Factors Contributing to Epidemic MRSA Clones Replacement in a Hospital Setting

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Abstract

The mechanisms governing the epidemiology dynamics and success determinants of a specific healthcare-associated methicillin-resistant S. aureus (HA-MRSA) clone in hospital settings are still unclear. Important epidemiological changes have occurred in Europe since 2000 that have been related to the appearance of the ST22-IV clone. Between 2006 and 2010, we observed the establishment of the ST22-IV clone displacing the predominant Italian clone, ST228-I, in a large Italian university hospital. To investigate the factors associated with a successful spread of epidemic MRSA clones we studied the biofilm production, the competitive behavior in co-culture, the capacity of invasion of the A549 cells, and the susceptibility to infection in a murine model of acute pneumonia of the two major HA-MRSA clones, ST22-IV and ST228-I. We showed that persistence of ST22-IV is associated with its increased biofilm production and capacity to inhibit the growth of ST228-I in co-culture. Compared to ST228-I, ST22-IV had a significantly higher capacity to invade the A549 cells and a higher virulence in a murine model of acute lung infection causing severe inflammation and determining death in all the mice within 60 hours. On the contrary, ST228-I was associated with mice survival and clearance of the infection. ST22-IV, compared with ST228-I, caused a higher number of persistent, long lasting bacteremia. These data suggest that ST22-IV could have exploited its capacity to i) increase its biofilm production over time, ii) maintain its growth kinetics in the presence of a competitor and iii) be particularly invasive and virulent both in vitro and in vivo, to replace other well-established MRSA clones, becoming the predominant European clone.

Introduction

Infections caused by antibiotic-resistant strains of Staphylococcus aureus have risen to epidemic proportions globally [1]. The overall burden of disease caused by methicillin-resistant S. aureus (MRSA) strains has reached alarming rates in many countries in both healthcare and community settings [2–4].

MRSA, for its ability to become resistant to virtually all antimicrobial agents available, to survive in hostile environments and to adapt to new ecological niches poses a major challenge to Infection Control Programs. After successfully spreading to hospitals and healthcare settings worldwide (HA-MRSA, healthcare-associated MRSA), two decades ago MRSA emerged in the community (CA-MRSA, community-acquired MRSA), causing infection in healthy people without predisposing risk factors for HA-MRSA infection. CA- and HA-MRSA strains can be distinguished for their genetic background, phenotype and virulence factors. CA-MRSA strains are susceptible to most non β-lactams antibiotics, and contain smaller, more mobile SCCmec IV, V and VII, and virulence factors genes, such as those coding for the Panton Valentine leucocidin (PVL), which is responsible, in combination with other exotoxins, for their enhanced pathogenicity. Since 2003, MRSA has been isolated from livestock and humans exposed to infected animals in several countries, revealing the zoonotic potential of MRSA. Such MRSA has been dubbed livestock-associated methicillin-resistant Staphylococcus aureus (LA-MRSA) [5,6]. Interactions between these different reservoirs have been reported, including nosocomial infections by CA-MRSA [7,8] and LA-MRSA [9,10].

Epidemiological studies using several molecular typing methods have shown that few highly epidemic MRSA (EMRSA) clones cause the majority of HA-MRSA infections worldwide. These EMRSA clones emerged upon acquisition of the SCCmec element by successful methicillin susceptible S. aureus clones, belonging to five phylogenetically distinct clonal complexes (CC): CC5 (sequence type ST5-I, -II, -IV, -V), CC8 (ST247-I, ST239-III, ST8-IV), CC22 (ST22-IV), CC30 (ST36-II) and CC45 (ST45-II, -IV) [11,12].

Since 2000, important epidemiological changes have occurred in Europe: the frequency of multiresistant (MDR) MRSA clones gradually decreased while the frequency of other epidemic clones still sensitive to non β-lactams, such as ST22-IV (EMRSA-15), increased [13–19].

A recent study, that traced the dynamic changes of HA-MRSA lineages in Italy, showed that over a 17-year period MDR-MRSA clones ST247-I/IA and ST239-III were replaced by ST220-I.
phobia of clone displacement in hospital environments, we
investigate the factors associated with the successful establish-
ment of clones ST22-IV and ST228-I. In particular, we
observed in several European countries [15–18,21].

