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Epidemiology, molecular and phenotypic typing of Methicillin Resistant Staphylococcus aureus (MRSA) strains isolated from multi-drug resistant screening program and blood culture.

S.S.D. MED/07

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Epidemiology, molecular and phenotypic typing of Methicillin Resistant Staphylococcus aureus (MRSA) strains isolated from multi-drug resistant screening program and blood culture-Liliana Galia

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A Mamma
A Nonna Murru
A Pietruccia
Nel mio cuore.

I. SOMMARIO

Staphylococcus aureus è un comune batterio presente sulla cute e sulle membrane mucose nel 20-30% delle persone sane. Talvolta può causare infezioni nell'uomo, solitamente infezioni della cute e suppurative a livello locale, ma anche infezioni più gravi a carico di diversi distretti dell'organismo. Alcuni ceppi di questo batterio, tuttavia, hanno sviluppato una resistenza agli antibiotici ß-lattamici, tra cui le penicilline, che sono utilizzati nella cura di numerose infezioni. Questi ceppi sono noti con il nome di *Staphylococcus aureus* meticillino-resistente (MRSA).

L'MRSA si trasmette all'uomo prevalentemente mediante contatto diretto con la persona infetta o con strumenti medici e apparecchiature medicali. L'MRSA è problematico soprattutto negli ospedali, dove i pazienti con un sistema immunitario indebolito sono più esposti al rischio di infezione rispetto alla popolazione generale.

La ricerca in questo ambito si occupa dello studio di nuove strumenti diagnostici e terapia salvavita che permettano di individuare velocemente ceppi multi-resistenti, in campioni clinici riducendo i tempi di refertazione, per dare garanzia di una corretta e immediata terapia.

Uno dei primi obiettivi di questo studio su ceppi di MRSA solati da campioni clinici è la tipizzazione molecolare della proteina A (specie-specifica) responsabile del legame con la porzione Fc delle immunoglobuline, rendendole cosi inattive al fine di individuare le caratteristiche epidemiologiche e molecolari di questi ceppi in pazienti colonizzati/infetti. La tipizzazione della proteina A è stata fatta tramite l'utilizzo della tecnica spa-typing.

Abbiamo inoltre caratterizzato la *cassetta cromosomica mobile SCCmec* e le rispettive correlazione con i diversi profili di antibiotico resistenza tra 6 spa-type più rappresentativi quali*: t032 CC22, t1036 CC22, t1214 CC22 ,t022 CC22, t041 CC5 ,t121 CC8,* ricercati su 135 ceppi di MRSA provenienti da tamponi faringei, rettali e emocolture di cui rispettivamente 94 da tamponi rettali e faringei e 41 da emocolture.

128 ceppi sono stati ritrovati appartenenti al tipo *SCCmec IV* e 7 appartenenti al tipo *t041* appartenenti a *SCCmec I,* rispettivamente tutti tutti di classe B. Abbiamo notato inoltre che nei 41 ceppi isolati da emocolture mancano gli *spa-type t1036 e t022* considerati tra i "*6 rappresentativi*" ritrovati in questo studio. Il nostro pensiero, per le ricerche future sarà quello di aumentare il numero di ceppi isolati da emocolture.

Abbiamo individuato un nuovissimo *spa-type t16026 CC22* sottomesso nell'anno 2016.

Solo 29 ceppi su 135 testati sono sensibili all'eritromicina ma gli altri ceppi hanno un alto livello di resistenza ai fluorochinoloni e macrolidi. Questo studio ha dimostrato inoltre che i valori di Mic ed E-test del Ceftobiprole (Basilea Pharmaceutica), una nuova cefalosporina di quinta generazione, designata a trattare le infezioni associate a MRSA, ha valori che rientrano nei breakpoint di sensibilità secondo le linee guida EUCAST. Questo è di notevole importanza poiché è, fino ad ora l'unica cefalosporina sensibile. La descrizione della suscettibilità agli antibiotici e dei tipi di *spa* è molto dettagliata e fornisce informazioni rilevanti sull'epidemiologia molecolare e sui fattori di virulenza di MRSA in Italia. Utilizzando la tecnica dello spa-typing, quindi, abbiamo potuto individurare la presenza di un clone principale E-MRSA 15 in questa parte dell'Italia.

Possiamo quindi concludere che i ceppi di MRSA isolati presso i reparti di anestesia e rianimazione, terapia intensiva, medicina interna, oncologia, pediatria, centro ustioni del policlinico di Verona "G. Rossi" hanno un profilo molecolare attribuibile per definizione ai ceppi comunitari CA-MRSA ma un profilo di multi- resistenza tipico dei ceppi ospedalieri e solo 6 ceppi presentano il gene *pvl.*

Quindi possiamo ipotizzare ad un cambiamento epidemiologico e microbiologico di ceppi di MRSA isolati in questa parte dell'Italia e si potrebbe inoltre proporre un cambiamento di definizione dei CA-MRSA.

Un altro obiettivo è stato quello di studiare una Real-time PCR che rilevasse rapidamente la resistenza alla meticillina , il fattore di virulenza PVL direttamente da un campione clinico e il gene *nuc* (*specie -specifico*) . Questa tecnica presentata in questo studio può identificare e differenziare MRSA, MSSA, resistente alla meticillina, Stafilococchi negativi alla coagulasi (MR-CNS) con un significato diagnostico e terapeutico di notevole importanza e una riduzione dei tempi di refertazione dalle 48h ad 1h 30 minuti.

Questa tecnica automatizzata e standardizzata ha come risultato una concordanza del 100% con le tecniche molecolari standard , il 100% di specificità e una sensibilità di 514 UFC/ml.

II. ABSTRACT

Staphylococcus aureus is a common bacterium found on the skin and mucous membranes in 20-30% of healthy people. Sometimes it can cause infections in humans, usually skin infections and suppuratives at the local level, but also more serious infections affecting different parts of the body. Some strains of this bacterium, however, have developed resistance to ß-lactam antibiotics, including penicillins, which are used in the treatment of numerous infections. These strains are known as *Methicillin-resistant Staphylococcus aureus* (MRSA*).* MRSA is transmitted to humans mainly through direct contact with the infected person or with medical instruments and medical equipment. MRSA is problematic especially in hospitals, where patients with a weakened immune system are more susceptible to infection than the general population. Research in this area deals with the study of new diagnostic tools and life-saving therapy that allow the rapid identification of multi-resistant strains, in clinical samples, reducing reporting times, to guarantee correct and immediate therapy. One of the first objectives of this study on MRSA strains solved by clinical samples is the molecular typing of protein A (species-specific) responsible for binding to the Fc portion of immunoglobulins, thus rendering them inactive in order to identify the epidemiological and molecular characteristics of these strains in colonized/infected patients. Protein A typing was done by using the spa-typing technique. We have also characterized the *SCCmec* mobile chromosomal cassette and the respective correlations with the different antibiotic resistance profiles among 6 more "representative" *spa-types* such as: *t032 CC22, t1036 CC22, t1214 CC22, t022 CC22, t041 CC5, t121 CC8*, sought from 135 strains of MRSA from pharyngeal, rectal and blood cultures of which 94 from rectal and pharyngeal swabs and 41 from blood cultures respectively. 128 strains were found belonging to the *SCCmec IV type* and 7 *t041* strains belonging to *SCCmec I*, all of class B respectively. We also noted that in the 41 strains isolated from blood cultures, the *t1036* and *t022* spa-types considered to be among the "6 representative " found in this

study are missing. Our think, for future research will be to increase the number of isolates isolated from blood cultures. We have identified a brand new *spa-type t16026* CC22 submitted in the year 2016. Only 29 out of 135 tested strains are sensitive to erythromycin but the other strains have a high level of resistance to fluoroquinolones and macrolides. This study also showed that the Mic and E-test values of Ceftobiprole (Basilea Pharmaceutica), a new fifth-generation cephalosporin, designed to treat infections associated with MRSA, have values that detect sensitivity breakpoints according to EUCAST guidelines. This is of considerable importance since it is, until now, the only sensitive cephalosporin. The description of susceptibility to antibiotics and spa types is very detailed and provides relevant information on the molecular epidemiology and virulence factors of MRSA in Italy. Using the *spa-typing* technique, therefore, we have been able to identify the presence of a main E-MRSA 15 clone in this part of Italy. We can therefore conclude that the MRSA strains isolated in the departments of anesthesia, intensive care, internal medicine, oncology, pediatrics, burns center of the "polyclinic G. Rossi" have a molecular profile attributable by definition to the CA-MRSA community strains but a multi-resistance profile typical of hospital strains with only 6 strains presenting the *pvl gene*. Thereore we can hypothesize an epidemiological and microbiological change of MRSA strains isolated in this part of Italy and it could also propose a change in the definition of CA-MRSA. Another objective was to study a Real-time PCR, which quickly detected the resistance to methicillin, the virulence factor PVL directly from a clinical sample and the *nuc gene* (species-specific). This technique presented in this study can identify and differentiate MRSA, MSSA, resistant to methicillin, coagulase-negative Staphylococci (MR-CNS) with a significant diagnostic and therapeutic significance and a reduction in reporting times from 48h to 1h 30 minutes.

This automated and standardized technique results in 100% agreement with standard molecular techniques, 100% specificity and a sensitivity of 514 CFU / ml.

Staphylococcus aureus is a major human pathogen that causes a wide range of clinical infections. It is the main cause of infectious bacteremia and endocarditis, as well as osteo-articular tissues, skin and soft tissue, and prosthetic devices. Over the last two decades there have been two net changes in the epidemiology of *S. aureus* infections: first, an increasing number of infections associated with healthcare, particularly observed in infectious endocarditis and prosthetic device infections, and second, a skin-related epidemic associated with the community and strain-driven soft tissue infections with certain virulence and resistance to β-lactam antibiotics. According to data from the European Antimicrobial Resistance Surveillance System (EARSS), there are a decrease in MRSA isolates from 18.8% in 2012 to 16.8% in 2015 in the European Union (EU) countries (EARSS 2015).

III. OBJECTIVES

Objectives of the study were: (i) molecular characterization of MRSA *S. aureus* clinical isolates by spa-typing; (ii) typing the SCC mec cassette; (iii) correlation between antibiotic resistance patterns and spa type t032 CC22, t1036 CC22, t1214 CC22 ,t022 CC22, t041 CC5 ,t121 CC8. antibiotic profile simultaneous identification by Real-time tecnique of *nuc* (specific species), *mecA* and *pvl* genes.

Background

New antibiotics are urgently needed due to the alarming development of resistance against all antibiotics on the market and in clinical use.

The pre-antibiotic era was the leading era of mortality and morbidity of humans and animals due to infectious diseases [1]. Among some of the successful pathogens was the Gram-positive *Staphylococcus aureus*, which had a mortality rate among infected patients that over 80%, while over 70% developed metastatic infections [2] MRSA infections are seldom eradicated by routine antimicrobial therapies. Evidence supports the use of *S. aureus* decolonization in surgical patients to prevent *S. aureus* infection, and this intervention has been associated with low rates of postoperative *S. aureus* infection. The staphylococcal carriage is most commonly eradicated by intranasal application of mupirocin either alone or in combination with antiseptic soaps or systemic antimicrobial agents. However, the major cause of nosocomial infection is methicillin-resistant *S. aureus* (MRSA), which is hard to eradicate despite reports of some cases treated by warming therapy.

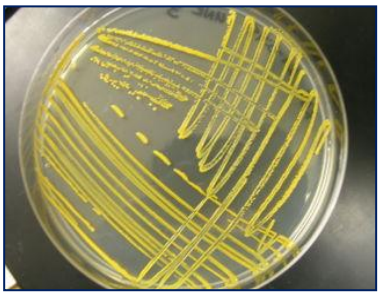
Nosocomial infection is a major cause of surgical morbidity and mortality, and SSIs have a reported incidence rate of 2%–20%. More concerning, some strains have become resistant to the newest antibiotics of last resort.

Furthermore, the efficacy of eradication in patients with community-associated MRSA has not been established, and the necessity of routine decolonization is not supported by data. MRSA outbreaks have created a significant challenge for surgery and clinical practice in recent decades; the failure of traditional antimicrobial treatments has gradually become a worldwide problem, especially in the developing world. Thus, effective therapeutic options to combat *S. aureus* infection, with an emphasis on MRSA, are urgently needed.

IV. STAPHYLOCOCCUS AUREUS

S. aureus was discovered in 1880s by the German surgeon Anton J. Rosenbach and since its discovery it has been emerged as an opportunist pathogen with the ability to cause a wide range of infections, ranging from mild-skin infections to a fatal outcome [3].

Staphylococci are catalase-positive and Gram-positive, with a diameter of 0.8-1.5 μm. no motile, no spore forming, without a capsule, grow well in common culture medium. On solid medium they produce colonies of 2-3 mm in diameter, rounded and marginal, convex, smooth, opaque and golden-yellow pigmentation. They develop between 10 and 45 ° C, with an optimum temperature ranging from 30 to 37 ° C, at a pH between 4 and 9, with an optimum obetween 7.0 and



7.5. Staphylococci are facultative anaerobes. Among the *Staphylococcal* genus, S. *aureus* is the organism that shows the highest pathogenic potential [5], [6]. The pathogenic potential of *S. aureus* lies in an array of factors, such as its great ability to establish successful infections independent of environmental conditions, its intrinsic virulence, quorum sensing mechanism, its genetic diversity, plasticity and ability to acquire exogenous DNA such as antibiotic resistance genes [4][7]. With the discovery of the antibiotic agent penicillin by A. Fleming, the success of this pathogen, and other pathogens, was dramatically reduced. In mid-1940s, penicillin was introduced into the clinical practice, which resulted in infections caused by these death-causing pathogens was easily treatable. However, the success of one of the greatest medical discovery did not last for long, as two years after its introduction to clinical practice, the first penicillin-resistant *S. aureus* strain was isolated and described by Patricia Jevons [2], [4]. By 1960s, more than 80% of all staphylococcal isolates were resistant to penicillin. As an attempt to

cope with the emerging number of penicillin-resistant *S. aureus* strains, the semi-synthetic antibiotic compound methicillin, among other, was discovered. However, as with penicillin, soon after the introduction of methicillin to clinical practice in 1961, the first methicillin-resistant *S. aureus* (MRSA) strain emerged [2]. Now, *S. aureus*, and especially MRSA is a major global health care concern, due to its ability to cause nosocomial infections and to its ability become resistance to multiple antibiotic compounds, and thus sought the importance of effective surveillance and control strategies of this pathogen is becoming more and more urgent.

The natural habitat of *S. aureus* is the skin and mucous membrane of humans and animals. It is estimated that approximately 30% of healthy individuals are asymptomatically carriers of *S. aureus* [8]. These patients do not have directly clinically relevance, but may act as a reservoir of *S. aureus*, from which *S. aureus* can transmit to other patients [9]. In fact, *S. aureus* have emerged as the leading pathogen that is the cause of more than 50% of healthcare associated infections, posing a great burden worldwide [5], [9], [10]. What might have helped *S. aureus* to emerge as leading pathogen is perhaps its capacity and capability to acquire antimicrobial resistance genes.

The prevalence of *S. aureus* and a high selective pressure of antibiotics in hospitals and healthcare institutions may have act as a dangerous cocktail. [8]. Especially the methicillin-resistant *S. aureus* (MRSA) become a global concern, as an infection caused by MRSA rather than a non-MRSA more often leads to a clinical infection. MRSA do not replace non-MRSA strains, but rather adds to the burden of infections caused by *S. aureus* [2] [11].

What differentiates methicillin-sensitive *S. aureus* (MSSA) strains from MRSA strains is the acquisition and insertion of the staphylococcal cassette chromosome *mec* (SCC*mec*) into *orfX* gene on the chromosome of MRSA strains (figure 1). The SCC*mec* element is a mobile genetic element, which harbors the single determinant for methicillin resistance, namely the *mecA* or *mecC* gene. Homology studies of the

mecA gene suggests that *mecA* may have its origin from *Staphylococcus sciuri* or *Staphylococcus fleurettii* with 88 % and 99.8 % nucleotide identity, respectively [3], [4]. Although, *S. fleurettii* shows a higher nucleotide identity, it does not show an *in vitro* resistance, which points to that *S. sciuri* as the prime candidate for the origin of *mecA*. However, the origin of the SCC*mec* still remains unclear. There have been postulations about that the *mecA* have been introduced into coagulase negative *S. aureus* (CoNS) isolates, together with other genes specific for the SCC*mec* element and from which the SCC*mec* element has been formed, and thus CoNS may serve as the origin and reservoir for the SCC*mec* element [12].

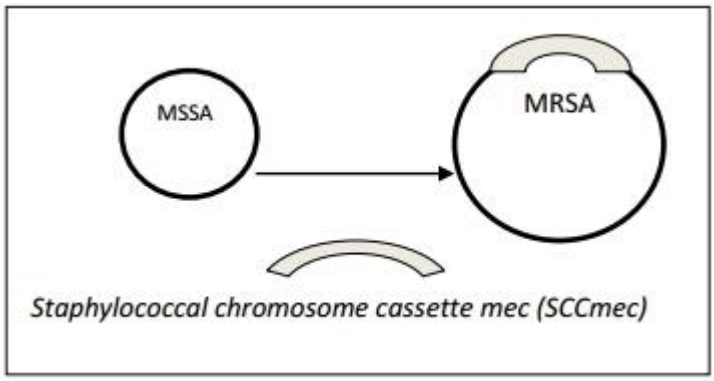


Figure 1: Schematic representation of the insertion of SCCmec into methicillin-resistance S. aureus (MRSA).

Some newly developed antibiotics exhibit high effectiveness in combating MRSA infection, as do candidates under development. With a combination of debridement and modern wound dressings, these agents can successfully treat MRSA wound infections limiting their usage. However, antibiotic resistance rapidly spreads, resulting in increasing numbers of multidrug- and even pan-drug-resistant strains. In addition to the development of novel antimicrobials and antibiotic-free treatments, the verification and validation of ethnomedical drugs is a feasible and cost-effective approach to address this issue.

MRSA is resistant to penicillin-like ß-lactam antibiotics. However, some drugs still retain activity against MRSA, including glycopeptides (vancomycin and teicoplanin), linezolid, tigecycline, daptomycin, and even some new ß-lactams, such as ceftaroline and ceftobiprole. However, MRSA has shown outstanding versatility at emerging and spreading in different epidemiological settings over time like hospitals, community, and, more recently, in animals.

Moreover, although resistance to anti-MRSA agents usually occurs through bacterial mutation, there have been reports of the transfer of resistance to linezolid and glycopeptide antibiotics, which is cause for major concern [13].

V. Antibiotics:

The term in the current common use indicates a drug, of natural or synthetic origin able to slow down or stop the proliferation of the bacteria. Antibiotics are therefore distinguished in bacteriostatic, blocking the reproduction of the bacterium, preventing its splitting and bactericides, killing directly the micro-organism.

The bacterial cell target of antibiotics could be different:

1) bacterial cell wall: **penicillins, cephalosporins, monobactams, carbapenems, bacitracin, glycopeptides (vancomycin) and cycloserine;**
2) cell membrane of the bacterium: **polymyxins, daptomycin;**
3)interfering with the synthesis of nucleic acids: **quinolones, rifampicin, nitrofurantoin, nitroimidazoles;**
4) interfering with protein synthesis: **aminoglycosides, tetracyclines, chloramphenicol, macrolides, clindamycin, spectinomycin, mupirocin**;
5)interfering with metabolism: **sulfonamides, trimethoprim, dapsone, isoniazid;**

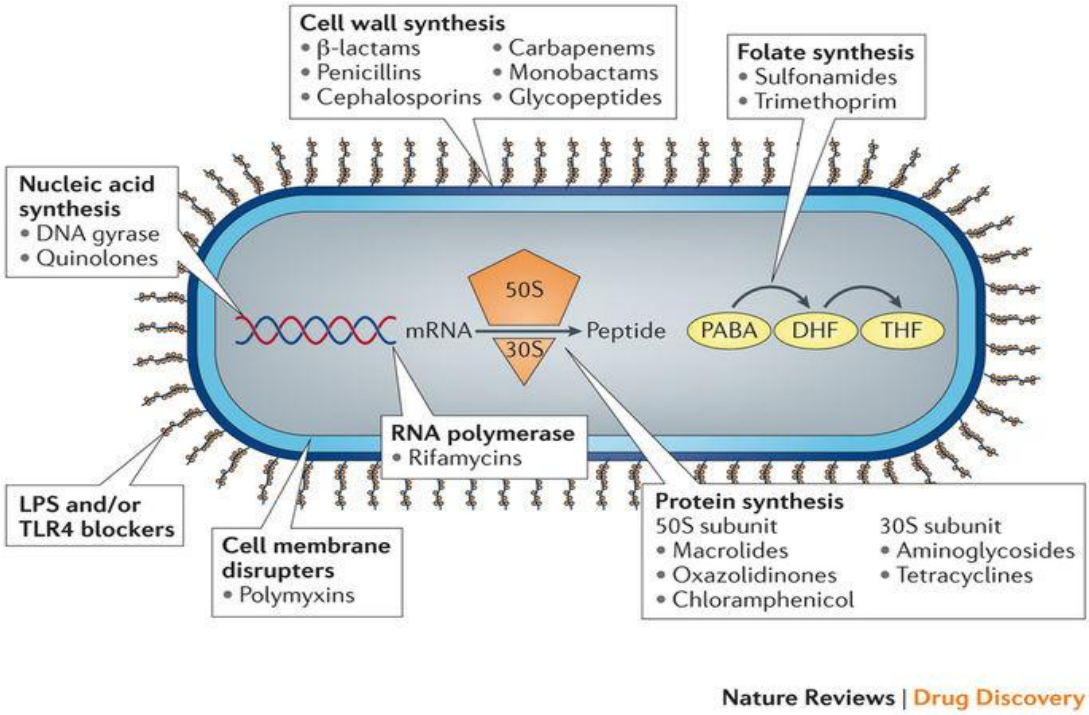


Figure 2: The bacterial cell target of antibiotics [14]

***Antibiotics acting on Cell wall synthesis*:**

Peptidoglycan, a component of the bacterial cell wall, confers form and rigidity to the cell. This molecule is formed by units of N-acetylglucosamine and N-acetyl-muramic. The mature glycine peptide is held together by picoidal peptide chains that cross wise connect the long glycan chains. This cross-linking process is the target of two major groups of antibiotics, **ß-lactams** and **glycopeptides (vancomycin and teicoplanin)**. [15].

β-lactam antibiotics: are a class of broad-spectrum antibiotics, consisting of all antibiotic agents that contain a ß-lactam ring in their molecular structures. This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems. Most β-lactam antibiotics work by inhibiting cell wall biosynthesis in the bacterial organism and are the most widely used group of antibiotics. Until

2003, when measured by sales, more than half of all commercially available antibiotics in use were β-lactam compounds [16].

They are bactericidal, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by DD-transpeptidases which are penicillin binding proteins (PBPs). PBPs vary in their affinity for binding penicillin or other β-lactam antibiotics. The amount of PBPs varies among bacterial species.

β-lactam antibiotics are analogues of D-alanyl-D-alanine the terminal amino acid residues on the precursor NAM/NAG-peptide subunits of the nascent peptidoglycan layer. The structural similarity between β-lactam antibiotics and D-alanyl-D-alanine facilitates their binding to the active site of PBPs. The β-lactam nucleus of the molecule irreversibly binds to (acylates) the Ser_{403} residue of the PBP active site. This irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis [17].

ß-lactams are classified according to their chemical structure: single ß-lactam ring (monobactam), or a fused ß-lactam ring with a 5 atoms penemic ring (penicillins and carbapenems) or fused with a cephalosporin ring 6 atoms (cephalosporins). Within these major groups, and differences in the site of attachment of the single or double-ring chains may have a significant effect on pharmacological properties and on the spectrum of ß-lactams

Penicillin is a group of antibiotics which include penicillin G (figure3)(intravenous use), penicillin V (oral use), procaine penicillin, and benzathine penicillin (intramuscular use). Penicillin antibiotics were among the first medications to be effective against many bacterial infections caused by *Staphylococci and Streptococci*. Penicillins resistant to penicillinases enzymes are methicillin, nafcillin, oxacillin, have a narrower spectrum of action but are very active against *S. aureus* that produces penicillases.

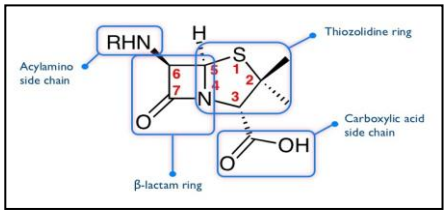


Figure3: Penicillin chemical structure.

Ampicillin penetrate the gram-negative outer membrane, Piperacillin and ticarcillin are active also against *Pseudomonas* spp but are less effective than ampicillins given against gram-negative bacteria.

Cephalosporins (figure 4) are resistant to the hydrolysis of the penicillinases of the *Staphylococci* and ß-lactamases of gram negative bacilli. They are classified according to generation I II III IV. The term generation indicates to discoveries that have historically allowed the expansion of the action spectrum by modifying the site

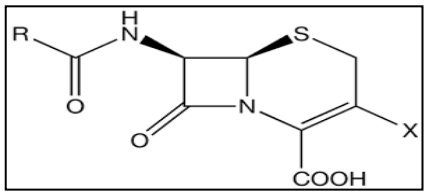


Figure 4: Cephalosporins chemical structure.

of attack of the chains. The last cephalosporin discovery of V generation is the ceftobiprole which is considered an anti-MRSA.

Carbapenems: Imipenem and meropenem are broad-spectrum antibiotics, that penetrate gram positive and gram-negative bacteria and resist to ß-lactamase action. However, imipenem (figure 5) is hydrolyzed by renal dehydropeptidase-1 therefore it must be administered in association with cilastatin which allows an increase in the concentration of the drug.

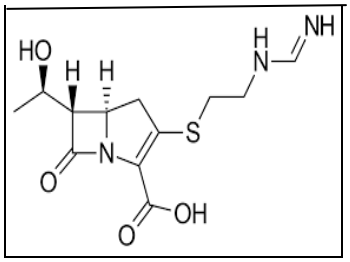


Figure 5: imipenem chemical structure

Glycopeptide antimicrobials are a class of drugs of microbial origin that are composed of glycosylated cyclic or polycyclic non ribosomal peptides. Significant glycopeptide antibiotics include the anti-infective antibiotics vancomycin, teicoplanin, telavancin, ramoplanin and decaplanin, and the antitumor antibiotic bleomycin. Vancomycin (figure 6) is used if MRSA infection is suspected.

Some members of this class of drugs inhibit the synthesis of cell walls in susceptible microbes by inhibiting peptidoglycan synthesis. They bind to the amino acids within the cell wall preventing the addition of new units to the peptidoglycan. They bind to acyl-D-alanyl-D-alanine in peptidoglycan.

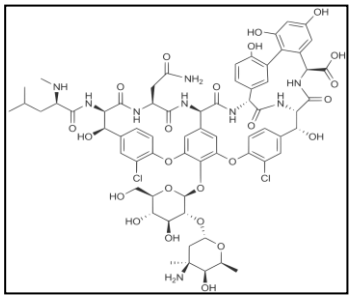
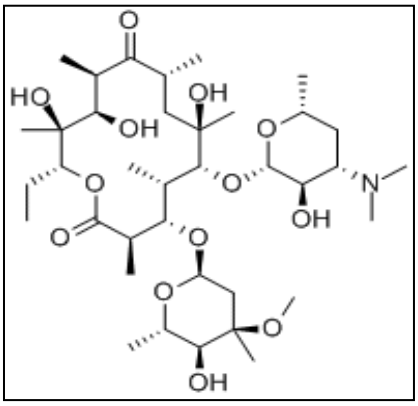


Figure 6: vancomycin chemical structure

2)Inhibitors of protein synthesis:

Macrolides: erythromycin (figure7) azitromycin claritromycin belong to a class of natural products that consist of a large macrocyclic lactone ring to which one or more deoxy sugars, usually cladinose and desosamine, may be attached. The lactone rings are usually 14-, 15-, or 16-membered. The antimicrobial spectrum of macrolides is slightly wider than that of penicillin, and, therefore, macrolides are a common substitute for patients with a penicillin allergy. ß-*hemolytic Streptococci, pneumococci, Staphylococci, and Enterococci* are usually susceptible to macrolides.



F*igure 7: erythromycin chemical structure*

Macrolides are protein synthesis inhibitors. The mechanism of action of macrolides is inhibition of bacterial protein biosynthesis, and they are thought to do this by preventing peptidyl transferase from adding the growing peptide attached to tRNA to the next amino acid as well as inhibiting ribosomal translation. [18]

Another potential mechanism is premature dissociation of the peptidyl-tRNA from the ribosome. [19]

Macrolide antibiotics do so by binding reversibly to the P site on the 50S subunit of the bacterial ribosome. This action is bacteriostatic.

