Elucidation of the Mechanism of Antibiotic Resistance Acquisition of Methicillin-Resistant Staphylococcus Aureus (MRSA) and Determination of Its Whole Genome Nucleotide Sequence

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Abstract: Although Staphylococcus aureus is a member of normal human flora, it may cause fatal infection to humans who underwent accidental injury or surgical operation. The bacteria is potent in acquiring antibiotic resistance, and is now a very important causative agent of hospital acquired infection. The most important mechanism of the antibiotic resistance acquisition is via staphylococcal cassette chromosome (SCC), a mobile DNA specific to staphylococci, which has had attained molecular evolution for the interspecies exchange of genetic information. Methicillin-resistant S. aureus (MRSA) has been born by acquiring penicillin/cephalosporin resistance that was carried by an SCC. Moreover, hospital MRSA has become multiply resistant to antibiotics, and finally acquired resistance to the “last resort” antibiotic vancomycin.

Key words: Staphylococcus aureus; Methicillin resistance; Vancomycin resistance; SCCmec; Genome

Introduction

Successful purification of penicillin G in 1941 marked the advent of the history of modern anti-microbial chemotherapy. However, it took only a few years before strains of Staphylococcus aureus producing penicillin-hydrolyzing enzyme (penicillinase) started to prevail. In the latter half of the 1950s, S. aureus multiply resistant to all the available antibiotics developed by that time (such as erythromycin, streptomycin etc.) prevailed the hospitals all over the world. In medical journals of those days we find articles reporting the critical situation of hospital infection which is very much similar to the recent situation of ours.

Development of a semi-synthetic penicillin, methicillin, which resists the hydrolysis by peni-
cillinase, together with subsequent launch of the first generation cephalosporins, have exerted a great power to expel these troublesome multi-resistant bacteria from the hospitals in the 1960s. However, as early as in 1960, the year of methicillin development, Jevons noticed an S. aureus strain that can grow in the presence of methicillin. In the following year she reported her observation that was the first isolation of methicillin-resistant S. aureus (MRSA).

MRSA did not cause much trouble to draw attention of clinicians in the following decade. However, it started to cause nosocomial infection frequently in European and US hospitals, followed by a steep rise of incidence of MRSA in Japanese hospitals in the early 1980s.

S. aureus is a part of normal human flora. The aim of my research was directed to understand how such a familiar bacterium turned out to be a treacherous human pathogen resisting all the fruits of modern antimicrobial chemotherapy.

How did S. aureus Acquire Methicillin Resistance?

1. meca gene

MRSA resists not only to methicillin but to all available beta-lactam antibiotics including penicillin and cephalosporin derivatives. The mechanism of resistance of MRSA has not been uncovered until the mid-1980s. Beta-lactam antibiotics kill bacteria by inhibiting the cell-wall synthesis. In the early 1980s, such researchers as P. Reynolds, T. Yokota, and A. Tomasz reported that some MRSA strains produce a strange penicillin-binding protein (PBP) besides the intrinsic sets of S. aureus PBPs. The PBP, now denoted PBP2’ or PBPa, seemed to have reduced binding affinity to beta-lactam antibiotics. Derivation of PBP2’ remained to be solved. In 1986, M. Matsushashi cloned the gene meca encoding the PBP2’, and has shown that the gene is not found in methicillin-susceptible S. aureus strains. The next question was whether meca is transmissible across S. aureus strains or not.

2. Staphylococcal cassette chromosome meca (SCCmec)

In 1995, by cloning of the chromosomal DNAs around meca gene, we showed that meca gene is carried by a big DNA fragment (mec region DNA) that corresponds to about 1–2% of the entire S. aureus chromosome, and that there are at least three distinct types in the mec region DNA that are found in the MRSA clinical isolates in the world. In 1999, we determined the nucleotide sequence of one of the mec region DNA, and have shown that the mec region DNA is a novel mobile DNA that is capable of site-specific integration into and precise excision from S. aureus chromosome. We designated the mec region DNA as staphylococcal cassette chromosome meca (SCCmec), and the two novel genes responsible for the movement of the element as cassette chromosome recombinase A and B (ccrA and ccrB).

We subsequently showed that there are three allotypes in the ccrA, B genes and more than four classes in the “mec gene complex” (which is composed of meca, and its regulator genes mecI and mecR1 located next to it), and that the SCCmec elements carried by the MRSA strains in the world are divided into three types defined by the combination of the type of ccr genes and class of meca gene complex.

