The molecular epidemiology and evolution of the Panton–Valentine leukocidin-positive, methicillin-resistant Staphylococcus aureus strain USA300 in Western Australia

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Abstract

Between 2003 and 2008, 76 clinical isolates of the Panton–Valentine leukocidin-positive Staphylococcus aureus strain ‘West Australian methicillin-resistant Staphylococcus aureus (MRSA)-12’ (WA MRSA-12) were recovered from 72 patients living in the Perth area in Western Australia. These isolates were found to belong to multilocus sequence type 8, and had a USA300-like pulsed-field gel electrophoresis pulsotype. All isolates were genotyped using diagnostic DNA arrays covering species markers, resistance factors, virulence-associated, as well as MSCRAMM (microbial surface components recognizing adhesive matrix molecules) genes to prove the identity between WA MRSA-12 and the pandemic strain USA300, as well as to detect possible genetic variability. In general, WA MRSA-12 isolates were similar to USA300, and the most common variant was identical to USA300-TC1516. From this clone, most of the other variants may have evolved by a limited number of gene losses or acquisitions. Variations in carriage of virulence and resistance-associated genes allow distinction of variants or sub-clones. Altogether, 16 variants could be distinguished. They differed in the carriage of resistance genes (blaZ/I/R, ermC, msrA + mppBM, aodD + mupR, aphA3 + sat, tetK, qacC, merA/B/R/T) of β-haemolysin-converting phages and of enterotoxins (sek + seq, which were deleted in four isolates). Notably, the arginine catabolic mobile element (ACME) was absent in 12 isolates (15.8%). The mercury resistance (mer) operon, which is usually associated with SCCmec type III elements, was found in several ACME-negative isolates. The present study emphasises the importance of genotyping in detecting the introduction and evolution of significant MRSA strains within a community.

Keywords: Arginine catabolic mobile element (ACME), diagnostic DNA microarray, molecular typing, MRSA, Panton–Valentine leukocidin (PVL), Staphylococcus aureus, USA300

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Introduction

In recent years, a pandemic unfolded of methicillin-resistant Staphylococcus aureus (MRSA) strains that carry novel and apparently highly mobile chromosomal staphylococcal chromosomal cassette elements harbouring mecA (SCCmec elements). Such strains have emerged outside the hospital setting and have been designated as ‘community-associated MRSA’ (CA-MRSA). Some of them harbour the prophage-encoded [1,2] virulence factor Panton–Valentine leukocidin (PVL). PVL is a bicomponent toxin that forms polymeric pores in leukocyte membranes [3], although its role in pathogenesis is still subject to discussion [4–6]. Several PVL-positive CA-MRSA strains from different clonal groups have evolved. Some have been confined to certain regions or localised outbreaks, whereas others have spread worldwide.

One of these strains, a clonal complex 8, spa type t008 MRSA carrying a SCCmec type IV element, has recently emerged as the dominant MRSA strain in North America, both in community and hospital settings [7–14]. Colloquially known as USA300, it has also been reported from Australia, Canada, Denmark, Germany, Japan, Switzerland and the UK [10,15–18]. Because of its rapid spread, it has drawn considerable attention, resulting in the sequencing of two complete
genomes (USA300-FPR3757, GenBank CP000255.1 [19] and USA300-TCH1516, CP000730.1 [20]). One remarkable finding was the detection of an arginine catabolic mobile element (ACME), which previously only has been found in Staphylococcus epidermidis. It was hypothesised that ACME contributed to the ability to metabolically alter the local pH on the skin. This could increase the ability of USA300 to persist on intact skin and, consequently, facilitate spread by skin contact [19,21].

Locally known as WA MRSA-12, several USA300-like isolates have been identified in Australia [22]. To determine whether WA MRSA-12 was identical to USA300, we applied previously developed DNA microarrays on a collection of Australian PVL-positive ST8-MRSA IV isolates. Variations detected within this strain may be relevant for typing or therapeutic interventions.

