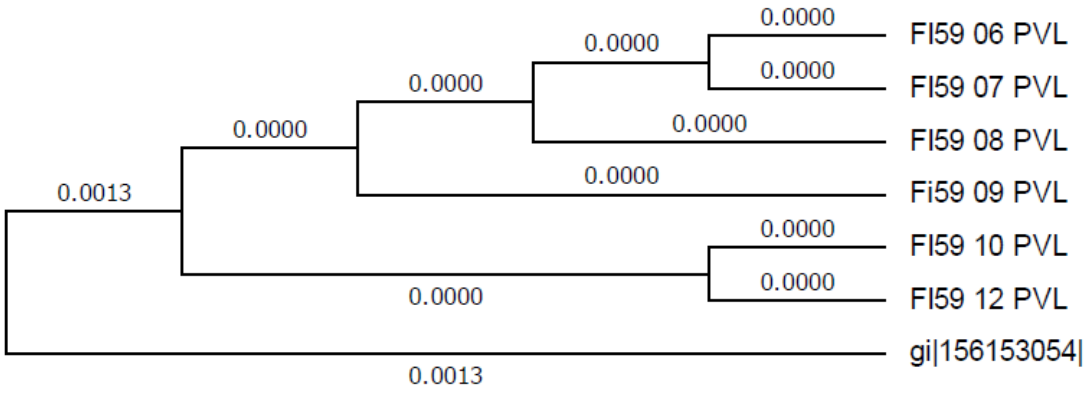

Figure 6. Diagram of PVL partial sequences belonging to a CF patient, and of the reference sequence gi|156153054|.

Partial PVL sequences of CF isolates appeared to be really conserved, according to the presence of a chronic infection. This may be the first report of a chronic infection sustained by a PVL-positive MRSA strain. Evolutionary analysis was set up with standard parameters following a recently published protocol[40] with bootstrap test. These data should not be taken into account for a phylogenic study due to their high-grade of similarity and, thus, conservation. Despite that, Figure 5 and 6 are suitable to illustrate that PVL partial sequencing are strongly conserved, being the more divergent sequence the one belonging to CF strain responsible for pulmonary infection. Moreover, this particular sequence appears to be highly conserved since 2006.

Molecular typing of both SA and MRSA PVL-positive strains gave interesting results. MRSA strains belonged to SWP ST30-MRSA-IV, and infected a family recently immigrated from Brazil. Two patients, a mother with recurrent soft tissue infections and her six-years old daughter, shared ST8-MRSA-IV clone. A 15 years-old boy had a trip in Venezuela and came back with a soft-tissue infection due to ST22-MRSA-IV or UK EMRSA-15. A one-year old boy reported an infection caused by ST5-MRSA-IV or Pediatric clone.

Chronic MRSA infection in ICU

This three-year survey allowed to monitor chronically infected patient, and to highlight the presence of epidemic lineages in ICU. Three patients were monitored during the entire study period, being positive for the presence of the same strain. One patient was infected by Southern Germany clone, another by ST22-MRSA-IV, and the last one maintained the strain, which was characterized by an unknown genotype.

First MRSA infection in CF patients

Since early eradication therapy for MRSA is still under debate[17], data concerning first infection are really important. In particular, determining if particular clones are cleared or able to establish a chronic infection should have a great relevance. Twenty-five strains responsible for first infection in CF patients were collected: 13 belonged to HA-MRSA, and 12 to CA-MRSA. CF patients were believed to have exposure to HA-MRSA more than CA-MRSA infection due to the clinical practices they usually undergo, but in this case the two groups are almost equal. Median age of patients infected by HA-MRSA is 21.3 years (range 2-49), CA-MRSA infected patients showed a median age of 13.9 years (range 0.4-35). This difference is not quite statistically significant (t-test, p 0.0830).