The mechanisms involved in the selection of pandemic MRSA
clones in nosocomial settings have not yet been clarified. In order
to investigate the factors associated with the successful establish-
ment and spread of an epidemic MRSA clone, as well as with the
phenomenon of clone displacement in hospital environments, we
studied both in vitro and in vivo virulence properties of the two
major HA-MRSA clones, ST22-IV and ST228-I. In particular, we
evaluated their ability to produce biofilm, their in vitro competitive
behavior in co-culture and their capacity to invade the A549 cell
line, as well as the virulence in a murine model of acute
pneumonia.

**Results**

**Biofilm Production**

Selected MRSA strains belonging to both epidemic (ST228-I,
ST22-IV, ST8-IV) and sporadic (ST3-III, ST229-III, ST1-IV,
ST8-IV USA300) clones were tested for their capacity to produce
biofilm in vitro (see Materials and Methods).

As shown in Figure 1, the sporadic MRSA clones ST3-III, ST1-
IV and ST8-IV USA300 were associated with low biofilm
production, the exception being ST239-III Brazilian clone that
was classified as a moderate biofilm producer. Accordingly, the
variants of epidemic clones isolated sporadically were weak
producers and their incidence decreased rapidly during the study
period (data not shown). On the contrary, the predominant
variants of clones ST228-I, ST22-IV and ST8-IV were associated
with high biofilm production. Interestingly, the clone ST22-IV
significantly increased its biofilm production during the study
period (p<0.01, Figure 1). In particular, the two predominant
variants of the clone ST22-IV, A and B, presented opposite
isolation trends, with variant A increasing from 22.2% in 2006 to
36.5% in 2010 and variant B decreasing from 44.4% to 30.7%,
and, while the variant B remained a weak biofilm producer, the
variant A significantly increased its biofilm production from weak
to moderate (p<0.05, data not shown).

**In vitro Competition Experiments**

The epidemic clones ST228-I and ST22-IV have different
background (respectively CC5 and CC22), SCC mec type (I
and IV) and resistance phenotype (gentamicin-resistant and
susceptible). In order to determine if the epidemiological changes
can be ascribed to different growth kinetics and/or competitive
behavior, we evaluated their growth rate in pure culture and in
culture. We selected two pairs of strains belonging to the variants
B3 (strains 67, isolated in 2006, and 442, isolated in 2009) and A
(strains 54, isolated in 2006, and 459, isolated in 2009) of the
clones ST228-I and ST22-IV respectively: during the 5-year
period, while the variant B3 exhibited a decreasing isolation trend,
the variant A was detected with increasing frequency.

Figure 2 shows the growth kinetics and the competition indexes
(CI) for mixed culture of one pair of strains; similar results were
obtained with the second pair of strains. As shown in Figure 2, the
two clones presented similar growth kinetics in pure culture; in
culture, while the variant A of the clone ST22-IV maintained the
growth rate observed in pure culture, the growth of the variant B3
of the clone ST228-I was significantly inhibited by the presence of
ST22-IV, as confirmed by the CI.

**Invasion in A549 Cell Line Model**

To investigate the virulence properties of the clones ST22-IV
and ST228-I, we used a well-characterized in vitro model, the
epithelial cell line A549. In particular, we evaluated the invasion
capacity of the major variants of the epidemic clones ST22-IV
(variants A and B) and ST228-I (variants B2 and B5) (Table 1).

Both MRSA clones were able to invade the epithelial cells A549
but at different efficiency (Figure 3). The percentages of invasion
associated with the variants A and B of the clone ST22-IV (48.3%
and 31% respectively) were significantly higher compared to the
ones associated with the variants B5 and B2 of the clone ST228-I
(16.5% and 17.1% respectively; ST22-IV A vs. ST228-I B5:
p<0.001; ST22-IV A vs. ST228-I B2: p<0.05; ST22-IV B vs.
ST228-I B5: p<0.05; ST22-IV B vs. ST228-I B2: p<0.05).