Material and methods

Isolates and patients
Between July 2003 and February 2008, 76 MRSA isolates from 72 patients living in the Perth area, Western Australia (WA), were characterised as PVL-positive WA MRSA-12 using pulsed-field gel electrophoresis (PFGE) [23] and PCR for the detection of PVL genes using previously published primers [24]. Representative PFGE patterns are shown in Fig. 1. MLST and SCCmec typing were performed on 16 isolates (see Supporting Information, Table S1) using previously published methods [25–28]. Some 62.5% of the patients were male, and approximately 55% were older than 30 years (Table S1).

Skin and soft tissue infections were reported in 94.4% of cases. The remaining cases were asymptomatic, and subjected to contact screening. Necrotising pneumonia was not observed.

To determine the stability of variants, follow-up isolates from individual patients were also characterised.

The sequenced strain USA300-FPR3757 was included in the study. Although the second sequenced strain, USA300-TCH1516, was not tested, a prediction of its hybridisation profile based on the published genome sequence allowed a comparison to be made with the WA isolates.

Array procedures
The DNA array used in the present study covers 334 target sequences. Depending on the nomenclature used, this corresponds to approximately 185 distinct genes and their allelic variants, and includes mainly clinically relevant genes on mobile elements that are not covered by whole-genome arrays derived from sequenced genomes [29]. The targets, related protocols, data interpretation and evaluation procedures used have been described previously [30,31].

Briefly, cultures were grown overnight on Columbia blood agar. Culture material was harvested, lysed using lysozyme, lysozyme and ribonuclease A and treated with proteinase K. DNA was purified using the Qiagen device EZ1 (Qiagen, Valencia, CA, USA) according to the manufacturer’s tissue lysis protocol. An iterated, linear primer elongation was employed for the simultaneous amplification of all targets. Within this step, amplicons were labelled by incorporation of biotin-16-dUTP.

The labelled sample was denatured and hybridised to the array. This was followed by washing steps and by the addition of a blocking reagent. Horseradish–peroxidase–streptavidin conjugate was then added to the array, followed by incubation and washing. The array tube was placed into the ATR01 reading device (Clondiag, Jena, Germany), and Sera- mun Green precipitating dye (Seramun, Heidesee, Germany)
was added. After 5 min, an image of the array was recorded and analysed.

Results

Virulence-associated genes and the ACME locus

Array hybridisation and PCR [24] demonstrated that all WA MRSA-12 isolates harboured the PVL genes, lukF-PV and lukS-PV.

All but four isolates (94.7%) yielded hybridisation signals for enterotoxin genes sek and seq. Negative results were confirmed by PCR (primers sek_forward, ACAGAGAATTT TCATTGGATGT and sek_reverse, CACATTGGCTTAT CCCTCCT, with a melting temperature during PCR of 55°C, as well as primers seq_forward_2 GCTTCAGAGGAGT TAGTTCTGG and seq_reverse_2 CTTGCAGATTCGG TGT, with a melting temperature of 54°C; see Supporting Information, Table S1).

One isolate was negative for genes encoding staphylokinase (sok), chemotaxis-inhibiting protein (chp) and staphylococcal complement inhibitor (scn). Two isolates were positive for sok and scn, but lacked chp. Carriage of set/ssl genes was identical to USA300-FPR3757 and USA300-TCH1516 genome sequences and to previously described USA300 isolates from Germany [30].

Genes of the ACME locus (arcA-SCC, arcB-SCC, arcC-SCC and arcD-SCC) were detected in 64 (84.2%) of the WA MRSA-12 isolates. An absence of ACME was confirmed using two different arcA PCRs [19,32].

Capsule, biofilm and microbial surface components recognizing adhesive matrix molecules (MSCRAMM) genes

Carriage of capsule genes (type 5), biofilm (icaA, icaC, icaD) and MSCRAMM genes (bbp, clfA, clfB, ebb, ebpS, eno, fib, fnbA, fnbB, map, sdrC, sdrD and wvh) was identical to USA300-FPR3757 and USA300-TCH1516.

Antibiotic resistance determinants

Apart from one isolate, all WA MRSA-12 isolates carried mecA as a part of the SCCmec type IV element. This isolate initially did not yield hybridisation signals for any of the SCCmec-associated genes.