Nucleic acid synthesis inhibitors:

Chinolones have a nucleus of two rings with six terms fused to each other and when the replacement in position 6 of the phenolic ring takes place with a fluorine atom they become fluochinolones, like ciprofloxacin, norflocacin, levofloxacin, ofloxacin Main target is DNA topoisomerase (gyrase) the enzyme responsible for cutting, supercoiling and welding of bacterial DNA during replication. DNA bacterial topoisomerase has four subunits, each of which is inhibited by every single quinolone.

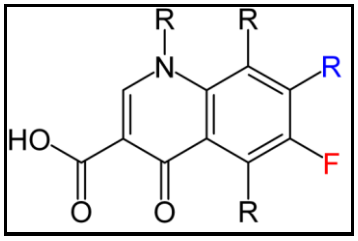


Figure:.8 fluoroquinolones chemical structure

The increased activity at a lower frequency of occurrence of resistant strains seems due to the ability of the more recent fluoroquinolones to bind to different enzyme fingers. They are bactericidal antibiotics.

Have a wide spectrum of aerobic and facultative anaerobic action including

P. aeruginosa. [15].

VI. Antibiotic resistance:

Resistance to antibiotics could be intrinsic or acquired.

Intrinsic or natural resistance is the constitutional insensitivity of a microorganism to a certain antibiotic. Immutable over time, genetically determined. It manifests itself in all strains of the same species.

It depends on:

- characteristics of the antibiotic
- microorganism structures
- lack of penetration of the drug in the microorganism.

Acquired resistance (informational variation) is the acquisition of new genetic determinant of resistance for a specific strain originally sensitive to a chemotherapy, that bring to emergence of antibiotic resistance.

It can be divided into:

- chromosomal or endogenous;
- extrachromosomal or exogenous;

Chromosomal resistance

- It is only 10-15% of all the acquired resistances (low frequency of onset)
- It is achieved through a spontaneous mutational alteration of chromosome genetic information.
- The antibiotic has a selective action (select the mutants resistant, inhibiting the sensitive cells. This resistance affects only antibiotic to which they are resistant mutants.
- The same mutants can also be resistant to other antibiotics with similar characteristics (cross-resistance).

- It is transmitted vertically through the offspring (from mother cell to daughter cell).

Extrachromosomal resistance

It constitutes 90% of all resistances (high frequency of onset).

- It originates for acquisition of new genetic information that it comes from other micro-organisms and enters the cell through the mechanisms of conjugation, transformation and transduction.
- It concerns more antibiotics simultaneously (resistance multiple).
- It is horizontal transmitted (genetic exchange).
- It can also be transferred to microorganisms belonging to different species (contagious resistance).
- It is due to genes present on plasmids or transposons (mobile genetics elements).

From an evolutionary perspective, bacteria use two major genetic strategies to adapt to the antibiotic "attack":

A) mutations in gene(s) often associated with the mechanism of action of the compound;

B) acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT);

A) In general, mutations resulting in antimicrobial resistance alter the antibiotic action via one of the following mechanisms,

a) modifications of the antimicrobial target (decreasing the affinity for the drug, see below);

b) a decrease in the drug uptake;

c) activation of efflux mechanisms to extrude the harmful molecule;

d) global changes in important metabolic pathways via modulation of regulatory; networks. Thus, resistance arising due to acquired mutational changes is diverse and varies in complexity. [20]

B) Horizontal gene transfer: classically, bacteria acquire external genetic material through three main strategies (figure 9);

a) transformation (incorporation of naked DNA)

b) transduction (phage mediated)

c) conjugation

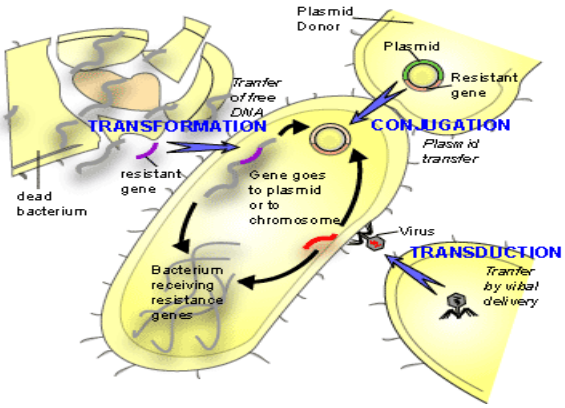


Figure 9: [21] Horizontal gene transfer Kennet todar, revew of bacteriology

Transformation is perhaps the simplest type of HGT, but only few clinically relevant bacterial species "naturally" incorporate naked DNA to develop resistance. Emergence of resistance in the hospital environment often involves conjugation, a very efficient method of gene transfer that involves cell-to-cell contact and is likely to occur at high rates in the gastrointestinal tract of humans under antibiotic treatment. Conjugation uses mobile genetic elements (MGEs) as vehicles to share valuable genetic information, although direct transfer from chromosome to chromosome has also been well characterized [22] .

The most important MGEs are plasmids and transposons, both of which play a crucial role in the development and dissemination of antimicrobial resistance among clinically relevant organisms.

Furthermore, this genetic exchange has been implicated in the dissemination of resistance to many frequently used antibiotics. Acquisition of foreign DNA material through HGT is one of the most important drivers of bacterial evolution and it is frequently responsible for the development of antimicrobial resistance. Most antimicrobial agents used in clinical practice are products naturally found in the environment. As mentioned before, bacteria sharing the environment with these molecules harbor intrinsic genetic determinants of resistance and there is robust evidence suggesting that such "environmental resistome" is a prolific source for the acquisition of antibiotic resistance genes in clinically relevant bacteria.

Finally, one of the most efficient mechanisms for accumulating antimicrobial resistance genes is represented by integrons, which are site-specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes. Integrons provide an efficient and rather simple mechanism for the addition of new genes into bacterial chromosomes, along with the necessary machinery to ensure their expression; a robust strategy of genetic interchange and one of the main drivers of bacterial evolution. [23]

Antibiotic resistance mechanisms can be classified in:

I) modifications of the antimicrobial molecule;

II) prevention to reach the antibiotic target (by decreasing penetration or actively extruding the antimicrobial compound);

III) changes and/or bypass of target sites;

IV) resistance due to global cell adaptive processes;

I) MODIFICATIONS OF THE ANTIMICROBIAL MOLECULE:

One of the most successful bacterial strategies to cope with the presence of antibiotics is to produce enzymes that inactivate the drug by adding specific chemical moieties to the compound or that destroy the molecule itself, rendering the antibiotic unable to interact with its target.

I.A) Chemical alterations of the antibiotic: the production of enzymes capable of introducing chemical changes to the antimicrobial molecule is a well-known mechanism of acquired antibiotic resistance in both gram-negative and gram-positive bacteria. Interestingly, most of the antibiotics affected by these enzymatic modifications exert their mechanism of action by inhibiting protein synthesis at the ribosome level [24]

I.B). Destruction of the antibiotic molecule: The main mechanism of β-lactam resistance relies on the destruction of these compounds by the action of β- lactamases. These enzymes destroy the amide bond of the β-lactam ring, rendering the antimicrobial ineffective.

Infections caused by penicillin-resistant *S. aureus* became clinically relevant after penicillin became widely available and the mechanism of resistance was found to be a plasmid-encoded penicillinase that was readily transmitted between *S. aureus* strains, resulting in rapid dissemination of the resistance trait. [25]

II. Decreased Antibiotic Penetration and Efflux

II.A) Decreased permeability

Many of the antibiotics used in clinical practice have intracellular bacterial targets or, in case of gram-negative bacteria, located in the cytoplasmic membrane (the inner membrane) This mechanism is particularly important in gram-negative bacteria. [26]

II.B) Efflux Pumps many classes of efflux pumps have been characterized in both gram-negative and gram-positive pathogens. These systems may be substrate-specific or with broad substrate specificity, which are usually found in MDR bacteria. The genes encoding efflux pumps can be in MGEs or in the chromosome.

III) Target sites modification

A common strategy for bacteria to develop antimicrobial resistance is to avoid the action of the antibiotic by interfering with their target site. To achieve this, bacteria

have evolved different tactics, including target protection and target modifications that result in decreased affinity for the antibiotic.

III.A) Target protection

Examples of drugs affected by this mechanism include tetracycline (Tet[M] and Tet[O]), fluoroquinolones (Qnr) and fusidic acid (FusB and FusC). One of the classic and best-studied examples of the target protection mechanism is the tetracycline resistance determinants Tet(M) and Tet(O). Tet(M) was initially described in *Streptococcus* spp. TetO and TetM interact with the ribosome and dislodge the tetracycline from its binding site in a GTP-dependent manner. These proteins belong to the translation factor superfamily of GTPases and act as homologues of elongation factors (EF-G and EF-Tu) used in protein synthesis. TetM directly dislodges and releases tetracycline from the ribosome by an interaction between the domain IV of the 16S rRNA and the tetracycline binding site. this interaction alters the ribosomal conformation, preventing rebinding of the antibiotic [27]

III.B.1) Target modification

Introducing target modifications is one of the most common mechanisms of antibiotic resistance in bacterial pathogens affecting almost all families of antimicrobial compounds. These target changes may consist of *i)* point mutations in the genes encoding the target site, *ii)* enzymatic alterations of the binding site (addition of methyl groups), and/or *iii)* replacement or bypass of the original target. As mentioned, regardless of the type of change, the final effect is always the same, a decrease in the affinity of the antibiotic for the target.

Fluoroquinolones kill bacteria by altering DNA replication through the inhibition of two crucial enzymes, DNA gyrase and topoisomerase IV. Development of chromosomal mutations in the genes encoding subunits of the above-mentioned enzymes (*gyrA-gyrB* and *parC-parE* for DNA gyrase and topoisomerase IV, respectively) is the most frequent mechanism of acquired resistance to these compounds. Importantly, since FQs interact with two enzymes (DNA gyrase and

topoisomerase), and both are essential for bacterial survival, the level of resistance achieved by developing changes in one of the enzymes will depend on the potency with which the antimicrobial inhibits the unaltered target.

III.B.2. Enzymatic alteration of the target

One of the best characterized examples of resistance through enzymatic target modification is the methylation of the ribosome catalyzed by an enzyme encoded by the *erm* genes (***e***rythromycin ***r***ibosomal ***m***ethylation), which results in macrolide resistance.

In *Staphylococci*, the most important *erm* genes are *erm*A (mostly distributed in a transposon in MRSA) and *erm*(C) (found in plasmids in methicillin-susceptible *S. aureus*).

These enzymes are capable of mono- or dimethylating an adenine residue in position A2058 of the domain V of the 23rRNA of the 50S ribosomal subunit. [28]

III.B.3. Complete replacement or bypass of the target site

Using this strategy, bacteria are capable of evolving new targets that accomplish similar biochemical functions of the original target but are not inhibited by the antimicrobial molecule. The most relevant clinical examples include methicillin resistance in *S. aureus* due to the acquisition of an exogenous PBP (PBP2a) and vancomycin resistance in *Enterococci* through modifications of the peptidoglycan structure mediated by the *van* gene clusters.

Resistance to methicillin (a semisynthetic penicillin stable against the staphylococcal penicillinase) in *S. aureus* results from the acquisition of a foreign gene (likely from *Staphylococcus sciuri*) designated *mecA* often located in a large DNA fragment designated staphylococcal chromosomal cassette *mec* (SCC*mec*). The *mecA* gene encodes PBP2a, a PBP that has low affinity for all β-lactams, including penicillins, cephalosporins (except for last generation compounds) and carbapenems. Acquisition

of *mecA* renders most β-lactams useless against MRSA and alternative therapies need to be used in serious infections. Of note, PBP2a carries a transpeptidase domain, but it does not function as a transglycosylase (class B PBP), therefore, it requires the activity of other native PBPs to perform the latter function and fully crosslink peptidoglycan. Specifically, the penicillin-insensitive transglycosylase domain of PBP2 (a class A PBP) is particularly important to achieve transglycosylation of peptidoglycan in the presence of β-lactams in *mecA*-carrying MRSA isolate.

mecA gene is usually found as part of a gene cassette inserted into a larger MGE (SCC*mec*), whose basic components include *mecA, mecR1* (encoding the signal transducer protein MecR1), *mecI* (encoding the repressor protein Mecl), and *ccr* (encoding a recombinase; *c*assette *c*hromosome *r*ecombinase). To date, 11 different SCC*mec* allotypes have been described with varying degrees of genetic homology and different sizes, insertion sequences and accompanying resistance genes [29]

Importantly, SCC*mec* types seem to differ between different MRSA clones. Indeed, community-associated MRSA strains appear to harbor shorter SCC*mec* cassettes (SCC*mec* type IV) and carry less antibiotic resistance determinants, whereas hospital-associated (HA) isolates possess longer elements (SCC*mec* type II) and are usually multidrug resistant.

VII. BETA-LACTAMS RESISTANCE MECHANISMS IN S. AUREUS

Mechanisms of resistance to β-lactam antibiotics is mediated in the production of β-lactamase, widely spread enzymes between Gram-positive and Gram-negative bacteria; they hydrolyze the amide linkage of the β-lattamic ring of penicillins and cephalosporins with the production of an β-lactam inactive derivative. [30] The β-lactamase production is plasmid encoded by the *blaZ* gene. *blaZ* is under the control of two adjacent regulatory genes, the *blaR1* antirepressor and the *blaI* repressor. [31] Following exposure to β-lactams, BlaR1, a transmembrane sensor-transducer, cleaves itself. The hypothesis is that the cleaved protein functions as a protease that cleaves

the repressor BlaI, directly or indirectly (an additional protein, BlaR2, may be involved in this pathway) and allows *blaZ* to synthesize enzyme. [32] (Fig 9).

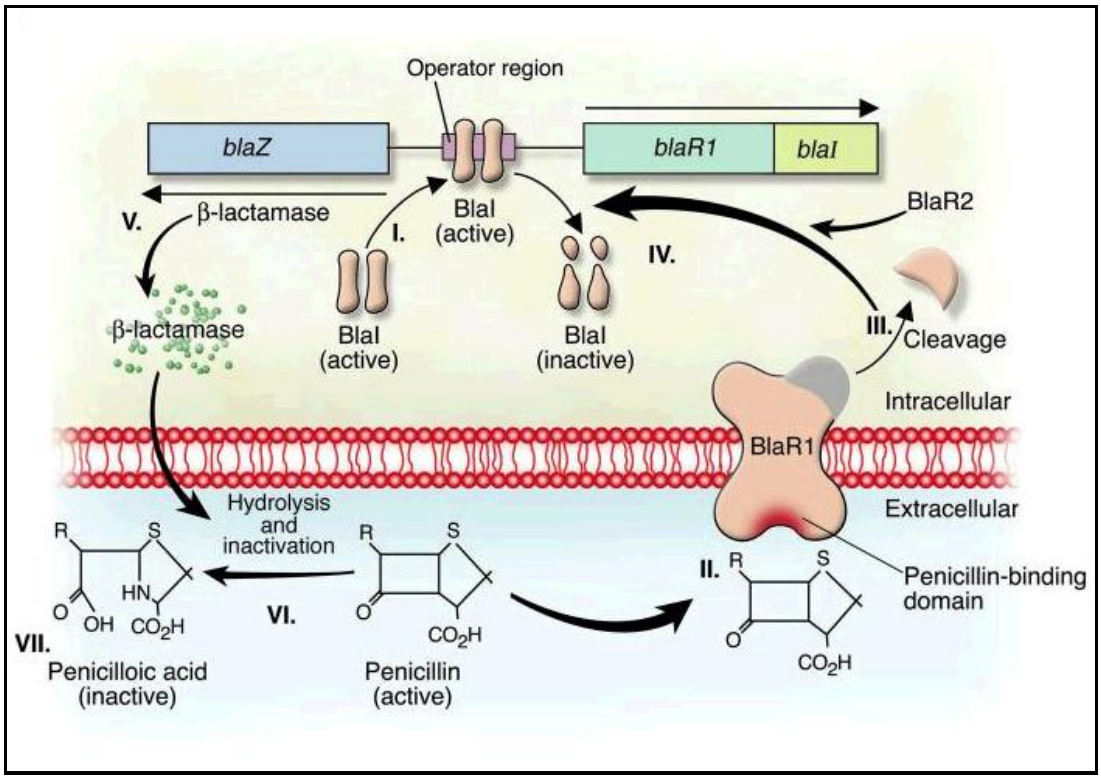


Figure 9: Induction of staphylococcal β-lactamase synthesis in the presence of the β-lactam antibiotic penicillin. Antimicrobial resistance [33]

The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both blaZ and blaR1-blaI. In the absence of penicillin, β-lactamase is expressed at low levels. II. Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation. III–IV. Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both blaZ and blaR1-blaI to commence. V–VII. β-Lactamase, the extracellular enzyme encoded by blaZ (V), hydrolyzes the β-lactam ring of penicillin (VI), thereby rendering it inactive (VII).

VIII. METHICILLIN RESISTANCE

The first MRSA strain appeared in 1961[34], one year after the introduction use of methicillin in therapy; MRSA strains subsequently spread to become a world-class problem. Several chromosomal genes are implicated in the phenotypic expression of methicillin-resistance, giving higher levels of staphylococcal resistance.

Level of resistance to β-lactams in MRSA is the result of the acquisition of the *mecA* gene, which encodes for penicillin-binding protein 2a (PBP2a).

The main mechanism of resistance is production of an auxiliary penicillin binding protein, PBP2a/PBP2c which renders the isolate resistant to all b-lactam except the novel class of specific 'anti-MRSA' cephalosporins. These agents have sufficiently hight affinity to PBP2a, and probably also the PBP encoded by *mecC*, to be active against MRSA. The auxiliary PBPs are encoded by the *mecA* gene or the recently described *mecC* gene. [35]

mec element is foreign to *S. aureus* and is not present in methicillin susceptible *S. aureus*. strains with marked heterogeneous expression of the *mecA* gene and frequently low MICs of oxacillin hamper the accuracy of susceptibility testing.

Some isolates express low level resistance to oxacillin, they are *mecA* and *mecC* negative and do not produce alternative PBPs (borderline susceptible *S aureus* BORSA.), these strains are relatively rare, and the mechanism of resistance is poorly characterized, but may include hyperproduction of b-lactamases or alteration of the pre-existing PBPs. [36]

IX. Recommended methods for detection of methicillin resistance in *S. aureus*

Methicillin/oxacillin resistance can be detected phenotypically by MIC determination and by disk diffusion. Agglutination can be used to detect PBP2a, but it will not reliably to detect PBPc. Genotypic detection with PCR is reliable.

Detection by MIC determination or disk diffusion.

The heterogeneous expression of resistance particularly affects MICs of oxacillin, which can appear susceptible. Cefoxitin is a very sensitive and specific marker of mecA/ mecC mediated methicillin resistance including in heterogeneous expressing strains and is the agent of choice. Disk siffusion using oxacillin is discouraged and interpretative zone diameters are no longer included in the EUCAST breakpoint table due to poor correlation with the presence of *mecA*.

A. Broth microdiluition:

Strandard methodology (ISO 20776-1) is used and strains with cefoxitin MICs ≥4 mg/L should be reported ad methicillin resistant.

B. Disk diffusion:

The EUCAST disk diffusion method is used. Strains with cefoxitin 30μg disk zone diameter ≥22 mm should be reported as methicillin resistant.

Detection with genotipyc and latex agglutination methods.

Genotypic detection of the *mecA* and *mecC* genes by PCR [37] and detection of the PBP2a protein with latex agglutination kits is possible using commercial or "in house" assays. PBP2c is not detected by most of commercial assays

Particularly interesting is the *mecA* gene encoding for methicillin-resistance. The *mecA* gene is part of a mobile genetic element, the SCC mec, which is incorporated into the bacterial. MRSA clones possess the *mecA* gene, and its *mecR1-mecI* regulatory genes. They are allocated on a genomic mobile island called staphylococcus chromosomal cassette mec (SCCmec, about 21-67 kb). This chromosomal cassette combines the entire operon mec (about 28kb) to the *ccr* gene, a complex that encodes for specific recombinase sites responsible for the mobility of SCC mec [38]. This mobility is essential for resistance, as strains of *S. aureus*

methicillin-sensitive (MSSA) capture SCC mec from MRSA strains. The SCC mec elements contain:

- the *mec* genes complex includes insertion sequences (IS431mec), the *mecA* gene, and the *mecR1* and *mecI* regulating genes;

- the complex of *ccr* genes encoding for recombinase (ccr) responsible for the precise excision and integration of *SCCmec* within the bacterial chromosome, and is responsible for its mobility;

-the flattening regions the mec and ccr complexes are referred to as J (junkyard) regions, which do not appear to be essential or useful for bacterial cells, except where they contain genes for resistance to other antibiotics. The SCC mec elements are classified in types and subtypes. To date, 11 types of SCC mec have been identified [39,40, 41, 42].

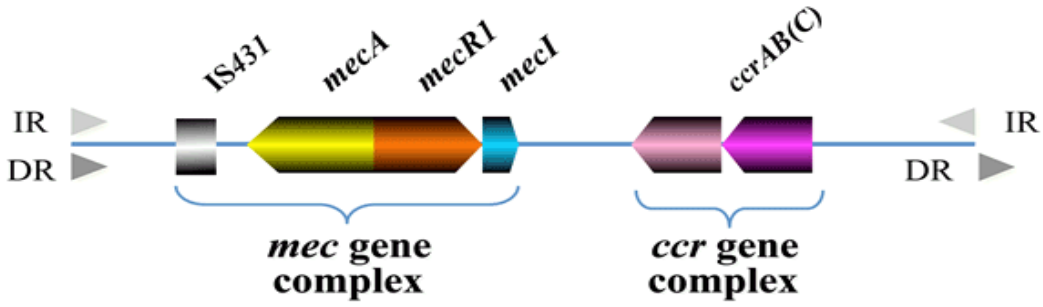


Figure 10: SCCmec is composed of mec-gene complex and ccr-gene complex. Hiramatsu K, et al. Infect Chemother. 2013;45:117-36. Tsubakishita S, et al. Antimicrob Agents Chemother 2010;54:1469-75. [43]

Most types of nosocomial MRSAs produce types I, II or III, while most of the EU-type CA-MRSA types are type IV or V, although EMRSA-15 codes Type IV. [44]

Three classes of mec (A, B and C) and four subtypes of ccr complexes are known, which, by combining, generate five different SCCmec (I to XI) boxes (Tab. 3), distinct in various subtypes depending on differences in junkyard regions.

SCC*mec* types	*ccr* gene complexes	*mec* gene complexes	strains
I	1 (A1B1)*	B	NCTC10442, COL
II	2 (A2B2)	A	N315, Mu50, Mu3, MRSA252, JH1, JH9
III	3 (A3B3)	A	85/2082
IV	2 (A2B2)	B	CA05, MW2, 8/6-3P, 81/108, 2314, cm11, JCSC4469, M03-68, E-MRSA-15, JCSC6668, JCSC6670
V	5 (C1)	C2	WIS(WBG8318), TSGH17, PM1,
VI	4 (A4B4)	B	HDE288
VII	5 (C1)	C1	JCSC6082
VIII	4 (A4B4)	A	C10682, BK20781
IX	1(A1B1)	C2	JCSC6943
X	7(A1B6)	C1	JCSC6945
XI	8(A1B3)	E	LGA251

Table 3: Type SCCmec. www.SCCmec.org

X. TYPING OF *S. AUREUS*

The *spa* typing technique uses the sequence of a polymorphic VNTR in the 3' coding region of the *S. aureus*-specific staphylococcal protein A (*spa*). Single locus DNA-sequencing of the repeat region of the *Staphylococcus* Protein A gene (spa) can be used for reliable, accurate and discriminatory typing of MRSA.Typing of *S. aureus* is crucial for preventing the spread of MRSA and for outbreak investigations [45]. A crucial factor in controlling MRSA is to know about the dissemination of MRSA and its clones, as *S. aureus* has a large clonal population structure, and thus a correct assignment of a strain to a clone is a highly important and essential part of the epidemiology and surveillance of MRSA. Typing of MRSA is used to support infection control measures. Different methods for *S. aureus* typing have been developed, all, which have their different strengths and weaknesses. A combination of different methods is necessary to obtain a correct assignment and have a high discriminatory power [45]. is the Genotypic methods consisting of multilocus sequence typing (MLST), *spa* typing, and SCC*mec* typing are among the mostly used typing method. The nomenclature is based on their sequence type (ST), *Staphylococcus* protein A (*spa)* type and SCC*mec* type. The sequence type is a profile of seven housekeeping genes, while the *spa* type is based on sequence polymorphism of the X-region in the *spa* gene. The sequence type is a multi-locus typing of *S. aureus*, while *spa* typing is a single-locus typing. SCC*mec* type is the typing of the mobile genetic element encoding the methicillin resistance in *S. aureus* strains [5].

A. SPA TYPING

The *spa* typing is based on sequencing of region X of the *spa* gene, a region that mainly consist of 24-bp repeats. These repeats are assigned a numerical code from which the *spa* type is determined. s*pa* typing is much more simple and accessible than MLST, as it only requires sequencing of a single locus, which often can be performed by an in-house sequencing platform. Because of its higher discriminatory power, *spa* typing is more specific compared to MLST. Due to its high discriminatory

gene marker, *spa* typing can further differentiate a collection of ST. An ST can consist of several *spa* types, and thus *spa* typing can be used for evolutionary purpose as well as under outbreak situations [3]. The diversity of *spa* types is due to deletions, duplication or point mutations of the repeats in the *spa* gene [3].

DNA sequences of the *spa* gene therefore provide portable and biologically meaningful molecular typing data that have demonstrated their utility for macro- and micro-epidemiological purposes from surveillance through to outbreak investigations at various geographical levels [46][47].

www.spaserver.ridem. last visited OCTOBER 12, 2017

The 20 most frequent spa types and multilocus sequence typing types among meticillin-sensitive *Staphylococcus aureus* and meticillin-resistant *S. aureus* isolates collected in 25 European countries in 2011

MSSA						MRSA					
Rank	spa type	Multilocus sequence type[a]	Frequency	%	Cumulative %	Rank	spa type	Multilocus sequence type[a]	Frequency	%	Cumulative %
1	t091	ST7	138	5.3	5.3	1	t032	ST22	202	17.9	17.9
2	t084	ST15	124	4.7	10.0	2	t003	ST225	99	8.8	26.6
3	t002	ST5	121	4.6	14.6	3	t008	ST8	95	8.4	35.0
4	t015	ST45	98	3.7	18.4	4	t002	ST5	87	7.7	42.7
5	t008	ST8	97	3.7	22.1	5	t067	ST125	50	4.4	47.2
6	t012	ST30	90	3.4	25.5	6	t041	ST228	24	2.1	49.3
7	t127	ST1	83	3.2	28.7	7	t777	ST5	21	1.9	51.2
8	t021	ST30	50	1.9	30.6	8	t018	ST36	20	1.8	52.9
9	t065	ST45	38	1.4	32.1	9	t022	ST22	20	1.8	54.7
10	t026	ST45	34	1.3	33.4	10	t037	ST239	19	1.7	56.4
11	t005	ST22	33	1.3	34.6	11	t127	ST1	18	1.6	58.0
12	t230	ST45	32	1.2	35.9	12	t747	ST22	17	1.5	59.5
13	t216	ST59	28	1.1	36.9	13	t044	ST80	15	1.3	60.8
14	t056	ST101	27	1.0	38.0	14	t2357	ST22	15	1.3	62.1
15	t148	ST72	25	1.0	38.9	15	t024	ST8	14	1.2	63.4
16	t024	ST8	23	0.9	39.8	16	t740	ST45	12	1.1	64.4
17	t346	ST15	23	0.9	40.7	17	t515	ST22	12	1.1	65.5
18	t571	ST398	23	0.9	41.5	18	t6057	ST22	11	1.0	66.5
19	t701	ST8	23	0.9	42.4	19	t030	ST239	9	0.8	67.3
20	t189	ST188	21	0.8	43.2	20	t014	ST225	9	0.8	68.1
Other	-	-	1,489	56.8	100.0	other	-	-	361	31.9	100.0
Total			**2,621**	**100**		**Total**			**1,130**	**100**	

MLST: multilocus sequence typing; MSSA: meticillin-sensitive *Staphylococcus aureus*; MRSA: meticillin-resistant *S. aureus*; %: percentage.
[a] Predicted from *spa* typing data.

Table 3: the 20 most frequent spa *types and multilocus sequence typing among meticillin-sensitive S. aureus and meticillin-resistant* S. aureus *isolates collected in 25 European countries in 2011.* [46]

For MSSA, the top 20 ranking *spa* types included 43.2% of all MSSA isolates (Table 3). Importantly, there was very little difference among the first 11 ranking *spa* types between the 2011 and 2006 datasets. Only changes in rank order were observed. Ranks 12 to 20 contained four new *spa* types in 2011.