**Virulence in a Mouse Model of Acute Lung Infection**

To test differences in virulence between the MRSA clones
ST22-IV and ST228-I, we used a murine model of acute
pneumonia. Variant A of the clone ST22-IV (strain 54) and
variant B5 of the clone ST228-I (strain 67) were used to infect
C57Bl/6NCrl mice. Lethality was assessed after intratracheal
challenge with 5*10^6 cfu/lung of each strain.

As shown in Figure 4, the two MRSA clones determined
opposite outcomes: the ST22-IV strain was fully lethal within 60
hours from infection, while the ST228-I strain was attenuated and
all mice survived for at least one week after challenge (ST22-IV
variant A) vs. ST228-I (variant B5): p<0.001).

Bacterial load was then determined in the lungs and spleen of
the infected mice (Figure 5). Moribund mice infected with ST22-
IV presented a significantly higher bacterial load in the lungs
(median value 8.5*10^6 cfu per lung) compared with mice infected
with ST228-I and sacrificed at day 3 (median value 4*10^6 cfu,
p<0.05). Complete clearance of ST228-I strain was observed
when the infection was prolonged for 7 days. The bacterial load
in the spleen was higher in mice of the ST22-IV group (median
2*10^5 cfu) and lower in ST228-I group (median 140 cfu), although
not statistically significant.

To assess clinical strain-specific traits of acute pneumonia, lung
histopathology was performed on mice challenged with different
MRSA clones (Figure 6). The lungs of mice infected with the
ST22-IV clone presented a heavily damaged structure and
noteworthy inflammatory infiltrate, while the lungs of mice
infected with the ST228-I clone and able to clear the infection
presented large areas where the normal pulmonary parenchyma
was preserved.

**Genes Coding for Virulence Factors**

The MRSA strains used for the competitive growth cultures and
for the experiments in the mouse model (strains 54 and 67
belonging to ST22-IV and ST228-I respectively) were character-
ized for the presence of genes encoding for virulence factors. Both
clones shared genes encoding for enterotoxins (sea, seb, sem, seg, sed),
for factors associated to tissue invasion (hla, hld, psm-m), for
the clumping factor B (clfB), and for the capsular polysaccharide type 5
caps (cap5). The variant B5 of the clone ST228-I also carried genes
encoding for other adhesins (fbsb, fbs, ebhB) and for an additional
factor of cellular lysis (luk E). The variant A of the clone ST22-IV
Figure 1. Biofilm production of the main sporadic and epidemic MRSA clones identified in hSR. Panel A: sporadic MRSA clones; panel B: epidemic MRSA clones. The dotted line indicates the threshold separating moderate and strong biofilm producers from non-producers and weak producers with Biofilm Unit (BU) value <0.46. Each symbol represents a single strain. For each clone the average BU value is indicated by the black bar. Three to eleven MRSA strains for sporadic clones and thirty-three to sixty-five MRSA strains for epidemic clones were tested. All MRSA strains tested originated from clinically significant samples (see Materials and Methods). For each epidemic clone (B) the biofilm production is reported for 2006–2007 and 2008–2009 for comparison purposes. Statistically significant differences are indicated by symbols when present: ** indicates a p value <0.01.
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harbored additional genes encoding for an enterotoxin \((\text{sen})\) and for two other toxins \((\text{hlb}, \text{hlg})\) (Table S1).

**Clinical Data**

Between 2009 and 2010 we identified and analyzed 16 cases of persistent infection caused by MRSA strains belonging to one of the predominant variants of the clones ST22-IV and ST228-I. As shown in Figure 7 and Table 2, 9 and 7 patients had a second MRSA isolate belonging to the ST22-IV and to the ST228-I clone respectively. The median of days between the first and the last MRSA detection for the clone ST228-I was 40 days, while for the ST22-IV was 55 days.

The average age of patients with persistent infection caused by the clone ST228-I and ST22-IV was 60.5 years and 59.7 years respectively.
respectively. Cases of resolving MRSA bacteremia, defined as an initial blood culture that yielded MRSA and subsequent blood cultures negative for MRSA after antibiotic treatment, were equally caused by both ST22-IV and ST228-I clones (data not shown). On the contrary, cases of persistent MRSA bacteremia were significantly associated to ST22-IV clone (ST22-IV vs. ST228-I: p < 0.001): four out of nine patients (44.4%) infected by the ST22-IV clone had persistent bacteremia, whose median length was 67.5 days, while only one (14.2%) of the seven patients infected by the ST228-I clone had persistent bacteremia that lasted 15 days (Table 2). ST228-I clone in the remaining 85.8% of the cases caused infections of the respiratory tract.