However, growth on a broth containing cefoxitin was observed. DNA from this culture yielded signals for mecA and all other SCCmec type IV probes (ugpQ, crrA-2, crrB-2, truncated mecR). Except for these markers, the hybridisation pattern remained unchanged and allowed assignment to variant L (Figure 2).

The β-lactamase operon (blaZ, blaI, blaR), a gene encoding a putative transport protein (SAUSA300_2128, USA300-HOU_2160), and the fosfomycin resistance gene fosB (SAUSA300_2280, USA300HOU_2313) were detected in all isolates.

The neo-ikanamycin resistance gene ahpA3 and the streptothricin resistance gene sat were jointly detected in 64 (84.2%) isolates. Two genes for macrolide efflux proteins, msrA and mupB were also always found together in 61 (80.3%) isolates.

Comparatively rare resistance determinants included the rRNA adenine N-6-methyltransferase gene emrC (in seven isolates, 9.2%), a gene for a tetracycline efflux protein, tetK (eight isolates, 10.5%), a gene encoding an unspecific efflux pump (qacC, one isolate, 1.3%), as well as the aminoglycoside adenyltransferase gene aadD and a gene conferring high level mupirocin resistance, mupR (two isolates, 2.6%). In WA MRSA-12 isolates, aadD and mupR genes occurred together, whereas, in USA300-FPR3757, only mupR was present.

MsrA/mupB or emrC were mutually exclusive in all isolates but one.

The mer operon

The genes merA (encoding mercuric reductase), merB (organonemurceral lyase), merR (regulatory protein) and merT (transport protein) were detected in eight (10.5%) isolates, with all of them being ACME-negative.

Variants of the WA MRSA-12 strain

Hybridization profiles identified 16 variants (variants A–P, Fig. 2) among 76 WA MRSA-12 isolates. Forty-seven isolates (61.8%) belonged to variant A. An analysis of the genome sequence of USA300-TCH1516 predicted the same hybridisation pattern as that observed in variant A. Strain CDC 2001-5114, which was used as reference for PFGE, represented a seventeenth variant. It differed from variant A only in one case, a patient initially infected with variant A cultured a follow-up isolate after 205 days, which differed in the presence of aadD and mupR (variant G).
In three separate episodes, USA300 was isolated from family members. In one case, a girl was diagnosed with a variant I infection, 295 days after the same variant was recovered from her mother. In another case, two isolates of variant A were sampled within 1 month from two siblings.

In a third family, both parents and their two children were, within a period of 7 months, infected with variant A.

**Discussion**

USA300 is a PVL-positive, CA-MRSA strain that has spread rapidly in North America. Sporadic cases and outbreaks have been reported from various European countries and in Australia. In WA, the first infection with USA300/WA MRSA-12 was reported in 2003. Subsequently, an increase in the number of cases has occurred, particularly within 2007 and in 2008 (Fig. 3).

USA300 isolates were not identical to each other. Thus, USA300 cannot be regarded as a genetically homogenous unit. Whether, for example, ACME-positive and -negative isolates should be regarded as variants of one strain, or as two different strains, is a matter of definition, raising the question of how to define a strain in general and USA300 in particular. Variations within USA300 affected virulence factors and antibiotic resistance determinants. MSCRAMM, capsule or set/ssl gene carriage was uniform, and closely resembled the other clonal complex 8 strains [30], including NCTC8325 [33]. It can be assumed that the lack of variability of these genes was due to the relatively short time-span of USA300 proliferation, whereas virulence- and resistance-associated genes varied on a faster time-scale because they are situated on mobile elements.
One isolate lacked the phage-born innate immune evasion cluster (which truncates the hib gene by introducing sak, chp, and scn [34,35]), and some isolates were negative for sak and seq. Because ST80-MRSA IV also lacks enterotoxin genes [36], it could be speculated that, for PVL-positive strains, some virulence factors were expendable without selective disadvantage.