To date, five first infections became chronic: one CA-MRSA, three HA-MRSA, and a LA-MRSA infection. All but two of these strains belonged to epidemic lineages: Southern Germany clone, ST152-MRSA with undetermined SCC*mec*, ST398-MRSA-V. Basing on these data, the possibility that the establishment of a chronic infection could depend on the better fitness characterizing epidemic clones appear to be more likely than other hypothesis.

Chronic infection and CF patients

Chronic MRSA infection in CF patients was demonstrated to have serious influence on survival[20]. Fourteen patients were chronically infected by MRSA at the time of the study. It was possible to characterize MRSA strains belonging to ten out of 14 chronically infected patients from first colonization until 2012. Figure 7 summarizes chronic patients monitored since first infection: there was only one strain replacement, and each infection is caused by a known epidemic clone.

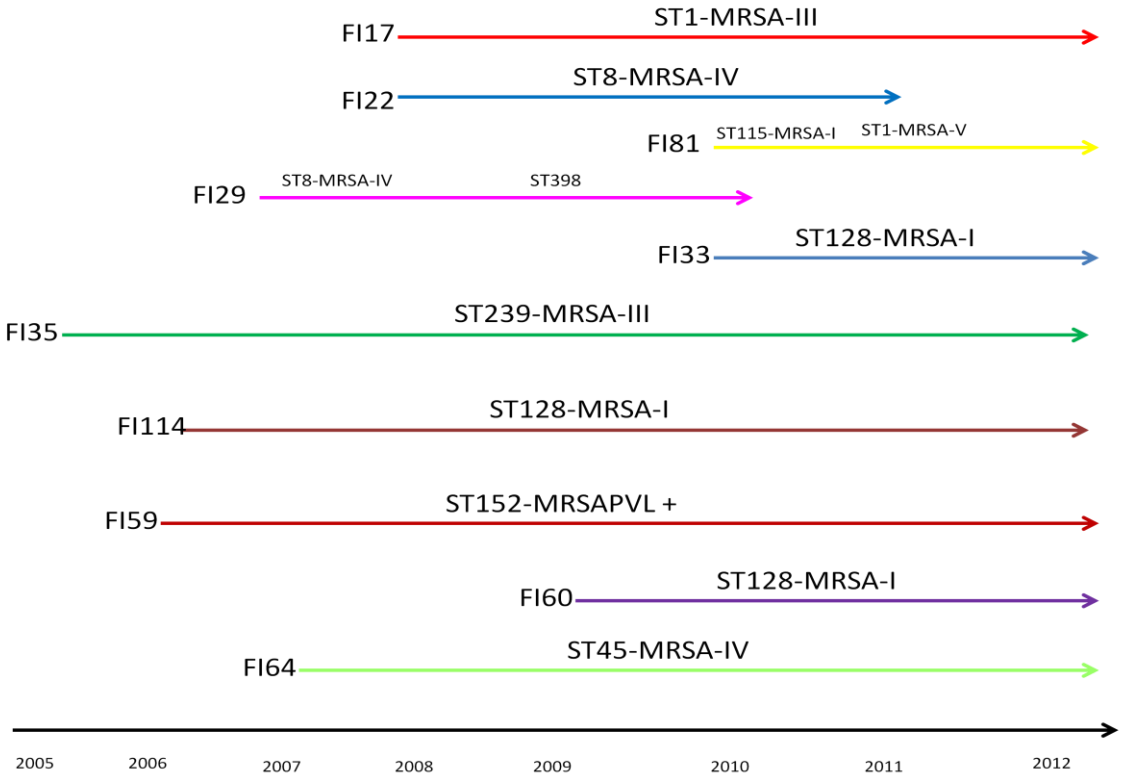

Figure 7. From first to chronic infection.

In one case only first infection strain was replaced after two years by a known epidemic clone establishing another chronic infection. In general, epidemic lineages are rarely cleared or replaced by another strain.

Two cases of siblings are part of chronically infected patients. The first two were adult, and shared Southern Germany clone until one of them died after a respiratory

failure. The other two are younger and still sharing a MRSA strain characterized by *spa* type 127, associated with LA-MRSA.