### Discussion

The mechanisms and factors governing the epidemiological dynamics and determining the establishment and successful spreading of a HA-MRSA clone in hospital settings are still unclear. Until the end of the 1990s, the international scenario was dominated by multidrug resistant (MDR) epidemic MRSA clones, suggesting a central role for multidrug resistance in the selection of successful MRSA clones, especially in the hospital environment where the selective antibiotic pressure is notoriously high. Since 2000, however, important epidemiological changes have occurred and classical MDR MRSA clones have been replaced by more susceptible clones. In Europe, these changes have been related to the gentamicin-susceptible ST22-IV clone, otherwise known as EMRSA-15, which has shown a particular ability to substitute other predominant clones in several countries, such as France, United Kingdom, Portugal, Spain, Hungary and Germany [16-19,21].

In line with the epidemiological changes observed in Europe in the last decade, in our institution during the five-year period 2006–2010, we detected the establishment of the ST22-IV clone that displaced the predominant Italian clone ST228-I. We investigated both in vitro and in vivo virulence properties associated with this phenomenon of clone replacement in hospital settings.

#### Table 1. Epidemic MRSA clones identified during the study period 2006–2010.

| MRSA clone (%)a | 2006  | 2007  | 2008  | 2009  | 2010  | Major variants of the clone (%)b |
|-----------------|-------|-------|-------|-------|-------|---------------------------------|
| ST22-IV (33.3%) | 20.4% | 26.5% | 35%   | 36.7% | 38.8% | A (28%); B (25%); C (7%)        |
| ST228-I (26%)   | 31.8% | 33.6% | 29.8% | 22.7% | 20.9% | B2 (16%); B5 (13%); B1 (8%)     |
| ST8-IV (15.8%)  | 6.8%  | 16.3% | 21.6% | 17.2% | 17.8% | A1 (23%); A2 (22%); B (21%); C (11%) |

aPercent of MRSA strains belonging to each clone, considering only the index cases (n = 499); 
bpercent of MRSA strains belonging to the major variants of each epidemic clone identified (n = 166 for ST22-IV; n = 131 for ST228-I; n = 80 for ST8-IV).

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Figure 3. Percentage of invasion of A549 cell line by MRSA clones ST22-IV and ST228-I. Three to six different strains for each predominant variant of the two clones (ST22-IV variants A and B, ST228-I variants B2 and B5) were tested. For ST22-IV strains tested originated from lower respiratory tract (LRT, 37%), pus (37%) and blood (26%); for ST228-I strains tested were isolated from LRT (55%) and blood (45%). The percentage of invasion was determined by plating cellular lysates after 2 hours contact between bacteria and cells’ monolayer, followed by 1 hour treatment with lysostaphin to kill extracellular bacteria. Each symbol represents a single strain; the black bar indicates the average percentage of invasion. Statistically significant differences are indicated by symbols when present: * indicates a p value <0.05; ***: p<0.001.

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Figure 4. Survival curves of mice infected with MRSA clones ST228-I (variant B5) and ST22-IV (variant A). C57Bl/6NCrl mice were infected intratracheally with $5 \times 10^8$ cfu per mouse of either the variant B5 of the clone ST228-I (strain 67, squares) or the variant A of the clone ST22-IV (strain 54, circles). Two to three independent experiments were pooled (n: 10 mice per MRSA strain). Statistical analysis of pairwise comparison is indicated (Mantel-Cox test): *** indicates a p value <0.001.

