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Molecular Microbial Diagnostic in Lung Transplant Recipients

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ABSTRACT

The appearing of microbial lineages carrying multiple dangerous loci are results from the extensive use of antibiotics and has brought a huge increase in the infections-derived morbidity and mortality, which is critical in the hospital setting. Etiologic agents of these non easily tractable infections include bacteria, such as the genus *Staphylococcus*, but also fungi and virus. Alterations in the immune system allow these organisms to invade and affect the functionality of any tissue, organ or system of the human being. Pulmonary infection occurs as result of deficient lung systemic defence mechanisms which could be altered by medical treatments or by environmental factors. The infective agents commonly gain access to the lung by air, but also by blood or lymphatic system. *Staphylococcus aureus* strains that share antibiotic resistance and virulence factors represent the aetiological agent responsible of many cases of bacterial pneumonia, thoracic surgery postoperative infections, and diverse tissue infections, resulting in significant disease and morbidity in recipient patients after lung transplantation. The control and treatment of *Staphylococcus* infections, especially methicillin resistant strains, need for developing reliable and rapid methods of detection and characterization of these microorganisms. Nowadays, new insights into the diagnostic and epidemiology of MRSA and other pathogenic staphylococci have been developed employing molecular methods. This has meant an important advance in the diagnostic and treatment plans of such infective bacteria.

Keywords: Molecular Microbial Diagnostic; Lung Transplant Recipients

1. Introduction

The progressive increment of microbial infections caused by highly virulent multiple resistant strains has become one of the most worrying health evils. Selective pressure resulting from the extensive use of antibiotics during last 35 years has brought to the emergence, selection and dissemination of resistance and virulence genes among pathogenic microorganisms. All these factors have brought us the risk of suffering non tractable infections. Clear examples are the growing appearance and spreading of resistance in Gram-positive bacteria such as the genus *Staphylococcus or Mycobacterium*; Gram-negative bacteria as *Pseudomonas*; fungi as *Aspergillus*; virus as *Cytomegalovirus* (CMV) and several parasites [1-7]. The constant and fast worldwide dispersion of microbial lineages carrying multiple dangerous loci has caused a huge increase in the infections-derived morbidity and mortality. These aspects were already observed by WHO in 2001 in the document "Infection control programs to control antimicrobial resistance" [8].

Infections caused by non easy tractable microbes are occurring globally and can affect any tissue, organ or system of the human being depending on the etiologic agent. Pulmonary infection occurs as result of the interference of immunosuppressive factors with the normal lung systemic defence mechanisms [9]. Several factors interfere with normal lung defence facilitating lung infection: depressed bactericidal and phagocyte function of macrophage caused by starvation, alcohol ingestion, hypoxia, uraemia, air pollutant, cigarette smoke and previous viral infection; drop off or loss of cough reflex leading to aspiration caused by drug, anaesthesias or coma; pulmonary edema; collection of secretion; poor mucociliary elevator function from smoking, infection, etc.; cystic fibrosis, airway obstruction, chronic bronchitis, and others [10-12].

In turn, pathogenic organisms gain access to the lung by different routes: through the airways; through the

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bloodstream; by traumatic implantation; or by a direct spread across the diaphragm through the lymphatic system. The most common route is the airways. Airway spread can result from inhalation of the organism as an aerosol on droplet nuclei. This is a major mechanism of spread of many viral infections and of tuberculosis [3,13, 14]. Development of infection often begins with colonization of the upper respiratory tract by potential pathogens followed by aspiration into the lower respiretory tract. A high diversity of microbes can be responseble of infection and associated decline of the respiratory system. Among all of them, our group has mainly specialized in the study of the Gram-positive bacterial genus *Staphylococcus*, principally *S. aureus* [15,16]. This microorganism is the aetiological agent responsible of many cases of bacterial pneumonia, thoracic surgery postoperative infections, and diverse tissue infections, resulting in significant disease and morbidity in recipient patients after lung transplantation.

2. Thoracic Surgery

Thoracic surgery is the field of medicine involved in the surgical treatment of diseases affecting organs inside the thorax (the chest). Generally, it involves treatment of conditions of the lungs, chest wall, and/or diaphragm.

