Typisierung und Expression von bakteriellen Exotoxinen am Beispiel von *Staphylococcus aureus*

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Abkürzungsverzeichnis

Abkürzung	Bedeutung
Ag	Antigen
Agr	accessory gene regulator
AS	ArrayStrip
AT	ArrayTube
ATR	ArrayTube-Reader
CA-MRSA	Community-acquired Methicillin-resistente *Staphylococcus aureus*
CC	klonaler Komplex
DNA	Desoxyribonukleinsäure
ELISA	Enzyme-linked Immunosorbent Assays
HA-MRSA	Hospital-acquired Methicillin-resistente *Staphylococcus aureus*
Hla	Hämolysin Alpha
LA-MRSA	Livestock-associated Methicillin-resistente *Staphylococcus aureus*
MALDI-TOF-MS	Matrix-unterstützte Laser-Desorption/Ionisation und Massen-spektrometrie mit Flugzeitanalysator
MGE	Mobiles genetisches Element
MLST	Multi-Locus-Sequence Typing
MRSA	Methicillin-resistente *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
PCR	Polymerase Kettenreaktion
PBP	Penicillin-bindendes Protein
PVL	Panton-Valentine Leukocidin
RT-PCR	Reverse Transkriptase Polymerase Kettenreaktion
S. aureus	*Staphylococcus aureus*
SCC	Staphylococcal Cassette Chromosome
Spa	Staphylococcal Protein A
ST	Sequenztyp

Zusammenfassung

Staphylococcus aureus (*S. aureus*) ist ein weltweit bei Menschen und Tieren vorkommendes Bakterium. Es ist ein fakultativ pathogener Keim, der gesunde Menschen asymptomatisch besiedeln, aber auch schwere invasive Erkrankungen wie Pneumonie, Endokarditis oder Sepsis auslösen kann. *S. aureus* Stämme können eine Vielzahl verschiedener Virulenz- und Resistenzgene besitzen und diese werden durch mobile genetische Elemente zwischen verschiedenen Stämmen übertragen. Methicillin-resistente *S. aureus* (MRSA) sind häufig auftretende Erreger, sowohl innerhalb als auch außerhalb von Krankenhäusern sowie in Tiermastanlagen. Ein vieldiskutierter Virulenzfaktor von *S. aureus* ist das Panton-Valentine Leukozidin (PVL). Dieser wird oft mit rezidivierenden Haut- und Weichteilinfektionen assoziiert, in seltenen Fällen auch mit lebensbedrohlichen Erkrankungen wie Pneumonien. Ein weiterer bedeutender Virulenzfaktor von *S. aureus* ist Hämolysin Alpha (Hla). Dieses Toxin hat eine hämolytische Wirkung und kann dermonekrotische Krankheitsbilder verursachen.

In der vorliegenden Arbeit wurden spezifisch hergestellte DNA-Mikroarrays zur Genotypisierung von *S. aureus* eingesetzt. Diese ermöglichen die Klassifizierung und Zuordnung einzelner *S. aureus* Isolate zu Verwandtschaftsgruppen, sogenannten klonalen Komplexen (CCs) sowie den Nachweis vorhandener Virulenz- und Resistenzgene. Diese Methodik hilft das Vorkommen und die Verbreitung von *S. aureus* Stämmen zu verstehen. Es können Infektionsketten erkannt und überwacht, sowie Populationsstudien durchgeführt werden. Diese Arbeit stellt die Anwendung der DNA-Mikroarrays in zwei separaten Studien dar. Es wurden zum einen *S. aureus* Stämme in Trinidad & Tobago, zum anderen die in Norwegen untersucht. Die Ergebnisse zeigen ein vermehrtes Auftreten von MRSA und PVL-positiven *S. aureus* Isolaten in Trinidad & Tobago im Vergleich sowohl zu früheren Studien aus dieser Region als auch zu der aus Europa bekannten Situation. Der in den USA epidemische Stamm CC8-MRSA-IV („USA300“) scheint sich zunehmend in Trinidad & Tobago zu verbreiten. Die *S. aureus* Population in Norwegen verhält sich anders. Dort existiert ein strenges Kontrollprogramm in den Krankenhäusern und vereinzelt auftretende *S. aureus* Isolate werden oft durch Reisen aus Regionen mit hoher PVL-Prävalenz eingeführt. Diese beiden Studien zeigen zwei epidemiologisch unterschiedliche *S. aureus* Populationen, die dem Anschein nach auf verschiedenen geografischen und sozioökonomischen Gegebenheiten beruhen.

In dieser Arbeit wurden ebenfalls Protein-Mikroarrays hergestellt, die sowohl dem qualitativen als auch quantitativen Nachweis von *S. aureus* Toxinen dienen. Zum qualitativen Nachweis von PVL wurde mit Hilfe spezifisch gefertigter Protein-Mikroarrays ein Lateral-Flow

(Schnell)-Test entwickelt und validiert. Dieser ermöglicht eine schnelle Diagnostik bei Verdachtsfällen und ggf. die Einleitung der passenden Therapie. Weiterhin wurden zwei Protein-Mikroarrays entwickelt, mit denen die *in vitro* Expressionen der Exotoxine PVL und Hla quantitativ messbar sind. Dafür wurden auf die Mikroarrays monoklonale anti-lukF-PV- bzw. anti-Hla-Antikörpern gespottet und kovalent gekoppelt. Für die quantitativen Auswertungen wurden Kalibrierungskurven, basierend auf Referenzexperimenten mit bekannten Toxinkonzentrationen, erstellt. Mit Hilfe dieser können anhand der Spotintensitäten auf dem prozessierten Mikroarray die Toxinkonzentrationen bestimmt werden. Mit der Anwendung dieser Protein-Mikroarray-Assays konnte der Zusammenhang zwischen Toxinexpression und genetischem Hintergrund der jeweiligen Stämme untersucht werden. PVL-positive *S. aureus* Isolate, die einem CC angehören, exprimieren ähnlich viel (z. B. CC8 und CC93) bzw. wenig (z. B. CC5 und CC80) PVL. Die Experimente zu Hla zeigen, dass Stämme einzelner CCs, wie z. B. CC22 oder CC30, unter den gegebenen Bedingungen kein Hla exprimieren. Stämme anderer CCs, wie CC1 oder CC5, hingegen weisen verschiedene Hla-Konzentrationen auf. Beim Vergleich der *in vitro* gebildeten Toxinkonzentrationen und den klinischen Krankheitsbildern, die die *S. aureus* Isolate zum Teil hervorgerufen haben, konnte weder bei PVL noch bei Hla eine Korrelation festgestellt werden. Die Toxine rufen also entweder bereits in geringen Konzentrationen klinisch schwere Erkrankungen hervor oder die *in vitro* Expression bzw. Regulation entspricht nicht der *in vivo* Expression. Die Virulenz eines *S. aureus* Stammes kann zusätzlich von weiteren inneren und äußeren Faktoren abhängen.