Figure 5. Bacterial load in lung and spleen of mice infected with MRSA clones ST228-I and ST22-IV. C57Bl/6NCrl mice were infected intratracheally with $5 \times 10^8$ cfu per mouse of either the variant B5 of the clone ST228-I (strain 67, squares) or the variant A of the clone ST22-IV (strain 54, circles). Moribund mice infected with ST22-IV clone (n = 5) were sacrificed between 6 and 60 hours from infection; surviving mice infected with ST228-I clone (n = 5) were sacrificed at 72 hours from infection. Each animal is represented by a different color; the black bar indicates the median cfu per organ. Statistically significant differences are indicated by symbols when present: * indicates a p value <0.05.
We found a correlation between the successful establishment and persistence in nosocomial settings of MRSA clones and their capacity to produce biofilm, so far demonstrated only for the epidemic Brazilian clone [22]. We also detected the ability of the predominant variant A of the clone ST22-IV to favorably modify its phenotype, thus acquiring a selective advantage over the variant B, a weak biofilm producer associated with a decreasing isolation trend over time. Biofilm constitutes a protective mode of growth that confers to bacteria resistance to antibiotic therapy and host immune response, thus probably increasing their persistence in the host, as well as in the environment and inanimate surfaces, and their pathogenic potential [23].

The role played by genetic background in establishing the success of a specific MRSA clone is unclear. Laurent et al. described a correlation between gentamicin susceptibility and the increased capacity of MRSA clones to replicate, establish and spread in nosocomial environments, compared with gentamicin-resistant clones [19]. Both ST22-IV and ST228-I clones presented similar in vitro growth kinetics when grown in pure culture, but different behavior in competitive culture as well as different virulence properties. In co-culture, the clone ST22-IV maintained an unaltered growth rate and was able to significantly inhibit the growth of the competitor clone ST228-I.

MRSA infections are often persistent and associated with a slow response to antibiotic therapy as well as with recurrences, and a consequent extended duration of antimicrobial therapy and hospital stay. *S. aureus* has classically been considered as an extracellular pathogen. Conversely, there is growing evidence suggesting the ability of *S. aureus* to internalize and survive in different cell types [24]. Intracellular presence of bacteria may explain the poor response to antibiotics and the development of chronic infections [25]. We noticed a significantly higher capacity of the clone ST22-IV to invade the human alveolar epithelial cells A549 compared to the clone ST228-I, that could enable this clone to elude both the host immune response and the effects of antimicrobial agents, and to cause persistent or recurrent infections.

**Figure 6. Histopathology in murine lung infected with MRSA clones ST22-IV (variant A) and ST228-I (variant B5).** C57Bl/6NCFr were infected intratracheally with 5\( \times \)10⁸ cfu per mouse of either the variant A of the clone ST22-IV (strain 54) or the variant B5 of the clone ST228-I (strain 67). Mice were sacrificed between 3 and 7 days from infection with ST22-IV (left panel) and ST228-I (right panel), respectively. Lungs were removed en bloc, fixed in 4% paraformaldehyde/PBS and processed for paraffin embedding. Longitudinal sections were stained with hematoxylin and eosin. Upper panel: 2.5 \( \times \) magnification; lower panel: 10 \( \times \) magnification. doi:10.1371/journal.pone.0043153.g006
infections. The analysis of sequential MRSA strains, isolated from patients with persistent MRSA infection, identified 16 patients with multiple isolates genotypically indistinguishable from the first (same pulstype, spa-type and sequence type) and belonging to one of the predominant variants of the clones ST22-IV and ST228-I. ST22-IV, compared with ST228-I, was able to cause a significantly higher percentage of persistent, long lasting bacteremia (44.4% vs. 14.2%, respectively), despite the presence of antimicrobial therapy. On the contrary, ST228-I clone in 85.8% of the cases determined infections of the respiratory tract. Further in vitro and in vivo studies that aim to evaluate the ability of these clones to persist intracellularly could confirm this data.

Considering that pneumonia caused by MRSA accounts for 20–40% of nosocomial infections and it is still one of the leading causes of death during flu epidemics [26], and considering that more than 35% of our MRSA strains have been isolated from clinically significant respiratory samples obtained from infected patients, we further evaluated the in vivo virulence associated with the MRSA clones ST22-IV and ST228-I using a murine model of acute lung infection. Mice challenged with either the ST22-IV or the ST228-I clone showed opposite clinical outcomes. The clone ST22-IV presented a higher virulence compared to clone ST228-I: it seriously damaged the lung, compromising its structure and causing severe inflammation, thus entering the bloodstream and determining sepsis and death in all the mice within 60 hours. This data correlated with the noteworthy in vitro invasion capacity associated with this clone. On the contrary, the reduced virulence of the clone ST228-I was associated with the survival of all animals and the clearance of the infection.