A Thoracic Surgeon provides the operative, per operative care and critical care of patients with acquired or congenital pathologic conditions within the chest. The thoracic surgeon function includes pathologic conditions of the lung, oesophagus and chest wall, abnormalities of the great vessels, tumours of the mediastinum, and diseases of the diaphragm and pericardium. Management of the airway and injuries of the chest are within the scope of the specialty [11,17-19].

Major complications following thoracic surgery fall into two categories: respiratory difficulties and wound infections. These postoperative infections (PIs) are serious complications of thoracic surgery and *S. aureus* infective agent has been described as one of the most important pathogens associated with surgical wound infections. Specifically methicillin-resistant *S. aureus* (MRSA) continues to pose a major threat to the lung and cardiovascular surgery patients [20].

3. Lung Transplantation

Lung transplantation is an important therapeutic treatment for many patients with life-threatening pulmonary diseases; however, long-term survival is still relatively limited compared with other solid organ transplants [21]. Lung transplantation has many potential posttransplant complications with PIs being a major contributor. Compared with recipients of other solid organ transplants (SOT) lung transplant recipients (LTR) are at particularly high risk of infectious complications due to a variety of factors associated to the higher state of immunosuppression, the direct and continuous contact with pathogens, bacterial contamination from the donor lung, the denervation of the allograft resulting in diminished cough reflex and mucociliary function, the impaired lymphatic drainage due to its disruption and the risk of cross-contamination of the transplanted lung by the native lung in single LTR [22,23]. Infectious complications substantially contribute to low-grade outcomes after lung transplantation (LTx) compared to other types of SOT, being the average 1-year survival rate approximately 85%, with a 5-year survival rate of 53% [17,18,22-25].

During the last years, several articles concerning infections in LTR have been published. In particular, important information has been published regarding CMV prophylaxis and treatment, and fungal infections following lung transplantation [11,14,23-27]. Furthermore, recent studies have shown that the incidence of MRSA has dramatically increased in lung transplant programs [18,28]. These studies highlight the importance of implementing preventive measures for MRSA colonization before, during and after lung transplantation.

4. Bacterial Infections

Pneumonia and influenza account for a high number of deaths all over the world and are the fifth leading cause of death, exceeded only by heart disease, cancer, cerebrovascular disease and accidents. Pneumonia can be broadly defined as any infection in the lung [29]. Acute pneumonias may be caused by pyogenic bacteria that primarily induce neutrophilic exudates in alveoli, bronchioles and bronchi or by a miscellaneous group of microorganisms that induce predominantly peribronchiolar and interstitial mononuclear inflammation [9,22,23,25,29].

Bacterial infections occur in two frequently overlapping morphologic patterns: bronchopneumonia and lobar pneumonia. They both can be caused by a variety of Gram-positive and Gram-negative organisms [4,30]. Depending on bacterial virulence and host resistance, the same organism may in one case cause bronchopneumonia and in another case lobar pneumonia and sometimes intermediate involvement.

In the first 3 months after transplantation, bacterial infections are responsible for most deaths. Pneumonia is the most common type of infection in LTR with Gram-negative bacteria (GNB) being responsible for the bulk of disease, most commonly with *Pseudomonas aeruginosa* and other GNB such as *Burkholderia* spp (formerly known as *Pseudomonas cepacia*) [23,30]. Cystic fibrosis (CF) patients infected with *B. cepacia* have historically been prone to postoperative infectious complications and poor outcomes. Because of this situation, many transplant centres consider infection with *Burkholderia* spe-

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cies a contraindication to lung trans- plant in CF [10,17]. However, recent publications have signalled that post-operative infections in CF after lung transplantation can caused by a variety of microorganisms: *Pseudomonas aeruginosa*, *Mycobacterium* spp, *Aspergillus* spp, and *Staphylococcus* spp [4,6,7,17,24,30].