Für den parallelen Nachweis mehrerer *S. aureus* Toxine in einem Experiment wurde ein Multiplex-Protein-Mikroarray entwickelt und validiert. Mit diesem ist es möglich sieben verschiedene Toxine (PVL, Toxic Shock Syndrome Toxin, Hämolysin alpha und beta, Enterotoxine A und B sowie Staphylokinase) als auch das Methicillin-Resistenz verursachende Penicillin-bindende Protein 2a (PBP2a) und der Speziesmarker Staphylococcal Protein A (Spa) in einem Multiplexverfahren phänotypisch nachzuwiesen. Das hat außer für Forschungs- und Referenzlabore auch in der Diagnostik eine besondere Bedeutung. Durch den schnellen und spezifischen Nachweis exprimierter Toxine kann für die betroffenen Patienten zügig eine adäquate Therapie eingeleitet werden.

Zusammenfassend ist zu sagen, dass *S. aureus* ein vielfältiger und potenziell lebensbedrohlicher Erreger ist. Die Vielzahl entstandener Resistenzen erschwert die Behandlung von *S. aureus* Infektionen zusätzlich. Umso wichtiger ist es, einen schnellen Überblick über vorhandene Virulenzfaktoren und Resistenzgene zu bekommen, um eine angepasste Therapie zu initiieren. Mikroarrays bieten eine optimale Plattform zur geno- und phänotypischen Charakterisierung von Bakterien und somit für die Entwicklung, Validierung und Herstellung von klinisch bedeutsamen Schnelltests.

Einleitung

Staphylococcus aureus – ein weit verbreitetes Bakterium

Das Bakterium *Staphylococcus aureus* (*S. aureus*) ist systematisch in die Familie der Staphylococcaceae einzuordnen. Sir Alexander Ogston beschrieb 1882 erstmals „Mikrokokken“ bei Abszessbildung und Sepsis und prägte den Namen „Staphylokokken“ (Ogston, 1882) (griech.: ‚kókkos‘ = Kügelchen, Kern; griech.: ‚Staphylos‘ = Traube). 1884 beschrieb F. J. Rosenbach die Spezies *Staphylococcus aureus* (Rosenbach, 1884; Orenstein, 2011). Es handelt sich dabei um Gram-positive Kokken, die meist haufen- bzw. traubenförmig angeordnet sind (Abbildung 1). Die Bakterienzellen haben einen Durchmesser von 0,8-1,2 µm, sind unbeweglich und bilden keine Sporen.

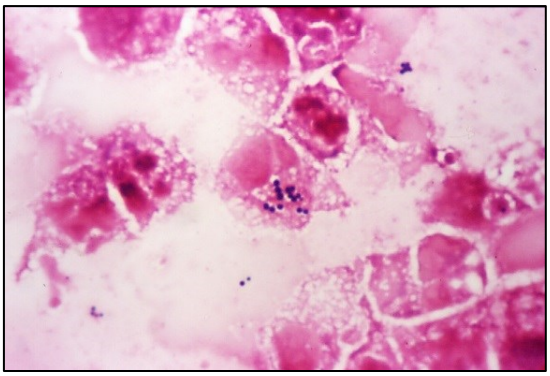

Abbildung 1: *S. aureus*, Gramfärbung von einem Eiterabstrich, 1000fache Vergrößerung (Foto: S. Monecke)

S. aureus Kolonien sind, auf Schafblut-Agar gewachsen, durch eine graugelbe bis goldgelbe (lat.: ‚aureus‘ = goldgelb), in einzelnen Fällen eine grauweißliche Färbung gekennzeichnet. Weiterhin sind viele Stämme zur Hämolyse in der Lage. Diese erscheint auf bluthaltigen Nährböden als entfärbter Hof um die einzelnen Bakterienkolonien (Abbildung 2).

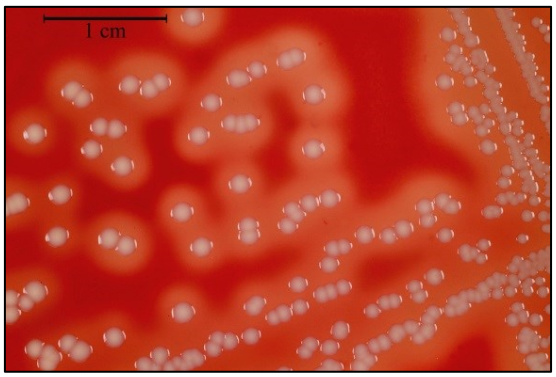

Abbildung 2: *S. aureus*, Kolonien mit Hämolyse auf Columbia Blutagar (Foto: S. Monecke)

Etwa 30 % der gesunden Bevölkerung (van Belkum, 2009) sind asymptomatisch auf der Haut und den Schleimhäuten mit *S. aureus* besiedelt. Diese Kolonisierung gilt als Risikofaktor, da unter entsprechenden Umständen (z. B. bei Verletzung der Haut oder durch chirurgische Eingriffe) ernsthafte, invasive Erkrankungen durch das Bakterium verursacht werden können (von Eiff et al., 2001). *S. aureus* ist ein klinisch relevanter Erreger, da er Krankheitsbilder auslöst, die von einer einfachen Haut- und Weichteilinfektion (z. B. Furunkel oder Abszess) (Hersh et al., 2008) bis hin zu lebensbedrohlicher Pneumonie, Endokarditis oder Sepsis reichen können (Parker und Prince, 2012; Miro et al., 2005; Lowy, 1998). *S. aureus* kann nosokomiale Ausbrüche verursachen, indem der Keim von einem auf den anderen Patienten übertragen wird. Dies wird durch mangelhafte Krankenhaushygiene begünstigt. Ein breites Repertoire an Staphylokokken-Toxinen kann zudem zu verschiedenen Toxikosen führen. Dabei ist unter anderem das Toxische Schock Syndrom Toxin 1 (TSST-1) von Bedeutung. Durch seine Immunsystem-stimulierende Wirkung kann dieses Exotoxin einen toxischen Schock auslösen (McCormick et al., 2001). Des Weiteren stellt die Staphylokokken-Enterotoxikose, bedingt durch den Verzehr *S. aureus* kontaminierter Nahrungsmittel, eine relativ häufige Erkrankung dar. Die dafür verantwortlichen Enterotoxine sind hitzestabil und können auch nach dem Kochen oder Braten toxisch wirken und zu Lebensmittelvergiftungen führen (Pinchuk et al., 2010).