The figure 2 shows for MRSA the top 20 ranking MRSA *spa* types contained 68.1% of all MRSA isolates (73.4% in 2006). There were no differences in the top six *spa* types. [48]

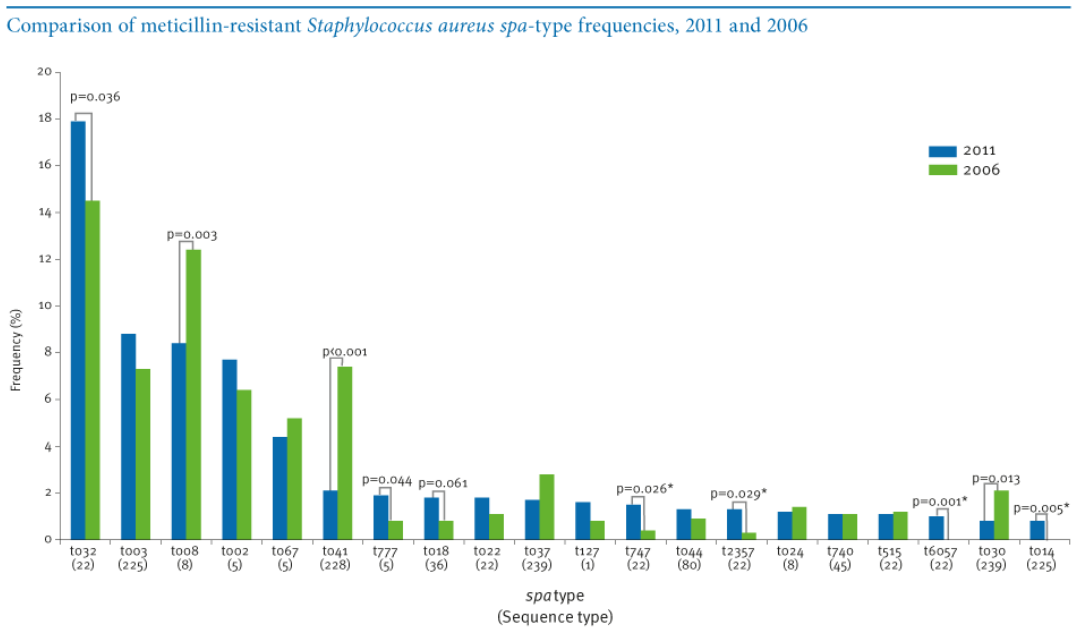


Figure 2: comparison of meticillin-resistant Staphylococcus aureus spa-type frequencies, 2011 and 2006[46]

Among MRSA isolates, a dynamic expansion was demonstrated for several *spa* types. MRSA isolates with *spa* types belonging to ST22 increased most markedly making ST22 the most critically expanding MRSA clone in Europe. This lineage (designated EMRSA-15) was first described during hospital outbreaks in England. [49].

SCCmec TYPING :

SCCmec elements are classified by a hierarchical system into "types" and "subtypes". "Types" are defined by the combination of (1) the type of *ccr* gene complex, which is represented by *ccr* gene allotype, and (2) the class of the *mec* gene complex. These are the key elements of the cassette responsible for integration and excision of SCCmec, and the beta-lactam resistance phenotype, respectively. To date, no excellent technique for SCC*mec* typing exists. After the SCC*mec* structure was recognized, different attempts for a SCC*mec* typing method have been developed. However, they are all not definitive typing methods as they often lack the ability to detect one or more types. The most promising SCC*mec* typing method was developed by Kondo *et al.* (2007) [50] and is based on conventional polymerase chain reaction (PCR), as conventional PCR remains the most convenient, common and easiest to implement in laboratories. This technique is based on a complex combination of multiplex-PCRs (M-PCR). The method is based on four M-PCRs; the first and second M-PCRs are used to recognize the SCC*mec* type, the third M-PCRs are used for subtyping purposes while the fourth M-PCRs is used for identification of transposons and plasmids. The primers are designed to target genes in the *mec* gene complex, *ccr* gene complex, genes relevant for subtyping, and for additional transposons and plasmids that are known to be found in SCC*mec* elements. Based on the amplicons from all four M-PCRs, a SCC*mec* typing can be determined. Often, using just M-PCR 1 and M-PCR 2 is sufficient as using these two M-PCRs can yield the SCC*mec* type. The advantage of the SCC*mec* typing method by Kondo *et al.* (2007) is that the nomenclature of identified SCC*mec* elements is based on the recommended nomenclature defined by The International Working Group on the Classification of *S*taphylococcal Cassette Chromosome Elements (IWG-SCC). The IWG-SCC was organized to: 1) form an intellectual network to contribute to the study of SCC elements; 2) establish a consensus on a uniform nomenclature system for SCC elements; 3) define minimum requirements for the description of new SCC

elements; and 4) establish guidelines for the identification of SCC elements for epidemiological study (i.e., SCCmec typing).

IWG-SCC was established for the development of a universal nomenclature system for SCC*mec* elements [5]. The group represented a nomenclature of SCC*mec* in which the SCC*mec* elements is designated by roman numerals followed with the *mec* gene complex and the *ccr* gene complex in parentheses. As an example, SCC*mec* type IV (2B), which indicates that it is a type IV SCC*mec* element, with a class 2 *ccr* gene complex and a class B *mec* complex (figure 11). The subtyping of SCC*mec* elements is based on the variation in the J1 region within the same SCC*mec* type. J1 region are designated based on the presence of specific DNA sequences, such as characteristic genes, pseudo genes, non-coding regions, and mobile genetic elements. However, a great disadvantage of this method is that it is rather time-consuming, quite sensitive and not fully developed, and should be further developed and evaluated. Another disadvantage is that, due to it is based on PCR, this method is unable to detect the presence of new alleles of the existing genes, and thus new SCC*mec* elements.

		M-PCR 1						M-PCR 2					SCC*mec* type
	Strain	Expected size (bp)					*ccr* gene complex	Expected size (bp)				*mec* gene complex	
		1791	1287	937	695	518		2827	1963	1799	804		
M													
1	PCR-H_2O												
2	COL				X		1	X				B	SCC*mec* type I
3	N315			X			2		X			A	SCC*mec* type II
4	85/2082	X				X	3+5			X		A	SCC*mec* type III
5	JCSA4469			X			2	X				B	SCC*mec* type IV
6	WIS					X	5				X	C2	SCC*mec* type V
7	HDE 238		X				4						SCC*mec* type VI

Figure 11. Representation of the SCCmec typing method by Kondo et al. (2007). [51]

XI. STAPHYLOCOCCAL CASSETTE CHROMOSOME *MEC*

Since 1980s where the SCC*mec* element was recognized, it also being categorized as a genomic island. SCC*mec*, opposite to the other GIs, encodes genes for antibiotic resistance rather than virulence genes [6]. To this date, 11 different types of SCC*mec* elements have been identified in *S. aureus* (type I to XI) and are reported in figure 12.

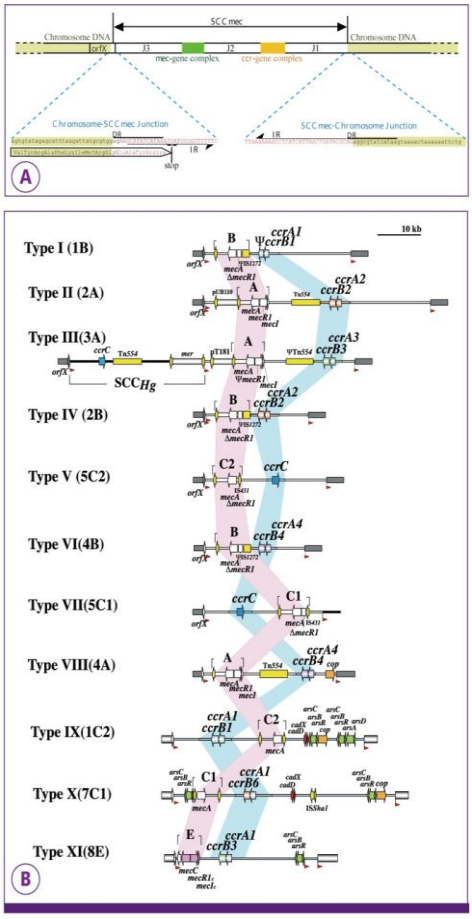


Figure 12. Basic structure of SCCmec.

SCCmec is bracketed by direct repeats (DRs) that contain integration site sequence (ISS) recognized by cassette chromosome recombinase (CCR). A pair of inverted repeats (IRs) are present at the termini of SCCmec. Two critical gene complexes, *ccr*

and *mec* are present, and the other regions are designated J1, J2, and J3. The type of SCCmec is defined by the combination of the type of *ccr*-gene complex and the class of *mec*-gene complex. Subtype of the SCCmec is based on the difference in the J (standing for junkyard) regions. (B) Various types of SCCmec. Direct repeats that comprise integration site sequences of SCC are located at both extremities of SCCmec (the red arrowheads). The location of five (A-E) classes of *mec*-gene complexes is indicated by pink belt. The locations of ccr-gene complexes are indicated by blue belt. Insertion sequences and transposons are indicated in yellow. Representative genes related to heavy metal resistance and integrated plasmids located in the J regions are also indicated. Type XI is a newly identified SCCmec found in the MRSA strains of bovine source [52], [53]

They all contain the same backbone structure, which consist of a *mec* gene complex, a *ccr* gene complex and three joining (J) regions (figure 13). The different types of SCC*mec* elements is due to difference in the gene complexes, however they are still organized in the same way. The SCC*mec* elements are classified into types based on the combination of the *mec* gene complex and *ccr* gene complex, while subtyping of SCC*mec* is based on the variation in the J1 region.

www.sccmec.org – last visited June 14, 2016

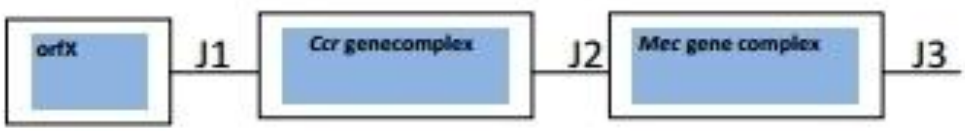


Figure 13. Schematic representation of the organization of the backbone structure of SCCmec

The SCC*mec* element makes up approximately 1-2% of the total genome size of *S. aureus*, and varies in size, ranging from approx. 0.1 kb to 34 kb3. They are composed of the *mec* gene complex, of which five different types of *mec* gene complex have

been characterized, and the *ccr* gene complex, of which eight different types have been characterized (table 4.2 and 4.3, respectively).

www.sccmec.org/Pages/SCC_ClassificationEN.html - last visited June 25, 2016

A. mec gene complex

The *mec* gene complex is composed of *mecA*, its regulatory genes, and associated insertion sequences. The class A mec gene complex (class A mec) is the prototype complex, which contains *mecA*, the complete *mecR1* and *mecI* regulatory genes upstream of *mecA*, and the hyper-variable region (HVR) and insertion sequence IS431 downstream of *mecA*. The class B mec gene complex (class B mec) is composed of *mecA*, a truncated *mecR1* resulting from the insertion of IS1272 upstream of *mecA*, and HVR and IS431 downstream of *mecA*. The class C mec gene complex (class C mec) contains *mecA* and truncated *mecR1* by the insertion of IS431 upstream of *mecA*, and HVR and IS431 downstream of *mecA*. There are two distinct class C mec gene complexes; in the class C1 mec gene complex, the IS431 upstream of *mecA* has the same orientation as the IS431 downstream of *mecA* (next to HVR), while in the class C2 mec gene complex, the orientation of IS431 upstream of *mecA* is reversed. C1 and C2 are regarded as different mec gene complexes since they have likely evolved independently. The class D mec gene complex (class D mec) is composed of *mecA* and *ΔmecR1*, it does not carry an insertion sequence downstream of *ΔmecR1* (as determined by PCR). *mec* gene complex is the complex responsible for the antibiotic resistance of MRSA stains. It encodes for the *mecA* gene, which is the single determinant for methicillin resistance or the *mecA*LGA251, also known as the *mecC* gene. Both genes encode for a penicillin-binding protein (PBP2a or PBP2'), which has a low affinity towards β-lactam antibiotics. The *mec* gene complex additional encodes for the regulatory genes, *mecR1* and *mecI*, and insertion sequence(s). *mecR1* is a transmembrane β-lactam-sensing signal transducer, which senses the absence or presence of β-lactam antibiotics, while *mecI* is a repressor that represses the transcription of *mecA* and *mecR1-mecI* complex in the absence of the β-lactam antibiotics. Differences in the *mec* gene complex is due to the insertion of

insertion sequences, IS431 and IS1272, in the *mecR1* and/or *mecI* genes resulting in truncated products. In total, there are six major classes of the *mec* gene complex (table 4.2), all which is a divergent type of the prototype gene complex (figure 14). The minor classes are variants within one of the major classes, such as class A3 and A4.

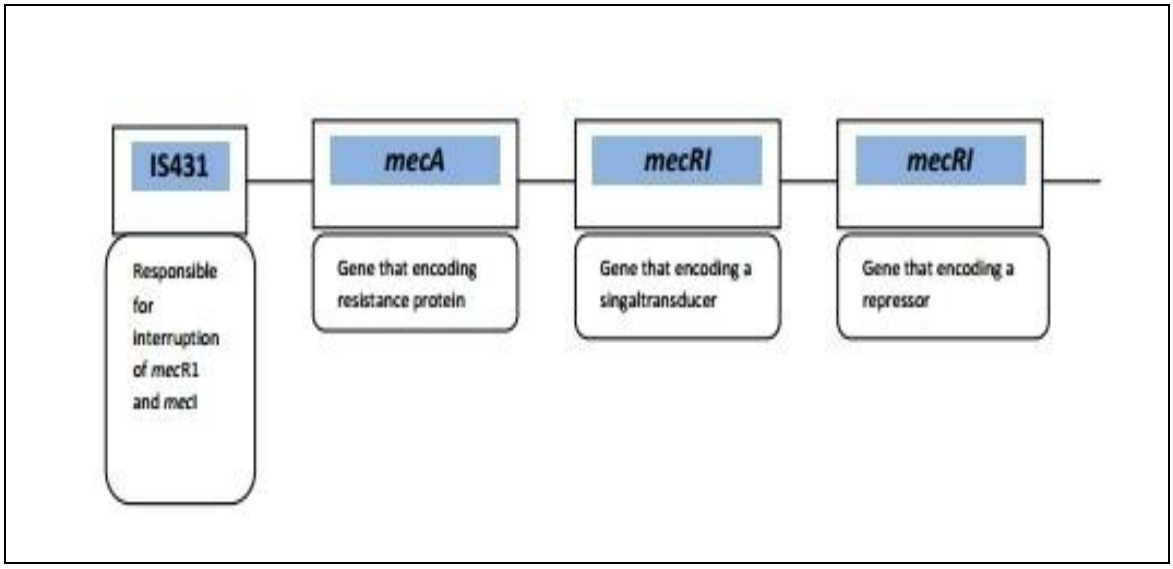


Figure 14: Schematic representation of the prototype mec gene complex.

B. ccr gene complex

The ccr gene complex is composed of the ccr gene(s) and surrounding open reading frames (ORFs) several of which have unknown functions. Currently, three phylogenetically distinct ccr genes, ccrA, ccrB, and ccrC, have been identified in *S. aureus* with DNA sequence similarities below 50%. To date, the *ccrA* and *ccrB* genes that have been identified in *S. aureus* have been classified into four allotypes. These allotypes are also found in other staphylococcal species as well as other allotypes have been described for these species only. In general, *ccr* genes with nucleotide identities of more than 85% are assigned to the same allotype, whereas, *ccr* genes that belong to different allotypes have lower nucleotide identities of between 60% and 82%, each other. All ccrC variants identified to date in staphylococcal strains have shown ≥87% similarity; thus, there is only one ccrC allotype. They suggest describing their differences as alleles by using previously used numbers, e.g., ccrC1 allele 2 or ccrC1 allele 8. The cassette chromosome recombinases (*ccr*) gene complex encodes gene(s) for the DNA recombinase enzyme of the invertase/resolvase family, which catalyzes the excision and insertion of the SCC*mec* element, and thus this complex is responsible for the movement of the SCC*mec* element into the staphylococcal chromosome. Integration of SCC*mec* elements into the chromosome of MSSA strains is a specific site integration. The integration happens at a unique 15-bp sequence called the integration site sequence (ISS) of the bacterial chromosomal attachment site (attBscc), which is located near the 3' end of orfX, which is an open reading frame of unknown function. [50]. Two groups of *ccr* genes have been reported: (1) homologous pairs of *ccrA* and *ccrB* gene and (2) one *ccrC* gene. As with the *mec* gene complex, different the *ccr* gene complex exists. To date there is eight different types; all which differ in their combination of either their homologue pairs, having only one ccrC genes or a mix of both (table 4).

C. Joining regions

Besides the mec and ccr gene complexes, the SCCmec element also contains three so-called J regions, which constitute nonessential components of the cassette. J1 (formerly L-C) is the region between the right chromosomal junction and the ccr gene complex; J2 (C-M) is between the ccr gene complex and the mec gene complex; and J3 (I-R) is between the mec gene complex and the left chromosomal junction. Variations in the J regions within the same mec-ccr gene complex are used for defining SCCmec subtypes. Joining (J) regions formerly known as junkyard regions, are as their name indicate regions joining the two gene complexes (*mec* and *ccr* gene complex) together. There are three J regions within each SCC*mec* element and encode for non-essential components of the cassette. Even though they constitute for non-essential components, they have importance for epidemiological and diagnostically purposes as these regions might carry additional resistance genes by the carriage of plasmid(s) and/or transposon(s), but also because subtyping of SCC*mec* element is based on difference in the J1 region within a SCC*mec* type [5].

Table 4. (1) List of currently identified SCCmec elements with ccr gene complex type and mec gene complex class (2) List of currently identified mec gene complexes and their composition. (3) List of currently identified ccr gene complexes and their composition

Sccme type	Ccr gene comple	mec gene complex
SccmecI	J1-------type 1 ccr gene complex -------J2------Class B mec gene complex --J3	
SCCmec II	J1------type 2 ccr gene complex-------J2------Class A mec gene complex ---J3	
SccmecIII	J1------ type 3 ccr gene complex-------J2------Class A mec gene complex ---J3	
SccmecIV	J1------ type 2 ccr gene complex------J2------ Class B mec gene complex ---J3	
SccmecV	J1------ type 5 ccr gene complex-------J2----- Class C2 mec gene complex --J3	
Sccmec VI	J1----- type 4 ccr gene complex-------J2------ Class B mec gene complex ---J3	
Sccmec VII	J1----- type 5 ccr gene complex-------J2--- Class C1 mec gene complex -----J3	
Sccmec VIII	J1------ type 4 ccr gene complex-------J2----- Class A mec gene complex ---J3	
Sccmec IX	J1------type 1 ccr gene complex-------J2------ Class C2 mec gene complex -J3	
Sccmec X	J1------type 7 ccr gene complex-------J2------ Class C1 mec gene complex -J3	
Sccmec XI	J1------type 8 ccr gene complex-------J2------ Class E mec gene complex ---J3	

1) List of currently identified Sccmec elements with ccr gene complex type and mec gene complex class.

Mec gene complex	
Class A	IS431—mecA—mecR1--mecI
Class B	IS431—mecA—дmecR1—IS1272
Class C1	IS431—mecA—дmecR1—IS431
Class C2	IS431—mecA—дmecR1—IS431
Class D	IS431—mecA—дmecR1
Class E	blaZ—mecALGA251—mecR1LGA251—mecILGA251

2)List of currently identified mec gene complexes and their composition

Ccr gene complex	
Type 1	ccrA1 and ccrB1
Type 2	ccrA2 and ccr B2
Type 3	ccrA3 and ccr B3
Type 4	Ccr A4 and ccr B4
Type 5	Ccr C1
Type 6	Ccr A5 and ccr B3
Type 7	Ccr A1 and ccr B6
Type 8	ccrA1 and ccr B3

3) List of currently ccr gene complexes and their composition.

XII. EPIDEMIOLOGY OF MRSA

Chambers & Deleo (2010) [8] describes the emerging of MRSA isolates through a series of waves (figure 15). The emergence of penicillin-resistant *S. aureus* marked the first wave of antibiotic resistance by *S. aureus*. The second wave was marked with the introduction of the semi-synthetic compound methicillin into clinical practice, of which soon after the emergence of MRSA harboring the SCC*mec* type I element was observed. The third wave was marked with the emergence of MRSA harboring the SCC*mec* type II or type III elements. MRSA have until recently often been associated with nosocomial infections, however the epidemiology of MRSA has changed; they are now also frequently found in the community and livestock settings, which Chamber & Deleo (2010) describes as the fourth and fifth wave of antibiotic resistance by *S. aureus*, respectively. (Fig. 15)

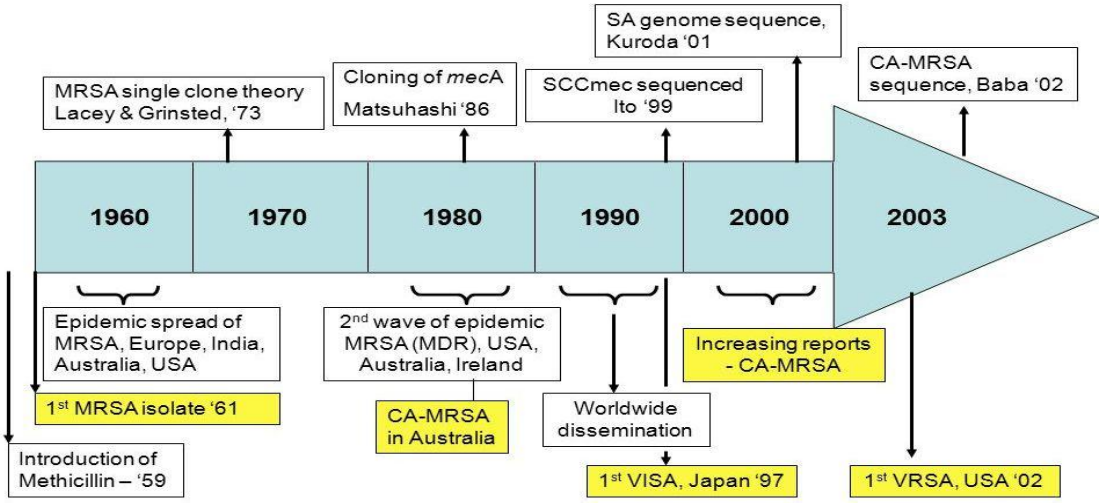


Figure15. Schematic representation of the timeline of development of antibiotic resistance in Staphylococcus aureus. [8]

A. Health Care Associated MRSA (HA-MRSA)

In the first decades MRSA was ranked among pathogenic microorganisms responsible for infections nosocomial and indicated as Health Care Associated MRSA (HA-MRSA); with the passing of years between the great variety of strains circulating began to recognize MRSA with epidemic potential (EMRSA). The origin of the various circulating MRSA strains is not yet clear, but the two theories now formulated seek to identify the time and contex of the introduction of SCCmec into the *S. aureus* genome and has taken place in one clone or multiple clones at the same time. One of the oldest strains seems to be a variation lower than the MRSA ST250 (ST247 MRSAI) known as the Iberian clone and one of the strains most popular in the world. [54].

A great contribution to the knowledge of the problem is given by the EARSS Surveillance System (European Microbial Resistance Surveillance System) also known as EARSnet (European Microbial Resistance Surveillance Network) a European surveillance system born in 1999 and focused on monitoring antibiotic resistance from the data of laboratory from cases of systemic / invasive infections.

It is interesting to note that not all MRSA strains have spread well within the hospitals. The main HA-MRSAs identified today are: CC5, CC8, CC22,
CC30, CC45 and CC8/ST239. [55]. All strains also tend to evolve and occupy new ecological niches like the ones EMRSA-16 and EMRSA-15 themselves. In fact, between 2001 and 2007, EMRSA-15 gradually replaced EMRSA-16 becoming the major HA-MRSA strain circulating in England. [56]. HA-MRSA isolates show high erythromycin resistance rates, tetracycline, ciprofloxacin, clindamycin, and particularly worrisome to vancomycin. The latter, the resistance to vancomycin, was acquired by enterococci for horizontal transfer of the *vanA* gene, initially in the HA-MRSA strain (USA100) then called VRSA (Vancomycin Resistant *Staphylococcus aureus*) and isolated for the first time in 2002. [57].

B. Community Associated MRSA (CA-MRSA)

MRSA infections in non-hospitalized population in the absence of particular risk factors have been recorded since the '90s, and isolated strains were designated as Community Acquired MRSA (CA-MRSA). Many studies show that the diffusion of CA-MRSA varies greatly from country to country; the High-circulation countries of HA-MRSA such as Italy are generally characterized by low percentages of CA-MRSA (1-2%) and vice versa low-circulating HA-MRSA countries as Denmark has high rates of CA-MRSA (29%). CDC investigations, between 2001 and 2002, assessed the percentage of CA-MRSA among all MRSA isolates to 8-20%. The spread of CA-MRSA deserves constant attention; the infections supported by CA-MRSA are generally infections to skin and soft tissues seldom have more serious infections such as necrotizing pneumonia associated with more than 50% in mortality Furthermore, it is not easy to identify specific risk factors for CA-MRSA to date because the studies carried out in this regard are very fragmentary. From a microbiological point of view CA-MRSA and HA-MRSA are deeply distinct for a variety of features:

- antibiotic resistance since CA-MRSA is usually susceptible to the most part of non-beta-lactam antibiotics and HA-MRSA are instead multi-resistant,
- SCCmec Type IV, V or VII are harbored in CA-MRSA while SCCmec Type I, II or III are harbored in HA-MRSA,
- Panton-Valentine leukocyte (PVL) generally produced by CA-MRSA.

At the global level, the most common and frequently isolated CA-MRSA strains are three: ST80, CC30 and CC8 (USA300). The ST80 circulating in Europe is generally resistant to fluoroquinolones, tetracyclines and fusidic acid while the the USA300 clones have already been identified as multi resistant. [58]

The impact of these CA-MRSA on human health is not limited to community but it should be extended to welfare facilities because these same strains can be introduced and stabilize in the hospital context. [59]

The main hospital clone in our day, UK-EMRSA-15, was originally a Community strain that acquired antibiotic resistances and succeded in replacing EMRSA-16 (CC30 ST36 SCCmecII) by spreading in hospitals around the world [60] [61] [62].

There is significant diversity in MRSA arising in communities worldwide. As CA-MRSA has become established in healthcare facilities, the range of infections caused by them has also increased. Although many CA-MRSA still maintain a nonmultidrug resistant antimicrobial profile, multiresistance to non-β-lactam agents has emerged in some clones, posing substantial problems for empirical and directed therapy of infections caused by these strains. The emergence of pandemic CA-MRSA clones not only limits therapeutic options but also presents significant challenges in infection control. Continued monitoring of global epidemiology and emerging drug resistance data is critical for the effective management of these infections. [62] [63]

XIII. MATERIAL AND METHODS

Bacterial Strains

A total of 135 *S. aureus* strains selected to be resistant to methicillin were used in the study. The strains were isolated from clinical samples at the Microbiology Laboratory of Verona Hospital during the period 2011-2016.

Strains were identified on gram staining, colony morphology and standard biochemical tests and MALDI-TOF (Vitek MS, Biomérieux).

The strains were divided in two groups. Out of 135 MRSA total strains, 94 were isolated from pharyngeal and rectal swabs during multi-drug resistant screening, and called MDR group, while 41 were isolated from blood cultures and called AMC group.

In table n° 5 and 6 are reported the strains under study with their clinical characteristics.