The analysis of the pathotypes associated with the two clones revealed that, while ST228-I presented a major number of genes encoding for adhesins (fnbA, efb, ebpS), ST22-IV presented a major number of toxins (hld, hlg), whose role is still unclear, although they probably contribute to its higher capacity to invade observed both in vitro and in vivo. Giese et al. have recently shown synergistic activity between the δ-toxin (hld) and the β-toxin (hlg) that allows S. aureus to escape from the phago-endosomes of human epithelial and endothelial cells, thus avoiding degradation [27]. It is not clear if the escape from the phago-endosome leads to the subsequent host cell’s death or if it allows bacteria to survive in the cytosol.

The data reported here suggest that the EMRSA clone ST22-IV could have exploited its capacity to i) increase its biofilm production over time, ii) maintain its growth kinetics in the presence of a competitor clone and iii) be particularly invasive and virulent both in vitro and in vivo, so as to replace other well-established EMRSA clones and become the predominant European clone. Furthermore, the ability of the clone ST22-IV to determine a higher number of persistent, long lasting bacteremia compared to ST228-I, revealed an additional factor that could help to explain its epidemiological success. In fact, although MDR clones have been progressively replaced by more susceptible clones, the treatment of MRSA infections is increasingly problematic: growing evidence suggests the ability of this pathogen to survive intracellularly, even for prolonged time periods, in the cytoplasm of both phagocytic and non-phagocytic cells [24,28]. It is presumable that intracellular persistence is a bacterial strategy to subvert immunological defense mechanisms, as well as extracellular bactericidal concentrations of antibiotics. An intracellular niche might then serve as a reservoir for chronic or relapsing infections and/or contribute to chronic carriage of the pathogen and to its spreading in both hospital settings and the community. Further studies using both in vitro and in vivo models of persistent infection could help to elucidate if these mechanisms of adaptation and persistence are also present in clinical EMRSA clones that we characterized for acute virulence.

Materials and Methods

Ethics Statement

Animal studies were conducted according to protocols approved by the San Raffaele Scientific Institute [Milan, Italy] Institutional Animal Care and Use Committee (IACUC) and adhered strictly to
Table 2. Clinical characteristics of patients with persistent MRSA infection identified between 2009 and 2010.

| Patient | Age (y)* | 1st sample | 2nd sample | 3rd sample | 4th sample | Days between first and last positive sample | MRSA clone | Underlying disease | Diagnosis | MRSA therapy |
|---------|----------|------------|------------|------------|------------|--------------------------------------------|------------|-------------------|-----------|--------------|
| 370     | 52       | biopsy     | BALb       | –          | –          | 40                                         | ST22-IV    | malignant tumour | MRSA infection | tigecycline |
| 425     | 76       | biopsy     | pus (wound)| –          | –          | 47                                         | ST22-IV    | –                 | MRSA decubitus ulcer | –   |
| 453     | 79       | blood      | blood      | pus (abscessus) + blood | – | 75                                         | ST22-IV    | chronic renal failure | MRSA bacteremia | vancomycin |
| 460     | 57       | BASPc      | BASP       | –          | –          | 35                                         | ST22-IV    | benign tumour | MRSA infection | teicoplanin |
| 527     | 55       | blood      | blood      | –          | –          | 60                                         | ST22-IV    | –                 | MRSA bacteremia | vancomycin |
| 537     | 64       | blood      | blood      | pericardial fluid | blood | 105                                        | ST22-IV    | –                 | MRSA bacteremia | cubicin |
| 575     | 65       | pus (wound)| biopsy     | –          | –          | 50                                         | ST22-IV    | peripheral arteriopathy | MRSA ulceration | levofloxacin |
| 614     | 28       | vaginal swab| BASP      | BAL        | –          | 135                                        | ST22-IV    | paraplegia | MRSA infection | levofloxacin + cotrimoxazole |
| 664     | 62       | blood      | blood      | blood      | –          | 55                                         | ST22-IV    | –                 | MRSA bacteremia | cotrimoxazole |
| 672     | 82       | BAL        | sputum     | –          | –          | 20                                         | ST22-8-I   | lung cancer | MRSA pneumonia | vancomycin |
| 539     | 59       | sputum     | BAL        | –          | –          | 65                                         | ST22-8-I   | –                 | MRSA infection | linezolid |
| 572     | 64       | BASP       | BASP       | BASP       | –          | 40                                         | ST22-8-I   | obstructive hydrocephalus | MRSA infection | * |
| 574     | 67       | BASP       | BASP       | BASP       | BASP       | 49                                         | ST22-8-I   | benign tumour | MRSA infection | vancomycin |
| 245     | 70       | blood      | blood      | –          | –          | 15                                         | ST22-8-I   | chronic renal failure + acute myeloid leukemia | MRSA bacteremia | teicoplanin |
| 441     | 41       | BASP       | BASP       | BASP       | blood     | 76                                         | ST22-8-I   | –                 | MRSA infection | vancomycin |
| 495     | 41       | BASP       | BASP       | –          | –          | 11                                         | ST22-8-I   | malignant tumour | MRSA infection | vancomycin |