Tiere bilden ebenso ein umfangreiches *S. aureus* Reservoir. Zum einen können Haustiere (z. B. Hunde oder Katzen) Träger des Bakteriums sein und durch engen körperlichen Kontakt die Erreger auf den Menschen übertragen (Weese, 2010). Zum anderen sind landwirtschaftliche Nutztiere und Tiere in Mastanlagen (Rinder, Schweine, Geflügel) betroffen. Auch hier tragen Menschen, die direkten Kontakt zu diesen Tieren haben (z. B. Tierärzte oder Landwirte), ein erhöhtes Risiko, nasal mit *S. aureus* besiedelt zu werden. Zudem können Nahrungsmittel wie Rohfleischprodukte kontaminiert sein und ggf. *S. aureus* bei entsprechender Handhabung oder Verzehr auf den Menschen übertragen (Argudin et al., 2010; Cuny et al., 2013). Von *S. aureus* besiedelte Wildtiere stellen eine potenzielle Infektionsgefahr dar (Porrero et al., 2014). Obwohl die Prävalenz von *S. aureus* in Wildtieren geringer ist als bei Haustieren, besteht die Möglichkeit, dass sie das Bakterium trotzdem auf andere Tiere und Menschen übertragen (Meyer et al., 2014). Schließlich können über die Ausscheidungen der Wildtiere, der Menschen, sowie Haus- und Zuchttiere *S. aureus* Zellen auch in Gewässer geraten und möglicherweise durch das Wasser weitergetragen werden (Porrero et al., 2014). Zunehmende Reiseaktivitäten, Flüchtlingsströme sowie Tiertransporte können zusätzlich zu einer globalen Verbreitung von Krankheitserregern wie *S. aureus* führen (RKI, Statistisches Bundesamt, 2003).

Staphylococcus aureus – Resistenzen und Virulenzen durch genetische Vielfalt

Das *S. aureus* Genom besteht zu ca. 80% aus dem sogenannten Kerngenom. Die Gene des Kerngenoms kodieren essentielle genetische Informationen wie zentrale Stoffwechselwege und regulatorische Funktionen (Fitzgerald et al., 2001). Innerhalb des Kerngenoms existieren variable Kernregionen. Deren Gene sind durch vergleichsweise höhere Sequenzvariabilität gekennzeichnet und kodieren Oberflächenproteine wie zum Beispiel das Staphylococcal Protein A (Spa), und deren Expression-steuernde Regulatoren wie z. B. den accessory gene regulator (*agr*) (Lindsay et al., 2006).
Einige Gene des Kerngenoms werden zu sequenzbasierten Typisierungen von *S. aureus* genutzt. Zu diesem Teil des Genoms gehören sieben „housekeeping" Gene: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* und *yqiL*, die immer vorhanden sind. Daher werden diese Gene beim Multilocus Sequence Typing (MLST) sequenziert und verglichen (Enright et al., 2000). Basierend auf Sequenzvariationen dieser sieben Gene werden *S. aureus* Stämme in sogenannte klonale Komplexe (CCs) und Sequenztypen (STs) eingeteilt. Dies hilft, Populationsstrukturen zu beschreiben und neu entdeckte Stämme zu definieren. Das Gen *spa*, das als zum variablen Kerngenom gehörig betrachtet wird, kann ebenfalls zur Typisierung von *S. aureus* eingesetzt werden, denn seine Sequenz beinhaltet eine variable repetitive Region (Harmsen et al., 2003).

Bis zu 20% des *S. aureus* Genoms sind hochvariabel (Fitzgerald et al., 2001). Diese Variabilität ergibt sich aus verschiedenen mobilen genetischen Elementen (MGEs), die eine Vielzahl an Virulenzfaktoren und Resistenzgenen tragen. Diese werden über horizontale Gentransfers zwischen den *S. aureus* Stämmen und, im Falle von Resistenzgenen, auch zwischen verschiedenen Spezies übertragen. Somit spielen sie in der Evolution neuer Klone, bei deren Anpassung an Wirte und dem Auslösen verschiedener Krankheiten eine entscheidende Rolle. Typische Vertreter solcher MGEs sind Bakteriophagen, Staphylococcal Cassette Chromosomes (SCC), Pathogenitätsinseln und Plasmide (Alibayov et al., 2014). So kodieren und übertragen bestimmte Bakteriophagen bedeutende Virulenzfaktoren wie das Panton-Valentine Leukocidin (PVL) (Boakes et al., 2011). SCC-Elemente können mehrere Funktionen übernehmen. Sie tragen Gene sowohl für Virulenzfaktoren und Rekombinasen (diese dienen dem Ausschneiden und Einfügen von Genen in die DNA) als auch für verschiedene antimikrobielle Resistenzen. Es gibt mehrere Typen von SCCs. SCC*mec*-Elemente kommen häufig vor und können das *mecA* Gen tragen, welches für die weit verbreitete Methicillin-Resistenz kodiert (Hiramatsu et al., 2001; http://sccmec.org).

Methicillin-Resistenz

Methicillin ist ein Penicillinase-resistentes Beta-Lactam-Antibiotikum. Es wurde in den 1950er Jahren entwickelt, nachdem Penicillinase-produzierende *S. aureus* Stämme auftraten und somit Penicillin als Medikament zur Behandlung von *S. aureus* Infektionen weitestgehend unwirksam wurde (Livermore, 2000).

Die Wirkung aller Beta-Lactam-Antibiotika zielt auf die Blockierung der bakteriellen Zellwandsynthese ab. Dort sitzen Penicillin-bindende Proteine 2 (PBP). Das sind Transpeptidasen, die die Bestandteile der Peptidoglycan-Schicht in der Zellwand katalytisch zusammensetzen. Beta-Lactam-Antibiotika binden die Transpeptidasen irreversibel und inhibieren damit den Zellwandaufbau und somit wiederum das Bakterienwachstum (Giesbrecht et al., 1998).