CF patients and lung transplantation

Lung transplantation represents a therapeutic option for the end stage of pulmonary disease. Patients undergoing this clinical practice are usually infected by MDR strains including mucoid *Pseudomonas aeruginosa*, other Gram-negative rods and molds such as *Aspergillus species*. MRSA is a part of this *plethora* of pathogens, and its effective role in pathogenic process is difficult to determine. Twelve CF patients who underwent lung transplantation were analyzed, 8 males and 4 females, median age 30.8 years (range 20-43 years-old). The chosen follow-up period started in 1984 and ended in 2012, allowing to monitor the presence of pathogens before and after lung transplantation. 958 culture tests results before and after lung transplantation were examined, showing the presence of several different pathogens as shown in Figure 8.

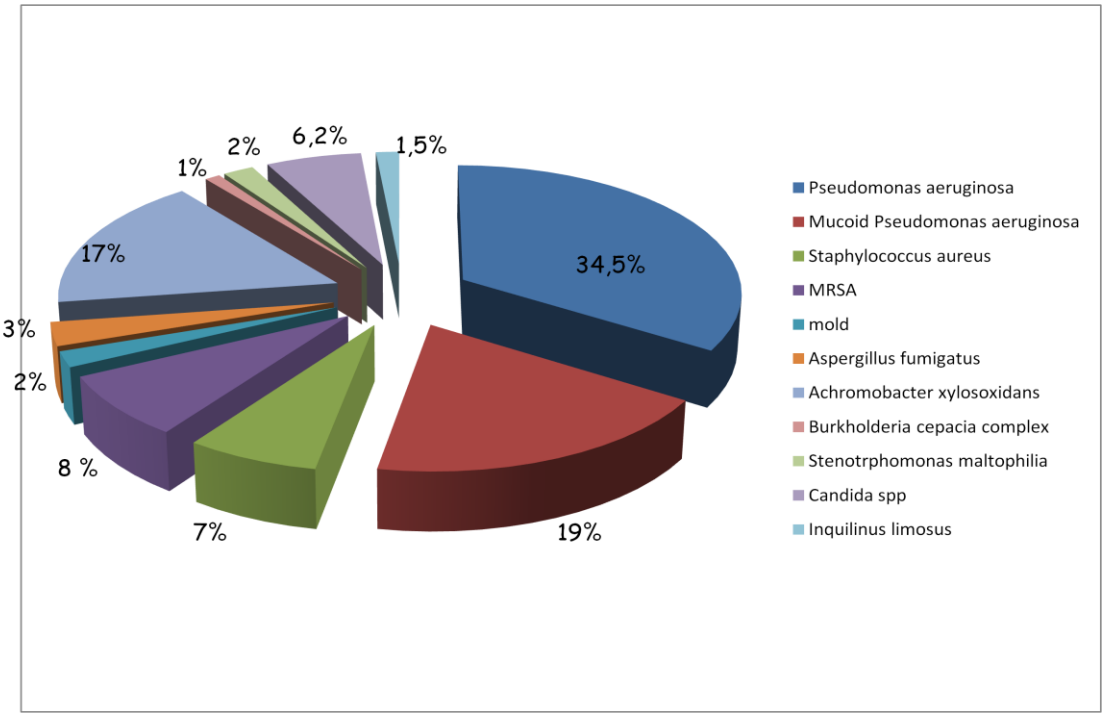

Figure 8: Pathogens recovered in respiratory samples of CF patients before and after lung transplantation.

Five out of 12 patients were chronically colonized by MRSA and Gram-negative *species* before lung transplantation, as shown in Figure 9.