Certain *Burkholderia* species, potentially including *B. gladioli* and some *B. cenocepacia*, cause a greater risk for death than do other *Burkholderia* species. Although *Burkholderia* infection by itself should not be considered an absolute contraindication for LTx, selection for transplant should consider the specific *Burkholderia* species present (9 genetically distinct species or genomovars are typified) [22], since there is an increased risk of death among patients infected with *B. cenocepacia*, *versus* those infected with non-*cenocepacia* species [23].

Bronchiolitis obliterans syndrome (BOS) is a major long-term complication following LTx, affecting approximately one-third of recipients by 3 years [31]. Colonization with GNB, particularly *Pseudomonas*, may play a role in the aetiology of BOS, so strategies aimed at reducing colonization may decrease rates of BOS and improve long-term outcomes [22]. Chronic pneumonia is caused by bacteria as *Nocardia* or *Actinomyces* species and some parasites, fungi and intracellular bacteria [2,13,25].

Nosocomial pneumonias develop in a number of hospitalized patients especially in critically ill patients admitted to intensive care units (ICUs). With the use of potent therapies that deliberately or incidentally produce immunosuppression there is a great risk of developing respiratory infection. The Gram-positive bacteria (GPB) *Streptococcus pneumoniae* is often colonizing the throat, and is the most frequent agent causing bacterial pneumonia. Second malevolence GPB causing pneumonia are *Staphylococcus* spp, especially *S. aureus* mainly if it harbours methicillin or other antibiotic resistance and/or virulent factors as Panton-Valentine Leukocydin (PVL), cell interfering clumping factors, etc. [27,32,33]. Other bacteria also involved in causing infectious in the LTR can be *Chlamydia pneumoniae*, and *Clostridium difficile.* Moreover, recently have been reported that nontuberculous mycobacterial (NTM) may be an underrecognized cause of posttransplant complications and certain candidates for LTx should be screened for NTM infections [3,17].

Fungal infections are also a frequent complication in LTR and among SOT recipients [51]. LTR appears to be the highest with an incidence between 15% and 35%, with an overall mortality of 80% [25,34]. *Aspergillus* and *Candida* species cause the majority of fungal infections in lung transplant recipients; *Cryptococcus* spp, the agents of mucormycosis, endemic fungi (*Histoplasma*, *Coccidioides*, and *Blastomyces* spp), *Scedosporium* spp, *Fusarium* spp, and dermatiaceous molds are other important causes [7,29]. The most common manifestations of aspergillosis in LTR are tracheobronchitis (37%), invasive pulmonary aspergillosis (32%), bronchial anastomosis infections (20%) and disseminated disease (10%) [6,7]. The incidence and timing of invasive aspergillosis (IA) in LTR has changed over time. Earlier publications had reported the incidence to be higher during the first year after transplantation but recent data suggest a much lower incidence during the first year and a more delayed presentation [11,25].

Some characteristics of Fungal Infections are unique in LTR [25]. Tracheobronchial aspergillosis occurs only in lung transplant recipients, typically within three months of transplantation [6]. Predisposing factors include pre-operative colonization with *Aspergillus*, early airway ischemia, stenotic airways, the presence of an airway stunt, single lung transplantation, hypogammglobulinemia, CMV infection, use of alemtuzumab or thymoglobulin induction therapies and acute rejection requiring augmentation of immunosuppression [6,23]. The diagnosis of IA is by biopsy with culture, accompanied by compatible clinical and radiographic abnormalities. Detection of galactomannan, a polysaccharide cell wall component that is released by *Aspergillus* organisms during fungal growth, by sandwich enzyme immunoassay in the bronchoalveolar lavage (BAL) appears as promising for its diagnosis [23]. Candidemia usually occurs during the first month following LTx as a result of intensive care unit exposure and recent surgery but in adults no cases of invasive pulmonary candidiasis have been reported following LTx [11,25].