Methicillin-resistente *S. aureus* (MRSA) tragen ein Allel des Gens *mecA* (Monecke et al., 2012), das für ein modifiziertes Penicillin-bindendes Protein 2a (PBP2a) kodiert. PBP2a übernimmt die Funktion vom PBP in der bakteriellen Zellwandsynthese, verhindert jedoch die Bindung von Methicillin an die bakterielle Zellwand und somit seine Wirkung (Hiramatsu et al., 2001). Das die Methicillin-Resistenz vermittelnde Gen *mecA* liegt auf SCC*mec*-Elementen, von denen es mehrere Typen gibt. Diese unterscheiden sich in der unmittelbaren Umgebung des *mecA* Gens inklusive seiner regulatorischen Gene, in den Allelen der *ccr* Rekombinase-Gene und dem Vorhandensein zusätzlicher Gene. Vor kurzem wurde ein zweites, verwandtes *mec* Gen entdeckt. Dieses sogenannte *mecC* Gen kodiert ebenfalls eine Resistenz gegenüber Beta-Lactam-Antibiotika (Garcia-Alvarez et al., 2011; Shore et al., 2011).

Nach der Einführung von Methicillin in den 1960er Jahren wurden alsbald MRSA Stämme detektiert (Jevons, 1961). Ab den 1970er Jahren kam es zu einem vermehrten Auftreten von MRSA in Krankenhäusern und Pflegeeinrichtungen. Die sogenannten hospital-acquired MRSA (HA-MRSA) tragen typischerweise die strukturell größeren SCC*mec*-Typen I, II oder III. Sie verursachen nosokomiale Infektionen und können durch ihre verschiedenen Antibiotikaresistenzen in Krankenhäusern überleben und sich verbreiten. Im Vergleich zu Methicillin-sensitiven *S. aureus* (MSSA) Stämmen in gesunden Trägern außerhalb von Krankenhäusern haben HA-MRSA eine längere Wachstumszeit und geringere Fitness (Ender et al., 2004). Daher ist deren Überleben unter bakteriellem Konkurrenzdruck, außerhalb von Gesundheitseinrichtungen und in Abwesenheit von Antibiotika, limitiert und sie verursachen selten Krankheiten bei gesunden Menschen.

Epidemiologisch sind die HA-MRSA von den Community-acquired (CA-) MRSA zu unterscheiden, die ab den 1990er Jahren verstärkt aufgetreten sind. Im Gegensatz zu HA-MRSA wurden sie zuerst außerhalb von Krankenhäusern gefunden, wobei sie jetzt in

Regionen, in denen sie häufig sind, sekundär in Krankenhäuser vordringen. Charakteristisch für CA-MRSA sind die kleineren SCC*mec*-Kassetten der Typen IV oder V. Resistenzgene wie *mecA* sichern den Bakterien einen Selektionsvorteil in Krankhäusern, wo sie sich zunehmend verbreiten (Liu et al., 2008). CA-MRSA tragen oft zusätzliche Pathogenitätsfaktoren wie beispielsweise das PVL, was deren Virulenz verstärkt (Vandenesch et al., 2003). In bisher gesunden Patienten ohne Risikofaktoren können PVL-positive Stämme schwere Krankheiten wie nekrotisierende Pneumonie oder Fasciitis auslösen (Lina et al., 1999; Labandeira-Rey et al., 2007; http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4832a2.htm) (siehe folgendes Kapitel). Seit 2004 werden MRSA auch in der Tierzucht beobachtet und spielen dort eine bedeutende Rolle. Diese MRSA, „Livestock-associated MRSA“ (LA-MRSA) genannt, verbreiten sich unter den Tieren in Mastanlagen und den dort arbeitenden Menschen (Graveland et al., 2011). LA-MRSA tragen typischerweise ebenfalls die kleineren SCC*mec*-Kassetten der Typen IV und V, häufig gekoppelt mit verschiedenen Schwermetall-Resistenzoperons. Der Grund dafür ist, dass zur Förderung der Tiermästung dem Futter Schwermetalle (Kupfer und Zink) beigesetzt werden. Dadurch kommt es zur Ko-Selektion gekoppelter Antibiotika- und Schwermetall-Resistenzgene (Zhu et al., 2012).

Weltweit betrachtet wurden in unterschiedlichen geografischen Regionen verschiedene *S. aureus* Stämme gefunden. Das gehäufte Auftreten eines *S. aureus* Stammes in einer Region war oft namensgebend. Beispiele dafür sind unter anderem die Klone „USA300“ in Nordamerika, der „Bengal Bay Clone“ in Indien und Umgebung oder der „European Clone“ in mehreren europäischen Ländern und dem Nahen Osten. Jedoch kam es aufgrund der nationalen Namensgebungen und einer fehlenden Standardnomenklatur zu verschiedenen Namen für den gleichen Stamm. So stehen z. B. „Rhein-Hessen-Epidemiestamm“, „USA100“ und „New York-Japan Clone“ für den gleichen Stamm. In den letzten Jahren wurden einige lokal typische und weitestgehend darauf begrenzte Stämme auch in weiteren geografischen Regionen beobachtet sowie genotypisch neue, virulente *S. aureus* Stämme entdeckt (Monecke et al., 2011).

Panton-Valentine Leukocidin

Das PVL ist ein Virulenzfaktor von *S. aureus*. Van de Velde beschrieb es 1894 erstmalig als Leukozyten-lysierende Substanz (Van de Velde, 1894). Das Leukocidin wurde nach Philip Panton und Francis Valentine benannt, die 1932 den Zusammenhang mit Haut- und Weichteilinfektionen nachgewiesen haben (Panton und Valentine, 1932). PVL ist ein Exotoxin und besteht aus zwei Untereinheiten, LukS-PV (33 kDa) und LukF-PV (34 kDa). Diese werden durch zwei benachbarte co-exprimierte Gene (*lukF-PV* und *lukS-PV*) kodiert. Diese Gene sind auf Prophagen lokalisiert und werden über diese in das Bakteriengenom