*years;  
bbroncho-alveolar lavage;  
cbronchial aspirate;  
*patient transferred to another hospital; - not present.

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the Italian Ministry of Health guidelines for the use and care of experimental animals.

### Bacterial Strains

A total of 575 MRSA non-duplicated strains (499 index cases and 76 secondary cases due to nosocomial transmission), isolated from 2006 to 2010 from hospitalized patients with severe MRSA infection, were included in the study. The strains were isolated from clinical samples originating from lower respiratory tract (LRT, 36.6%), blood (27%), pus (13.8%), biopsy (9.1%) and other sites (11.5%). All these strains were characterized by pulsed-field gel electrophoresis and SCCmec typing, which allowed identifying epidemic and sporadic MRSA clones. Epidemic clones were constantly detected during the whole study period and their frequency of isolation was >5% (Table 1); on the contrary, sporadic clones were rarely detected with a frequency of isolation <5%. Each epidemic clone presented major and minor variants (or sub-clones) associated to different pulserotypes that were detected at a frequency >5% and <5% respectively.

### DNA Extraction and PCR for Virulence Factors Genes

Bacterial DNA was prepared from isolated colonies suspended in 100 μl Triton X-100 lysis buffer with 1% Triton and 50 μg/ml lysostaphin, incubated at 37°C for 15 min, heated at 95°C for 15 min. After centrifugation, 5 μl of the supernatant was used for PCR. Selected virulence factors were detected by PCR using primers and protocol described by Diep BA et al. [29].

### Biofilm Production

Quantitative determination of biofilm production was performed in a microtiter plate under static conditions following the procedure previously described by Stepanovic S et al. [30], and using SH1000 as positive control and TSB as negative control. The biofilm units (BU) were calculated using the formula: ODbiofilm/ODgrowth. Classification: BU≥0.230 non-producers, BU>0.230 and ≤0.460 weak producers, BU>0.460 and ≤0.920 moderate producers, BU>0.920 strong producers. Each MRSA strain was tested in triplicate and each experiment was repeated three times independently. For ST22-IV clone the isolates tested belonged to major variants A1, A2, B, C and A1, A2, B, C respectively and originated from blood (42%), LRT (32%), pus (13%) and biopsy (13%). For ST5-NY, a Japan clone strains originated from LRT (45%), blood (20%), pus (17.5%) and biopsy (17.5%); for ST239-Brazilian clone strains originated from blood (42%), LRT (42%) and pus (16%); for ST1 clone isolates originated from pus (66.6%) and LRT (33.3%); ST8-USA300 and ST3-USA300-related, being community-acquired MRSA clones, caused mainly skin and soft tissue infections (SSTI) and 62% of isolates tested for biofilm production originated from pus, 25% from LRT and 13% from blood. Figure 1 reports the average BU value for each MRSA strain tested.