Carriage of ACME was also variable. This is especially intriguing because this locus is assumed to be involved in facilitating the spread of USA300 by skin contact [19,21]. ACME-negative variants of USA300 appear to be common in WA. They also exist in the USA but appear to be extremely rare (one study found none [37] and another identified a single isolate [38]), and they have been reported in Germany [39]. Thus, they are not restricted to WA, and a strategy to identify USA300 by multiplex PCR detection of PVL, mecA and ACME can confirm, but not rule out, the presence of USA300. Because of the limited time of the presence of USA300 in WA, further studies should focus on possible changes of the ratio of ACME-positive to -negative variants. This may improve our understanding of the clinical significance of this element and its proposed role in the rapid spread of USA300.

The variability of resistance genes was not unexpected because these genes are subjected to a high, but variable selective pressure.

In one isolate, negative signals for SCCmec probes were observed but, after passage on a cefoxitin-containing medium, the resulting culture was positive for these genes. We assume that a majority of cells lost the SCCmec element, but that a small mecA-positive subpopulation below the detection limit of linear amplification was still present. In the presence of cefoxitin, this subpopulation had a selective advantage resulting in displacement of the deletion variant. A loss of SCCmec elements from MRSA has occasionally been observed [40–42], emphasising the mobility of the SCCmec gene cluster.

Other common, but variable resistance genes included the β-lactamase operon and a fixed combination of sat and aphA3. The latter genes are frequently detected in diverse MRSA strains, including ST8-MRSA IV, ST45-MRSA IV, ST80-MRSA IV and ST228-MRSA I [30]. Because neomycin is commonly used as topical preparation, aphA3 might confer an advantage to a strain usually associated with skin infections.

Another apparently fixed combination of resistance genes comprised aadD and mupR, encoding resistance to neomycin, tobramycin and mupirocin. Although mupR was rare, it deserves further attention because mupirocin is crucial for the eradication of MRSA. Macrolide resistance genes were common. The genes mrsa/mpbBM or ermC, apart from a single exception, proved to be mutually exclusive. This might indicate that the maintenance of multiple genes conferring similar resistance properties could result in an unnecessary fitness cost. Because ermC encodes not only macrolide, but also clindamycin resistance, it might confer a more significant advantage. In other MRSA strains, erm genes are more abundant than msrA [43] but, in USA300, mrsa/mpbBM positive isolates predominate. Thus, clindamycin can be considered as a therapeutic option, although a widespread application might favour ermC-positive variants of USA300.

Surprisingly, the mercury resistance (mer) operon was found in ACME-negative USA300 isolates. It can be plasmid-borne (GenBank L29436) but, similar to ACME, it can also be associated with recombinases (GenBank AB037671, [44]) forming some kind of SCC element. Thus, its genetic background and its position in the USA300 genome remains to be clarified.

Further isolates from diverse regions and over a longer time-span need to be studied to determine whether the described variability represents random variations, or an early stage of a rift into separate strains. It will also be interesting to observe the competition of variants which are—except for a small number of genes—essentially isogenic. An especially successful variant can be expected to combine genetic traits that also are responsible for the success of USA300, regardless of whether this might be the carriage of PVL, ACME [19,21] or another factor yet to be identified. In the present study, variant A appeared to be most successful. Because it was the first USA300 variant detected in WA, it can be regarded as the founder variant, from which variants B–L, and possibly M, may have been derived by a limited number of gene losses or acquisitions. Variant A appears to be geographically widespread because it has been found in Texas (USA300-TC1516), in the German states Saxony [16,30] and Brandenburg, and in Switzerland (isolates courtesy of T. Juratzek and B. Berger-Baech).

The explosive expansion of USA300 still warrants further study. DNA microarray technology might contribute to the understanding of this phenomenon by resolving variants below strain level. This might be helpful for tracing chains of transmissions and elucidating the sources of importation of that strain into a given region.

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Transparency Declaration

R. Ehricht and P. Slickers are employees of Clondiag. The authors declare no other conflicting interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:
Table S1. Patient data and isolate characteristics.

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References


5. Schwartzman WA, Beenhouwer DO, Schaberg DR. How relevant were the models used to measure the impact of Panton–Valentine leukocidin in human staphylococcal infections? J Infect Dis 2007; 195: 1726–1727; author reply 1727–1728.