integriert (Kaneko und Kamio, 2004). PVL-tragende Prophagen können sowohl in die Genome von MSSA als auch von hoch virulenten CA-MRSA integriert sein. Das Toxin bildet Polymere und formt Poren in den Membranen polymorph-kerniger Leukozyten (Granulozyten). Dies bewirkt eine erhöhte Interleukin-Produktion der befallenen Zellen und führt zum Zelltod durch Apoptose oder Nekrose (Kaneko und Kamio, 2004; Gillet et al., 2002; Genestier et al., 2005; Boyle-Vavra und Daum, 2007). Das klinische Bild einer Infektion mit PVL-positiven *S. aureus* ist häufig von chronischen und rezidivierenden Haut- und Weichteilinfektionen (Furunkel, Karbunkel) gekennzeichnet. In seltenen Fällen können selbst bei jungen und bis dahin gesunden Menschen schwere, lebensbedrohliche Erkrankungen wie nekrotisierende Pneumonien auftreten (Gillet et al., 2002; Lina et al., 1999; Labandeira-Rey et al., 2007). PVL-positive *S. aureus* Stämme kommen weltweit vor und verbreiten sich zunehmend. Neben pandemischen PVL-positiven MSSA Stämmen (die oft zu den CC30 und CC121 gehören) wurden MRSA-Isolate ST 8 (USA300) hauptsächlich in den USA, ST 80-MRSA-IV (European Clone) Isolate meist in Europa und dem Nahen Osten und Isolate vom ST 93-MRSA-IV (Queensland Clone) in Australien detektiert (Vandenesch et al., 2003). Eine aktuellere Studie zeigt die Ausbreitung dieser Stämme über Länder- und Kontinent-Grenzen hinaus sowie neu erfasste Klone (wie z. B. ST 766 oder ST 377) (Tristan et al., 2007).

Hämolysin Alpha

Hämolysin Alpha (Hla), auch Alpha-Toxin genannt, ist ein weiterer Virulenzfaktor von *S. aureus*. Es wird von den Allelen des Gens *hla* kodiert, ist chromosomal lokalisiert und in allen CCs vorhanden. *Hla* wurde zu Beginn der 1980er Jahre entdeckt (Kehoe et al., 1983) und 1984 vollständig sequenziert (Gray und Kehoe, 1984). Das exprimierte Protein ist ein Exotoxin (33 kDa) mit hämolytischer Wirkung. Es werden 1-2 nm große transmembrane Poren gebildet, indem das Bakterium Hla-Monomere sekretiert und diese sich zu zylindrischen Heptameren in der Membran der Zielzelle formen. Zielzellen sind vorwiegend Erythrozyten (Valeva et al., 1997). Durch die entstandenen Poren kommt es, bedingt durch den Efflux von K^+, Na^+ und Ca^{2+}, zu einem Ungleichgewicht im Ionenhaushalt. Klinisch kann das zu dermonekrotischen Krankheitsbildern führen (Bhakdi und Tranum-Jensen, 1991; McCormick et al., 2009; Bubeck Wardenburg et al., 2007). Eine Infektion mit einem Hla-exprimierenden *S. aureus* bei Tieren, wie z. B. bei Kaninchen, zeigt oft eine noch stärkere Lyse der Erythrozyten als bei Menschen (Dinges et al., 2000).

Mikroarray-Technologie – eine vielseitig anwendbare Plattform

Die Mikroarray-Technologie ist eine molekularbiologische Methode, die den gleichzeitigen Nachweis vieler verschiedener Parameter (Multiplex) in einer biologischen Probe ermöglicht. Zudem können Untersuchungen auf DNA- und Proteinebene mit derselben Technik zeitgleich durchgeführt werden. Die Experimente dieser Arbeit zur Genotypisierung von *S. aureus* (Publikation I und II) und der Analyse von Staphylokokken-Toxinen (Publikation III-VI) wurden mit solchen Mikroarrays durchgeführt. Generell sind Mikroarrays im Vergleich zu anderen Methoden schnell und einfach zu handhaben, kostengünstig und ohne größere Laborgeräte einsetzbar.

DNA-Mikroarrays

Speziell entwickelte DNA-Mikroarrays ermöglichen eine detaillierte Untersuchung des *S. aureus* Genoms. Dafür sind DNA-Fragmente von Speziesmarkern, Resistenz- und Virulenzgenen als Sonden auf den Mikroarray gespottet. Durch Hybridisierung der markierten Bakterien-DNA werden diese Gene geprüft und bei Anwesenheit nachgewiesen. Zusätzlich kann über das entstandene Hybridisierungsmuster auf dem Mikroarray eine Typisierung des getesteten Isolats vorgenommen werden, indem das Muster mit Referenzexperimenten einer internen Datenbank abgeglichen und zugeordnet wird. Mit der gleichzeitigen Information über klinisch relevante Gene und der Typisierung eines Isolates haben DNA-Mikroarrays einen deutlichen Vorteil gegenüber klassischen Typisierungsmethoden wie MLST oder Spa-Typisierung. Diese beruhen auf der Analyse von Sequenzpolymorphismen einzelner Gene und liefern keine Information über die Anwesenheit relevanter MGEs (wie z. B. PVL-Phagen oder SCC*mec*-Elementen). Diese müssen in separaten Tests nachgewiesen werden (Monecke und Ehricht, 2005; Monecke et al., 2008). Die Sequenzierung vollständiger bakterieller Genome kann alternativ ebenso Informationen über alle Virulenz- und Resistenzgene liefern und auch über typisierungsrelevante Gene Aufschluss bringen. Aufgrund der Menge der Daten ist deren Auswertung jedoch sehr aufwändig und zeitintensiv.

Das Prinzip des Nachweises der Hybridisierung auf den DNA-Mikroarrays beruht auf der Bindung zwischen Biotin und Streptavidin sowie einer Peroxidase-katalysierten lokalen Fällungsreaktion. Mit der bakteriellen DNA wird eine lineare Amplifikationsreaktion durchgeführt. Dabei werden in die amplifizierten DNA-Abschnitte biotinylierte dUTPs eingebaut. Diese biotinylierte DNA wird auf den Mikroarray gegeben und komplementäre DNA-Fragmente binden aneinander. Diese Bindung wird durch Streptavidin-Peroxidase-Komplexe visualisiert. Das Streptavidin bindet die hybridisierte biotinylierte DNA und die

Peroxidase setzt das zugegebene Substrat zu einem schwer löslichen Produkt um. Der sichtbare Niederschlag auf dem Mikroarray (dunkler Spot) stellt ein positives Testergebnis für dieses bestimmte Gen dar (Monecke und Ehricht, 2005) (Abbildung 3 A).