	MDR	**id**	**specimen**	**section**
1	Mdr 1	15-04-2013	Pharyngeal swab	Pediatric and oncology and haematology
2	N°2	3-12-2014	Pharyngeal swab	anesthesia
3	N°3	3-12-2014	Pharyngeal swab	anesthesia
4	Mdr 4	10-12-2014	Pharyngeal swab	Anesthesia
5	Mdr 6	10-12-2014	Rectal swab	anestesia
6	Mdr 8		Pharyngeal swab	ICU
7	Mdr 12L	10-02-2015	Rectal swab	ICU
8	Mdr 16	10-12-2014	Pharyngeal swab	ICU
9	N°20	16-12-2014	Rectal swab	ICU
10	Mdr 007	13-04-2013	Pharyngeal swab	Vascular surgery
11	Mdr 0089	--	Pharyngeal swab	burns

12	Md r44/20	--	Pharyngeal swab	burns
13	Mdr 016	24-04-2013	Pharyngeal swab	geriatry
14	Mdr 023	30-04-2013	Pharyngeal swab	general medicine
15	Mdr 058	15-05-2013	Rectal swab	ICU
16	Mdr 062	20-05-2013	Pharyngeal swab	ICU
17	Mdr 068	23-05-2013	Rectal swab	General surgery
18	Mdr 091	10-06-2013	Pharyngeal swab	ICU
19	Mdr 092	10-06-2013	Pharyngeal swab	ICU
20	Mdr 093	10-06-2013	Pharyngeal swab	ICU
21	Mdr131	19-06-2013	Rectal swab	ICU
22	Mdr 139	24-06-2013	Pharyngeal swab	ICU
23	Mdr140	24-06-2013	Pharyngeal swab	ICU
24	Mdr 144	24-06-2013	Pharyngeal swab	ICU
25	Mdr 145	24-06-2013	Pharyngeal swab	ICU
26	Mdr 150	24-06-2013	Pharyngeal swab	burns
27	Mdr 157	25-06-2013	Pharyngeal swab	General medicine
28	Mdr 169	02-07-2013	Pharyngeal swab	ICU
29	Mdr 181	02-07-2013	Pharyngeal swab	ICU
30	Mdr 199	09-07-2013	Pharyngeal swab	ICU
31	Mdr 204	09-07-2013	Rectal swab	ICU
32	Mdr 212	10-07-2013	Rectal swab	gastoenterology
33	Mdr 241	17-07-2013	Pharyngeal swab	ICU
34	Mdr 243	18-07-2013	Pharyngeal swab	ICU
35	Mdr 268	30-07-2013	Pharyngeal swab	ICU
36	Mdr 270	30-07-2013	Pharyngeal swab	ICU
37	Mdr 281	05-08-2013	Pharyngeal swab	Liver transplatation
38	Mdr 322	02-09-2013	Pharyngeal swab	ICU
39	Mdr 378	10-09-2013	Pharyngeal swab	ICU
40	Mdr 405	12-09-2013	Pharyngeal swab	ICU
41	Mdr 416	18-09-2013	Pharyngeal swab	ICU
42	Mdr 470	18-10-2013	Rectal swab	ICU
43	Mdr 533	27-11-2013	Pharyngeal swab	ICU
44	Mdr 537	28-11-2013	Pharyngeal swab	ICU
45	Mdr 541	18-09-2013	Nasal swab	Cardiac surgery
46	Mdr 543	01-08-2013	pharyngeal	geriatrics

47	Mdr 545	28-09-2013	Pharyngeal swab	ICU
48	Mdr 560	11-12-2013	Rectal swab	endocrinology
49	Mdr 561	11-12-2013	Rectal swab	medicine
50	Mdr 571	18-12-2013	Pharyngeal swab	ICU
51	Mdr 591	27-12-2013	Rectal swab	ICU
52	Mdr 601	31-12-2013	Rectal swab	ICU
53	Mdr 613	10-01-2014	Pharyngeal swab	ICU
54	Mdr 632	18-02-2014	Pharyngeal swab	ICU
55	Mdr 641	22-01-2014	Pharyngeal swab	ICU
56	Mdr 643	23-01-2014	Pharyngeal swab	ICU
57	Mdr 665	07-03-2014	Rectal swab	cardiology
58	Mdr 669	08-03-2014	Pharyngeal swab	ICU
59	Mdr 670	06-05-2014	Pharyngeal swab	General surgery
60	Mdr 674	28-02-2014	Rectal swab	Infectious Diseases
61	Mdr 838	---	---	ortopedia
62	Mdr 849	16-06-2014	Pharyngeal swab	ICU
63	Mdr 850	16-06-2014	Rectal swab	ICU
64	Mdr 851	----	---	Infectious disease
65	Mdr 874	23-06-2014	Pharyngeal swab	ICU
66	Mdr 891	26-06-2014	Pharyngeal swab	neurosurgery
67	Mdr 911	07-07-2014	Pharyngeal swab	ICU
68	Mdr 915	07-07-2014	Rectal swab	Neuro surgery
69	Mdr 919	08-07-2014	Pharyngeal swab	Pediatric onco-haematology
70	Mdr 949	14-07-2014	Pharyngeal swab	neurosurgery
71	Mdr 997	26-07-2014	Pharyngeal swab	ICU
72	Mdr 1007			oncology
73	Mdr 1050	08-08-2014	Pharyngeal swab	ICU
74	Mdr 1051	08-08-2014	Pharyngeal swab	ICU
75	Mdr 1096	19-08-2014	Pharyngeal swab	ICU
76	Mdr 1111	22-08-2014	Pharyngeal swab	ICU
77	Mdr 1147	----	-----	unknown
78	Mdr 1251	29-09-2014	Rectal swab	ICU
79	Mdr 1260	30-09-2014	Rectal swab	ICU
80	Mdr 1265	01-10-2014	Rectal swab	General medicine
81	Mdr 1275	02-10-2014	Pharyngeal swab	ICU
82	Mdr 1294	08-10-2014	Rectal swab	ICU

83	Mdr 1310	13-10-2014	Pharyngeal swab	ICU
84	Mdr 1305	10-10-2014	Rectal swab	psichiatry
85	Mdr 1330	17-10-2014	Pharyngeal swab	ICU
86	Mdr 1678	07-01-2015	Rectal swab	ICU
87	Mdr 1698	08-01-2015	Pharyngeal swab	ICU
91	Mdr 1713	12-01-2015	Pharyngeal swab	Pancreatic surgery
88	Mdr 1729	15-01-2015	Pharyngeal swab	burns
89	Mdr 1745	19-01-2015	Pharyngeal swab	Cardiac surgery
90	Mdr 1756	21-01-2015	Pharyngeal swab	ICU
92	Mdr 3636	---	---	unknown
93	Mdr 3734	---	---	unknown
94	Mdr 3740	---	---	unknown

Table 5. Clinical characteristics MDR strains.

	AMC	**Id**	**Specimen**	**Section**
1	Amc 597	05-12-2011	Blood culture	Medicine
2	Amc 584	01-12-2011	Blood culture	Haematology
3	Amc 602	24-11-2011	Blood culture	Surgery
4	Amc 622	13-12-2011	Blood culture	haematology
5	Amc 720	08-03-2012	Blood culture	ICU
6	Amc 772	05-07-2012	Blood culture	Medicine
7	Amc 783	20-07-2012	Blood culture	Urology
8	Amc 787	23-07-2012	Blood culture	Medicine
9	Amc 794	24-07-2012	Blood culture	Medicine
10	Amc 937	13-11-2012	Blood culture	Neurology
11	Amc 994	26-11-2012	Blood culture	nefrology

12	Amc 1073	28-01-2013	Blood culture	haematology
13	Amc 1074	28-01-2013	Blood culture	ICU
14	Amc 3319	01-07-2015	Blood culture	Medicine
15	Amc 3364	19-07-2015	Blood culture	Cardiology
16	Amc 3672		Blood culture	
17	Amc 6537	09-06-2016	Blood culture	Medicine
18	Amc 6553	15-06-2016	Blood culture	Surgery
19	Amc 6559	17-06-2016	Blood culture	Medicine
20	Amc 6666		Blood culture	unknown
21	Amc 6668	21-09-2016	Blood culture	Geriatry
22	Amc 6730	12-09-2016	Blood culture	Medicine
23	Amc 6761	20-06-2016	Blood culture	Surgery
24	Amc 6767	28-06-2016	Blood culture	Geriatry
25	Amc 6781	07-07-2016	Blood culture	ICU
26	Amc 6784	04-07-2016	Blood culture	Infectious diseases
27	Amc 6797	21-07-2016	Blood culture	Surgery
28	Amc 6801		Blood culture	unknown
29	Amc 6822	08-11-2016	Blood culture	Geriatry
30	Amc 6826	10-11-2016	Blood culture	Medicine
31	Amc 6834		Blood culture	unknown
32	Amc 6846	12-11-2016	Blood culture	Medicine
33	Amc 6857	25-10-2016	Blood culture	Medicine
34	Amc 6862	29-10-2016	Blood culture	medicine
35	Amc 6871	--	Blood culture	unknown
36	Amc 6872	--	Blood culture	unknown

37	Amc 6880	--	Blood culture	unknown
38	Amc 6894	--	Blood culture	unknown
39	Amc 6895	--	Blood culture	unknown
40	Amc 7047	--	Blood culture	unknown
41	Amc 7022	---	Blood culture	unknown

Table 6: clinical characteristics AMC strains.

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed both by Etest and broth microdilution methods. The results were interpreted by following the latest European Committee Antimicrobial Susceptibility Testing (EUCAST) breakpoints (www.eucast.org). *S. aureus* ATCC 25923 was used as quality control

E-test: epsilometric test

The epsilometric test, also called Etest, is a widely used variant of the diffusion method. This test is done by applying in the Mueller Hinton Agar one or more rectangular bibula strips (about 0.4 cm x 8 cm) containing scalar concentrations of the antibiotic to test. E-test employs strips of an impregnated antibiotic polymer placed on the agar surface Muller hinton agar (MHA) that can gradually release it. After incubation we obtain an elipsoidal inbition zone and the ellipse will intersect the reading scale (in μg/ml) of the MIC at the point where the concentration of the antibiotic tested inhibits the growth of microorganisms. The method allows quick evaluation of the minimum inhibiting concentration; in fact, the point where the tip of the drop meets the strip, corresponds to the smallest antibiotic concentration still able to inhibit bacterial growth.

Broth microdilution test.

The broth microdilution method is based on the use of 96-well curved microplates. Each well was filled with 100 µl of Mueller-Hinton (MH) broth, 5.12 µl from a 10 mg/ml mother solution was pipetted into the first well and made serial dilution, to obtain final 100 µl in each well. Finally, 100 µl of bacterial inoculum in MH broth, with 10^5 cfu/ml was added in each well, obtaining antibiotic concentrations of 128 µg/ml in the first well and 0.06 µg/ml in the last one. The microdilution plates were incubated overnight at 37 ºC. MIC is the lowest drug concentration that does not allow visible growth.

MRSA screening

MRSA screening was performed by the cefoxitin disk diffusion test (30µg). According to the Eucast Institute, a zone of growth inibithion around the cefoxitin disk of <22 mm indicates the MRSA phenotype and the isolate should be reported as MRSA.

Multiplex detection of *mec, pvl, scn* and *spA* genes

MRSA isolates were typed using the protocol of Stegger et al [64].

The *mecA, mecC, scn, pvl* and *spA* genes were amplified using the primers described in table 7.

Primers	**Sequence 5'→3'**	**Amplicon(bp)**
Spa-1113f	TAAAGACGATCCTTCGGTGAGC	Variable (200-600bp)
Spa-1514r	CAGCAGTAGTGCCGTTTGCTT	
mecA F	TCCAGATTACAACTTCACCAGG	162 bp
mecA R	CCACTTCATATCTTGTAACG	
MecA lga251 F	GAAAAAAAGGCTTAGAACGCCTC	138 bp
MecA lga 251 R	GAAGATCTTTTCCGTTTTCAGC	
Scn F	ATATTTTGCTTCTGACATTTTCT	112 bp
Scn R	AGCTACTGGAAGTTTAAACACT	
Pvl F	GCTGGACAAAACTTCTTGGAATAT	~85 bp
Pvl R	GATAGGACACCAATAAATTCTGGATTG	

Table 7. List of primers sequence used for PCR analysis in this study.

DNA extraction were performed with 3-4 bacteria colonies suspended in 200µl sterile water from a fresh subculture of *S. aureus* isolate. Subsequently boiled for 10 min and centrifuged for 5 min at 20000 rpm. 2 µl of supernatant was used as template. *S. aureus* 50148 and lga251 were used as positive controls. This M-PCR protocol was used to type five genes. The amplification mixture contained 10 µM of each primer on final volume of 25µl mixed multiplex 2x the genes *spA*, *mecA*, *pvl*, *meclga251*, *scn.* Amplification conditions were one cycle at 94°C for 15 min, 25 cycles at 94°C for 1 min, 59°C for 1 min, 72°C for 60 min and one final cycle at 72 °C for 10 min.

SpA repeats detection:

We developed a Microsoft Window application coded in Delphi 7 program called SpA Finder. This language (Embarcadero) uses the Pascal-based programming. SpA Finder is a program to locate and display sequence variable number repeat (VNR) of 21-27 base pair in DNA sequence. The output file in a memo box contain information about each repeat, including its location, size, number of copy nucleotide.

PCR and sequence analysis of the X region of the *spA* gene was amplified by PCR primer indicated in table 7. DNA sequences were obtained with ABI 377 Sequencer (Applied Biosystem CA). SPA types were determined with our software called SpA Finder described above.

The program is very fast, analyzing sequence on the order of 20000 nucleotide in just a few seconds. In addition, SpA Finder is a free open source software available in our Institution by mail request.

Rapid Identification System for mec, ccr by Combination of Multiplex PCRs for Staphylococcal Cassette Chromosome mec Type Assignment

PCR based SCCmec typing.

All MRSA isolates were typed using the method described by Kondo *et al*. (2007). Prior to the SCCmec typing according to Kondo, DNA templates were produced according to Kumari *et al*. [65]

Colonies (3-4 colonies) from each isolate were dissolved with 25µL lysostaphin (100 µg/mL) and incubated for 10 minutes at 37 °C. Each isolate was mixed with 25 µL proteinase K (100 µg/mL) and 75 µL Tris-HCl (pH 8.0) and incubated at 37 °C and 97 °C for 10 min and 5 minutes, respectively. After incubation, all isolates where centrifuged at 20.000 x g for 5 min. Together with six/seven reference genomes for SCCmec cassette type I-VII (COL, N315, 85/2085,

JCSA4459, WIS, HDE288) for validation, they were subjected to M-PCR 1 and M-PCR 2 for the amplification of ccr gene complex and mec gene complex.

Primers used for SCCmec typing were listed in table 8 and 9.

Primers	**5→ 3'**	**Gene**	**Size bp**
mA1 mA2	TGCTATCCACCCTCAAACAGG AACGTTGTAACCACCCCAAGA	mecA	286
α1 ßc	AACCTATATCATCAATCAGTACGT ATTGCCTTGATAATAGCCITCT	ccrA1-ccrB	695
α 2 ßc	TAAAGGCATCAATGCACAAACACT ATTGCCTTGATAATAGCCITCT	ccrA2-ccrB	937
α3 ßc	AGCTCAAAAGCAAGCAATAGAAT ATTGCCTTGATAATAGCCITCT	ccrA3-ccrB	1791
α4.2 ß4.2	GTATCAATGCACCAGAACTT TTGCGACTCTCTTGGCGTTT	ccrA4-ccrB4	1287
ɣR ɣf	CCTTTATAGACTGGATTATTCAAAATAT CGTCTATTACAAGATGTTAAGGATAAT	ccrC	518

Table 8: primers used in this study M-PCR 1

Primes	**5->3**	**Gene**	**Size bp**
mA7 mI6	ATATACCAAACCCGACAACTACA CATAACTTCCCATTCTGCAGATG	mecA-mecI	1963
mA7 IS7	ATATACCAAACCCGACAACTACA ATGCTTAATGATAGCATCCGAATG	mecA-IS1272 upstream of mecA	2827
mA7 IS2(iS-2)	ATATACCAAACCCGACAACTACA TGAGGTTATTCAGATATTTCGATGT	mecAiS431 upstream of mecA	804

Table 9: primers used in this study M-PCR 2.

Each PCR reaction contained 10× PCR Buffer minus Mg, $MgCl_2$ 50mM, dNTP mix (2,5 mM of each dNTP) primers mix (0,5 µM) and 5U/µL of Platinum Taq DNA polymerase (Invitrogen) and DNA template to a total volume of 25µL

Amplification conditions were the following: one cycle 94°C for 2 min; 30 cycles at 94 °C for 4 min. 57°C for 1 min and 72°C for 2 min and finally one cycle at 72 °C for 2 minutes. The PCR amplicons were visualized on E-gels 2% agarose gel.

Triplex Real-time PCR assay for detection of S. aureus genes encoding Panton-Valentine Leukocidin, Methicillin Resistance directly from clinical samples*:*

We develop a triplex assays real-time PCR to quickly detect *S. aureus*, methicillin resistance and the virulence factor *pvl* directly from a clinical sample without culture. This assay identifies and differentiate MRSA, MSSA, Methicillin-Resistant Coagulase Negative Staphylococci (MR-CNS) and Methicillin-Sensitive Coagulase Negative Staphylococci (MS-CNS) The TaqMan PCR method was used for the detection of *pvl* and *mecA* encoding genes and the amplification of *nuc* gene specific for identification of *S. aureus* species.

Strains were incubated for 24 h on MSA agar plates, and cultures adjusted to the McFarland (McF) 0.5 standard suspension (1,5 × 10^8 CFU/mL). Nucleic acids were extracted with a Microlab Nimbus apparatus (Hamilton Robotics, NV, USA). from 350 µl of McF suspension according to the manufacturer's instructions. 340 µl of lysis buffer containing proteinase K at the concentration of 20µg/ml (Sigma, Milan Italy) was added. This mixture was incubated at 56°C for 5 min with 25 µl of silica followed by automatic magnetic separation. Nucleic acid was then recovered in 100 µl of elution buffer. In table 10 are reported the sequence of labeled probes designed for the assay.

Target gene	**Primer/Probe**	**Sequence (⇨ 3')**	**Amplicon size (bp)**
	Forward	CAATGCCAAAATCTCAGGTAAAGTG	
mecA	Reverse	AACCATCGTTACGGATTGCTTC	107
	Probe	**FAM**-ATGAGCTATATGAGAACGG-**MGBNFQ**	
	Forward	AAATGCTGGACAAAACTTCTTGG	
pvl	Reverse	TTTGCAGCGTTTTGTTTTCG	108
	Probe	**VIC**-AAATGCCAGTGTTATCC-**MGBNFQ**	
	Forward	GGCATATGTATGGCAATTGTTTC	
nuc	Reverse	CGTATTGCCCTTTCGAAACATT	73
	Probe	**NED**-ATTACTTATAGGGATGGCTATC-**MGBNFQ**	

Table 10: probes designed for triplex RT-PCR

In the table 11, 12, 13 are reported the sequence of gene and the primer FW and RW and probe position.

>Nuc DQ507380
ATGACAGAATACTTATTAAGTGCTGGCATATGTATGGCAATTGTTTCA**ATATTACTTA** **TAGGGATGGCTAT**CAGTAATGTTTCGAAAGGGCAATACGCAAAGAGGTTTTTCTTTT TCACTACTAGTTGCTTAGTGTTAACTTTAGTTGTAGTTTCAAGTCTAAGTAGCTCAGCA AATGCATCACAAACAGATAACGGCGTAAATAGAAGTGGTTCTGAAGATCCAACAGTA TATAGTGCAACTTCAACTAAAAAATTACATAAAGAACCTGCGACATTTATTAAAGCGA TTGATGGTGATACGGTTAAATTAATGTACAAAGGTCAACCAATGACATTCAGACTATT ATTGGTTGATACACCTGAAACAAAGCATCCTAAAAAAGGTGTAGAGAAATATGGTCC TGAAGCAAGTGCATTTACGAAAAAAATGGTAGAAAATGCAAAGAAAATTGAAGTCGA GTTTGACAAAGGTCAAAGAACTGATAAATATGGACGTGGCTTAGCGTATATTTATGCT GATGGAAAAATGGTAAACGAAGCTTTAGTTCGTCAAGGCTTGGCTAAAGTTGCTTATG TTTATAAACCTAACAATACACATGAACAACTTTTAAGAAAAAGTGAAGCACAAGCAA AAAAAGAGAAATTAAATATTT
fW 5’-GGCATATGTATGGCAATTGTTTCA-3’ 59°C Tm
rev 5’-CGTATTGCCCTTTCGAAACATT-3’ 59 °C Tm
probe NED 5’-ATTACTTATAGGGATGGCTATC-3’ 68°C Tm

Table 11: sequence gene Nuc e primer fw reW and probe.

>mecA KC243783.1
ATGAAAAAGATAAAAATTGTTCCACTTATTTTAATAGTTGTAGTTGTCGGGTTTGGTATATATTT TTATGCTTCAAAAGATAAAGAAATTAATAATACTATTGATGCAATTGAAGATAAAAATTTCAAA CAAGTTTATAAAGATAGCAGTTATATTTCTAAAAGCGATAATGGTGAAGTAGAAATGACTGAAC GTCCGATAAAAATATATAATAGTTTAGGCGTTAAAGATATAAACATTCAGGATCGTAAAATAAA AAAAGTATCTAAAAATAAAAAACGAGTAGATGCTCAATATAAAATTAAAACAAACTACGGTAA CATTGATCGCAACGTTCAATTTAATTTTGTTAAAGAAGATGGTATGTGGAAGTTAGATTGGGATC ATAGCGTCATTATTCCAGGAATGCAGAAAGACCAAAGCATACATATTGAAAATTTAAAATCAGA ACGTGGTAAAATTTTAGACCGAAACAATGTGGAATTGGCCAATACAGGAACAGCATATGAGAT AGGCATCGTTCCAAAGAATGTATCTAAAAAAGATTATAAAGCAATCGCTAAAGAACTAAGTATT TCTGAAGACTATATCAAACAACAAATGGATCAAAATTGGGTACAAGATGATACCTTCGTTCCAC TTAAAACCGTTAAAAAAATGGATGAATATTTAAGTGATTTCGCAAAAAAATTTCATCTTACAAC TAATGAAACAAAAAGTCGTAACTATCCTCTAGAAAAAGCGACTTCACATCTATTAGGTTATGTT GGTCCCATTAACTCTGAAGAATTAAAACAAAAAGAATATAAAGGCTATAAAGATGATGCAGTT ATTGGTAAAAAGGGACTCGAAAAACTTTACGATAAAAAGCTCCAACATGAAGATGGCTATCGT GTCACAATCGTTGACGATAATAGCAATACAATCGCACATACATTAATAGAGAAAAAGAAAAAA GATGGCAAAGATATTCAACTAACTATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGA AAAATGATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATTATTAGCACTTGTAAG CACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGTAACGAAGAATATAATAAATTA ACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCCAGATTACAACTTCACCAGGTTCAACTC AAAAAATATTAACAGCAATGATTGGGTTAAATAACAAAACATTAGACGATAAAACAAGTTATA AAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGGGGTGGTTACAACGTTACAAGATATG AAGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAGAATCATCAGATAACATTTTCTTTGC TAGAGTAGCACTCGAATTAGGCAGTAAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGG TGAAGATATACCAAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAGATAATG AAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAAATACTGATTAACCCAGTACAGATCCT TTCAATCTATAGCGCATTAGAAAATAATGGCAATATTAACGCACCTCACTTATTAAAAGACACG AAAAACAAAGTTTGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTA TGCAACAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTTATGCAAACTTAATTGG CAAATCCGGTACTGCAGAACTCAAAATGAAACAAGGAGAAACTGGCAGACAAATTGGGTGGTT TATATCATATGATAAAGATAATCCAAACATGATGATGGCTATTAATGTTAAAGATGTACAAGAT AAAGGAATGGCTAGCTACAATGCCAAAATCTCAGGTAAAGTGT**ATGATGAGCTATATGAGAA** **CGG**TAATAAAAAATACGATATAGATGAATAACAAAACAGTGAAGCAATCCGTAACGATGGTTG CTTCACTGTTTTATTATGAATTATTAATAAGTGCTGTTACTTCTCCCTTAAATACAATTTCTTCAT TTTCATTGTATGTTGAAAGTGACA
mecA fW 5’ CAATGCCAAAATCTCAGGTAAAGTG 3’ Tm 59,9 °C
mecA rW 5’ AACCATCGTTACGGATTGCTTC 3’ Tm 59 °C
mecA probe FAM **5’ ATGAGCTATATGAGAACGG 3’** **Tm 68 °C**

Table 12 sequence gene mecA e primer fw reW and probe.

>X72700.1 S. aureus gene F component of Panton-Valentine leucocidins ORF *luk* F
ATGAAAAAAATAGTCAAATCTAGAGAAGTTACATCAATTGCATTGCTTTTGCTATCCA ATACACTTGATGCAGCTCAACATATCACACCTGTAAGTGAGAAAAAGGTTGATGATA AAATTACTTTGTACAAAACAACTGCAACATCAGATTCCGATAAGTTAAAAATTTTGGA AACATTTATTCTGGCTATACAAAGCCAAATCCAAAAGACACTATTAGTTCTCAATTTT ATTGGGGTTCTAAGTACAACATTTCAATTAATTCAGATTCTAATGACTCAGTAAACGT TGTAGATTATGCACCTAAAAATCAAAATGAAGAATTTCAAGTACAACAAACGGTAGG TTATTCTTATGGTGGAGATATTAATATCTCTAACGGCTTGTCAGGTGGAGGTAATGGT TCAAAATCTTTTTCAGAGACAATTAACTATAAACAAGAAAGCTATAGAACTAGCTTAG ATAAAAGAACTAATTTCAAAAAAATTGGTTGGGATGTTGAAGCACATAAAATTATGA ATAATGGTTGGGGACCATATGGCAGAGATAGTTATCATTCAACTTATGGTAATGAAAT GTTTTTAGGCTCAAGACAAAGCAACTTAAATGCTGGACAAAACTTCTTGGAATATCAC **AAAATGCCAGTGTTATCC**AGAGGTAACTTCAATCCAGAATTTATTGGTGTCCTATCT CGAAAACAAAACGCTGCAAAAAAATCAAAAATTACTGTTACTTATCAAAGTGAAATG GATAGATATACAAACTTTTGGATCAACTTCAACTGGATAGGTAATAATTATAAAGATC ACATAAGAGCAACTCATACATCAATTTATGAAGTTGATTGGGAAAATCATACAGTTAA ATTAATAGATACTCAATCTAAGGAAAAAAATCCTATGAGCTAA
Pvl fW 5’AAATGCTGGACAAAACTTCT 3’ Tm 59°C
Pvl reW 5’TTTGCAGCTTTTGTTTTCG 3’ Tm 59°C
Pvl probe VIC 5’ AAAATGCCAGTGTTATCC 3’ Tm 68°C

Table 13 sequence gene pvl and primer fw reW and probe

The reaction mixture contained 10 µl of DNA template, 900nM primers, 250 nM probe, 12µl 2X Master mix (Applied Biosystem CA, USA) and nuclease free water with a final volume of 20 µl. Multiplex real-time PCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems). The thermocycler condition consisted in initial denaturation at 94 °C for 5 min, followed by amplification that was performed during 38 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 30 sec. Multiple fluorescent signals were obtained once for cycle upon completion of extension step. Data acquisition and

analysis of the real-time PCR assay were performed using ABI 7500 real-time PCR system (Applied Biosystems).

Nucleic acid extraction directly from clincal samples were carried out with a Microlab Nimbus apparatus (Hamilton Robotics, NV, USA) as described above starting from 350 µl of medium.

The nucleotide sequence of the methicillin resistance (*mecA*), Panton valentine leucocidine (*pvl*) and *nuc* genes of *S. aureus* were obtained by Gene bank database. To design the probes for *S. aureus* RT- assay we used specific sequence deposited on GeneBank. The sequence DQ507380 for the *nuc* gene was used (GeneBank). Probe design of the methicillin resistance was based on the sequence KC243783.1 and Panton-Valentine leucocidins was based on the sequence AB006796.

Select primers and probes sequence were compared with sequence submitted to the GeneBank nucleotide database using a standard nucleotide comparision tool: BLASTN (www.ncbi.nlm.nih.gov). The probes were labeled with minore grove binding and two different fluorescent dyes (FAM, VIC, NED) at the 5' end, so that the multiple genes could been detected simultaneously in a single tube.

The analytical sensitivity was evaluated using different cells known concentration. The sensitivity of the assay was evaluated using 12 MRSA clinical isolates, 3 MSSA clinical isolates, 58 MRCoNS,7 MSCoNS and the reference strains ATCC 25923 (MSSA) and ATCC 700699 (MRSA).

All strains were incubated for 24 h on MSA agar plates, some colonies were adjusted to the McFarland 0.5 standard suspension ($1{,}5 \times 10^8$ CFU/mL). The suspension was then serially diluted ten-fold in lysis buffer with sterile NaCl until to a final concentration of 10 CFU/mL. DNA from each dilution was extracted according to method described above for the Triplex Real-time PCR assay. A positive quantitative PCR signal for the lowest concentration of the suspension was defined as the lower limit of detection for the assay.

Validation of RT-PCR directly from clinical samples

Nucleic acids from clinical samples were extracted from 350 µl of Easy swabs (Copan, Italy) buffer and was carried out with a Microlab Nimbus apparatus (Hamilton Robotics, NV, USA). according to the manufacturer's instructions. Briefly, 350 µl of pretreated sample was added to 340 µl of lysis buffer containing proteinase K. This mixture was incubated at 56°C for 5 min with 25 µl of silica followed by automatic magnetic separation. Nucleic acid was then recovered in 100 µl of elution buffer.

RESULTS

We characterized 135 MRSA strains regarding their antimicrobials susceptibility, the SCCmec typing and SpA typing. 94 MRSA out of 135 strains, indicated as MDR strains, have been isolated during MDR screening and 41 MRSA strains, indicated as AMC strains, have been isolated from blood culture samples.