Viruses' infections in lung transplant recipients are preferentially represented by CMV viriasis [24]. LT recipients have the highest risk of developing CMV disease among all SOT recipients, although the risk of CMV has decreased over time [14]. CMV infection contributes to a significant proportion of post-LTx morbidity and mortality but since the introduction of more effective CMV prophylaxis, the burden of CMV infection and disease in recipients of SOT has diminished significantly as well as all-cause mortality in SOT recipients [21]. CMV infection requires evidence of CMV viral replication trough laboratory testing, whereas CMV disease requires not only CMV viral replication but any evidence of typical symptoms or tissue-invasive disease. CMV infection or disease is initially diagnosed based on the detection of viremia, lately quantitative nucleic acid testing, via polymerase chain reaction (PCR), which has become the most widely accepted method of CMV viral load monitoring [26,35]. Most authorities favours the use of universal CMV prophylaxis (administration of antivirals to

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all at-risk individuals, positive donor or recipient serologic status), including lung transplant recipients, being oral valganciclovir either alone or after a short course of intravenous ganciclovir the usual accepted strategy [5]. A recent trial comparing short-course (3 months) with long-course (12 months) prophylaxis with valganciclovir in at-risk LTx showed that only 4% of long-course prophylaxis patients developed CMV disease versus 32% of short-course patients [5,17].

5. *Staphylococcus aureus* Pulmonary Infections. Molecular Diagnostics

Currently, one of the most worrisome examples about infections caused by pathogenic bacteria is the rising emergence and spreading of antibiotic resistance and virulence factors in *Staphylococcus aureus* and other members of the genus *Staphylococcus* [36]. Several population groups are under high risk of suffering infections by pathogenic staphylococci. *Staphylococcus* spp infections are usual in immunocompromised patients: e.g. postsurgery patients, transplanted patients, persons affected of diseases such as flu, bronchopulmonar pathologies. The control and treatment of *Staphylococcus* infections is an extremely relevant subject from its health and clinical implications [15]. Moreover, already in the XXI century, the rising dissemination of methicillin resistant strains, antibiotic that was considered the basic agent against these infections, has extremely complicated their erradication. Thereby, there is a growing frequency of appearance of MRSA strains worlwide, especially in hospital settings, although emergence of community acquired infections is also rising up. Furthermore, the frequency of resistance in MRSA against the so called *last weapons*, as e.g. vancomycin, mupirocin, linezolid, daptomycin, is also increasing. In this context, we have carried out the selection, design and assay of new natural or synthetic molecules with antibiotic activity against staphylococci (patent pending Ref. ES-2498.1, Casero C., Estévez-Braun A., Gutiérrez-Ravelo A., Demo M., Méndez-Álvarez S., Machín-Concepción F.).

The need for developing reliable and rapid methods of detection and characterization of these pathogenic microorganisms has led to new insights into the diagnostic and epidemiology of MRSA and other pathogenic staphylococci. Our working group has been focused on the development of molecular protocols for a sensitive, specific and fast identification of *S. aureus* from clinical samples simultaneously detecting resistance and/or virulence genes [37,38]. The succeed in this goal has meant an important advance in the diagnostic and treatment plans of such infective bacteria. The protocols developed allowed to elucidate the existing association between the presence of the Panton-Valentine leukocidin-encoding gene and a lower rate of survival among hospitalized pulmonary patients with staphylococcal infection [12]. Moreover, combination of different molecular typing methods with classical microbiology ones has permitted us to build up solid MRSA epidemiology studies at both global level and local stage [33,39-44]. These studies constitute a corner stone for the establishment of prevention and treatment measures and can be divided according to the utilization of DNA amplification [45,46].

5.1. Methods without DNA Amplification

5.1.1. Restriction Fragment Length Polymorphisms (RFLPs)

Since the chromosome is the most fundamental component of identity of the cell, methods measuring this molecule represents a preferred approximation for assessing strain interrelatedness. Restriction patterns originated after chromosomal DNA enzymatic digestion generates a restriction fragment length polymorphism (RFLP), after pulsed field gel electrophoresis (PFGE), which efficiently and accurately allows the differentiation of strains and compare following conventional agarose gel electrophoresis [47].