Protein-Mikroarrays

Protein-Mikroarrays bieten die Möglichkeit zur Analyse von Protein-Expression, -Regulation und -Interaktion. Speziell entwickelte Antikörper-Arrays erlauben sowohl den qualitativen (Publikation III und VI) als auch quantitativen Nachweis (Publikation IV und V) bestimmter Staphylokokken-Toxine. Diesem liegt jeweils eine spezifische Antigen-Antikörper-Bindung, gefolgt von einer Fällungsreaktion bzw. Fluoreszenzfärbung, zugrunde.

Für den Toxinnachweis wird eine Suspension, die das Toxin (Antigen) enthält, auf den Mikroarray gegeben und dieses wird spezifisch vom gespotteten Fangantikörper auf dem Mikroarray gebunden. Die Detektion erfolgt mit einem biotinylierten Detektionsantikörper, an den wiederum ein Streptavidin-Peroxidase-Komplex bindet. Die Peroxidase katalysiert ein zugegebenes Substrat und macht ein positives Ergebnis durch einen dunkel gefärbten Spot auf dem Mikroarray sichtbar (Abbildung 3B).

Eine alternative Färbung für Protein-Mikroarrays ist die Fluoreszenzfärbung. Dafür wird das Toxin (Antigen) ebenso auf den Mikroarray gegeben und spezifisch vom Fangantikörper und zusätzlich von einem biotinylierten Detektionsantikörper gebunden. Dann werden Streptavidin-gekoppelte Fluoreszenzbeads zugegeben, die über das Streptavidin an das Biotin im Antigen-Antikörper-Komplex binden. Die fluoreszierenden Beads können mit geeigneten optischen Geräten sichtbar gemacht und als positives Ergebnis detektiert werden (Abbildung 3 C).

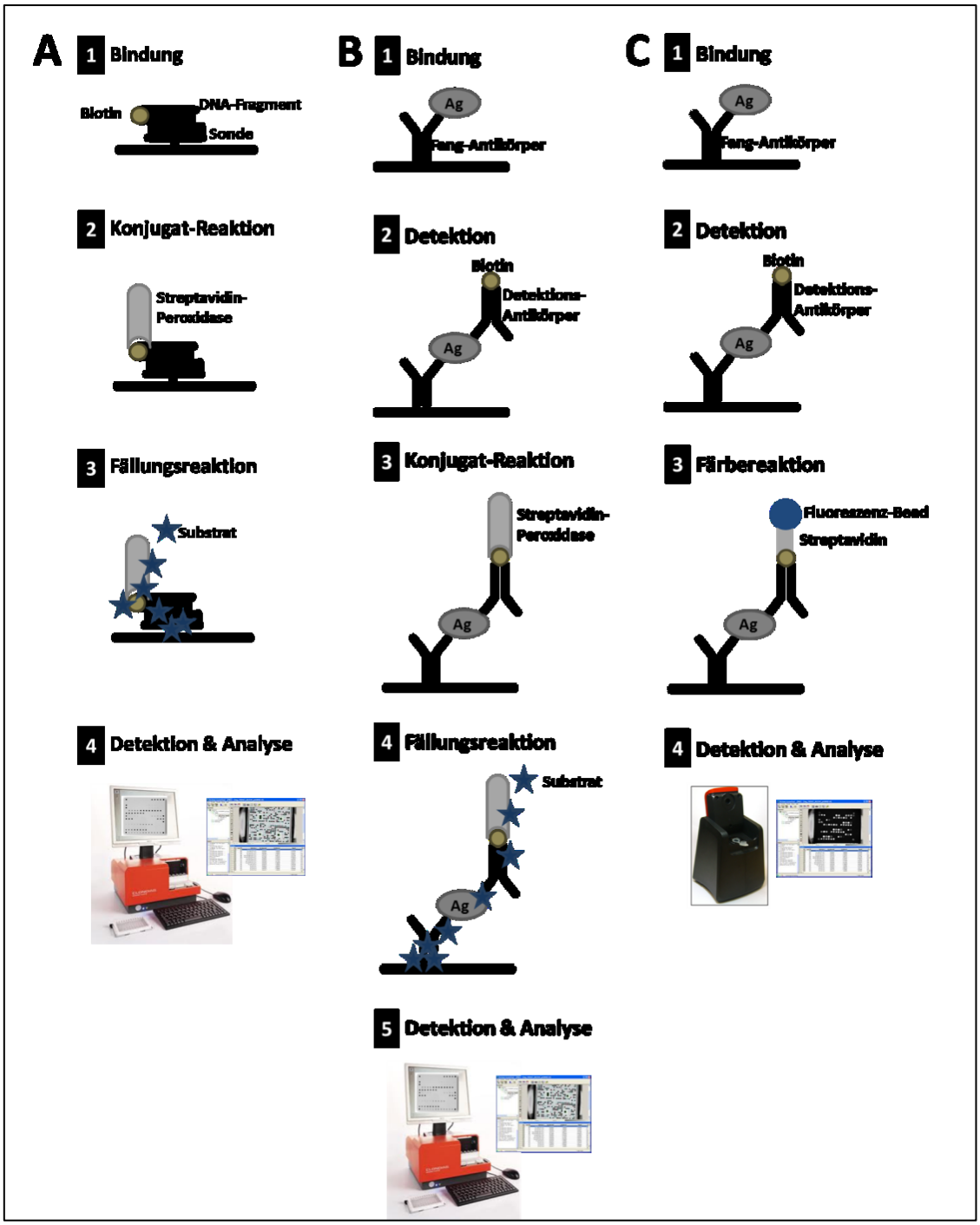

Abbildung 3: Verschiedene Nachweisreaktionen auf den Mikroarrays

A: Prinzip DNA-Mikroarray/ lokale Fällung; Hybridisierung Biotin-markierter DNA-Fragmente und Streptavidin-Peroxidase gekoppelte Fällungsreaktion

B: Prinzip Protein-Mikroarray/ lokale Fällung; Hybridisierung des Proteins (Antigen, Ag), Detektion über Biotin-markierten Detektionsantikörper und Streptavidin-Peroxidase gekoppelte Fällungsreaktion

C: Prinzip Protein-Mikroarray/ lokale Fluoreszenz; Hybridisierung des Proteins (Antigen, Ag), Detektion über Biotin-markierten Detektionsantikörper und Streptavidin-Fluoreszenz gekoppelte Färbereaktion

Technologie

Aus der Vielzahl verfügbarer Mikroarray-Systeme unterschiedlicher Anbieter (Heller, 2002; Jaluria et al., 2007) wurde für die Experimente dieser Arbeit die Mikroarray-Plattform der Firma Alere Technologies GmbH (Jena, Deutschland) verwendet. Je nach Array-Typ wurden spezifische DNA-Fragmente oder Proteine auf die modifizierte Glasoberfläche der Arrays gespottet. Abhängig vom Layout umfasst ein Array die Sonden in zwei- bis dreifacher Redundanz, zusätzliche Reaktionskontrollen und Referenzmarken.