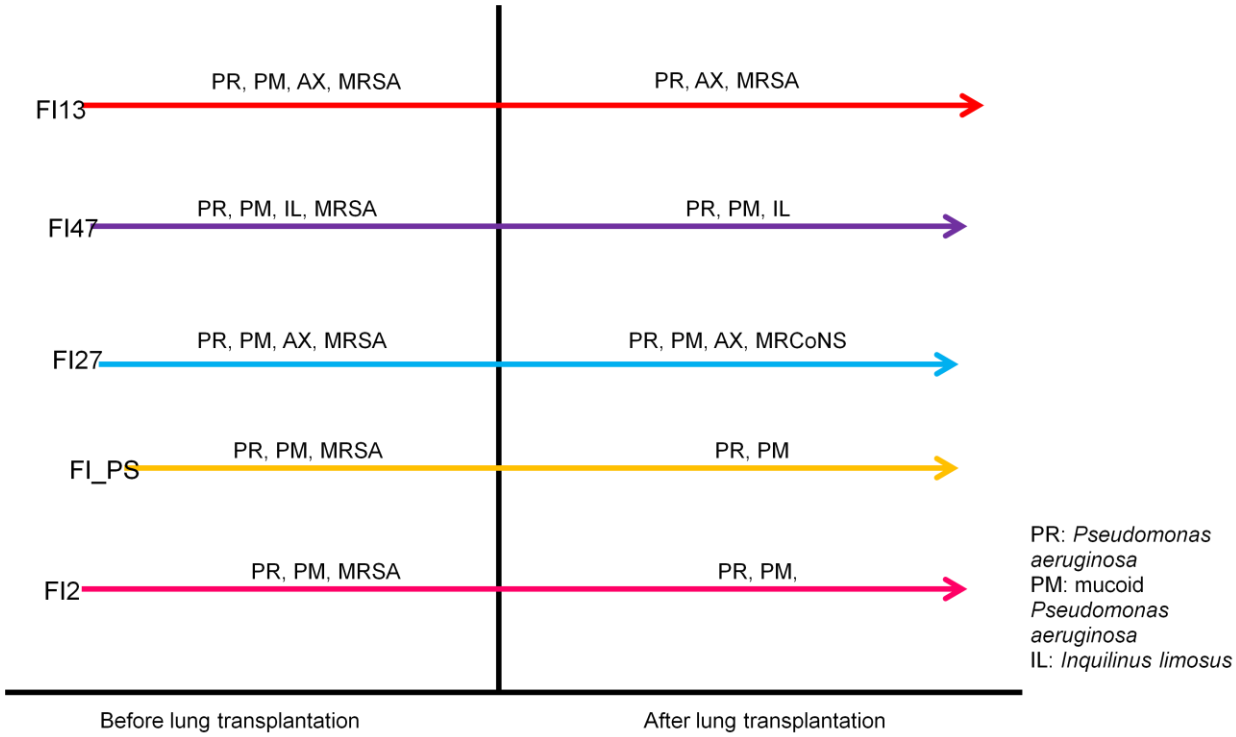

Figure 9: Chronic infection before and after lung transplantation.

MRSA strains infecting patients undergoing lung transplantation belonged to ST128-MRSA-I or Southern Germany clone, ST5-MRSA-I or UK EMRSA-3, ST8-MRSA-IV. All MRSA strains were cleared after lung transplantation, since patient FI13 showed MRSA before and after lung transplantation, but the genotypes of such strains were different, suggesting a replacement. Gram-negative rods such as *Pseudomonas aeruginosa* and *Inquilinus limosus* were maintained.

Discussion

Since its discovery, SA represented a serious threat for public health due to its great capacity to develop antimicrobial resistance in a short period of time. Four different waves of resistance were recently described by Chambers[1]. Wave 1 started after penicillin introduction, and was caused by a PVL-producer strain. Wave 2 was started by Archaic clone (ST250-MRSA-I) after the introduction of methicillin into clinical practice. Wave 3 began in 1970s in healthcare settings, and was caused by MRSA harboring the novel SCCmec types II and III. Vancomycin usage for treatment of such infections led to emergence of VISA strains. Wave 4, started in 1990s, marked the emergence of CA-MRSA[1].