Step-Wise Expression of Methicillin Resistance by S. aureus

1. pre-MRSA

S. aureus does not express methicillin resistance even if it has acquired SCCmec. A strain that has acquired meca gene together with its regulator genes, mecI and mecR1, remains susceptible to methicillin. We identified such clinical strain and designated it pre-MRSA, and proposed the idea that mutational inactivation of mecI gene (that encodes potent repressor of meca gene transcription) is necessary for pre-MRSA to become MRSA.
2. hetero-MRSA

When mecI-mediated repression is released by mutation, “hetero-MRSA” emerges. This class of MRSA still remains susceptible to higher concentrations of methicillin (and low concentrations of potent beta-lactam antibiotics such as imipenem). This is explained by the hypothesis that the physiological status of the S. aureus cell is not appropriate to take a full advantage of the function of the exogenous cell-wall synthesis enzyme PBP2'. Hetero-MRSA is named as such since the cells of various degrees of methicillin-resistance spontaneously emerge within its cell population.

3. homo-MRSA

With high frequencies of one in 10,4–5 mutant strains emerge from hetero-MRSA whose cell population is composed of the cells with “homogeneously high” methicillin-resistance. Many researchers including B. Berger-Bachi and A. Tomasz groups have been working for the mechanism how this mutational conversion occurs. We have also cloned two genes, hmrA and hmrB, whose overexpression raise methicillin resistance of a hetero-MRSA strain to the level of homo-MRSA. However, we still do not know how they function in raising methicillin resistance.

Vancomycin Resistance of MRSA

Besides beta-lactam, MRSA is resistant to practically all antibiotics belonging to other classes of anti-microbial function. Vancomycin had been considered as the last resort antibiotic before 1996 when we identified the first vancomycin-resistant MRSA strain from a 4-months-old baby whose surgical wound infection did not respond to vancomycin therapy. Strain Mu50, which we designated vancomycin-resistant S. aureus (VRSA), recorded vancomycin MIC of 8 mg/l. (The strains of MIC 8 and 16 mg/l are called vancomycin-intermediate S. aureus [VISA] by some researchers, but it should be pointed out that the infection with MRSA strains with MIC 4 and above are difficult to treat by vancomycin therapy, thus are clinically “resistant”.)

1. Low-level vancomycin-resistant S. aureus (L-VRSA)

Now that highly vancomycin-resistant S. aureus with a different mechanism has been reported in US in 2002, we call Mu50 and other strains in the world with MIC levels of 8 and 16 mg/l low-level vancomycin-resistant S. aureus (L-VRSA). After our report of Mu50, L-VRSA strains have been isolated from all over the world including US, England, France, Greece, Brazil, Korea, South Africa, and Taiwan, indicating that the resistance acquisition is a global issue.

2. hetero-L-VRSA

Our discovery of L-VRSA was preceded by that of hetero-L-VRSA (or hetero-VRSA) in 1996. We isolated MRSA strain Mu3 from a 65-year-old patient whose MRSA pneumonia resisted vancomycin therapy. Mu3 recorded susceptible level of vancomycin MIC (2 mg/l). However, using a more sensitive susceptibility test called “population analysis”, we recognized that the Mu3 strain contains the cells with various levels of vancomycin resistance (capable of growth in the presence of 4–8 and 9 mg/l of vancomycin). We named the strain, hetero-VRSA (now better be called hetero-L-VRSA due to the reason described above) in analogy with the hetero-resistance in methicillin resistance. Mu50 happened to have the same pulse-field gel electrophoresis (PFGE) pattern with that of Mu3, indicating that Mu50 was closely related to Mu3.

3. Conversion from hetero-L-VRSA to L-VRSA

The conversion from hetero-L-VRSA to L-VRSA is due to spontaneous mutation (Kapi, M. in preparation). By analyzing 16 L-VRSA strains isolated from 7 countries, we noticed that the cell-wall of the L-VRSA strains was sig-
nificantly thicker than those of control strains. By utilizing a defined cell-wall synthesis media, we demonstrated the importance of increased number of peptidoglycan layers in the cell wall to prevent the penetration of vancomycin molecules to the cytoplasmic membrane where the targets of vancomycin are present. Based on these data, we proposed an affinity trapping model for vancomycin resistance.8)

Evolution of MRSA into the New Direction

As a hospital pathogen, MRSA continues to evolve into multiple resistance; the culmination of it being the acquisition of vancomycin resistance. There is always an environmental pressure before the bacteria evolves itself. Diverse classes of antibiotics used in the hospital obviously constitute the selective pressure exerted on the nosocomial pathogens.