Es gibt zwei unterschiedliche Plattformen der Mikroarrays: ArrayTubes und ArrayStrips (Abbildung 4). Bei den ArrayTubes ist der Mikroarray in ein 1,5 ml-Reaktionsgefäß eingebaut und es kann eine Probe pro ArrayTube bearbeitet werden. Der Mikroarray hat eine Größe von 3 mm x 3 mm und bietet Platz für bis zu 17 x 17 Sonden.
Bei den ArrayStrips sind 8 Mikroarrays pro ArrayStrip eingebaut. Bis zu 12 dieser ArrayStrips („96 well-Format“) können – ähnlich wie bei einer Mikrotiterplatte – parallel bearbeitet werden. Diese eignen sich somit besonders für einen hohen Probendurchsatz. Die Mikroarrays in ArrayStrips sind 4 mm x 4 mm groß und bieten Platz für bis zu 28 x 28 Sonden.

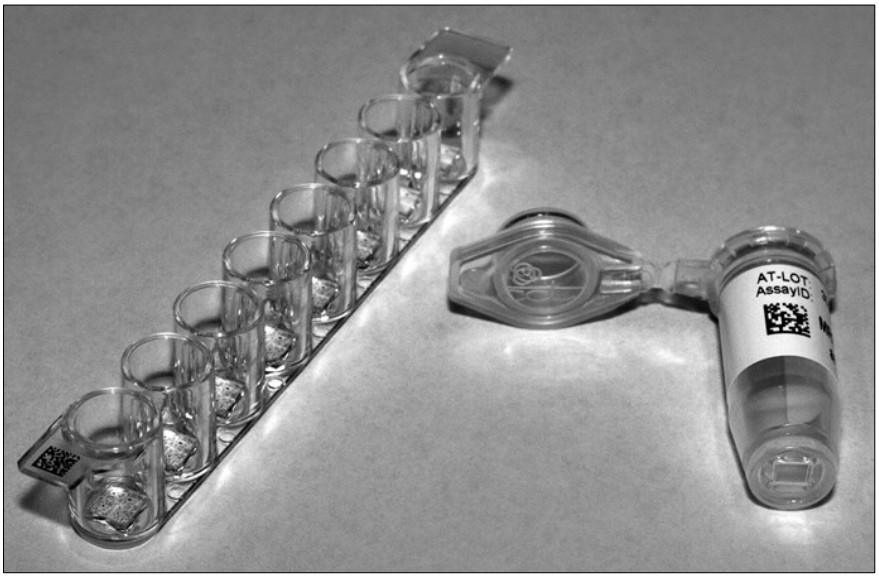

Abbildung 4: ArrayStrip (links) und ArrayTube (rechts). (Foto: S. Monecke)

Zum Auslesen der Mikroarrays werden eigens dafür entwickelte Geräte genutzt. Der ArrayMate-Reader (Abbildung 5A) nimmt die Arraybilder von ArrayTubes und ArrayStrips auf und wertet über einen integrierten PC mit der speziell dafür entwickelten IconoClust-Software die Bilder automatisch aus. Beim ATRFluo-Reader (Abbildung 5B) handelt es sich um ein Fluoreszenz-Auslesegerät für ArrayTubes. Die aufgenommenen Bilder werden über eine USB-Verbindung an einen PC geleitet und durch die IconoClust-Software ausgewertet.

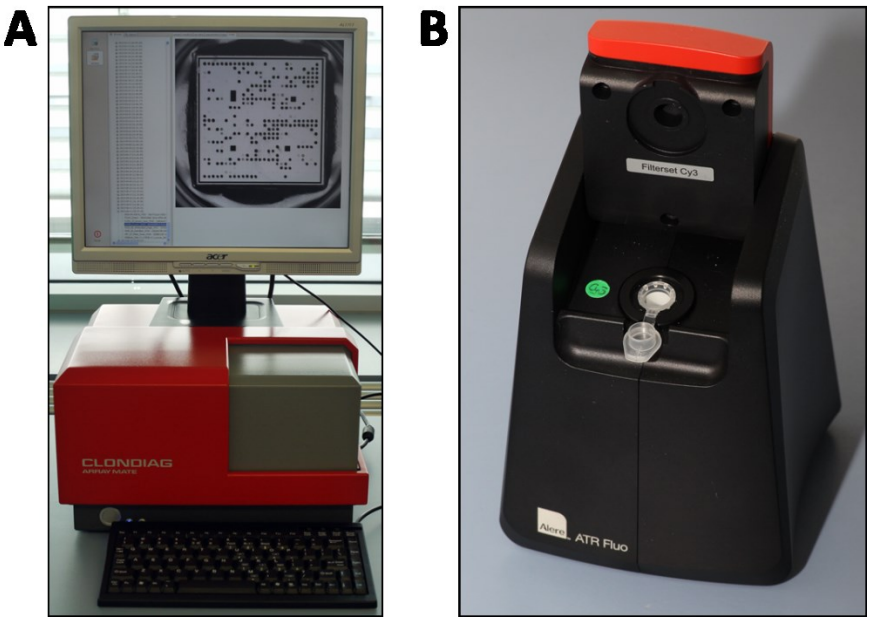

Abbildung 5: Auslesegeräte für die Mikroarrays

A: ArrayMate-Reader; **B:** ATRFluo-Reader (Fotos: S. Monecke)