The antimicrobials susceptibility test results are reported in table 14 and 15 for MDR and AMC strains, respectively. MICs of erythromycin, levofloxacin and ciprofloxacin were measured by broth microdilution method. The new 5th generation cephalosporin anti-MRSA, Ceftobiprole MICs were measured both by Etest and broth microdilution.

Table 14 MICs of MDR strains under study for erythromycin, levofloxacin, ciprofloxacin and ceftobiprole.

	N° strains	Spa-type	CC	id	specimen	section	E-test Ceftoµg/ml	MIC Cefto µg/ml	Mic levo µg/ml	Cipro µg/ml	Erytromycin µg/ml
1	Mdr 1	t 127	CC1	15-04-2013	Pharyngeal swab	Pediatric and oncology and haematology	0,5	0,125	16	128	16
2	N°2	t3441	Cc22	3-12-2014	Pharyngeal swab	anesthesia	1,5	0.5	32	128	128
3	N°3	t032	Cc22	3-12-2014	Pharyngeal swab	anesthesia	1	1	8	128	128
4	Mdr 4	t3441	Cc22	10-12-2014	Pharyngeal swab	Anesthesia	0.38	1	32	128	2
5	Mdr 6	t032	CC22	10-12-2014	Rectal swab	anestesia	0,5	1	8	128	128
6	Mdr 8	t032	CC22		Pharyngeal swab	ICU	0.5	0,25	64	128	128
7	Mdr 12L	t008 pvl +	CC8	10-02-2015	Rectal swab	ICU	0,5	0.5	64	128	128
8	Mdr 16	t032	CC22	10-12-2014	Pharyngeal swab	ICU	0.5	0.5	32	128	32
9	N°20	t032	Cc22	16-12-2014	Rectal swab	ICU	0.75	0.5	4	128	128
10	Mdr 007	t032	CC22	13-04-2013	Pharyngeal swab	Vascular surgery	0.75	2	64	128	0,5
11	Mdr 0089	t1214	Cc22 Scn-		Pharyngeal swab	burns	0.5	0.25	32	128	128
12	Mdr 44/20	t041	CC5		Pharyngeal swab	burns	2	2	32	64	128
13	Mdr 016	t032	CC22	24-04-2013	Pharyngeal swab	geriatry	0,5	0,25	16	128	128
14	Mdr 023	t1214	CC22	30-04-2013	Pharyngeal swab	general medicine	1	0,25	16	128	128
15	Mdr 058	t032	CC22	15-05-2013	Rectal swab	ICU	1	1	128	128	128
16	Mdr 062	t022	CC22	20-05-2013	Pharyngeal swab	ICU	0,75	2	64	128	128
17	Mdr 068	t032	CC22	23-05-2013	Rectal swab	General surgery	1	0.5	32	128	32
18	Mdr 091	t1036	Cc22	10-06-2013	Pharyngeal swab	ICU	0.75	0.5	16	128	16
19	Mdr 092	t1036	Cc22	10-06-2013	Pharyngeal swab	ICU	1	0,5	16	128	16
20	Mdr 093	t015	CC45	10-06-2013	Pharyngeal swab	ICU	1	1	4	8	16
21	Mdr 131	t032	Cc22	19-06-2013	Rectal swab	ICU	1	0,5	128	128	128

22	Mdr 139	t1036	Cc22	24-06-2013	Pharyngeal swab	ICU	1,5	1	8	64	8
23	Mdr 140	t1036	Cc22	24-06-2013	Pharyngeal swab	ICU	1.5	1	16	128	128
24	Mdr 144	t1036	Cc22	24-06-2013	Pharyngeal swab	ICU	1,5	1	4	128	128
25	Mdr 145	t1036	Cc22	24-06-2013	Pharyngeal swab	ICU	1,5	1	4	128	128
26	Mdr 150	t032	CC22	24-06-2013	Pharyngeal swab	burns	0,75	0,5	64	128	64
27	Mdr 157	t032	CC22	25-06-2013	Pharyngeal swab	General medicine	1	0,5	4	128	128
28	Mdr 169	t1171	Cc8	02-07-2013	Pharyngeal swab	ICU	1	1	8	128	128
29	Mdr 181	t1036	Cc22	02-07-2013	Pharyngeal swab	ICU	1	0,5	8	64	8
30	Mdr 199	t1036	Cc22	09-07-2013	Pharyngeal swab	ICU	1	1	64	128	128
31	Mdr 204	t1036	Cc22	09-07-2013	Rectal swab	ICU	1	1	16	64	128
32	Mdr 212	t1036	Cc22	10-07-2013	Rectal swab	gastoenterology	0,75	0,5	16	128	16
33	Mdr 241	t1036	Cc22	17-07-2013	Pharyngeal swab	ICU	1	0,5	64	128	128
34	Mdr 243	t1036	Cc22	18-07-2013	Pharyngeal swab	ICU	1,5	0,5	8	128	128
35	Mdr 268	t1036	Cc22	30-07-2013	Pharyngeal swab	ICU	2	1	32	128	128
36	Mdr 270	t032	Cc22	30-07-2013	Pharyngeal swab	ICU	0,25	1	8	128	0,25
37	Mdr 281	t127	Cc1	05-08-2013	Pharyngeal swab	Liver transplatation	1	0,25	0.06	0.06	128
38	Mdr 322	t032	Cc22	02-09-2013	Pharyngeal swab	ICU	0.5	0,25	8	128	0.125
39	Mdr 378	t223	Cc22	10-09-2013	Pharyngeal swab	ICU	0.5	0,25	4	64	0,125
40	Mdr 405	t032	Cc22	12-09-2013	Pharyngeal swab	ICU	0,5	0,25	32	128	32
41	Mdr 416	t032	Cc22	18-09-2013	Pharyngeal swab	ICU	0,38	0,25	8	128	0,5
42	Mdr 470	t032	Cc22	18-10-2013	Rectal swab	ICU	0,75	0,5	8	128	32
43	Mdr 533	t032	Cc22	27-11-2013	Pharyngeal swab	ICU	1	0,5	64	128	2
44	Mdr 537	t032	Cc22	28-11-2013	Pharyngeal swab	ICU	1.5	0.5	32	128	128
45	Mdr	t1214	Cc22	18-09-	Nasal	Cardiac surgery	1	0.5	32	128	0,5

	541		scn-	2013	swab						
46	Mdr 543	t1036	Cc22	01-08-2013	pharyngeal	geriatrics	0,5	0,25	8	128	8
47	Mdr 545	t1036	Cc22	28-09-2013	Pharyngeal swab	ICU	0.38	0,25	4	64	4
48	Mdr 560	t032	Cc22	11-12-2013	Rectal swab	endocrinology	1	0,25	32	128	32
49	Mdr 561	t032	Cc22	11-12-2013	Rectal swab	medicine	0,5	0,25	32	128	8
50	Mdr 571	t032	Cc22	18-12-2013	Pharyngeal swab	ICU	1,5	0,5	16	128	16
51	Mdr 591	t1171	Cc8 Scn-	27-12-2013	Rectal swab	ICU	0.5	0.5	16	128	16
52	Mdr 601	t032	Cc22	31-12-2013	Rectal swab	ICU	1	2	8	64	8
53	Mdr 613	t041	Cc5	10-01-2014	Pharyngeal swab	ICU	2	2	16	32	16
54	Mdr 632	t041	Cc5	18-02-2014	Pharyngeal swab	ICU	2	2	16	64	128
55	Mdr 641	t032	Cc22	22-01-2014	Pharyngeal swab	ICU	0,5	0,5	16	128	1
56	Mdr 643	t032	Cc22	23-01-2014	Pharyngeal swab	ICU	0,75	1	8	128	128
57	Mdr 665	t022	Cc22	07-03-2014	Rectal swab	cardiology	0.50	0,50	32	128	128
58	Mdr 669	t1214	Cc22 Scn-	08-03-2014	Pharyngeal swab	ICU	1	0,5	32	128	128
59	Mdr 670	t1214	Cc22 Scn-	06-05-2014	Pharyngeal swab	General surgery	0,5	0,5	128	128	128
60	Mdr 674	t1214	Cc22	28-02-2014	Rectal swab	Infectious Diseases	1	0,5	16	128	128
61	Mdr 838	t032	Cc22			ortopedia	0,5	0,25	32	128	128
62	Mdr 849	t022	Cc22	16-06-2014	Pharyngeal swab	ICU	1	0,5	8	128	128
63	Mdr 850	t022	Cc22	16-06-2014	Rectal swab	ICU	1	0,5	4	128	128
64	Mdr 851	t121 pvl+	Cc8			Infectious disease	1	1	4	64	128
65	Mdr 874	t008	Cc8	23-06-2014	Pharyngeal swab	ICU	0,5	0,5	16	64	128
66	Mdr 891	t1171	Cc8	26-06-2014	Pharyngeal swab	neurosurgery	0,75	0,5	128	128	0,06
67	Mdr 911	t1214	Cc22 Scn-	07-07-2014	Pharyngeal swab	ICU	0,5	0,5	32	128	0,25
68	Mdr 915	t032	Cc22	07-07-2014	Rectal swab	Neuro surgery	1	0,5	8	128	4

69	Mdr 919	t127	Cc1	08-07-2014	Pharyngeal swab	Pediatric onco-haematology	0,75	1	8	128	128
70	Mdr 949	t032	Cc22	14-07-2014	Pharyngeal swab	neurosurgery	0,5	0,5	32	128	8
71	Mdr 997	T022	Cc22	26-07-2014	Pharyngeal swab	ICU	0.75	0,5	16	28	128
72	Mdr 1007	t032	CC22			oncology	0.5	0,25	8	64	32
73	Mdr 1050	t032	Cc22	08-08-2014	Pharyngeal swab	ICU	0.5	0,25	32	128	0.06
74	Mdr 1051	T790	Cc5	08-08-2014	Pharyngeal swab	ICU	0,25	0,25	128	128	32
75	Mdr 1096	t041	Cc22	19-08-2014	Pharyngeal swab	ICU	1	2	8	128	128
76	Mdr 1111	t022	Cc22	22-08-2014	Pharyngeal swab	ICU	0.5	0.5	16	128	128
77	Mdr 1147	t515	Cc22			unknown	0,5	0,5	128	64	2
78	Mdr 1251	t032	Cc22	29-09-2014	Rectal swab	ICU	0,25	0,25	8	128	128
79	Mdr 1260	t1214	Cc22	30-09-2014	Rectal swab	ICU	0.5	0.5	8	8	32
80	Mdr 1265	t1214	Cc22	01-10-2014	Rectal swab	General medicine	0.5	0,5	16	128	128
81	Mdr 1275	t032	Cc22	02-10-2014	Pharyngeal swab	ICU	0,25	0,125	16	128	1
82	Mdr 1294	t121	Cc8	08-10-2014	Rectal swab	ICU	0.5	0.5	8	128	128
83	Mdr 1310	t032	Cc22	13-10-2014	Pharyngeal swab	ICU	0,5	0,5	16	128	0.125
84	Mdr 1305	t121 pvl+	Cc8	10-10-2014	Rectal swab	psichiatry	0,5	0,5	16	128	32
85	Mdr 1330	t032	Cc22	17-10-2014	Pharyngeal swab	ICU	0,5	0,25	32	128	128
86	Mdr 1678	t022	Cc22	07-01-2015	Rectal swab	ICU	0,5	0,25	32	128	0,125
87	Mdr 1698	t032	Cc22	08-01-2015	Pharyngeal swab	ICU	1	1	16	128	0,25
91	Mdr 1713	t032	Cc22	12-01-2015	Pharyngeal swab	Pancreatic surgery	0,5	0,5	8	64	128
88	Mdr 1729	t032	Cc22	15-01-2015	Pharyngeal swab	burns	1	1	32	128	128
89	Mdr 1745	t041	Cc5	19-01-2015	Pharyngeal swab	Cardiac surgery	2	2	16	128	128
90	Mdr 1756	t032	Cc22	21-01-2015	Pharyngeal swab	ICU	1	1	32	32	128

92	Mdr 3636	t022	Cc22	----	---	unknown	1	0,25	32	128	0,5
93	Mdr 3734	t041	Cc5	-----	---	unknown	1,5	1	128	128	128
94	Mdr 3740	t032	Cc22	----	---	unknown	1	0,5	32	128	128

Table 14: MIC results of MDR strains.

	AMC	**Spa type**	**CC**	**Id**	**specimen**	**Section**	**E-Test Cefto**	**Mic Cefto**	**Levo µg/ml**	**Cipro µg/ml**	**Erythro µg/ml**
1	Amc 597	t032	Cc22	05-12-2011	Blood culture	Medicine	0,75	1	8	64	128
2	Amc 584	t024	Cc8	01-12-2011	Blood culture	Haematology	0,5	0,5	32	128	128
3	Amc 602	t032	Cc22	24-11-2011	Blood culture	Surgery	0,75	1	16	128	0,06
4	Amc 622	t024	Cc8	13-12-2011	Blood culture	Haematology	0.5	0,5	16	128	128
5	Amc 720	t008	Cc8	08-03-2012	Blood culture	ICU	0.75	1	8	32	128
6	Amc 772	t041	Cc5	05-07-2012	Blood culture	Medicine	1	1	16	128	128
7	Amc 783	t121	Cc8	20-07-2012	Blood culture	Urology	0,5	0.5	16	128	128
8	Amc 787	t020	Cc22	23-07-2012	Blood culture	Medicine	0.75	1	16	32	0,25
9	Amc 794	t16026	Cc22	24-07-2012	Blood culture	Medicine	0,75	0,5	32	128	128
10	Amc 937	t790	Cc22	13-11-2012	Blood culture	Neurology	0.75	1	4	64	128

11	Amc 994	t304	Cc8 Scn-	26-11-2012	Blood culture	Nefrology	0.094	0,5	8	32	128
12	Amc 1073	t032	Cc22	28-01-2013	Blood culture	Haematology	0.5	2	16	64	128
13	Amc 1074	t121 pvl +	Cc8	28-01-2013	Blood culture	ICU	0.125	0,5	16	32	128
14	Amc 3319	t032	Cc22	01-07-2015	Blood culture	Medicine	1,5	2	128	128	128
15	Amc 3364	t002 pvl +	Cc5	19-07-2015	Blood culture	Cardiology	0,5	1	0,125	0,25	128
16	Amc 3672	t032	Cc22		Blood culture		1	1	32	64	128
17	Amc 6537	t1214	Cc22	09-06-2016	Blood culture	Medicine	0.75	1	32	128	128
18	Amc 6553	t11920	Cc22	15-06-2016	Blood culture	Surgery	0,75	1	32	128	0,25
19	Amc 6559	t1214	Cc22	17-06-2016	Blood culture	Medicine	1	1	64	128	128
20	Amc 6666	t2892	Cc22		Blood culture	Unknown	1	2	32	128	0.5
21	Amc 6668	t121	Cc22	21-09-2016	Blood culture	Geriatry	0.5	1	4	16	128
22	Amc 6730	t1214	Cc22	12-09-2016	Blood culture	Medicine	1	1	32	128	128
23	Amc 6761	t223	Cc22	20-06-2016	Blood culture	Surgery	1	0.5	0,25	0,5	0,5
24	Amc 6767	t032	Cc22	28-06-2016	Blood culture	Geriatry	1	0,5	32	128	0,5
25	Amc 6781	t032	Cc22	07-07-2016	Blood culture	ICU	1	1	16	128	128
26	Amc 6784	t024	CC8	04-07-2016	Blood culture	Infectious diseases	1	0.5	4	4	0,06
27	Amc	t032	Cc22	21-	Blood	Surgery	1	1	16	32	0,06

	6797			07-2016	culture						
28	Amc 6801	t032	Cc22		Blood culture	Unknown	0.75	1	64	128	0,5
29	Amc 6822	t121	Cc8	08-11-2016	Blood culture	Geriatry	0,75	1	8	16	128
30	Amc 6826	t127	Cc1	10-11-2016	Blood culture	Medicine	1	1	16	64	128
31	Amc 6834	t121/CC8	CC8		Blood culture	Unknown	1,5	1	64	128	64
32	Amc 6846	t032	Cc22	12-11-2016	Blood culture	Medicine	1	1	64	128	0,25
33	Amc 6857	t1214	CC22	25-10-2016	Blood culture	Medicine	1	0,5	16	128	128
34	Amc 6862	t121	CC8	29-10-2016	Blood culture	Medicine	1	0,5	16	128	128
35	Amc 6871	t3213 CC22	CC22		Blood culture	Unknown	0.38	0,5	16	128	128
36	Amc 6872	t718/	Cc22		Blood culture	Unknown	1,5	1	16	128	0.125
37	Amc 6880	t121	CC8 **Scn -**		Blood culture	Unknown	1	1	8	128	128
38	Amc 6894	t657pvl +	CC22		Blood culture	Unknown	1	1	8	64	64
39	Amc 6895	t718	CC22 **Scn-**		Blood culture	Unknown	2	1	16	128	0,125
40	Amc 7047	t024	CC22		Blood culture	Unknown	0.75	0,5	8	64	128
41	Amc 7022	t657	CC22		Blood culture	Unknown	0.75	0,5	16	64	128

Table 15: MIC results of AMC strains.

We can observe that only 25 strains out of 135 resulted susceptible to erythromycin, the other had high level of resistance.

Fluoroquinolones also present high level of resistance, only three strains resulted fully susceptible to this class of antibiotics. Note of worth is that levofloxacin MICs are usually two or more time less than ciprofloxacin MICs.

The high level of resistance to other class than beta-lactams of these MRSA strains characterized them as multi-resistant strains.

We perform ceftobiprole susceptibility testing with two methods, either broth microdilution and E-test, comparing the results obtained.

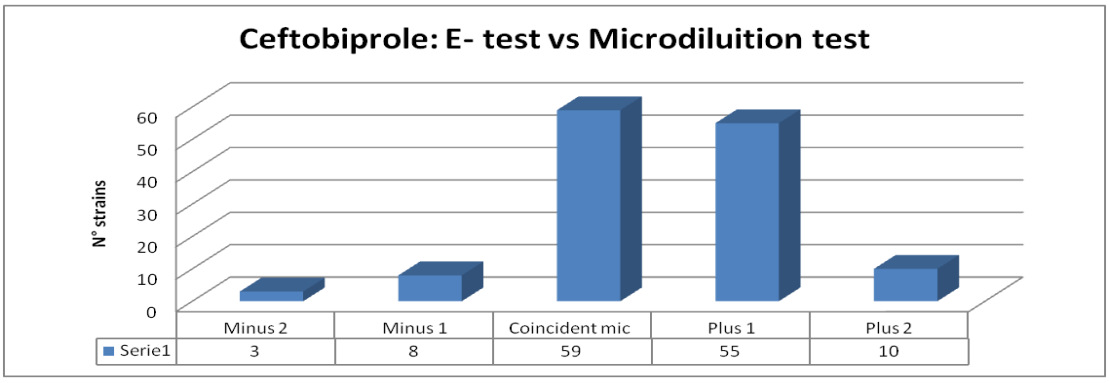
In table 14 and 15 are reported the results of susceptibility testing of 135 MRSA strains against ceftobiprole performed with broth microdilution and E-test, and results interpretation followed the EUCAST recommendations.

All 135 MRSA strains tested against Ceftobiprole, using E-test and microdilution technique, presented MICs in the susceptible range. According to the EUCAST breakpoint, *S. aureus* can be defined resistant to ceftobiprole with a MIC >2 µg/ml and susceptible with a MIC ≤ 2µg/ml.

The MIC_{50} value was 0.75 mg/L using E-test and 0.5 mg/L using the microdilution method. MIC_{90} value was 1.5 mg/L using E-test and 1 mg/L using the microdilution method.

The agreement between the two methods used, using microdilution as gold standard, was 97.5%.

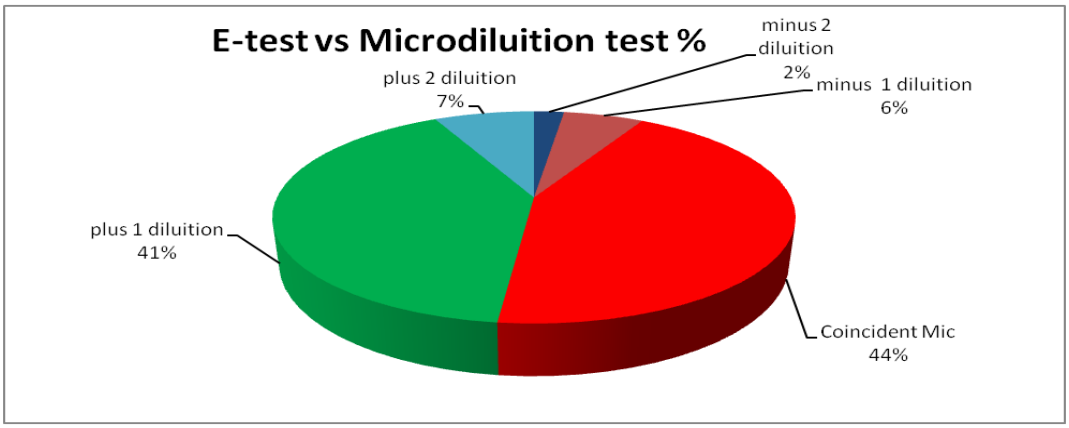
Analysis of results are reported in the graph 1:



Graph 1: n° strains with value MIC concident and/or differ to 1 and 2 diluition on 135 strains

We compared the MIC values obtained with the E-test technique with the values obteined with broth microdilution test and we observed that 55 strains have a coincident MIC value, 55 differ by of +1 dilution respect to the broth microdilution technique, 10 strains differ of +2 dilutions. Only 8 strains differ from minus 1 diluition and 3 differ minus 2 diluitions.

In graph 2 we reported the distribution of MIC obtained with Etest respect broth microdilution method.



Graph 2: % MIC value E-test vs Microdiluition test on 135 MRSA strains

we have therefore observed that the 44% of the MIC value is coincident, 41% differ by plus 1 diluition; the 7% differ by plus 2 diluitions and only rispectlivly 2% and 6% differ by minus 2 and minus 1 diluitions.

Molecular characterization of *S. aureus* strains

The characterization of strains was continued with SCCmec typing following Kondo protocol. M-PCRs results are reported in table 16,17.

Table n° 16 molecular characterization of 135 *S. aureus* strains under study with SCCmec typing by Kondo protocol.

	MDR	Spa-type	CC	id	specimen	section	Kondo 1 Ccr type	Kondo 2 Classes	SCCmecType
1	Mdr 1	t 127	CC1	15-04-2013	Pharyngeal swab	Pediatric and oncology and haematology	Ccr type2 937 bp	Class B (2827 bp)	IV
2	N°2	t3441	Cc22	3-12-2014	Pharyngeal swab	anesthesia	Ccr type2 937 bp	Class B (2827 bp)	IV
3	N°3	t032	Cc22	3-12-2014	Pharyngeal swab	anesthesia	Ccr type2 937 bp	Class B (2827 bp)	IV
4	Mdr 4	t3441	Cc22	10-12-2014	Pharyngeal swab	Anesthesia	Ccr type2 937 bp	Class B (2827 bp)	IV
5	Mdr 6	t032	CC22	10-12-2014	Rectal swab	anestesia	Ccr type2 937 bp	Class B (2827 bp)	IV
6	Mdr 8	t032	CC22		Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
7	Mdr 12L	t008 pvl +	CC8	10-02-2015	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
8	Mdr 16	t032	CC22	10-12-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
9	N°20	t032	Cc22	16-12-2014	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
10	Mdr 007	t032	CC22	13-04-2013	Pharyngeal swab	Vascular surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
11	Mdr 0089	t1214	Cc22 Scn-		Pharyngeal swab	burns	Ccr type2 937 bp	Class B (2827 bp)	IV
12	Mdr 44/20	t041	CC5		Pharyngeal swab	burns	**Ccr type 1 695 bp**	**Class B (2827 bp)**	**I**
13	Mdr 016	t032	CC22	24-04-2013	Pharyngeal swab	geriatry	Ccr type2 937 bp	Class B (2827 bp)	IV
14	Mdr 023	t1214	CC22	30-04-2013	Pharyngeal swab	general medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
15	Mdr	t032	CC22	15-05-2013	Rectal	ICU	Ccr	Class B	IV

	058				swab		type2 937 bp	(2827 bp)	
16	Mdr 062	t022	CC22	20-05-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
17	Mdr 068	t032	CC22	23-05-2013	Rectal swab	General surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
18	Mdr 091	t1036	Cc22	10-06-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
19	Mdr 092	t1036	Cc22	10-06-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
20	Mdr 093	t015	CC45	10-06-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
21	Mdr 131	t032	Cc22	19-06-2013	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
22	Mdr 139	t1036	Cc22	24-06-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
23	Mdr 140	t1036	Cc22	24-06-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
24	Mdr 144	t1036	Cc22	24-06-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
25	Mdr 145	t1036	Cc22	24-06-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
26	Mdr 150	t032	CC22	24-06-2013	Pharyngeal swab	burns	Ccr type2 937 bp	Class B (2827 bp)	IV
27	Mdr 157	t032	CC22	25-06-2013	Pharyngeal swab	General medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
28	Mdr 169	t1171	Cc8	02-07-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
29	Mdr 181	t1036	Cc22	02-07-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
30	Mdr 199	t1036	Cc22	09-07-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV

31	Mdr 204	t1036	Cc22	09-07-2013	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
32	Mdr 212	t1036	Cc22	10-07-2013	Rectal swab	gastoenterology	Ccr type2 937 bp	Class B (2827 bp)	IV
33	Mdr 241	t1036	Cc22	17-07-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
34	Mdr 243	t1036	Cc22	18-07-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
35	Mdr 268	t1036	Cc22	30-07-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
36	Mdr 270	t032	Cc22	30-07-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
37	Mdr 281	t127	Cc1	05-08-2013	Pharyngeal swab	Liver transplatation	Ccr type2 937 bp	Class B (2827 bp)	IV
38	Mdr 322	t032	Cc22	02-09-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
39	Mdr 378	t223	Cc22	10-09-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
40	Mdr 405	t032	Cc22	12-09-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
41	Mdr 416	t032	Cc22	18-09-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
42	Mdr 470	t032	Cc22	18-10-2013	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
43	Mdr 533	t032	Cc22	27-11-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
44	Mdr 537	t032	Cc22	28-11-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
45	Mdr 541	t1214	Cc22 scn-	18-09-2013	Nasal swab	Cardiac surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
46	Mdr 543	t1036	Cc22	01-08-2013	pharyngeal	geriatrics	Ccr type2	Class B (2827	IV

							937 bp	bp)	
47	Mdr 545	t1036	Cc22	28-09-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
48	Mdr 560	t032	Cc22	11-12-2013	Rectal swab	endocrinology	Ccr type2 937 bp	Class B (2827 bp)	IV
49	Mdr 561	t032	Cc22	11-12-2013	Rectal swab	medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
50	Mdr 571	t032	Cc22	18-12-2013	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
51	Mdr 591	t1171	Cc8 **Scn-**	27-12-2013	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
52	Mdr 601	t032	Cc22	31-12-2013	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
53	Mdr 613	t041	Cc5	10-01-2014	Pharyngeal swab	ICU	**Ccr type 1 695 bp**	**Class B (2827 bp)**	**I**
54	Mdr 632	t041	Cc5	18-02-2014	Pharyngeal swab	ICU	**Ccr type 1 695 bp**	**Class B (2827 bp)**	**I**
55	Mdr 641	t032	Cc22	22-01-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
56	Mdr 643	t032	Cc22	23-01-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
57	Mdr 665	t022	Cc22	07-03-2014	Rectal swab	cardiology	Ccr type2 937 bp	Class B (2827 bp)	IV
58	Mdr 669	t1214	Cc22 **Scn-**	08-03-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
59	Mdr 670	t1214	Cc22 **Scn-**	06-05-2014	Pharyngeal swab	General surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
60	Mdr 674	t1214	Cc22	28-02-2014	Rectal swab	Infectious Diseases	Ccr type2 937 bp	Class B (2827 bp)	IV
61	Mdr 838	t032	Cc22			ortopedia	Ccr type2 937 bp	Class B (2827 bp)	IV
62	Mdr	t022	Cc22	16-06-2014	Pharyngeal	ICU	Ccr	Class B	IV

	849				swab		type2 937 bp	(2827 bp)	
63	Mdr 850	t022	Cc22	16-06-2014	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
64	Mdr 851	t121 pvl+	Cc8			Infectious disease	Ccr type2 937 bp	Class B (2827 bp)	IV
65	Mdr 874	t008	Cc8	23-06-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
66	Mdr 891	t1171	Cc8	26-06-2014	Pharyngeal swab	neurosurgery	Ccr type2 937 bp	Class B (2827 bp)	IV
67	Mdr 911	t1214	Cc22 **Scn-**	07-07-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
68	Mdr 915	t032	Cc22	07-07-2014	Rectal swab	Neuro surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
69	Mdr 919	t127	Cc1	08-07-2014	Pharyngeal swab	Pediatric onco-haematology	Ccr type2 937 bp	Class B (2827 bp)	IV
70	Mdr 949	t032	Cc22	14-07-2014	Pharyngeal swab	neurosurgery	Ccr type2 937 bp	Class B (2827 bp)	IV
71	Mdr 997	t022	Cc22	26-07-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
72	Mdr 1007	t032	CC22			oncology	Ccr type2 937 bp	Class B (2827 bp)	IV
73	Mdr 1050	t032	Cc22	08-08-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
74	Mdr 1051	T790	Cc5	08-08-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
75	Mdr 1096	t041	Cc22	19-08-2014	Pharyngeal swab	ICU	**Ccr type 1 695**	Class B (2827 bp)	**I**
76	Mdr 1111	t022	Cc22	22-08-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV

77	Mdr 1147	t515	Cc22			unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
78	Mdr 1251	t032	Cc22	29-09-2014	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
79	Mdr 1260	t1214	Cc22	30-09-2014	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
80	Mdr 1265	t1214	Cc22	01-10-2014	Rectal swab	General medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
81	Mdr 1275	t032	Cc22	02-10-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
82	Mdr 1294	t121	Cc8	08-10-2014	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
83	Mdr 1310	t032	Cc22	13-10-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
84	Mdr 1305	t121 pvl+	Cc8	10-10-2014	Rectal swab	psichiatry	Ccr type2 937 bp	Class B (2827 bp)	IV
85	Mdr 1330	t032	Cc22	17-10-2014	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
86	Mdr 1678	t022	Cc22	07-01-2015	Rectal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
87	Mdr 1698	t032	Cc22	08-01-2015	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
91	Mdr 1713	t032	Cc22	12-01-2015	Pharyngeal swab	Pancreatic surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
88	Mdr 1729	t032	Cc22	15-01-2015	Pharyngeal swab	burns	Ccr type2 937 bp	Class B (2827 bp)	IV
89	Mdr 1745	t041	Cc5	19-01-2015	Pharyngeal swab	Cardiac surgery	**Ccr type 1 695 bp**	**Class B (2827 bp)**	**I**
90	Mdr 1756	t032	Cc22	21-01-2015	Pharyngeal swab	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
92	Mdr 3636	t022	Cc22	----	---	unknown	Ccr type2	Class B (2827	IV

							937 bp	bp)	
93	Mdr 3734	t041	Cc5	---	---	unknown	**Ccr type 1 695 bp**	**Class B (2827 bp)**	**I**
94	Mdr 3740	t032	Cc22	---	---	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV

Table n°16 MDR molecular characterization M-PCR-1.