On the other hand, in the past several years, some researchers became aware of a novel trends in MRSA epidemiology. Center of Disease Control and Prevention (CDC) reported the death of four children in the two US states in 1999. The children independently contracted severe pneumonia caused by MRSA whose antibiogram patterns were different from those of hospital MRSA strains. We identified a new SCCmec (type IV) in such community-acquired MRSA (C-MRSA) strains, and thus proved that distinct MRSA strains have been prevailing outside the hospital.5)

As shown in Fig. 1, the type-IV SCCmec is much smaller in size than the other three types of SCCmec found in the hospital, and has few genes other than those with the cardinal function, ccr genes and mec gene complex.

The phenotypic characteristics of C-MRSA strains are rapid growth rate, susceptibility to multiple antibiotics other than beta-lactam,
and high virulence reported with some strains. As described below, we could correlate these features of C-MRSA to its genome structure in comparison with that of hospital-acquired MRSA (H-MRSA).

**The Feature of *S. aureus* as Inscribed in Its Genome**

To explore how multi-antibiotic resistance is acquired by H-MRSA strains and how the high virulence of strain is expressed by a certain C-MRSA strains, we have determined whole genome sequence of L-VRSA strain Mu50, pre-MRSA strain N315, and MW2, a C-MRSA strain isolated from one of the four victims of paediatric deaths. This turned out to be the first determination of the entire genome sequence of *S. aureus*. The feature of *S. aureus* genome is as follows (Fig. 2).

About 2,600 genes are found on the chromosome of about 2.8 Mbp in size. More than 90% of the sequence are conserved among the three. Medically important feature of individual *S. aureus* strain such as pathogenicity and antibiotic resistance is determined by the sum of the allotype of genomic islands (Gislands). The names of the Gislands and their encoded function in parentheses, in clockwise rotation, are: SCCmec (methicillin and other drug resistance), ρSaα (superantigen), ρSa3 (enterotoxins), φSa1mu (unknown), φSa2mw (Panton-Valentine leukocidins), φSa3 (enterotoxins), ρSaβ (enterotoxins, bacteriocin), ρSa4 (unknown). IS, insertion sequence; Tn (transposon).
S. aureus and Bacillus species share a common ancestor in the early days of species diversification. The feature of S. aureus as a human pathogen is determined by another functional domain of the chromosome. S. aureus chromosome contains several genomic islands (Gislands) that are found at least at seven different loci thus interrupting the domain of chromosome encoding house-keeping function which descended from the ancestor. In these Gislands, most of the genes involved in pathogenesis and antibiotic resistance are identified.

1. Gislands
SCC is one of the Gislands. Besides the SCCmec carrying most of the chromosomally localized antibiotic resistance genes, SCC can carry such genes involved in capsule formation that constitutes a part of virulence potential of the organism. Prophages as a subfamily of Gisland are integrated at specific chromosomal loci encode virulence genes. In MW2, one of the prophages carries Panton-Valentine leukocidin genes that encode potent cytotoxin against human white blood cells. Thus, Gislands are the regions of the chromosome that have been laterally (in contrast to vertical transmission) acquired from other bacterial species or strains. Gislands are discriminated from the surrounding chromosome by their subtle difference in the nucleotide composition (GC contents etc.) or the codon usage preference. Further identification of Gislands by comparison with other staphylococcal species and close examination of the gene function carried by the Gislands will reveal the history how S. aureus has evolved itself as a human pathogen.

2. Gisland allotyping
Comparison of the chromosome of C-MRSA strain MW2 with those of H-MRSA strains N315 and Mu50 reveals striking difference in the genes present in Gislands. For example, on the Saα island, we find a cluster of genes encoding superantigens. While N315 and Mu50 had almost identical repertoire of the superantigen genes (though, one of the 10 genes in N315 was missing in Mu50). However, none of the peptides encoded by 11 genes of MW2 was identical with those of N315/Mu50. In another island Saβ of N315 and Mu50, a cluster of enterotoxin genes (responsible for food poisoning) are present. The corresponding island of MW2, however, does not contain enterotoxin genes. Instead a novel operon involved in the production of bacteriocin (the toxin against other bacteria) was identified in the island. This seems to support the idea that MW2 is really a community pathogen which is required to compete with other bacterial species for successful colonization to human mucous membranes without the help of antibiotics which MRSA strains in the hospital enjoy.

As exemplified as above, there are several allotypes for each Gisland, and the pathogenicity potential and antibiotic resistance profile of each S. aureus clinical isolate are determined as the sum of the function of all the Gisland allotypes possessed by the strain. This signifies the importance of Gisland allotyping as a rapid diagnostic method to infer the disease course and to determine therapy against the infection caused by individual S. aureus strain.

Conclusion
After our publication of genome sequence information, the research targeting on the conquer of S. aureus infection has been tremendously accelerated. We are also trying our best in the same direction of efforts towards the development of new therapeutic and preventive measures for S. aureus infection.

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