PFGE methods have been used to evaluate the spread of various antimicrobial resistant bacteria [48]. The finding of isolates that have identical or related restriction endonuclease patterns suggests spread from single strains. Guidelines proposed by Tenover *et al.* (1997) are often used to for the interpretation of PFGE [45]. With these guidelines, a banding pattern difference of three fragments could have occurred due to a single genetic event and thus these isolates are classified as highly related; differences of four to six restriction fragments are likely due to two genetic events, and differences of greater than seven restriction fragments are due to three or more genetic events. Isolates that differ by three fragments in PFGE analysis may represent epidemiologically related subtypes of the same strain. Conversely, isolates differing in the positions of more than three restriction fragments may represent a more tenuous epidemiologic relation. A number of studies using PFGE and other typing methods indicate that single genetic events, such as those that may alter or create a new restriction endonuclease site or DNA insertions/deletions associated with plasmids, bacteriophages, or insertion sequences, can occur unpredictably even within the time span of a well-defined outbreak (One to three months) [49].

In general, strains are considered identical if they show 100% similarity and are considered clonally related if they show greater than 80% similarity (generally comparable to a three-fragment difference as noted above). The typical phylogenic output is the dendrogram, which provides a visual representation of strain lineages, plus genetic similarities and differences between groups.The accuracy and typing MRSA isolates [48].

5.1.2. Plasmid Profiling and Restriction Endonuclease Analysis of Plasmid (REAP)

Plasmids are DNA molecules that are separate from, and can replicate independently of, the chromosomal DNA. Plasmid sizes vary enormously and can be considered part of the mobilome because they are often associated with conjugation, a mechanism of horizontal gene transfer which typically provides a selective advantage under a given environmental state since may carry genes that provide resistance to antibiotics or the ability to produce proteins that act as toxins [40].

Plasmids are present as variable components of many staphylococcal genomes, and are classified into 4 classes and 15 incompatibility groups which are often responseble for antibiotic resistance. This fact is exploited for typing of multi-resistant staphylococci by separating in agarose gel electrophoresis the isolated intact plasmidic DNA [34]. Thus, the number and size of plasmids is determined in a plasmid profiling. Since extrachromosomal DNA content shows considerable variation, plasmid profiling is suitable for the study of relatively recent epidemiological relationships, whereas analysis of chromosomal DNA reflects more reliably relationships over a longer period of time.

In restriction endonuclease analysis of plasmid (REAP) the isolated plasmid DNA of *S. aureus* is digested separately with the restriction enzymes *Hind*III and *EcoR*I. The DNA digests obtained are separated by electrophoresis in agarose gels generating patterns of restriction fragments which allow to determine composite strain types. This method can be complemented with specific gene detection using probes targeting for instance, genes encoding antibiotic resistance [36]. Owing to the possible instability of plasmid, the method shows only moderate reproducibility and therefore an additional typing method is to be used for analysis [37,40].

5.2. Methods with DNA Amplification

5.2.1. Gene-Specific PCR

Amplification and sequencing conserved genes of bacterial genomic DNA are used for identification of staphylococcal species and their genotypes. In *S. aureus* the genes encoding 16S rRNA, factor A essential for methicillin resistance (*fem*A), and staphylococcal thermonuclease (*nuc*) (among others) are frequently used for identification at the species level [43].

5.2.2. Multiplex PCR Assays (MPCR)

The multiplex PCR (MPCR) assays allows simultaneous amplification of several genes in one reaction mixture. As explained above, MRSA strains constitute a major health care problem; therefore, the availability of sensitive and specific methods for the accurate detection of antibiotic resistance in these bacteria has become an important tool in clinical diagnosis. Since phenotypic typing methods are not discriminating enough and are highly dependent on growth conditions, it is essential to use molecular techniques to stop the spread of multiple-antibiotic-resistant *S. aureus*. These techniques allow a rapid, accurate identification of staphylococci and their resistance type. Thus, fast, sensitive, and specific molecular methods will be an essential diagnostic tool for microbiology laboratories. The use of PCR for the sensitive and specific detection of microorganisms and antibiotic resistance genes is increasing in clinical microbiology laboratories. There are several reports in the literature describing the use of MPCR for detection of MRSA strains, but most of these protocols are designed to detect only one or two gene fragments from overnight liquid cultures [37].