	AMC	Spa type	CC	Id	specimen	section	ccr type	classes	SCCmetype
1	Amc 597	t032	Cc22	05-12-2011	Blood culture	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
2	Amc 584	t024	Cc8	01-12-2011	Blood culture	Haematology	Ccr type2 937 bp	Class B (2827 bp)	IV
3	Amc 602	t032	Cc22	24-11-2011	Blood culture	surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
4	Amc 622	t024	Cc8	13-12-2011	Blood culture	haematology	Ccr type2 937 bp	Class B (2827 bp)	IV
5	Amc 720	t008	Cc8	08-03-2012	Blood culture	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
6	Amc 772	t041	Cc5	05-07-2012	Blood culture	Medicine	**Ccr type 1 695**	Class B (2827 bp)	I
7	Amc 783	t121	Cc8	20-07-2012	Blood culture	Urology	Ccr type2 937 bp	Class B (2827 bp)	IV
8	Amc 787	t020	Cc22	23-07-2012	Blood culture	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
9	Amc 794	t16026	Cc22	24-07-2012	Blood culture	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
10	Amc 937	t790	Cc22	13-11-2012	Blood culture	Neurology	Ccr type2 937 bp	Class B (2827 bp)	IV
11	Amc 994	t304	Cc8 **Scn-**	26-11-2012	Blood culture	nefrology	Ccr type2 937 bp	Class B (2827 bp)	IV
12	Amc 1073	t032	Cc22	28-01-2013	Blood culture	haematology	**Ccr type 1 695**	Class B (2827 bp)	**I**
13	Amc 1074	t121 pvl +	Cc8	28-01-2013	Blood culture	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
14	Amc 3319	t032	Cc22	01-07-2015	Blood culture	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
15	Amc 3364	t002 pvl +	Cc5	19-07-	Blood culture	Cardiology	Ccr type2 937 bp	Class B (2827 bp)	IV

				2015					
16	Amc 3672	t032	Cc22		Blood culture		Ccr type2 937 bp	Class B (2827 bp)	IV
17	Amc 6537	t1214	Cc22	09-06-2016	Blood culture	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
18	Amc 6553	t11920	Cc22	15-06-2016	Blood culture	Surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
19	Amc 6559	t1214	Cc22	17-06-2016	Blood culture	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
20	Amc 6666	t2892	Cc22		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
21	Amc 6668	t121	Cc22	21-09-2016	Blood culture	geriatry	Ccr type2 937 bp	Class B (2827 bp)	IV
22	Amc 6730	t1214	Cc22	12-09-2016	Blood culture	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
23	Amc 6761	t223	Cc22	20-06-2016	Blood culture	surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
24	Amc 6767	t032	Cc22	28-06-2016	Blood culture	geriatry	Ccr type2 937 bp	Class B (2827 bp)	IV
25	Amc 6781	t032	Cc22	07-07-2016	Blood culture	ICU	Ccr type2 937 bp	Class B (2827 bp)	IV
26	Amc 6784	t024	CC8	04-07-2016	Blood culture	Infectious diseases	Ccr type2 937 bp	Class B (2827 bp)	IV
27	Amc 6797	t032	Cc22	21-07-2016	Blood culture	surgery	Ccr type2 937 bp	Class B (2827 bp)	IV
28	Amc 6801	t032	Cc22		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
29	Amc 6822	t121	Cc8	08-11-2016	Blood culture	geriatry	Ccr type2 937 bp	Class B (2827 bp)	IV
30	Amc 6826	t127	Cc1	10-11-2016	Blood culture	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
31	Amc 6834	t121/CC8	CC8		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
32	Amc 6846	t032	Cc22	12-11-	Blood	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV

				2016	culture				
33	Amc 6857	t1214	CC22	25-10-2016	Blood culture	Medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
34	Amc 6862	t121	CC8	29-10-2016	Blood culture	medicine	Ccr type2 937 bp	Class B (2827 bp)	IV
35	Amc 6871	t3213 CC22	CC22		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
36	Amc 6872	t718/	Cc22		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
37	Amc 6880	t121	CC8 Scn -		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
38	Amc 6894	t657pvl +	CC22		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
39	Amc 6895	t718	CC22 Scn-		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
40	Amc 7047	t024	CC22		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV
41	Amc 7022	t657	CC22		Blood culture	unknown	Ccr type2 937 bp	Class B (2827 bp)	IV

Table 17:AMC molecular characterization M-PCR-2

In figures 15, 16 are reported electrophoresis gel of some strains to detect SCCmec type by Kondo protocol:

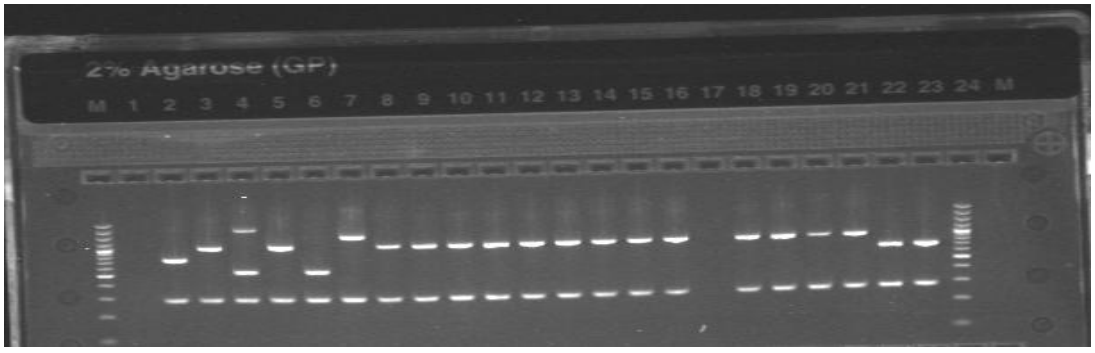


Figure 15: electrophoresis gel of some strains to detect SCCmec type by Kondo protocol: Multiplex PCR 1. MW marker. Lane1) Negative control, Lane 2) COL ontrol ccr1, lane

3) N315 control ccr2, lane 4) 85/2082 control ccr3+5, lane 5) JCSA4469 control ccr2, lane 6) WIS control ccr5, lane 7) HDE 288 ccr4.

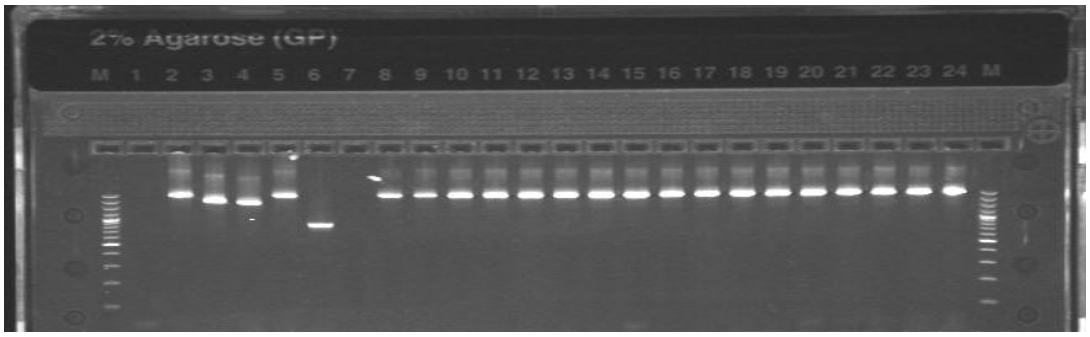


Figure 16 M-PCR 2. Multiplex PCR 2. MW marker. Lane1) Negative control, Lane 2) COL control classB, lane 3) N315 control class A, lane 4) 85/2082 control class A, lane 5) JCSA4469 control class B, lane 6) WIS control class C2.

As we can see 88 (94%) strains out of 94 of MDR group belong to the SCC*mec* IV and only 6 strains out of 94 (6%) belong to SCC*mec* I. All MDR strains indeed belong to the class B.

39 strains out of 41 AMC strains (95%), coming from blood culture, belong to SCC*mec* IV and only 2 strains (5%) belong to SCC*mec* I. All AMC strains, as for MDR strains, belong to class B.

MRSA strains characterization was performed also by SpA typing by M-PCR. Other than *spA* gene were searched also *mecA*, *mecC*, *pvl* and *scn* genes. Results of SpA typing M-PCR are reported in table n° 18 for MDR strains and in table 19 for AMC strains.

All strains are resulted negative to *meC* gene.

Table 18: SpA type molecular characterization of 94 MDR MRSA strains

	MDR	Spa-type	CC	mecA	mecC	scn	pvl	id	specimen	section
1	Mdr 1	t 127	CC1	Pos.	Neg.	Pos.	neg	15-04-2013	Pharyngeal swab	Pediatric and oncology and haematology
2	N°2	t3441	Cc22	Pos.	Neg.	Pos.	neg	3-12-2014	Pharyngeal swab	anesthesia
3	N°3	t032	Cc22	Pos.	Neg.	Pos.	neg	3-12-2014	Pharyngeal swab	anesthesia
4	Mdr 4	t3441	Cc22	Pos.	Neg.	Pos.	neg	10-12-2014	Pharyngeal swab	Anesthesia
5	Mdr 6	t032	CC22	Pos.	Neg.	Pos.	neg	10-12-2014	Rectal swab	anestesia
6	Mdr 8	t032	CC22	Pos.	Neg.	Pos.	neg	---	Pharyngeal swab	ICU
7	Mdr 12L	t008 pvl +	CC8	Pos.	Neg.	Pos.	Pos.	10-02-2015	Rectal swab	ICU
8	Mdr 16	t032	CC22	Pos.	Neg.	Pos.	neg	10-12-2014	Pharyngeal swab	ICU
9	N°20	t032	Cc22	Pos.	Neg.	Pos.	neg	16-12-2014	Rectal swab	ICU
10	Mdr 007	t032	CC22	Pos.	Neg.	Pos.	neg	13-04-2013	Pharyngeal swab	Vascular surgery
11	Mdr 0089	t1214	Cc22 Scn-	Pos.	Neg.	Neg.	neg	---	Pharyngeal swab	burns
12	Mdr 44/20	t041	CC5	Pos.	Neg.	Pos.	neg	----	Pharyngeal swab	burns
13	Mdr 016	t032	CC22	Pos.	Neg.	Pos.	neg	24-04-2013	Pharyngeal swab	geriatry
14	Mdr 023	t1214	CC22	Pos.	Neg.	Pos.	neg	30-04-2013	Pharyngeal swab	general medicine
15	Mdr 058	t032	CC22	Pos.	Neg.	Pos.	neg	15-05-2013	Rectal swab	ICU
16	Mdr 062	t022	CC22	Pos.	Neg.	Pos.	neg	20-05-2013	Pharyngeal swab	ICU
17	Mdr 068	t032	CC22	Pos.	Neg.	Pos.	neg	23-05-2013	Rectal swab	General surgery
18	Mdr 091	t1036	Cc22	Pos.	Neg.	Pos.	neg	10-06-2013	Pharyngeal swab	ICU
19	Mdr 092	t1036	Cc22	Pos.	Neg.	Pos.	neg	10-06-2013	Pharyngeal swab	ICU
20	Mdr 093	t015	CC45	Pos.	Neg.	Pos.	neg	10-06-2013	Pharyngeal swab	ICU
21	Mdr 131	t032	Cc22	Pos.	Neg.	Pos.	neg	19-06-2013	Rectal swab	ICU

22	Mdr 139	t1036	Cc22	Pos.	Neg.	Pos.	neg	24-06-2013	Pharyngeal swab	ICU
23	Mdr 140	t1036	Cc22	Pos.	Neg.	Pos.	neg	24-06-2013	Pharyngeal swab	ICU
24	Mdr 144	t1036	Cc22	Pos.	Neg.	Pos.	neg	24-06-2013	Pharyngeal swab	ICU
25	Mdr 145	t1036	Cc22	Pos.	Neg.	Pos.	neg	24-06-2013	Pharyngeal swab	ICU
26	Mdr 150	t032	CC22	Pos.	Neg.	Pos.	neg	24-06-2013	Pharyngeal swab	burns
27	Mdr 157	t032	CC22	Pos.	Neg.	Pos.	neg	25-06-2013	Pharyngeal swab	General medicine
28	Mdr 169	t1171	Cc8	Pos.	Neg.	Pos.	neg	02-07-2013	Pharyngeal swab	ICU
29	Mdr 181	t1036	Cc22	Pos.	Neg.	Pos.	neg	02-07-2013	Pharyngeal swab	ICU
30	Mdr 199	t1036	Cc22	Pos.	Neg.	Pos.	neg	09-07-2013	Pharyngeal swab	ICU
31	Mdr 204	t1036	Cc22	Pos.	Neg.	Pos.	neg	09-07-2013	Rectal swab	ICU
32	Mdr 212	t1036	Cc22	Pos.	Neg.	Pos.	neg	10-07-2013	Rectal swab	gastoenterology
33	Mdr 241	t1036	Cc22	Pos.	Neg.	Pos.	neg	17-07-2013	Pharyngeal swab	ICU
34	Mdr 243	t1036	Cc22	Pos.	Neg.	Pos.	neg	18-07-2013	Pharyngeal swab	ICU
35	Mdr 268	t1036	Cc22	Pos.	Neg.	Pos.	neg	30-07-2013	Pharyngeal swab	ICU
36	Mdr 270	t032	Cc22	Pos.	Neg.	Pos.	neg	30-07-2013	Pharyngeal swab	ICU
37	Mdr 281	t127	Cc1	Pos.	Neg.	Pos.	neg	05-08-2013	Pharyngeal swab	Liver transplatation
38	Mdr 322	t032	Cc22	Pos.	Neg.	Pos.	neg	02-09-2013	Pharyngeal swab	ICU
39	Mdr 378	t223	Cc22	Pos.	Neg.	Pos.	neg	10-09-2013	Pharyngeal swab	ICU
40	Mdr 405	t032	Cc22	Pos.	Neg.	Pos.	neg	12-09-2013	Pharyngeal swab	ICU
41	Mdr 416	t032	Cc22	Pos.	Neg.	Pos.	neg	18-09-2013	Pharyngeal swab	ICU
42	Mdr 470	t032	Cc22	Pos.	Neg.	Pos.	neg	18-10-2013	Rectal swab	ICU
43	Mdr 533	t032	Cc22	Pos.	Neg.	Pos.	neg	27-11-2013	Pharyngeal swab	ICU
44	Mdr 537	t032	Cc22	Pos.	Neg.	Pos.	neg	28-11-2013	Pharyngeal swab	ICU

45	Mdr 541	t1214	Cc22 scn-	Pos.	Neg.	Neg.	neg	18-09-2013	Nasal swab	Cardiac surgery
46	Mdr 543	t1036	Cc22	Pos.	Neg.	Pos.	neg	01-08-2013	pharyngeal	geriatrics
47	Mdr 545	t1036	Cc22	Pos.	Neg.	Pos.	neg	28-09-2013	Pharyngeal swab	ICU
48	Mdr 560	t032	Cc22	Pos.	Neg.	Pos.	neg	11-12-2013	Rectal swab	endocrinology
49	Mdr 561	t032	Cc22	Pos.	Neg.	Pos.	neg	11-12-2013	Rectal swab	medicine
50	Mdr 571	t032	Cc22	Pos.	Neg.	Pos.	neg	18-12-2013	Pharyngeal swab	ICU
51	Mdr 591	t1171	Cc8 Scn-	Pos.	Neg.	Neg.	neg	27-12-2013	Rectal swab	ICU
52	Mdr 601	t032	Cc22	Pos.	Neg.	Pos.	neg	31-12-2013	Rectal swab	ICU
53	Mdr 613	t041	Cc5	Pos.	Neg.	Pos.	neg	10-01-2014	Pharyngeal swab	ICU
54	Mdr 632	t041	Cc5	Pos.	Neg.	Pos.	neg	18-02-2014	Pharyngeal swab	ICU
55	Mdr 641	t032	Cc22	Pos.	Neg.	Pos.	neg	22-01-2014	Pharyngeal swab	ICU
56	Mdr 643	t032	Cc22	Pos.	Neg.	Pos.	neg	23-01-2014	Pharyngeal swab	ICU
57	Mdr 665	t022	Cc22	Pos.	Neg.	Pos.	neg	07-03-2014	Rectal swab	cardiology
58	Mdr 669	t1214	Cc22 Scn-	Pos.	Neg.	Neg.	neg	08-03-2014	Pharyngeal swab	ICU
59	Mdr 670	t1214	Cc22 Scn-	Pos.	Neg.	Neg.	neg	06-05-2014	Pharyngeal swab	General surgery
60	Mdr 674	t1214	Cc22	Pos.	Neg.	Pos.	neg	28-02-2014	Rectal swab	Infectious Diseases
61	Mdr 838	t032	Cc22	Pos.	Neg.	Pos.	neg	---	----	ortopedia
62	Mdr 849	t022	Cc22	Pos.	Neg.	Pos.	neg	16-06-2014	Pharyngeal swab	ICU
63	Mdr 850	t022	Cc22	Pos.	Neg.	Pos.	neg	16-06-2014	Rectal swab	ICU
64	Mdr 851	t121 pvl+	Cc8	Pos.	Neg.	Pos.	Pos.	---	---	Infectious disease
65	Mdr 874	t008	Cc8	Pos.	Neg.	Pos.	neg	23-06-2014	Pharyngeal swab	ICU
66	Mdr 891	t1171	Cc8	Pos.	Neg.	Pos.	neg	26-06-2014	Pharyngeal swab	neurosurgery
67	Mdr 911	t1214	Cc22 Scn-	Pos.	Neg.	Neg.	neg	07-07-2014	Pharyngeal swab	ICU

68	Mdr 915	t032	Cc22	Pos.	Neg.	Pos.	neg	07-07-2014	Rectal swab	Neuro surgery
69	Mdr 919	t127	Cc1	Pos.	Neg.	Pos.	neg	08-07-2014	Pharyngeal swab	Pediatric onco-haematology
70	Mdr 949	t032	Cc22	Pos.	Neg.	Pos.	neg	14-07-2014	Pharyngeal swab	neurosurgery
71	Mdr 997	T022	Cc22	Pos.	Neg.	Pos.	neg	26-07-2014	Pharyngeal swab	ICU
72	Mdr 1007	t032	CC22	Pos.	Neg.	Pos.	neg	---	----	oncology
73	Mdr 1050	t032	Cc22	Pos.	Neg.	Pos.	neg	08-08-2014	Pharyngeal swab	ICU
74	Mdr 1051	T790	Cc5	Pos.	Neg.	Pos.	neg	08-08-2014	Pharyngeal swab	ICU
75	Mdr 1096	t041	Cc22	Pos.	Neg.	Pos.	neg	19-08-2014	Pharyngeal swab	ICU
76	Mdr 1111	t022	Cc22	Pos.	Neg.	Pos.	neg	22-08-2014	Pharyngeal swab	ICU
77	Mdr 1147	t515	Cc22	Pos.	Neg.	Pos.	neg	---	---	unknown
78	Mdr 1251	t032	Cc22	Pos.	Neg.	Pos.	neg	29-09-2014	Rectal swab	ICU
79	Mdr 1260	t1214	Cc22	Pos.	Neg.	Pos.	neg	30-09-2014	Rectal swab	ICU
80	Mdr 1265	t1214	Cc22	Pos.	Neg.	Pos.	neg	01-10-2014	Rectal swab	General medicine
81	Mdr 1275	t032	Cc22	Pos.	Neg.	Pos.	neg	02-10-2014	Pharyngeal swab	ICU
82	Mdr 1294	t121	Cc8	Pos.	Neg.	Pos.	neg	08-10-2014	Rectal swab	ICU
83	Mdr 1310	t032	Cc22	Pos.	Neg.	Pos.	neg	13-10-2014	Pharyngeal swab	ICU
84	Mdr 1305	t121 pvl+	Cc8	Pos.	Neg.	Neg.	Pos.	10-10-2014	Rectal swab	psichiatry
85	Mdr 1330	t032	Cc22	Pos.	Neg.	Pos.	neg	17-10-2014	Pharyngeal swab	ICU
86	Mdr 1678	t022	Cc22	Pos.	Neg.	Pos.	neg	07-01-2015	Rectal swab	ICU
87	Mdr 1698	t032	Cc22	Pos.	Neg.	Pos.	neg	08-01-2015	Pharyngeal swab	ICU
91	Mdr 1713	t032	Cc22	Pos.	Neg.	Pos.	neg	12-01-2015	Pharyngeal swab	Pancreatic surgery
88	Mdr 1729	t032	Cc22	Pos.	Neg.	Pos.	neg	15-01-2015	Pharyngeal swab	burns
89	Mdr 1745	t041	Cc5	Pos.	Neg.	Pos.	neg	19-01-2015	Pharyngeal swab	Cardiac surgery

90	Mdr 1756	t032	Cc22	Pos.	Neg.	Pos.	neg	21-01-2015	Pharyngeal swab	ICU
92	Mdr 3636	t022	Cc22	Pos.	Neg.	Pos.	neg	---	--	unknown
93	Mdr 3734	t041	Cc5	Pos.	Neg.	Pos.	neg	---	---	unknown
94	Mdr 3740	t032	Cc22		Neg.	Pos.	neg	---	---	unknown

Table 18: SpA type molecular characterization of 94 MDR MRSA strains

	AMC	**Spa type**	**CC**	**mecA**	**mecC**	**scn**	**pvl**	**Id**	**specimen**	**section**
1	Amc 597	t032	Cc22	Pos.	Neg.	Pos.	Neg.	05-12-2011	Blood culture	Medicine
2	Amc 584	t024	Cc8	Pos.	Neg.	Pos.	Neg.	01-12-2011	Blood culture	Haematology
3	Amc 602	t032	Cc22	Pos.	Neg.	Pos.	Neg.	24-11-2011	Blood culture	surgery
4	Amc 622	t024	Cc8	Pos.	Neg.	Pos.	Neg.	13-12-2011	Blood culture	haematology
5	Amc 720	t008	Cc8	Pos.	Neg.	Pos.	Neg.	08-03-2012	Blood culture	ICU
6	Amc 772	t041	Cc5	Pos.	Neg.	Pos.	Neg.	05-07-2012	Blood culture	Medicine
7	Amc 783	t121	Cc8	Pos.	Neg.	Pos.	Neg.	20-07-2012	Blood culture	Urology
8	Amc 787	t020	Cc22	Pos.	Neg.	Pos.	Neg.	23-07-2012	Blood culture	Medicine
9	Amc 794	t16026	Cc22	Pos.	Neg.	Pos.	Neg.	24-07-2012	Blood culture	Medicine
10	Amc 937	t790	Cc22	Pos.	Neg.	Pos.	Neg.	13-11-2012	Blood culture	Neurology
11	Amc 994	t304	Cc8 **Scn-**	Pos.	Neg.	Neg.	Neg.	26-11-2012	Blood culture	nefrology
12	Amc 1073	t032	Cc22	Pos.	Neg.	Pos.	Neg.	28-01-2013	Blood	haematology

									culture	
13	Amc 1074	t121 pvl +	Cc8	Pos.	Neg.	Pos.	Pos.	28-01-2013	Blood culture	ICU
14	Amc 3319	t032	Cc22	Pos.	Neg.	Pos.	Neg.	01-07-2015	Blood culture	Medicine
15	Amc 3364	t002 pvl +	Cc5	Pos.	Neg.	Pos.	Pos.	19-07-2015	Blood culture	Cardiology
16	Amc 3672	t032	Cc22	Pos.	Neg.	Pos.	Neg.		Blood culture	
17	Amc 6537	t1214	Cc22	Pos.	Neg.	Pos.	Neg.	09-06-2016	Blood culture	Medicine
18	Amc 6553	t11920	Cc22	Pos.	Neg.	Pos.	Neg.	15-06-2016	Blood culture	Surgery
19	Amc 6559	t1214	Cc22	Pos.	Neg.	Pos.	Neg.	17-06-2016	Blood culture	Medicine
20	Amc 6666	t2892	Cc22	Pos.	Neg.	Pos.	Neg.	----	Blood culture	unknown
21	Amc 6668	t121	Cc22	Pos.	Neg.	Pos.	Neg.	21-09-2016	Blood culture	geriatry
22	Amc 6730	t1214	Cc22	Pos.	Neg.	Pos.	Neg.	12-09-2016	Blood culture	Medicine
23	Amc 6761	t223	Cc22	Pos.	Neg.	Pos.	Neg.	20-06-2016	Blood culture	surgery
24	Amc 6767	t032	Cc22	Pos.	Neg.	Pos.	Neg.	28-06-2016	Blood culture	geriatry
25	Amc 6781	t032	Cc22	Pos.	Neg.	Pos.	Neg.	07-07-2016	Blood culture	ICU
26	Amc 6784	t024	CC8	Pos.	Neg.	Pos.	Neg.	04-07-2016	Blood culture	Infectious diseases
27	Amc 6797	t032	Cc22	Pos.	Neg.	Pos.	Neg.	21-07-2016	Blood culture	surgery
28	Amc 6801	t032	Cc22	Pos.	Neg.	Pos.	Neg.	---	Blood culture	unknown
29	Amc 6822	t121	Cc8	Pos.	Neg.	Pos.	Neg.	08-11-2016	Blood culture	geriatry
30	Amc 6826	t127	Cc1	Pos.	Neg.	Pos.	Neg.	10-11-2016	Blood	Medicine

									culture	
31	Amc 6834	t121/CC8	CC8	Pos.	Neg.	Pos.	Neg.	---	Blood culture	unknown
32	Amc 6846	t032	Cc22	Pos.	Neg.	Pos.	Neg.	12-11-2016	Blood culture	Medicine
33	Amc 6857	t1214	CC22	Pos.	Neg.	Pos.	Neg.	25-10-2016	Blood culture	Medicine
34	Amc 6862	t121	CC8	Pos.	Neg.	Pos.	Neg.	29-10-2016	Blood culture	medicine
35	Amc 6871	t3213 CC22	CC22	Pos.	Neg.	Pos.	Neg.	---	Blood culture	unknown
36	Amc 6872	t718/	Cc22	Pos.	Neg.	Pos.	Neg.	---	Blood culture	unknown
37	Amc 6880	t121	CC8 **Scn -**	Pos.	Neg.	Neg.	Neg.	---	Blood culture	unknown
38	Amc 6894	t657pvl +	CC22	Pos.	Neg.	Pos.	Pos.	---	Blood culture	unknown
39	Amc 6895	t718	CC22 **Scn-**	Pos.	Neg.	Neg.	Neg.	---	Blood culture	unknown
40	Amc 7047	t024	CC22	Pos.	Neg.	Pos.	Neg.	---	Blood culture	unknown
41	Amc 7022	t657	CC22	Pos.	Neg.	Pos.	Neg.	----	Blood culture	unknown

Table 19: SpA type molecular characterization of 41 AMC MRSA strains

In figure 17 is shown electrophoresis gel of some strains for these PCR reactions.

