Genomic Analysis of the Emergence of Vancomycin-Resistant *Staphylococcus aureus*

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ABSTRACT ***Staphylococcus aureus* is a human commensal bacterium and a prominent cause of infections globally. The high incidence of *S. aureus* infections is compounded by the ability of the microbe to readily acquire resistance to antibiotics. In the United States, methicillin-resistant *S. aureus* (MRSA) is a leading cause of morbidity and mortality by a single infectious agent. Therapeutic options for severe MRSA infections are limited to a few antibiotics to which the organism is typically susceptible, including vancomycin. Acquisition of high-level vancomycin resistance by MRSA is a major concern, but to date, there have been only 12 vancomycin-resistant *S. aureus* (VRSA) isolates reported in the United States and all belong to a phylogenetic lineage known as clonal complex 5. To gain enhanced understanding of the genetic characteristics conducive to the acquisition of vancomycin resistance by *S. aureus*, V. N. Kos et al. performed whole-genome sequencing of all 12 VRSA isolates and compared the DNA sequences to the genomes of other *S. aureus* strains. The findings provide new information about the evolutionary history of VRSA and identify genetic features that may bear on the relationship between *S. aureus* clonal complex 5 strains and the acquisition of vancomycin resistance genes from enterococci.**

Staphylococcus aureus is a leading cause of human bacterial infection worldwide and is endemic in both hospitals and the community (1). The ubiquity of *S. aureus* disease is facilitated by a commensal lifestyle, and the bacterium is frequently found on the skin and anterior nares of healthy individuals. *S. aureus* is a predominant cause of nosocomial infections, which arise often in individuals with predisposing risk factors such as hemodialysis or surgery. The clinical spectrum of *S. aureus* infection ranges from relatively benign skin and soft tissue infections to severe and life-threatening systemic disease (1). Management of severe *S. aureus* disease is confounded by the penchant of the pathogen to develop antibiotic resistance.

The ability of *S. aureus* to develop drug resistance is well documented and is perhaps best exemplified by the emergence of the penicillin-resistant *S. aureus* lineage known as phage type 80/81 shortly after the introduction of penicillin in the 1940s (1). Resistance is conferred by a plasmid-encoded penicillinase that hydrolyzes the β-lactam ring of penicillin. *S. aureus* phage type 80/81 spread rapidly and became pandemic in both hospitals and the community. Similarly, methicillin-resistant *S. aureus* (MRSA) strains were first reported in the early 1960s—soon after the introduction of methicillin. Methicillin resistance is mediated by a low-affinity penicillin-binding protein (PBP2a) encoded by *mecA* residing on a mobile genetic element known as staphylococcal cassette chromosome *mec* (SCC*mec*) (1). In contrast to *S. aureus* plasmid-encoded penicillin resistance, which confers resistance to penicillins only, SCC*mec* confers a broad spectrum of resistance to the entire β-lactam class of therapeutic agents and frequently non-β-lactam therapeutics. As a result, vancomycin, a glycopeptide antibiotic that inhibits cell wall synthesis, has become one of the last viable options for treating severe MRSA infections (2).

The high prevalence of MRSA in hospitals has led to the increased use of vancomycin for the treatment of severe MRSA infections, making vancomycin one of the most commonly used antibacterial agents in academic health centers in the United States (3). In 1997, the first report of an *S. aureus* isolate with reduced susceptibility to vancomycin was published (4), and resistance was found to be associated with structural changes in the cell wall (5). According to the Clinical and Laboratory Standards Institute (CLSI), these strains are classified as vancomycin-intermediate *S. aureus* and are defined by vancomycin MICs of 4 to 8 μg/ml. In comparison, vancomycin-resistant *S. aureus* (VRSA) is defined by the CLSI as having vancomycin MICs of ≥16 μg/ml. Over the past decade, 12 cases of VRSA infection have been reported in the United States (6, 7-10). Resistance in VRSA occurs via specific modifications of the peptidoglycan cell wall target and is mediated by the VanA operon (11). VanA is carried on the mobile genetic element Tn*1546* in *Enterococcus* and can be transferred to a recipient *S. aureus* strain by transconjugation (12, 13). Notably, all characterized U.S. VRSA strains belong to the *S. aureus* clonal complex 5 (CC5) phylogenetic lineage.

To better understand the genetic characteristics of the CC5 lineage that may permit or favor the acquisition of the vancomycin resistance plasmid from enterococci, Kos et al. sequenced the genomes of all 12 known U.S. VRSA strains and compared them to the genomes of other *S. aureus* lineages (6). The authors showed that all VRSA strains are closely related in DNA sequence and thus the genomes have a fairly recent ancestor in common. Inasmuch as vancomycin has been used as an antimicrobial agent since the late 1950s (11), and given that the ancestral strain is predicted by Kos et al. to have existed in the early 1960s (6), it may seem surprising that VRSA was first isolated in 2002 (14). Why did it take 40 years for VRSA to emerge, when in comparison, MRSA

Published 26 June 2012

Citation Kobayashi SD, Musser JM, DeLeo FR. 2012. Genomic analysis of the emergence of vancomycin-resistant *Staphylococcus aureus*. mBio 3(4):e00170-12. doi:10.1128/mBio.00170-12.

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emerged within 2 years after methicillin was introduced to treat human infections (15)? Several factors unrelated to the composition of the VRSA genome likely contributed, including the restricted use of vancomycin until the 1980s, the need for an appropriate Tn*1546*-containing plasmid donor (in this case, vancomycin-resistant enterococcus [VRE]), and conditions that favor polymicrobial infection with VRE and *S. aureus* (11).