Unfortunately the present study was set up and started just before VITEK acquisition and EUCAST venue, becoming impossible to properly compare data obtained in 2010, 2011 and 2012 with the exception of the lack of resistance to glycopeptides or linezolid. A recent study, dating from 2012, compared EUCAST and CLSI and their different effects on rods responsible for acute infection, stating that minor discrepancies occurred between the two methods[45]. In spite of that study, the choice to compare antimicrobial susceptibility tests obtained by means of manual techniques and automated methods appear inappropriate, thus these results represents only a description.

SA and MRSA infections represent a global public health issue even due to the high cost of management. MRSA infections cause societal costs such as expenses caused by extension of hospital stay, additional diagnostics or therapeutic procedures and therapies; moreover there are indirect social costs, like loss of productivity, long-term disability and mortality. Other financial repercussions include costs for containment of outbreaks, and changes of empirical antibiotic prescribing habits[46]. Nasal colonization still represent a risk factor for MRSA infection[25], and the

primary natural *reservoir* of SA. Moreover, domestic animals, livestock and fomites may serve as adjunctive *reservoirs*[2]. The anterior nasal mucosa has traditionally been thought to be the most frequent site for the detection of SA colonization of healthy carriers, although recent studies demonstrated that screening at multiple body sites are more accurate[2]. Three patterns of asymptomatic SA carriers were individuated in the general population: persistent carriers, intermittent carriers and non-carriers who rarely harbor SA. The asymptomatic carriage of SA can be dynamic, and there were recently described asymptomatic colonized patients harboring more than one MRSA strain, and only a few of these patients had persistent colonization at the same anatomical site[2]. Moreover, a recent Portuguese study illustrated the presence of the major MRSA epidemic clones on bus[47] allowing to consider surfaces as a vehicle of MRSA acquisition, while another study described waterborne transmission routes for SA and MRSA[48].

Due to the lack of a commonly accepted procedure for MRSA screening, and cost-effectiveness benefits of such practice remains under debate, this Pediatric hospital chose to screen patients in case of necessity, e.g. outbreaks or particular cases. Healthcare givers of ICU wards are usually screened once/year, giving positive results in two cases, being the colonizing MRSA strains still under investigation.

During this study, nasal screening was usually used in case of PVL infection: household contacts of positive patients were screened to search for *reservoir*. This procedure allowed to determine several cases of intra-familial transmission. In one case it was documented the spreading of a PVL-positive SWP[49], from the father showing a recurrent soft tissue infection, to the mother showing a nasal colonization; their infant developed an invasive infection with a pyopneumothorax. It was possible to document other two intra-familial cases, concerning mothers with reported recurrent soft tissue infections, transmitted to their childs. A PVL-positive SA was shared by two siblings, colonizing one and infecting the other. This situation was reflected in two different cases, the characterization of which is still ongoing. Soft

tissue infections should be taken into account, to avoid the spread of potentially dangerous PVL-positive strains.

Hospital-acquired infections represent an important global healthcare problem, and PICU patients are traditionally at risk for pulmonary infections acquisition, especially in case of assisted ventilation. Almost all PICU patients monitored during this study already underwent a tracheotomy, and maintained the same MRSA strain during the whole study period, suggesting the possibility of a chronic infection. Those patients were admitted to the ICU with diagnosis compatible with a previously acquired infection. Although the small number of strains is not helpful defining the presence of endemic strains, ST128-MRSA-I or Southern Germany clone is the most represented epidemic lineage in PICU, according to recently published Italian data demonstrating that this clone account for 57% of studied isolates[3]. Interestingly, CA-MRSA strains were also found: ST22-MRSA-IV PVL-negative, and ST148-MRSA-V PVL-negative. ST22-MRSA-IV represent a known epidemic clone, while ST148-MRSA-V belongs to CC5; strains with the same genetic background but PVL-positive were sporadically isolated in Germany and Abu Dhabi[9].