In 2001, our group described a multiplex PCR assay for the detecting clinically relevant antibiotic resistance genes harbored by some *S. aureus* isolates. Conditions were optimized for the simultaneous detection of regions of the *mecA* (encoding high-level methicillin resistance), *ileS*-2 (encoding high-level mupirocin resistance), and *femB* (encoding a factor essential for methicillin resistance) genes, respectively, from a single colony in a single reaction tube [35,37,42].

These MPCRs offers a rapid, simple, feasible, specific, sensitive, and accurate identification of mupirocin-resistant MRSA clinical isolates and is suitable for diagnostic test in clinical microbiology laboratories, facilitating the design and use of antibiotic therapy [38,50].

5.2.3. Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA)

A relatively new method for typing *S. aureus* strains is the multiple-locus variable-number tandem-repeat analysis (MLVA). This technique consists of simultaneous amplification of variable-number tandem repeats of different genes. Several works have tried to determine if MLVA provides enough information to be performed routinely instead of PFGE or MLST, which are more laborious, in the clinical setting. In our laboratory, MLVA was performed as previously described [51], but slightly modified to obtain optimal results and to accelerate the process [33,52,53] concluding that the utilization of MLVA allows to distinguish among different MRSA reservoirs and other circulating MRSA strains. The proven simplicity, low cost, and speed of MLVA enable the performance of routine checkups in patients, mainly via admission screening on surgical wards and in intensive care units, hampering the spread of these strains and therefore reducing the morbidity, mortality, and costs [28,44,54, 55].

5.2.4. Spa Typing of MRSA

DNA sequence analysis of the protein A gene variable

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repeat region (*spa*) typing provides a rapid and accurate method to discriminate *S. aureus* outbreak isolates from those deemed epidemiologically unrelated. This technique involves DNA sequencing of short sequence repeats in the polymorphic X region of the protein A gene of *S. aureus*. The region consists of a variable number of these repeat units which are usually 24 bp in length (Laboratory of HealthCare Associated Infection). Each new base composition of a repeat is assigned an alphanumerical code (r01, r02, etc.) and the repeat succession determines the *spa* type (e.g. t001, t002, etc.). *Spa* typing is used throughout Europe and world-wide for reliable, accurate and discriminatory typing of *S. aureus* [both methicillin sensible *S. aureus* (MSSA) & MRSA]. Over 7000 *spa* types have been described to date; *spa* non-typable strains are rare (less than 0.1%). There is a standard international nomenclature which is web-enabled and the data are directly comparable between centers and countries. For some *S. aureus* lineages, the technique has a discriminatory index approaching that of PFGE [56].

5.3. Multi-Locus Sequence Typing (MLST)

Multi-locus sequence typing is the molecular method with highest discriminatory capability and is based in characterizing the sequences of 450 bp internal fragments of 7 housekeeping genes: *carbamate kinase* (*arcC*), *shikimate* 5*-dehydrogenase* (*aroE*), *glycerol kinase* (*glpF*), *guanylate kinase* (*gmk*), *phosphate acetyltransferase* (*pta*), *triose-phosphate isomerase* (*tpi*) and *acetyl-CoA C-acetyltransferase* (*yqiL*). The sequences obtained are assigned to allele numbers after comparison with a DNA sequences database (www.mlst.net). The allele numbers at each of the seven *loci* define the allelic profile or sequence type (ST). Novel alleles and STs not found on the MLST website are confirmed by repeating both the PCR and sequencing [49,57].

MLST has been employed for identifying the MRSA and MSSA clones among isolates from patients with serious community- and hospital-acquired infections. This method is suitable for studying both the evolution of MRSA pandemic clones and local epidemiology, and has similar discriminatory power to PFGE [58]. MLST provides generally highly reproducible and comparable results. However, it has the disadvantage of being expensive and technically demanding. Therefore, there are attempts to detect sequence polymorphisms of the 7 loci by other method instead of DNA sequencing (like DNA microarrays).