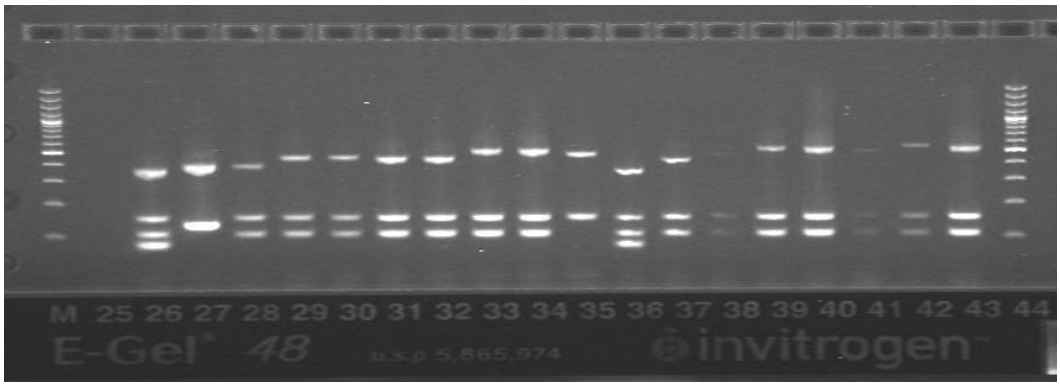


Figure 17: Multiplex PCR for detection of mecA, pvl, and spa gene; Marker (100bp). Line 25) negative control; line26) 50148 control spa meca scn pvl genes positive; line 27) lga251 control spa mecC positive.

As we can see from table 18 and 19 for the 94 MDR strains we could identify 24 spA types, 6 of them we can considere as "representative": 40 MDR out of 94 (43%) were SpA t032 CC22, (16%) 15 Spa t1036 CC22; (10%) 9 Spa t1214 CC22; (9%) 8 Spa t022;(6%) Spa 6 t041 CC5; (3%) 3 Spa t121CC8 (PVL-positive).

All 94 MDR strains were positive for *mecA* gene, while no one amplified the *mecC* gene.

Only 3 strains were *pvl* positive, while only 6 strains were *scn* negative (6%) and 88 strains were *scn* positive (94%).

All strains belonging to spaA t041 CC5 were SCCmec I. In these strains lack spA type t024 CC8, t020 CC22, t16026 CC22, t304 CC8, t002 CC5, t11920CC22, t2892 CC22, t657 CC22, t718 CC22, t3113 CC22.

The other spA type no-representative are spA type t127 CC1(3 strains), t008 CC8(1), t3441 CC22(1), t015 CC45 (1), t1171 CC8 (3), t223 CC22 (1), t790 CC22 (1), t515 CC22(1).

AMC strains isolated from blood culture presented 5 spA type that we can considered "representative": 10 strains out of 41 (24%) belong to spA type t032 CC22, of these 9 strains were SCCmec IV and only one belong to SCCmec type I; 4 Spa t1214 CC22 (10%); 1 Spa t041 CC5 (2%); 7 CC8 Spa t121(17%) also pvl-positive); 4 spa t024 CC8 (10%); Only 7 % were *pvl* gene positive (3 strains) and 93%(38) were *scn* gene positive.

Lack in the AMC group of the SpA t1036 CC22, the spA type more representative in MDR strains is note of worth. spa t022, spa t3441, spa t015, spa t11171, t515.

All strains belonging to the spa t041 CC5 were SCCmec I.

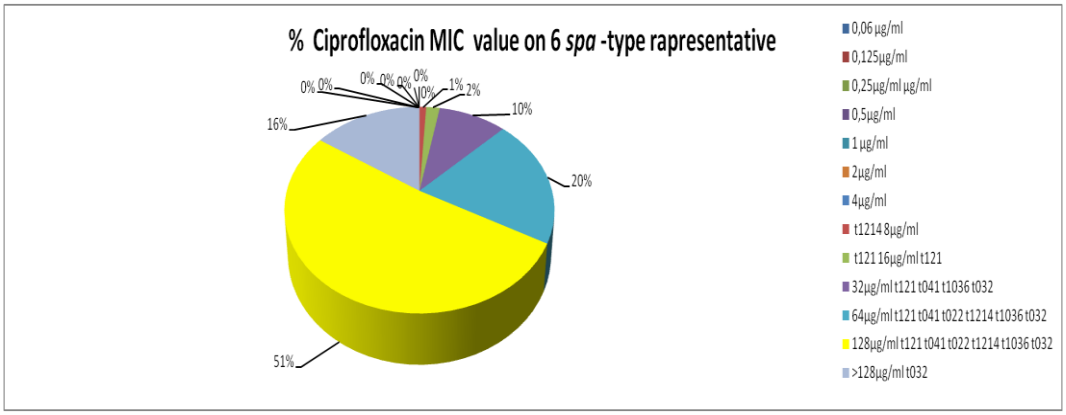
The “no-representative” spA type in the AMC group were t127 CC1 (1), t008 CC8 (1), t223 CC22 (1), t790 CC22 (1), t020 CC22 (1), t16016 CC22 (1), t304 CC8 (1) *scn* negative, t002 CC5(1) *pvl* positive, t11920 CC22 (1), t657 CC22 (2) *pvl* positive and *scn* negative, t718 CC22 (2), t3213 CC22(1).

Of these 135 strains we have identified 6 most representative spA-types: t032 CC22, t1214 CC22, t1036 CC22, t022 CC22, t041 CC5, t121 CC8. We then studied antibiotic susceptibility on 6 representative spA types with a total of 103 strains tested for the sensitivity to ciprofloxacin levofloxacin and erythromycin.

The 6 spA types representative on 135 MRSA strains that we choosed for analysis were:

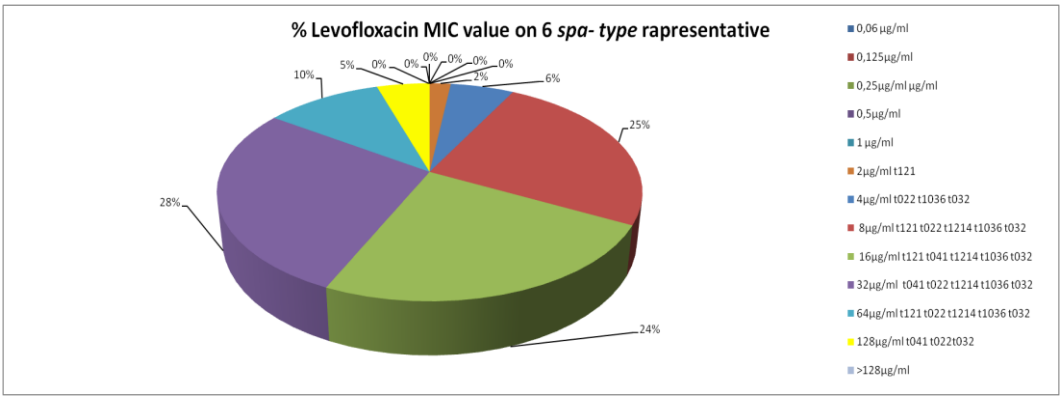
t032 CC22 (50 strains), t1036 CC22 (15 strains), t1214 CC22(13 strains), t022 CC22 (8 strains), t041 CC5 (7 strains), t121 CC8 (10 strains).

In graph 3 we can see the MIC distribution against ciprofloxacin for the 6 more representative spA types:



Graph 3:% ciprofloxacin MIC values distribution for the 6 more representative spA types.

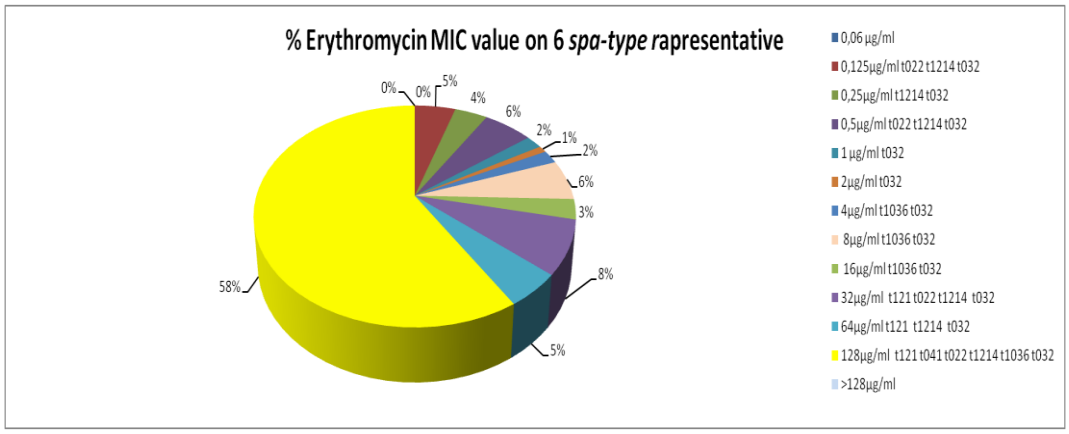
We have therefore seen, as reported in the graph 3, that 53 (51%) of the strains tested with ciprofloxacin had MIC of 128 µg/ml belonging to all 6 spA types that we found more representative. 21 strains (20%) with a MIC of 64 µg/ml belonging to all 6 representative spA types. 16 samples (16%) with a MIC> 128µg/ml belonging to the t032 type. 10 samples (10%) with a MIC equal to 32µg/ml (t121 t041 t1036 t032). 2 strains (2%) with a MIC of 16µg/ml (t121). 1 strain (1%) with a MIC equal to 8 µg/ml (t1214). None of the tested strains had MIC equal or less than 4 µg/ml.



Graph 4: % levofloxacin MIC values on 6 spa type rapresentative

In the graph n° 4 we can see that 29 (28%) strains tested have a MIC equal to 32µg/ml with spa type t041 t022 t1214 t1036 t032; 26 tested strains (25%) with a

MIC equal to 8 µg/ml spa t121 t022 t1214 t1036 t032; 25 strains with a MIC equal to 16 µg/ml (t121 t041 t1214 t1036 t032). No strains were found with a value of MIC lower than 2 µg/ml.

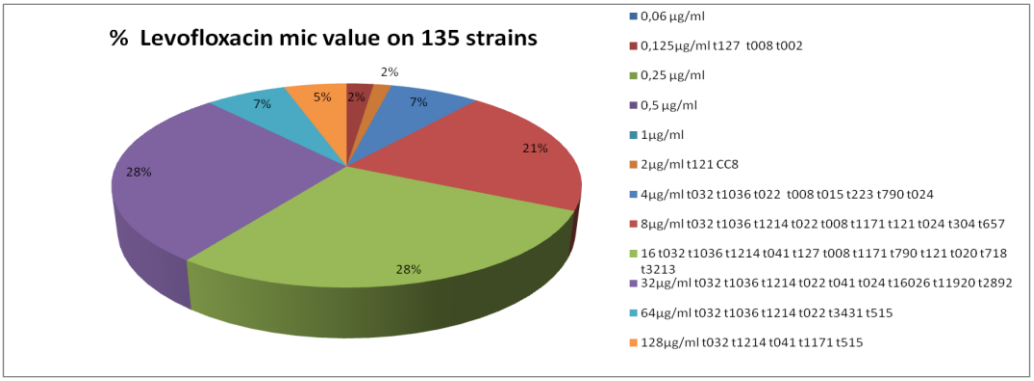


Graph 5:% Erythromycin MICs value on 6 spa type rapresentative.

In the graph 5 we can see that most of tested strains (58%) have a MIC of 128µg/ml belonging to spa type, t121 t041 t022 t1214 t1036 t032. No sample has a MIC equal to 0.06 µg/ml.

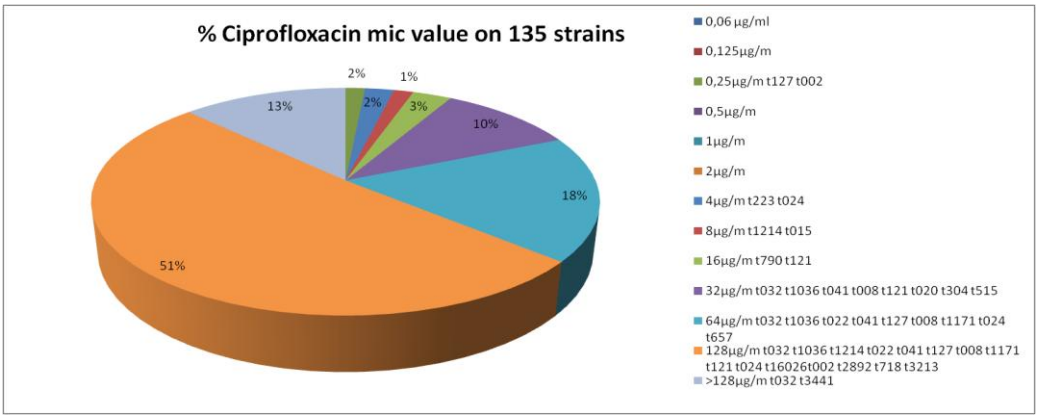
We also compared the MIC results obtained between these three antibiotics and we observed that only 18 on 103 (17,4%) samples were sensitive to erythromycin. These 18 samples that are sensitive to erythromycin, are resistant to ciprofloxacin levofloxacin. In the graph 5, are reported the MIC values, from left to right, of levofloxacin ciprofloxacin and erythromycin, respectively. In the table 20, we can observe the levofloxacin and ciprofloxacin MIC values, according to EUCAST guideline, of all strains (100%) are within the resistance range, while 18 samples tested with erythromycin were sensitive. this in agreement to Das *et al* [66] and Amorim *et al* [67].

We also observed, as reported in graphs 6, 7, 8 that 100% of the strains that belong to the 6 rapresentative spa-type and clonal complex were resistant to levofloxacin ciprofloxacin and erythromycin.



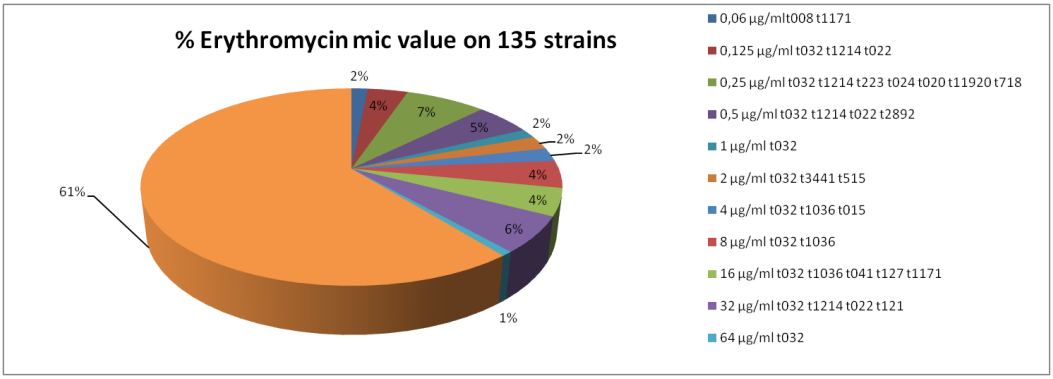
Graph 8: levofloxacin MIC value in %

We also analyzed the sensitivity to levofloxacin on 135 strains tested and we observed that only 3 strains had mic equal to 0,125µg/ml and belong to type t127 t008 t002. These spa-types have not been defined as representative because they were found very rarely among the tested strains. The 96% of the strains tested for levofloxacin were resistant.



Graph 7 :ciprofloxacin MIC value in %

We also analyzed the sensitivity to ciprofloxacin on 135 strains tested and we observed that only 2 strains had mic equal to 0,25µg/ml and belong to type t127 t002. These spa-types have not been defined as representative because they were found very rarely among the tested strains. The 98% of the strains tested for ciprofloxacin were resistant.



Graph 8: erythromycin MIC value in %

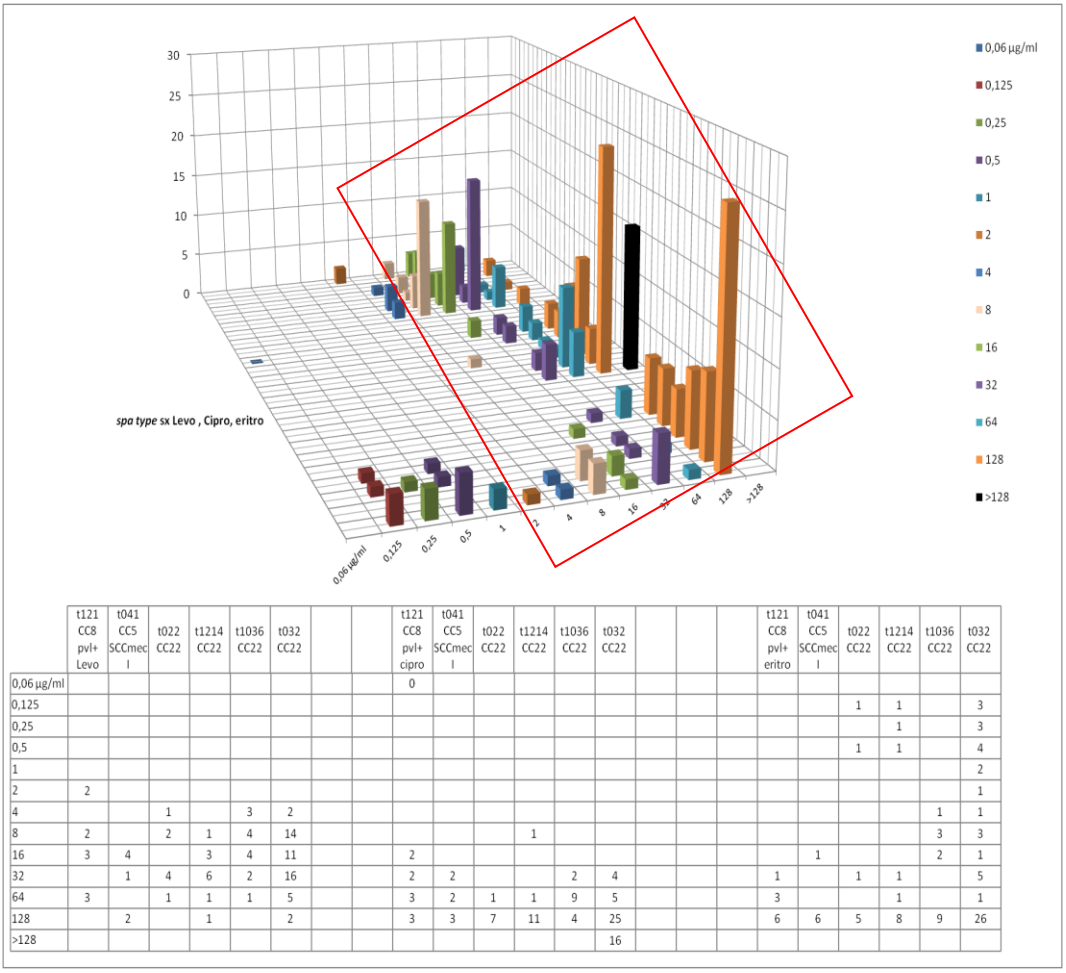
We also analyzed the sensitivity to erythromycin on 135 strains tested and we observed that only 2 strains (2%) had MIC equal to 0,06 µg/ml t008 t1171,

5 strains (4%) had MIC equal to 0,125 µg/ml and belong to type t032 t1214 t022; 11 strains (7%) had MIC 0,25 µg/ml belong to spa t 032 t1214 t223 t024 t020 t11920 t718. 7 strains (5%) had MIC 0,5 belong to spa t 032 t1214 t022 t2892.

2 strains (2%) had mic 1 µg/ml belong to spa t032; 3 strains (2%) had MIC 2 µg/ml belong to spa type t032 t3441 t515. The 98% of the strains tested for ciprofloxacin were resistant. The 79% of the strains tested were resulted resistant to erythromycin. Only 29 strains on 135 total strains (21,5%) are resulted sensitive to erythromycin.

In the graph n°9 is reported compared results of levofloxacin, ciprofloxacin and erythromycin. Note of worthy is that the trend of the mic values is shifted towards the values of the resistance.

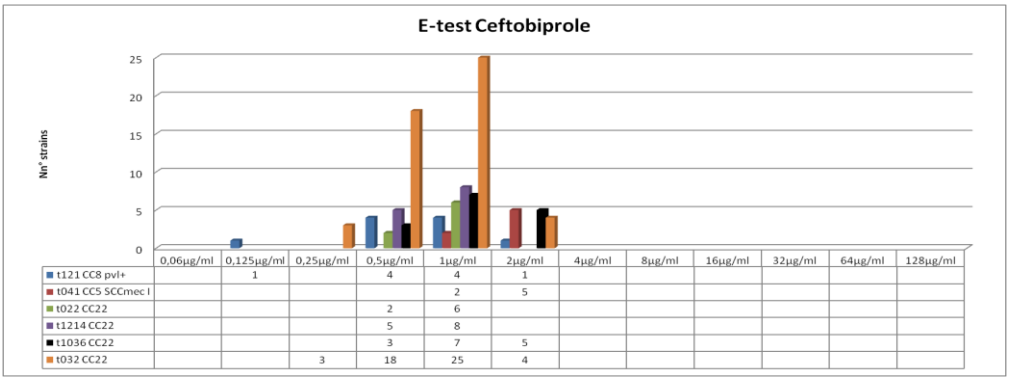
We can therefore define these strains tested not only resistant to all ß-lactam antibiotic by definition, but also to other antibiotics of clinical use and importance. From antibiotic susceptibility profile we can say that they have a hospital-type profile caused by the selective pressure to use antibiotics.



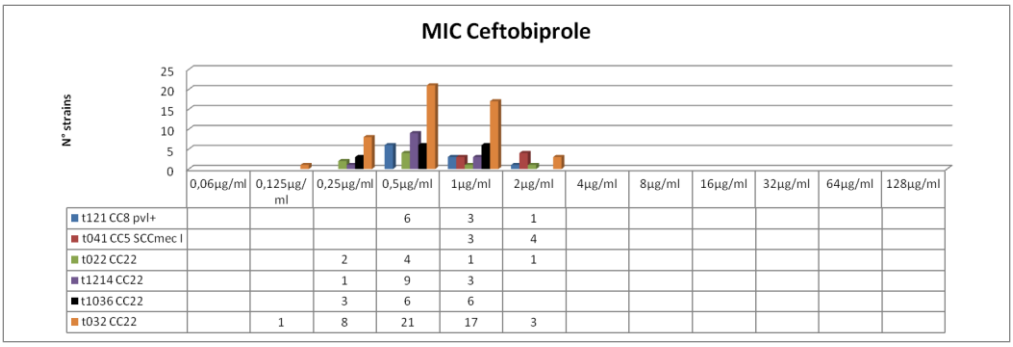
	t121 CC8 pvl+ Levo	t041 CC5 SCCmec I	t022 CC22	t1214 CC22	t1036 CC22	t032 CC22			t121 CC8 pvl+ cipro	t041 CC5 SCCmec I	t022 CC22	t1214 CC22	t1036 CC22	t032 CC22				t121 CC8 pvl+ eritro	t041 CC5 SCCmec I	t022 CC22	t1214 CC22	t1036 CC22	t032 CC22
0,06 µg/ml									0														
0,125																				1	1		3
0,25																					1		3
0,5																				1	1		4
1																							2
2	2																						1
4			1		3	2																1	1
8	2		2	1	4	14						1										3	3
16	3	4		3	4	11			2										1			2	1
32		1	4	6	2	16			2	2			2	4				1		1	1		5
64	3		1	1	1	5			3	2	1	1	9	5				3			1		1
128		2		1		2			3	3	7	11	4	25				6	6	5	8	9	26
>128														16									

Graph n°9: levofloxacin, ciprofloxacin, erythromycin MIC value on 6 spa type rapresentative.

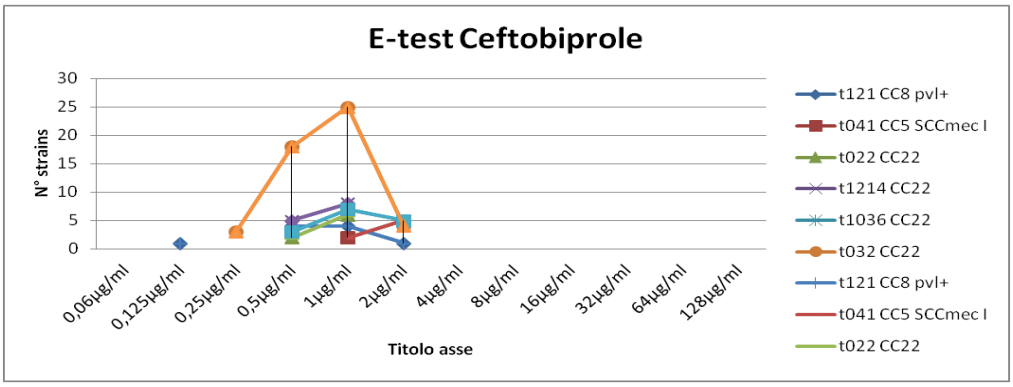
We then compared the 6 *spa*-types represented with the MIC obtained with the E-test and the standard Gold technique and we have seen E-test, as reported in the graph 12, 13 and 10, 11 the histograms overestimate the microdilution technique. in fact, the graph curve moves to the right in favor of the E-test technique.



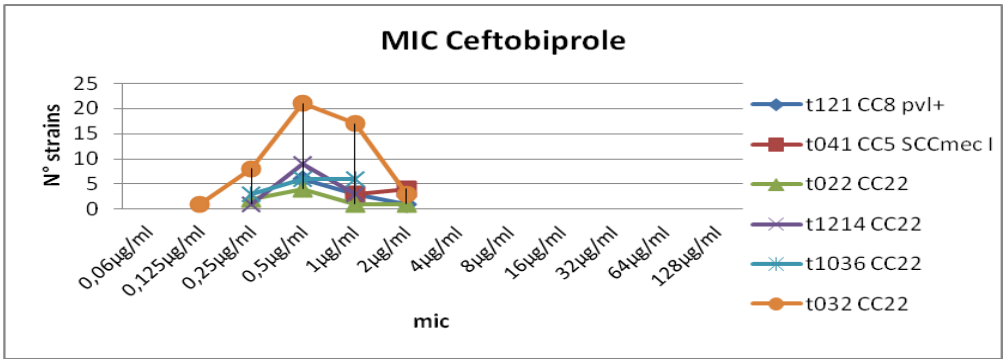
Graph n° 10 E-test MIC value on 6 spa type rapresentative.



Graph n° 11 MIC value on 6 spa type rapresentative.



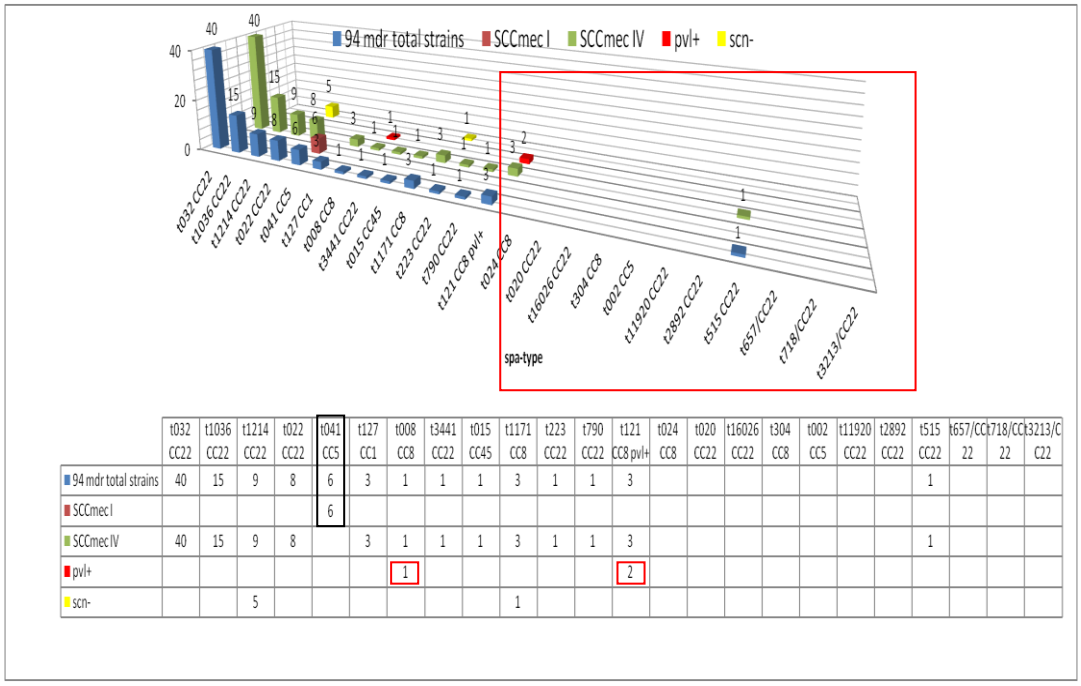
Graph n°12 E-test MIC value on 6 spa type rapresentative.