During a previous prospective multi-center study involving CF center from all-over Italy and 178 patients[4], SCC*mec* types were distribute as follows: 49.5% type I, 1.2% type II, 3.9% type III and 31.4% type IV. Several chronically infected patients were monitored until 2012, becoming part of the present study. Changes occurred in SCC*mec* distribution, probably due to the differences in number of examined strains and geographic provenience. However, SCC*mec* type I remained the most represented HA-MRSA type. Although SCC*mec* type V appeared in 2010, CA-MRSA percentage appears to be comparable with the previous study, being 29.6% in 2010, while in 2011 was 25.9% and 27.2% in 2012. Three strains belonging to the same patient showed an undetermined SCC*mec* type, testing positive for different SCC*mec* types using different methods, thus remaining undetermined. Epidemic clones were found in examined CF patients, particularly those chronically

infected. Chronic infection represent one of the main aspect of MRSA and CF: longitudinal study comprehending genetic background of isolate strains are rarely performed. To date, data obtained during this study represent an important starting point to understand molecular epidemiology of MRSA infecting CF patients. The importance of these findings is connected to the possibility to monitor patients consecutively for years, to have a continue surveillance on strains and their behavior. Eleven patients were monitored from first infection, as shown in Figure 7, three of which were infected by a CA-MRSA epidemic clone, which demonstrated the capability to establish chronic infections.

Some unusual genetic background are shown in Figure 7: such as ST152 and ST8-MRSA-V. ST152 is not common in CF Italian patients, it was recently described in 2010 causing infective endocarditis[50]. Another patient showed to be chronically infected by the same strain, but PVL-negative. ST152 was recently described in Africa[51], and previously in Denmark[52], ex-Yugoslavia and Switzerland[53]. African isolates demonstrated to be SA, not MRSA, whilst strain from Denmark was MRSA with an impossible-to-type SCC*mec*. ST152 strains isolated during a seven-years chronic CF infection are characterized by *agr* type I, as previously described strains[53]. The presence of a PVL-negative homologue strain may be explained with the excision of PVL, which is carried on a phage[54] and thus to be excised. More studies are required to confirm this hypothesis.

Another interesting case is represented by two siblings chronically infected by *spa* type t127, ST1, PVL-negative MRSA. This particular genotype was described as a porcine infective agent[55]. Noteworthy, another pig-related strain is responsible for a chronic infection in a CF patient: ST398-MRSA-V. This LA-MRSA was described in the Netherlands in people with frequent contact with farm animals; infections were only sporadically reported in farm workers and their family members[56]. The presence of this strains represents an important change in MRSA Italian epidemiology of CF patients.

Five CF patients were monitored before and after lung transplantation. The role played by MRSA in lung decline is not determinable due to the co-infection with Gram-negative species characterized by an high impact on lung function, such as *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex. Although these species, including the rarely isolated *Inquilinus limosus* were isolated before and after lung transplantation, MRSA disappeared after lung transplantation. An explanation may be found in nasal *sinus* where *Pseudomonas aeruginosa* and other pathogens including MRSA are harbored. A recent report[57] demonstrated persistence of identical *P. aeruginosa* genotypes in CF upper airways prior to and after lung transplantation, underlining risks of descending colonization of transplanted lungs with *P. aeruginosa*. MRSA presence was not cited, suggesting two different *scenario*: *P. aeruginosa* strains physically occupy nasal cavities avoiding the co-colonization with MRSA; those patients were not colonized by MRSA, thus after lung transplantation descending colonization appeared unlikely.

The need of more detailed studies to shade a light on this question appears clear.

Virulence factors represent an important aspect in SA and MRSA pathogenesis, and these species are able to produce an *armamentarium* of virulence factors including structural and secreted products, the regulation of which play a central role in pathogenesis played by *agr* quorum sensing system[58]. This system was recently investigated to determine its real importance in USA300 pathogenesis, showing that in USA300 there is an up-regulation of virulence factor genes comparing to HA-MRSA[59]. At the moment obtained data should be integrated and correlated with clinical data, in order to assess correlation with patients' *status*. Table 14 showed *agr* typing of collected strains; both PICU and CF chronically infected patients maintained the same *agr* type during the study period, suggesting that rearrangements are not common in a successful strain capable of a stable infection.