Based on the genotype of the first VRSA isolate from Michigan (VRS1 in the study by Kos et al.), the acquisition of Tn*1546* by *S. aureus* likely involves acquisition of the Tn*1546*-containing plasmid from VRE and subsequent transfer of Tn*1546* from the VRE donor plasmid to a staphylococcal multiresistance plasmid (9). These transfer events have not been reproduced *in vitro* using selection with vancomycin (9, 13), perhaps due to the time required for the induction of vancomycin resistance. In addition, VRS1 did not retain the Tn*1546*-containing enterococcal plasmid (9). However, several subsequent VRSA isolates, for example, the VRSA isolate from New York (VRS3a in the paper by Kos et al.), maintained the Tn*1546*-containing plasmid from VRE (10), and therefore, high-level vancomycin resistance in *S. aureus* does not require transfer of Tn*1546* into a staphylococcal plasmid or integration into the chromosome. Based on these findings, Weigel et al. suggested that eliminating the need for a second transfer step significantly increases the probability of the emergence of VRSA from VRE-*S. aureus* coinfections (10). One possible explanation for the selective transfer of the Tn*1546*-containing plasmid from VRE to CC5 strains is that CC5 strains have a defect in one or more restriction modification systems. However, in their analyses of VRSA genomes, Kos et al. found that restriction modification systems were largely intact. The authors identified several genetic features that distinguish the CC5 phylogenetic lineage from other *S. aureus* lineages, including a truncating frameshift mutation in the gene encoding DprA—a molecule known to increase DNA transformation efficiency in bacteria—in all but one North American VRSA strain, the lack of a lantibiotic bacteriocin operon (*bsa*), and the presence of a large repertoire of genes encoding lipoproteins and superantigens that may detrimentally alter host immune function (6). Whether these characteristics predispose CC5 strains to the acquisition of vancomycin resistance and enhance the spread of such strains is unknown and merits further investigation.

A critical question that whole-genome sequencing and subsequent phylogenetic analyses might be able to address is whether transfer of the Tn*1546*-containing plasmid from VRE to *S. aureus* has occurred multiple independent times (i.e., a transfer event in each patient) or if there was a single VRE-to-*S. aureus* transfer event followed by horizontal (conjugal transfer) or vertical transmission to other *S. aureus* strains (and transmission of the same VRSA strain among multiple patients). Previous molecular genetic analyses of the VRSA isolates from three distinct geographic locations in North America, Michigan, Pennsylvania, and New York, suggested that there has been independent acquisition of vancomycin resistance from VRE at each geographic location (8, 10, 16). For example, Tenover and colleagues reported that DNA sequences of Tn*1546* obtained from the first Michigan VRSA isolate (VRS1) and the Pennsylvania VRSA isolate (VRS2) were distinct, as Tn*1546* in VRS2 had two insertion elements and a deletion relative to that from VRS1 (16). In a subsequent study, Zhu et al. found that four VRSA isolates from patients in Michigan had unique pulsed-field gel electrophoresis patterns, albeit all were pulsed-field type USA100 or related to USA100. The authors concluded that each Michigan VRSA isolate arose by a unique genetic event (17), that is, arose multiple times independently. Although collectively these data provide support to the idea that VRSA isolates have arisen independently, a comprehensive analysis of the VRSA genome sequences was needed to better understand this process.

In their whole-genome sequence analyses, Kos et al. found that the Tn*1546* DNA sequences obtained from VRSA strains segregate by region of isolation (Michigan, Pennsylvania, New York, and Delaware) rather than by time of isolation from the patient, which suggests there has been independent acquisition of vancomycin resistance at each of these locations (6). By comparison, all nine Tn*1546* DNA sequences in the Michigan VRSA isolates were either identical to each other or different by one single-nucleotide polymorphism, a finding consistent with the occurrence of a common donor strain (i.e., a single acquisition event). Moreover, four Michigan isolates contained identical Inc18 enterococcal donor plasmids that contain Tn*1546*, an observation consistent with previous restriction enzyme analysis by Zhu et al. (17). On the other hand, the content of *S. aureus* plasmids and, to some extent, other plasmids of enterococcal origin was varied among the VRSA strains. This finding is perhaps not surprising, since there is some variance in plasmid content even among *S. aureus* isolates with the same pulsed-field type (e.g., among some USA300 isolates) (18). One possible explanation for the occurrence of multiple (and seemingly unrelated) VRSA infections in Michigan is that there was a single Tn*1546* transfer event (from VRE to *S. aureus*) and then subsequent horizontal transfer of the enterococcal plasmid to other closely related *S. aureus* clones or integration of Tn*1546* into an *S. aureus* plasmid, followed by a horizontal transfer event. Horizontal transfer of the vancomycin resistance plasmid from VRSA to MRSA has been demonstrated *in vitro*, providing support for the latter hypothesis (9). Such a phenomenon might also explain the phylogeny-based observations of Kos et al., in which analysis of hypervariable repeat regions of several *S. aureus* molecules failed to reveal a single common line of VRSA descent (beyond the recent common ancestor, ca. 1960). Taken together, the genomic data reported by Kos et al. and others suggest that VRSA arose independently at each geographic location, whereas the argument for independent acquisition of vancomycin resistance in each of the Michigan VRSA isolates is less compelling and, in fact, provides evidence for a single acquisition event. Regardless, the work by Kos et al. provides new insight into the molecular basis of the emergence of VRSA and raises many important questions that require further investigation.

ACKNOWLEDGMENT

S.D.K. and F.R.D. are supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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