### Competitive Growth Experiments

Two MRSA isolates belonging to each of the ST22-IV and ST228-I clones were tested against each other in both pure and mixed cultures for competitive growth. Each pair of strains were inoculated at equal ratio (1 OD600, optical density) from mid-exponential phase pure cultures in a final volume of 30 ml of Tryptic Soy Broth and incubated at 37°C with agitation (180 rpm) for 24 hours. Growth rates of pure and mixed cultures were monitored every 2 hours up until 8 hours and at 24 hours, by dilution plating on Tryptic Soy agar (TSA) plates with or without gentamicin (32 μg/mL), to discriminate the two MRSA clones, and CFU count. Each experiment was repeated three times independently. The competition index (CI) for mixed culture was calculated as the ST22-IV/ST228-I CFU ratio for the output (obtained at the different time points) divided by the corresponding ratio for the input ( inoculum at time t = 0) [31].

### Invasion Assay

Bacterial invasion was investigated using the A549 cell line as previously described by Liang X et al [32]. A549 cell line (ATCC number CCL-185) was obtained from LGC Standards. Briefly, 2×10⁶ cells were seeded on 24-well flat-bottom plates and incubated for 2 hours at 37°C with 1–2×10⁶ bacteria from the mid-exponential phase; adherent and extracellular bacteria were killed by lysostaphin treatment (20μg/ml) for 1 hour at 37°C; after washing wells and treating with Trypsin-EDTA, followed by Triton X-100 0,025% to detach and lyse cells, internalized bacteria were serially diluted and plated on TSA for counting. Each MRSA strain was tested in duplicate and each experiment was repeated three times independently. Figure 3 reports the average percentage of invasion for each MRSA strain tested.

### Mouse Model of Acute Lung Infection

C57Bl/6NJackson mice (20–22 g) were purchased by Charles River. Mice were housed in filtered cages under specific-pathogen conditions and permitted unlimited access to food and water. Prior to animal experiments, the clinical MRSA strains were grown for 5 h to reach exponential phase. Next, the bacteria were pelleted by centrifugation (2700 g, 15 min), washed twice with sterile PBS and the OD of the bacterial suspension was adjusted by spectrophotometry to 0.66. The intended number of cfu was extrapolated from a standard growth curve [33,34]. Appropriate dilutions with sterile PBS were made to prepare the inoculum of 5×10⁴ cfu per mouse. Mice were anesthetized with 375 mg/kg Avertin (2,2,2-tribromoethanol, 97%; Aldrich), the trachea directly visualized by a ventral midline incision, exposed and intubated with a sterile, flexible 22-g cannula attached to a 1 ml syringe according to established procedures [35,36]. Mice were monitored for mortality and sacrificed by CO₂ administration up to 7 days. To determine the bacterial load murine lungs and spleens were aseptically excised, homogenized, and plated onto TSA agar with or without gentamicin (32 μg/mL).

### Histological Examination

Moribund mice were sacrificed by CO₂ administration while surviving mice were sacrificed after 7 days from infection, lungs were removed en bloc and fixed in 4% paraformaldehyde/PBS, at 4°C for 24 h, and processed for paraffin embedding. Longitudinal 2-μm sections taken at regular intervals were obtained using a microtome from the proximal, medial and distal lung regions. Sections were stained with Haematoxylin-Eosin according to standard procedures and examined blindly.

### Statistical Analysis

Data were analyzed with the software GraphPad Prism 5. Data concerning biofilm production, competitive cultures and invasion analysis were analyzed using two-tailed non-parametric Student’s t test. For in vivo experiments survival data were analyzed with the Log Rank test (Mantel-Cox). Value of p<0.05 was considered to be statistically significant.
Supporting Information

Table S1 Virulence characteristics of the MRSA strains used for in vivo experiments.

(DOC)

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Author Contributions

Conceived and designed the experiments: RB DMC NIL A. Bragonzi. Performed the experiments: RB FT NIL PO CO. Analyzed the data: RB FT DMC NIL A. Bragonzi A. Biancardi PN MM. Contributed reagents/materials/analysis tools: DMC A. Bragonzi. Wrote the paper: RB DMC A. Bragonzi MM.