Graph n°13 MIC value on 6 spa type rapresentative.

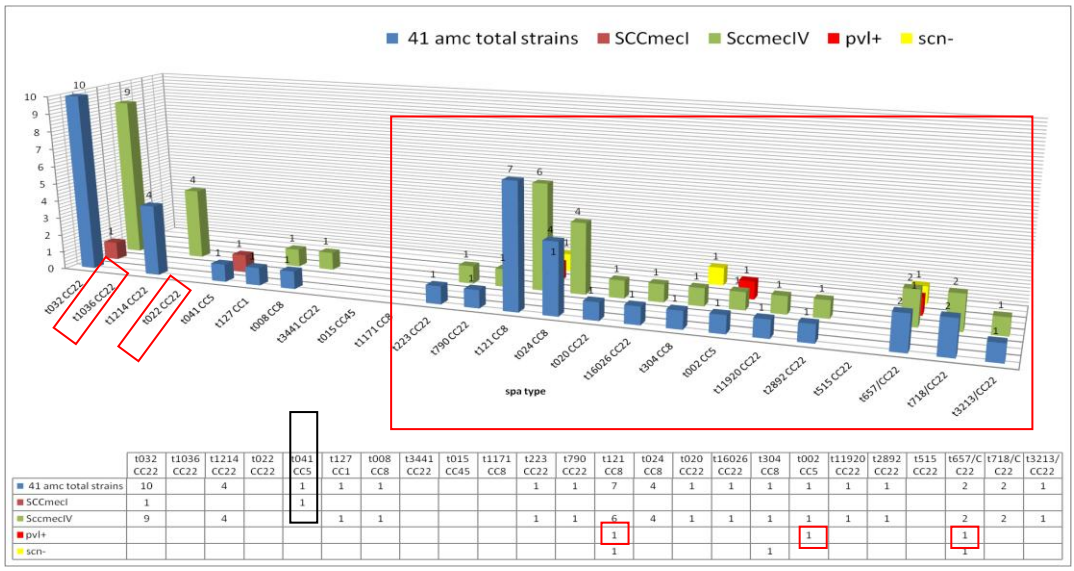
In graphs 12 and 13 we can see both the distribution of MIC between E-test and broth microdiluition and we can observe that the trend of MIC values are shifted to the right of 1dilution.
In the graph 14 and 15 is reported the distribution of blood culture and MDR strains based on all typed spa-types, the identification of the *pvl* gene and the *scn* gene and the typing of the class SCCmec I and IV.

In the graphs 14 and 15 we can see, compared, that in the AMC strains tested is missing the spa-types t1036 and t022 (highlighted in red) considered two of the most representative spa-types, which instead we find in MDR strains. This is the first observation we can make by comparing these histograms. The second observation we can make is that among the 24 spa-types found, 10 (t024 t020 t16026 t304 t002 t11920 t2892 t515 t657 t718 t3213) are missing in MDR strains. A third observation is that the spa-type t041 CC5 (highlighted in black) present in both MDR and ACM strains, belongs to the type SCCmec type I. This is the first molecular correlation we can underline in this study. The fourth observation is that out of 135 tested strains only 6 are *pvl* positive. 129 strains tested belong to the type class IV and by molecular definition they are CA-MRSA, but overall have, on the whole, an hospital profile.



	t032 CC22	t1036 CC22	t1214 CC22	t022 CC22	t041 CC5	t127 CC1	t008 CC8	t3441 CC22	t015 CC45	t1171 CC8	t223 CC22	t790 CC22	t121 CC8 pvl+	t024 CC8	t020 CC22	t16026 CC22	t304 CC8	t002 CC5	t11920 CC22	t2892 CC22	t515 CC22	t657/CC22	t718/CC22	t3213/CC22
94 mdr total strains	40	15	9	8	6	3	1	1	1	3	1	1	3								1			
SCCmec I					6																			
SCCmec IV	40	15	9	8		3	1	1	1	3	1	1	3								1			
pvl+							1						2											
scn-			5							1														

Graph 14:MDR strains SCCmec type, pvl gene, scn gene.

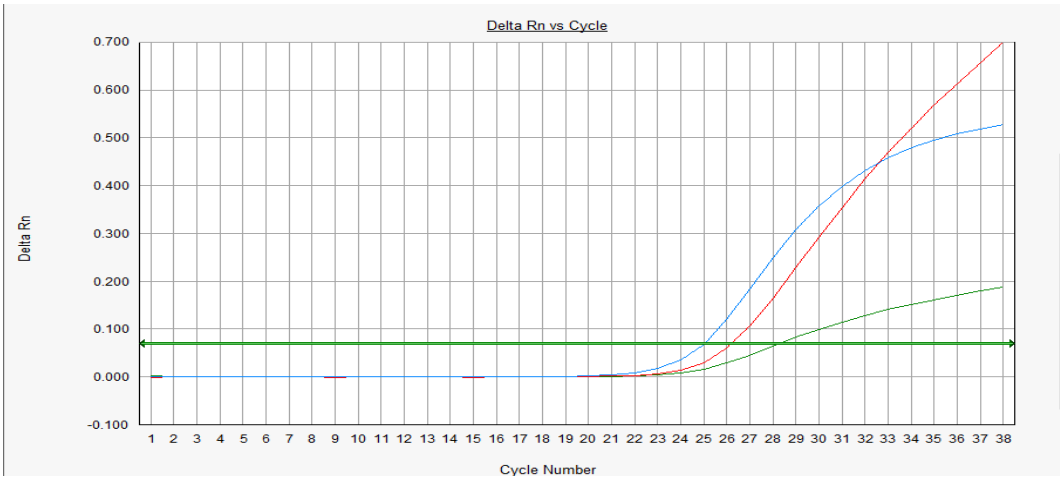


	t032 CC22	t1036 CC22	t1214 CC22	t022 CC22	t041 CC5	t127 CC1	t008 CC8	t3441 CC22	t015 CC45	t1171 CC8	t223 CC22	t790 CC22	t121 CC8	t024 CC8	t020 CC22	t16026 CC22	t304 CC8	t002 CC5	t11920 CC22	t2892 CC22	t515 CC22	t657/CC22	t718/CC22	t3213/CC22
41 amc total strains	10		4		1	1	1				1	1	7	4	1	1	1	1	1	1		2	2	1
SCCmecI	1				1																			
SccmecIV	9		4			1	1				1	1	6	4	1	1	1	1	1	1		2	2	1
pvl+													1					1				1		
scn-													1				1					1		

Graph 15: AMC strains SCCmec type, pvl gene, scn gene.

REAL-TIME TRIPLEX ASSAY RESULTS

The graph 16 showed the amplification plot of the three genes under study of control strains.



Graph n°16 Amplification plot obtained with a detection system based on the use of three fluorophores with reference strains S. aureus ATCC 25923: FAM (red line) for the detection of mecA gene, VIC (blu line) for the detection of pvl gene and NED (green line) for for the detection of Nuc gene.

In the table n° 20 are reported the results of PCR-Real Time assay.

	AMC	**Spa type**	**CC**	**mecA**	**mecC**	**scn**	**pvl**	**specimen**	**section**
4	S.aureus	t032	CC22	Pos.	Neg.	Pos.	Neg.	Swab pharingeal	Medicine
6	S.aureus	t032	CC22	Pos.	Neg.	Pos.	Neg.	Pharingeal swab	Haematology
8	S.aureus	t022	Cc22	Pos.	Neg.	Pos.	Neg.	Rectal swab	surgery
9	S.aureus	t1036	CC22	Pos.	Neg.	Pos.	Neg.	Pharingeal swab	haematology
16	S.aureus	t1214	CC22	Pos.	Neg.	Pos.	Neg.	Pharingeal swab	ICU

23	S.aureus	t1171	CC22	Pos.	Neg.	Pos.	Neg.	Pharingeal swab	Medicine
24	S.aureus	t041	CC8	Pos.	Neg.	Pos.	Neg.	Pharingeal swab	Urology
33	S.aureus	t1214	Cc22	Pos.	Neg.	Pos.	Neg.	Rectal swab	Medicine
38	S.aureus	t127	Cc22	Pos.	Neg.	Pos.	Neg.	Pharingeal swab	Medicine
46	S.aureus	t032	CC8	Pos.	Neg.	Pos.	Neg.	Pharingeal swab	--
52	S.aureus	t032	CC8	Pos.	Neg.	Pos.	Neg.	Rectal swab	--
56	S.aureus	t032	CC22	Pos.	Neg.	Pos.	Neg.	Pharingeal swab	--
67	S.aureus	t1036	CC22	neg	Neg.	Pos.	Neg.	Pharingeal swab	--
70	S.aureus	t1036	CC22	neg	Neg.	Pos.	Neg.	Pharingeal swab	--
71	S.aureus	t1214	CC22	neg	Neg.	Pos.	Neg.	Pharingeal swab	--
ATCC 700699	S.aureus	--	--	pos	neg	pos	neg	--	--
ATCC 25923	S.aureus	--	--	neg	neg	pos	pos	--	--

Table 20: results of the strains used for RT-PCR triplex assay

As we can note all *S. aureus* strains were recognized from the nuc probe. *mecA* and *pvl* genes were also corrected recognize from respective probes.

In table n°21 are reported the results obtained directly from clinical samples.

Samples	**Culture Identification**			**Molecular Identification**			**Interpretation**
	Sample	ID MALDI		nuc	mecA	pvl	
1	R	*S.haemol*		-	+	-	MRCoNS
2	R	*S.haemol*		-	+	-	MRCoNS
3	R	*S.haemol*		-	+	-	MRCoNS
4	F	*S aureus*		+	+	-	MRSA
5	U	*S cohnii*		-	-	-	MRCoNS
6	F	*S aureus*		+	+	-	MRSA
7	R	*S.haemol*		-	+	-	MRCoNS
8	R	*S aureus*		+	+	-	MRSA
9	F	*S aureus*		+	+	-	MRSA
10	R	*S.haemol*		-	+	-	MRCoNS
11	F	*S.haemol*		-	+	-	MRCoNS
12	R	*S.haemol*		-	+	-	MRCoNS
13	F	*S.haemol*		-	+	-	MRCoNS
14	PA	*S.haemol*		-	+	-	MRCoNS
15	U	*S epidermid*		-	-	-	MRCoNS
16	F	*S aureus*		+	+	-	MRSA
17	R	*S.haemol*		-	+	-	MRCoNS
18	R	*S.haemol*		-	-	-	MRCoNS
19	R	*S.haemol*		-	+	-	MRCoNS
20	R	*S.haemol*		-	+	-	MRCoNS

21	R	*S.haemol*		-	-	-	MRCoNS
22	R	*S.haemol*		-	+	-	MRCoNS
23	F	*S aureus*		+	-	-	MSSA
24	F	*S aureus*		+	-	-	MSSA
25	F	*S.haemol*		-	+	-	MRCoNS
26	R	*S.haemol*		-	+	-	MRCoNS
27	R	*S.haemol*		-	+	-	MRCoNS
28	R	*S.haemol*		-	+	-	MRCoNS
29	R	*S.haemol*		-	+	-	MRCoNS
30	R	*S.haemol*		-	+	-	MRCoNS
31	R	*S.haemol*		-	+	-	MRCoNS
32	R	*S.haemol*		-	+	-	MRCoNS
33	R	*S aureus*		+	+	-	MRSA
34	R	*S.haemol*		-	+	-	MRCoNS
35	R	*S.haemol*		-	+	-	MRCoNS
36	R	*S.haemol*		-	+	-	MRCoNS
37	F	*S.haemol*		-	+	-	MRCoNS
38	F	*S aureus*		+	+	-	MRSA
39	R	*S.haemol*		-	+	-	MRCoNS
40	R	*S.haemol*		-	+	-	MRCoNS
41	R	*S.haemol*		-	+	-	MRCoNS
42	R	*S.haemol*		-	+	-	MRCoNS
43	R	*S.haemol*		-	-	-	MRCoNS
44	R	*S.haemol*		-	+	-	MRCoNS
45	R	*S.haemol*		-	+	-	MRCoNS

46	F	*S aureus*		+	+	-	MRSA
47	R	*S.haemol*		-	+	-	MRCoNS
48	R	*S.haemol*		-	+	-	MRCoNS
49	R	*S.haemol*		-	-	-	
50	R	*S.haemol*		-	+	-	MRCoNS
51	R	*S.haemol*			+	-	MRCoNS
52	R	*S aureus*		+	+	-	MRSA
53	R	*S.haemol*		-	+	-	MRCoNS
54	R	*S.haemol*		-	+	-	MRCoNS
55	R	*S.haemol*		-	+	-	MRCoNS
56	F	*S aureus*		+	+	-	MRSA
57	R	*S.haemol*		-	+	-	MRCoNS
58	R	*S.haemol*		-	+	-	MRCoNS
59	PA	*S.haemol*		-	+	-	MRCoNS
60	R	*S.haemol*		-	+	-	MRCoNS
61	R	*S.haemol*		-	+	-	MRCoNS
62	R	*S.haemol*		-	+	-	MRCoNS
63	R	*S.haemol*		-	+	-	MRCoNS
64	F	*S.haemol*		-	+	-	MRCoNS
65	R	*S.haemol*		-	+	-	MRCoNS
66	R	*S.haemol*		-	+	-	MRCoNS
67	F	*S aureus*		+	+	-	MRSA
68	R	*S.haemol*		-	+	-	MRCoNS
69	R	*S.haemol*		-	+	-	MRCoNS

70	F	*S aureus*		+	+	-	MRSA
71	F	*S aureus*		+	-	-	MSSA
72	PA	*S.haemol*		-	+	-	MRCoNS
73	R	*S.haemol*		-	+	-	MRCoNS
74	R	*S.haemol*		-	+	-	MRCoNS
75	R	*S.haemol*		-	+	-	MRCoNS
76	R	*S.haemol*		-	+	-	MRCoNS
77	R	*E faecalis*		-	-	-	-----
78	F	*S.haemol*		-	+	-	MRCoNS
79	R	*S.haemol*		-	+	-	MRCoNS
80	R	*S.haemol*		-	-	-	MRCoNS
ATCC 700699				+	+	-	
ATCC 25923				+	-	+	

Table 21: list of strains used in Real time.

In table 21 are reported results of the triplex RT-PCR assay applied directly to 80 clinical samples after DNA extraction. 15 samples out of 80 (18,7%) were *nuc* positive indicating that there was a presence of *S. aureus* strain. 12 of them (80%) amplified also the *mecA* gene and were classified as sample with MRSA indeed 3 (20%) didn't show the *mecA* amplification and were classified as sample with MSSA strains. 65 samples out of 80 were *nuc* negative and were classified as no presence of *S. aureus* strains. 58 strains out of 65 (89%) amplified anyway the *mecA* gene and were classified as sample with the presence of MR-CoNS while 7 samples (11%) were negative for the presence of all gene tested and were classified as negative samples. There were no samples positive for the presence of *pvl* gene.

The agreement with the molecular standard technique was of 100%.

Analytical specificity was evaluated using DNA lysates prepared from clinical samples after conventional culture methods. 10 phenotypically and genotypically well-characterized *Staphylococcus* spp. and 10 other Gram-positive from pharyngeal swabs and some Gram-negative strains obtained from rectal swabs such as *Escherichia coli* (20), *Proteus vulgaris* (8), *Enterococcus faecalis* (9), *Enterococcus faecium* (4), *Enterobacter cloacae* (8), *Klebsiella pneumoniae* (10) were used.

The analytical specificity of the assay was determined using 30 methicillin-resistant CoNS and methicillin-susceptible CoNS samples from our laboratory, including five strains of methicillin-resistant *S. epidermidis*, five samples of methicillin-susceptible *S. epidermidis*, five samples of *S. haemolyticus*, and one each of *S. hominis*, *S. lugdunesis*, *S. capitis*, *S. carnosus*, *S. cohnii*, *S. sciurii*, and *S. warneri*. In addition, seven *Streptococcus* spp strains, reported to be common in colonization or infection of the throat and respiratory tract were analyzed, including two samples of *S. mitis*, two of *S. salivarius*, two of *S. pneumoniae*.

The sensitivity and specificity of the Triplex RT-PCR were both 100% for these targets when compared with the culture and conventional methods.

We found an analytical sensitivity of this current Triplex PCR assay of 514 CFU/mL. PCR assays, with three replicas per sample, consistently detected MRSA alone at 18 copies per reaction mixture in 20µL. We retain that this analytical sensitivity might be high enough to perform the assay directly from clinical specimens

We therefore want, with our Real-time assay, to underline its importance. The ability to simultaneously identify the species, the meticillin resistance and the presence of the necrotizing toxin present in the community strains in an hour of time, directly from the clinical sample, eg the species, thus reducing reporting times and activating immediately clinical therapy.

DISCUSSION AND COCLUSIONS

S. aureus is a major human pathogen causing skin and tissue infections, pneumonia, septicemia, and device-associated infections. The emergence of MRSA and resistance to other antibacterial agents has become a major concern, especially in the hospital environment, because of the high mortality of the infections caused by these strains.

Infection is the most important factor in increase of morbidity and mortality in hospitalized patients. The spread of multi-drug resistant *S. aureus* strains have become a serious challenge in community and healthcare systems. The prevalence of MRSA isolates has been reported more than 25-50% in different regions of Italy. [68]

The defining feature of MRSA is the staphylococcal cassette chromosome mec (SCCmec). This is a mobile genetic element that carries the central determinant for broad-spectrum beta-lactam resistance encoded by the *mecA* gene. The emergence of methicillin-resistant staphylococcal lineages is due to the acquisition and insertion of the SCCmec element into the chromosome of susceptible strains. SCCmec elements are highly diverse in their structural organization and genetic content and have been classified into types and subtypes.

Many types, sub-types, and variants of SCCmec elements and SCC elements lacking *mecA* have been reported without following any standardized, internationally agreed rules of nomenclature. Consequently, there are ambiguities and inconsistencies in the classification of SCC elements in the published literature to date.

Single locus DNA-sequencing of the repeat region of the *Staphylococcus* protein A gene (*spA*) can be used for reliable, accurate and discriminatory typing of MRSA. Repeats are assigned a numerical code and the spA-type is deduced from the order of specific repeats. However, spa-typing was hampered in the past by the lack of a consensus on assignments of new spa-repeats and -types.

We used sequence typing of the *spa* gene repeat region to study the epidemiology of MRSA at Verona University Hospital. Therefore, single-locus DNA sequencing of repeat regions the *spa* gene (protein A), respectively, could be used for reliable and accurate typing of MRSA. [69] [70] [71] [72] [73]

Spa-typing is especially interesting for rapid typing of MRSA in a hospital setting since it offers higher resolution than coa typing. [74]

MRSA isolates are serious threat for public health. [75] [76]

These strains are typically hospitalized and include multi-resistance to ciprofloxacin, levofloxacin and erythromycin in according to the Budimir *et al* [77] and Kalenić *et al* [78].

In the present study, resistance to antibiotics in community was high, these findings are very important because the increase of antibiotic resistance in community can be lead to failure in empirical therapy. 94% of the strains (127) were SCCmec type IV and only 6% (8) were SCCme I. also 6 *pvl* positive and 129 strains *pvl* negative. About *scn* gene 9 out of 135 strains were scn negative. [79] [80] [81] [82] [83]

Inappropriate use of antibiotic, ineffective infection control, hygiene practices and extensive use of antibiotics in agriculture are all factors that might be caused the increase of antibiotic resistance in community. [84] [85]

In this study we have completely characterized 135 MRSA strains, using both SSCmec and spA-typing. 94 out of 135 were isolated during screening of multidrug resistant strains, the other 41 were isolated from blood culture. We observed the presence of 6 “representative” spa- types, namely t032 CC22 t1036 CC22, t1214 CC22, t022 CC22, t041 CC5, t121 CC8.

We also noted the presence of 3 clonal complex that representing this population of MRSA tested, namely CC22, CC5, CC8.

We have observed that most of the MRSA strains tested (127) belong to a type SCCmec IV and only 8 belong to a SCCmec type I. From literature, the definition

of a Community strain (CA-MRSA) presents a SCCmec of the type IV or V, instead of an Hospital acquired (HA-MRSA) strain usually belong to Sccmec Type I II and III.

It has been established that SCCmec types I, II, III are related to HA-MRSA while the SCCmec types IV and V are prominent types of CA-MRSA. [86]

It has been reported that HA-MRSA exhibits different genetic characteristics in different geographic regions. Several clones seem to have emerged in Europe. For example, the ST8, ST247, ST239 and ST228 clones are predominant in Italy. [87] We also noted a different distribution of spa-types in MDR strains and in strains derived from blood culture. Among the strains coming from blood culture we have observed the lack of a spa-type which is instead considered to be representative in screening strains, namely t1036 CC22.

There are several different spa-types, including one spa-type completely new: t16026 CC22 that we submitted to the Ridom Spa server site in the year 2016.

Among the 24 types that we have found, there are 10 spa-type "no-representative" that lacking in screening strains t024 CC8, t020 CC22, t16026 CC22, t304 CC8, t002 CC5, t11920 CC22, t2892 CC22, t657 CC22, t718 CC22, t3213 CC22.

Vice versa in the samples coming from blood culture we have observed that there are 6 spa types missing; of these 6 spa types, the t1036 CC22 that is one of the "most representative' spa-type types found in the screening; t022 CC22 is among the most expressed, while the other 4 are "not -representative" and were t3441 CC22, t015 CC45, t1171 CC8, t515 CC22.

A first consideration is that data indicate a different distribution of different spa-types between specimens isolated from blood culture and screening ones.

We need to continue with this hypothesis increasing the number of characterized strains isolated from blood culture.

We further noted that a type of "spa -type representative" but more experienced in strains isolated from screening belongs to a type Sccmec I; all the strains

expressing the t041 in both (screening strains and blood culture) are SCCmec I. This is the first association to do in this type of study. Of these strains 7 strains belonging to t041 SCCmec I (6%) (HA-MRSA), no one was positive for *pvl* gene. According to definition of community acquired MRSA (CA-MRSA), i.e. strains isolated in an outpatient setting, or from patients within 48h of hospital admission. [88]

This strain belongs to the Sourthen German clone MRSA (ST-111 ST228) with a frequency of 0.24% with about 928 isolates. The last isolation was submitted in the Ridom spa server site had been identified in Italy and in Croatia.

In this study the 94% of the strains belong to Sccmec IV. Spa type t032 Clonal complex 22 (CC22) SCCmec IV has a frequency of 10.42%. About 40.469 strains were isolated in different parts of the world and in different parts of Italy including the Friuli Venezia Giulia. The last isolation was submitted in the Ridom spa server site have been identified in Sweden, Austria and Germany.

50 strains t032 CC22 were isolated in this study and these strains belongs to the Barnim MRSA (prototype & subclone), EMRSA-15, prototype of ST-22, CC22 in according to Evolutionary models of the emergence of methicillin-resistant *S. aureus*. Robinson DA1, Enright MC.

SCCmec type IV was first discovered in recent studies that examined isolates of community-acquired MRSA. [89] [90] Several new clones that carry SCC*mec* type IV have also been identified from samples from patients with community-acquired MRSA. [91] Our results, based on inferences from evolutionary models, show that SCC*mec* type IV is also the most frequently acquired element within the five major lineages responsible for most hospital-acquired MRSA infections. While the prevalence of disease caused by clones that carry SCC*mec* types I to III at present may be higher than that caused by clones that carry SCC*mec* type IV, the more frequent acquisition of SCC*mec* type IV has markedly increased the genetic diversity of MRSA and suggests that the prevalence of disease caused by clones that carry this element will increase.

The other most representative spa-types such as t1036 t1214 t022 belong to the clonal complex CC22 and are SCCmec IV and therefore belong to the EMRSA - 15 clone.

Several major MRSA clones have emerged, which involved independent SCC*mec* acquisitions by distinct *S. aureus* lineages such as CC5, CC8, CC22, CC30 and CC45.

In fact we have isolated two other important lines CC8 with a spa type t121 *pvl* positive more experienced than the other spa types CC8 (t008, t1171, t024, t304) and CC5 with the spa type t041 more expressed and t002 with the frequency 6,82% [92], belong to the Rhine Hesse MRSA (prototype), EMRSA-3 clone, New York clone, Japan clone.

The other CC lines as CC45 belong to the spa-type t015 and CC1 with the clonal complex t127 with the frequency 2,5%.in according to Jamrozy *et al* [93] Spa-type t008 (2 strains) *pvl* positive with the clonal complex CC8, frequency 6 %, belong to the clone Northern German MRSA subclone, USA300 ORSA IV, Archaic/Iberian, ST250. We have observed that the CC8 line expresses very frequently the presence of the *pvl* gene. Only one strain belonging to the CC5 line was positive for the *pvl* gene, only another strain CC22 was positive for *pvl* gene. Then on 6 strains that we have found to be positive for the *pvl* gene on 135 total strains, 4 belong to the CC8 line. [94].

More recently, whole-genome sequencing (WGS) and reconstruction of phylogenetic relationships between MRSA isolates derived from the same CC has demonstrated that MRSA has become widespread predominantly through a process of clonal expansion. [95] [96]. However, acquisition of SCC*mec* is not the sole event involved in the emergence of MRSA clones. Other evolutionary changes occur such as the acquisition of additional MGEs that collectively constitute molecular markers of a new MRSA clone.

Contemporary MRSA clones include the epidemic MRSA-15 (EMRSA-15), which belongs to CC22. The first reported isolation of EMRSA-15 was in the UK in the early 1990s, and it has since become the dominant hospital-associated

MRSA (HA-MRSA) in the country. [97] [98] EMRSA-15 subsequently spread beyond the UK, with rapid expansion across Europe to become the dominant HA-MRSA lineage in Australia and Singapore. Whole genome analysis of CC22 isolates did not reveal a single prominent genetic element that could explain the success of EMRSA-15 clone, with a combination of genetic variations observed, of which the most notable were determinants of antimicrobial resistance. [99]

Additionally, EMRSA-15 was found to suffer a lower fitness cost due to fluoroquinolone resistance than other MRSA clones. [100]

The major predominant line is the CC22, the major predominant spa-type is t032 belonging to the E-MRSA 15 clone.

Spa type t008 CC8 (2 strains) in blood culture and screening strains belong to the clone Northen German MRSA.

Spa type t002 CC5 (1 strains) that we found only in the blood culture strains belong to the clone E-MRSA 3, Rhine Hesse MRSA (prototype), USA 800 ORSA IV. According to Emergence of clonal complex 5 (CC5) methicillin-resistant *Staphylococcus aureus* (MRSA) isolates susceptible to trimethoprim-sulfamethoxazole in a Brazilian hospital [101] Spa- type t022 CC22 (8 strains) isolated only in screening strains belong to the E-MRSA 15

Spa t 041 CC5 belong to the Sourthen German MRSA.

According to the Ridom spa server

Five major lineages of MRSA (CC5, CC8, CC22, CC45 and CC30) circulate internationally and cause most nosocomial MRSA infections worldwide [102] [103] [104] [105]

Five predominant clones (Brazilian, Iberian, Hungarian, pediatric and New York/Japan (NYJ) clones) were identified among 3000 MRSA strains collected in surveillance studies and outbreak investigations from 1994 to 2000 (the CENMET initiative); The authors hypothesized that these major clones have a unique ability to cope with changing clinical environments.

We develop a triplex assays real-time PCR to quickly detect *S. aureus,* methicillin resistance and the virulence factor *pvl* directly from a clinical sample without culture. This assay can identify and differentiate MRSA, MSSA, Methicillin-Resistant Coagulase Negative Staphylococci (MR-CNS).

Results obtained with this RT-PCR presented a 100% of agreement compared with endpoint PCR, both starting from colonies and clinical samples. These is very important because we validate the system starting from clinical samples usually heavy contaminated as throat and rectal swab. Assay showed a 100% of sensibility been able to detect *S. aureus* species also in presence of other CNS or other bacteria. We found an analytical sensitivity of this current Triplex PCR assay of 514 CFU/mL. PCR assays, with three replicas per sample, consistently detected MRSA alone at 18 copies per reaction mixture in 20µL. We retain that this analytical sensitivity might be high enough to perform the assay directly from clinical specimens. Specificity also is 100% as reported from our results, since we performed test in presence of different species and results from clinical samples confirmed this. This home-made RT-PCR assay could be useful in the screening of carrier patients that must undergoing to decolonization therapy, reducing time and cost of screening and reducing MRSA infections.

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