IEC proteins are considered as virulence factor as well, being responsible for counteracting innate immunity. Those determinants are frequently encountered in

different MRSA genetic background[9], but the effectiveness of their role remains unclear. Chronically infected patients tend to conserve the same IEC type, as well as *agr* type, but due to the number of tested strains is not possible to assess a correlation between IEC types and chronic infection. All types are represented both in chronic and in cleared first infections, suggesting the need of further studies on a larger number of MRSA isolates.

ACME presence was tested in MRSA strains with compatible genetic background, and on all CoNS strains. Only one CF MRSA strain tested positive for ACME. The remaining 11 positive strains were CoNS, belonging to different ACME types. CoNS strains analysis did not give the expected results, being difficult to define CoNS as a virulence factor *reservoir*. Thus, CoNS role should be more carefully investigated with a larger number of strains, to confirm or to contest these preliminary data.

PVL is a further virulence factor representing an important chapter in SA and MRSA history of virulence and pathogenesis. Several different bacteriophages encode PVL, and recent studies investigated the possibility of a correlation between phages and MRSA lineages[54]. Each SA lineage, and therefore MRSA, had *lineage*-specific restriction-modification systems, causing their independent evolution[11]. Figure 5 and 6 show the evolutionary distances between different PVL partial sequences. It is interesting to note that pulmonary CF strains are in a separated branch comparing to the other strains, characterized by a different genotype. Both SA and MRSA PVL-positive strains showed highly represented genetic backgrounds, such as ST22, ST8 (both MRSA and SA), ST5, being ST8 the most represented clone. CF strain belonged to ST152, a drastically different background described as SA in France, Togo and French West Indies[60]. These data concerning epidemiology of PVL-positive strains confirmed the theory basing on which there is an association between PVL and successful lineages[54]. Moreover, the problem of unknown epidemiology and diffusion of PVL-producer strains spreading in the community remains an important issue.

This study allowed to collect strains and data for three years, being possible to investigate various aspects of 149 SA and MRSA strains. Molecular epidemiology delineated a picture representing all the major MRSA epidemic clones infecting CF patients together with new LA-MRSA strains isolated for the first time in Florence. PICU MRSA percentages confirmed good practice of infection control and surveillance, and the peculiarity of this third-level Pediatric hospital allowed to monitor MRSA chronically infected PICU patients. Surprisingly, these patients were infected both by HA- and CA-MRSA, although the more represented clone remained Southern Germany. CoNS isolates remained an un-resolved question, and their effective role should be investigate more intensively.

Molecular epidemiology of PVL-positive strains revealed the presence of the most represented genetic backgrounds, shared by both MRSA and SA.

It was interesting to note intra-familiar spreading starting from nasal colonization or soft tissue infections, with different repercussion on clinical status of pediatric patients, suggesting to pay attention to recurrent furuncles and soft tissue infections. New genotypes emerged, leading to the need of more accurate investigations involving phage insertion sites and complete genomes, to determine the origin and evolution of such strains, other than independent PVL acquisition in different lineages.

This three-year study may be a starting point to examine the effectiveness of infection control measures as well as therapies and antimicrobial usage in a third-level Pediatric hospital. Diffused clones may have been influenced in antimicrobial resistance by different therapeutic approaches, so that global epidemiology may represent a further advantage to set up improved therapies. Moreover, particular clones may be associated with chronic infections whilst other clones to transient ones, may be requiring different antimicrobial strategies. In conclusion, more studies are needed to evaluate the possible association between particular MRSA or SA

clones and clinical conditions in order to improve treatment strategies and possibly set up *ad hoc* therapeutic options.

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