5.4. Staphylococcal Cassette Chromosome Mec Typing (SCCmec)

A remarkable phenomenum that occurs is that MSSA strains become MRSA strains by the acquisition of a staphylococcal cassette chromosome *mec* (SCC*mec*) element carrying the *mecA* gene, which is responsible for methicillin resistance. SCC*mec* elements are unique genomic islands that are found in staphylococci. These elements have two essential components, the *ccr* gene complex (*ccr*) and the *mec* gene complex (*mec*) [59]. The *ccr* gene complex is composed of *ccr* genes and surrounding open reading frames (ORFs), and the *mec* gene complex is composed of the *mecA* gene, regulatory genes, and insertion sequences upstream or downstream of *mecA*. Several *mec* and *ccr* allotypes have been found among SCC*mec* elements, what has led to the specific classification. Oliveira and Lencastre (2002) developed a novel method to identify the structural types of SCC*mec* in MRSA strains based on a MPCR aproximation [59]. To date, several structural-differences in SCCmec elements have been identified. SCC*mec* typing classifies SCC*mec* elements in the basis of their structural-differences and is used in epidemiological studies to discriminate MRSA strains or to define a MRSA clone in combination with the genotype of MSSA strain in which a SCC*mec* element has integrated [53].

The different techniques available for typing MRSA serves to emphasize that none has yet been recognized as the definitive method [54]. In many cases it is necessary use a battery of them, so our group have followed this work line. In 2003, we communicated that we had detected the UK EMRSA-16 multiresistant clone as an epidemic clone in a university hospital in Tenerife, Spain (Hospital Universitario Ntra. Sra. de Candelaria, HUNSC) [43]. To our knowledge, this was the first time that hospital establishment of the EMRSA-16 clone in Spain had been reported. We have ensured the clonal nature of our isolates by development and integrative analyses of different molecular approaches: PFGE, SCC*mec* multiplex PCR assay, MLST, MLVA and *spa* typing. Results from these analyses showed that the clone was ST36-MRSA-II. Subsequently, we tracked MRSA clones during a 5-year period in the HUNSC [36].

As explained above, MRSA is the most common cause of serious hospital-acquired infections. Infections of the respiratory tract by *S. aureus* can be more severe if the infecting strain produces the Panton-Valentine leukocidin (PVL) [32]. The serious impact of PVL-positive *S. aureus* infections seems to be associated with pulmonary complications. In 2007, we hypothesized that PVL-positive MRSA is associated with mortality in patients with *S. aureus* pneumonia [12]. The presence of PVL differed significantly between dead and living patients, since all of the PVL-positive patients died. The findings of this study may have some implications for clinical decision making. PVL-positive MRSA strains seem to be dangerous for pulmonary patients, so we recommend screening

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for the presence of PVL when an MRSA is detected in such patients. The presence of PVL could be detected by simple PCR amplification and sequencing specific genes.

In other study [52], MLVA was used to perform 292 MRSA isolates previously characterized by PFGE, MLST, and SCC*mec* typing. This study demonstrated the ability of MLVA to distinguish among different MRSA reservoirs and other circulating MRSA strains in the HUNSC. The proven simplicity, low cost, and speed of MLVA enable the performance of routine checkups in patients, mainly via admission screening on surgical wards and in intensive care units, hampering the spread of these strains and therefore reducing the morbidity, mortality, and costs.

In a recent published article [53], a wide variety of MRSA clones was revealed, including an emergent ST and two new *spa* types. The PVL genes were found in isolates belonging to unrelated lineages, what could indicate different independent introductions of PVL-positive strains in Tenerife. Moreover, we detected that hospital MRSA clones had spread to the community and are now circulating in both environments [44, 53].

Definitely, laboratory screening for MRSA is a complex balance between speed of result, sensitivity, specificity and cost. The development of high-throughput methods for typing bacterial pathogens requires careful assessment of the qualities of candidate systems. Therefore, it is extremely important not only to compare methods with each other, but also to define the robustness or weakness of individual methods [45,60,61]. The use of molecular methods for epidemiological typing of nosocomial bacterial pathogens has become a standard service for hospital infection control programs. It is also an important tool for the surveillance of antibiotic-resistant pathogens, such as MRSA strains causing epidemics in health care facilities or other unexpected staphylococci, as a multiresistant *Staphylococcus hominis* infecting a dyalisis patient (Macía M., Méndez-Alvarez S. *et al*., 2012, unpublished results).

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