Ziele der Arbeit

Der Entwicklung, Validierung und Herstellung von Schnelltests sind von großer Bedeutung, um klinisch relevanter Keime in Referenz- und Diagnostiklaboren bestimmen zu können. Für Erregernachweise bei Infektionskrankheiten, Infektionskontrollen sowie Überwachung und Verfolgung von Ausbrüchen in Krankenhäusern sind geeignete Testverfahren erforderlich. Die geno- und phänotypische Charakterisierung von Bakterien wie *S. aureus* kann mittels spezifisch hergestellter Mikroarrays durchgeführt werden und dient in dieser Dissertation zur Erarbeitung folgender Zielstellungen:

- Genotypisierung von *S. aureus* Isolaten zweier verschiedener Populationen, um zur Aufklärung der geografischen Verteilung von *S. aureus* Stämmen beizutragen (Publikationen I & II)

- Entwicklung eines Lateral Flow (Schnell)-Tests zum Nachweis des *S. aureus* Virulenzfaktors PVL (Publikation III)

- Entwicklung eines Protein-Mikroarray-Assays zur quantitativen Messung der Toxin-Expressionen von PVL und Hla (Publikationen IV und V)

- *In vitro*-Untersuchung der Zusammenhänge zwischen Toxin-Expression und genetischem Hintergrund sowie der Schwere des ausgelösten klinischen Bildes einzelner Isolate (Publikationen IV und V)

- Entwicklung eines Multiplex-Protein-Mikroarrays zum parallelen phänotypischen Nachweis mehrerer *S. aureus* Toxine (Manuskript I)

Wesentliche Beiträge zu den Arbeiten leisteten Kollegen aus den folgenden Institutionen:

- Institut für Medizinische Mikrobiologie und Hygiene, Technische Universität Dresden

- Alere San Diego Inc., California, USA

- Alere Scarborough Inc., Maine, USA

- Department of Microbiology and Infectious Diseases, PathWest Laboratory Medicine WA, Royal Perth Hospital, Perth, Australia

- Department of Paraclinical Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago

- Department of Microbiology and Infection Control, Akershus University Hospital, Akershus, Norwegen

Übersicht der Publikationen

Publikation I

Population Structure of Staphylococcus aureus from Trinidad & Tobago

Stefan Monecke (SM), Bettina Stieber (BS), Rashida Roberts (RR), Patrick Eberechi Akpaka (PEA), Peter Slickers (PS) & Ralf Ehricht (RE)

PLoS ONE, 2014, 9(2): e89120.

(Publiziert)

In dieser Publikation beschreiben wir die Untersuchung von *S. aureus* Isolaten, die in verschiedenen Krankenhäusern in Trinidad und Tobago aufgetreten sind. Zur Typisierung der vorkommenden *S. aureus* Stämme wurden spezifische DNA-Mikroarrays benutzt. Die Auswertung der Daten zeigt im Vergleich zu früheren Daten ein vermehrtes Auftreten von MRSA- und PVL-positiven Stämmen. Die beobachtete Populationsstruktur lässt auf eine Verbreitung afrikanischer Stämme (ST72 und CC152) sowie des in Nordamerika epidemischen Stamms CC8-MRSA-IV „USA300“ in dieser Region schließen.

Autorenbeiträge:

Idee und Planung: SM, PEA, RE

Durchführung der Experimente: BS, RR

Datenanalyse: SM, RE

Bereitstellung von Materialien/Isolaten: PEA, RR

Bioinformatik: PS

Schreiben des Manuskripts: SM, BS, RR, PEA, PS, RE

Zusätzlich ergänzende Informationen („Full demographic data and hybridisation profiles“) zum Manuskript sind online unter folgendem Link zu finden:

http://s3-eu-west-1.amazonaws.com/files.figshare.com/1391822/File_S1.pdf

Publikation II

Characterization of PVL-positive MRSA from Norway

Stefan Monecke (SM), Hege Vangstein Aamot (HVA), Bettina Stieber (BS), Antje Ruppelt (AR) & Ralf Ehricht (RE)

Acta Pathologica; Microbiological et Immunologica Scandinavica (APMIS), 2013, 122(7): 580-4.

(Publiziert)

In dieser Publikation haben wir im Krankenhaus von Akershus aufgetretene *S. aureus* Isolate mittels DNA-Mikroarrays typisiert. Dabei lag der Fokus auf dem Vorkommen und der Verbreitung PVL-positiver MRSA. In Norwegen spielen HA-MRSA aufgrund strikter Kontrollen keine bedeutende Rolle. CA- und/oder PVL-positive MRSA treten gelegentlich auf, ein epidemisch auftretender *S. aureus* Stamm konnte in dieser Population nicht gefunden werden. Viele Isolate gehörten zu Stämmen, die in verschiedenen Reiseländern als epidemisch bekannt sind und für 14% der Patienten konnten entsprechende Reiseanamnesen erfasst werden.

Autorenbeiträge:

Idee und Planung: SM, HVA, RE

Durchführung der Experimente: BS, AR

Datenanalyse: SM, RE

Bereitstellung Materialien/Isolaten: HVA

Schreiben des Manuskripts: SM, HVA, BS, AR, RE

Zusätzlich ergänzende Informationen („Clinical and epidemiological data as well as complete microarray hybridization profiles for all isolates included“) zum Manuskript sind online unter folgendem Link zu finden:

http://onlinelibrary.wiley.com/store/10.1111/apm.12181/asset/supinfo/apm12181-sup-0001-SupplementalFile.pdf?v=1&s=467e8d93854e6820c4298fe1789bf42d475b282a

Publikation III

Rapid Detection of Panton-Valentine Leukocidin in *Staphylococcus aureus* Cultures by Use of a Lateral Flow Assay Based on Monoclonal Antibodies

Stefan Monecke (SM), Elke Müller (EM), Joseph Buechler (JB), John Rejman (JR), Bettina Stieber (BS), Patrick Eberechi Akpaka (PEA), Dirk Bandt (DB), Rob Burris (RB), Geoffrey Coombs (GC), G. Aida Hidalgo-Arroyo (GAHA), Peter Hughes (PH), Angela Kearns (AK), Sonia Molinos Abós (SMA), Bruno Pichon (BP), Leila Skakni (LS), Bo Söderquist (BSö) & Ralf Ehricht (RE)

Journal of Clinical Microbiology, 2013, 51(2): 487-95.

(Publiziert)

In dieser Publikation beschreiben wir die Entwicklung eines phänotypischen Nachweistests für PVL. Für die lukF-PV Komponente wurden dafür monoklonale Antikörper hergestellt. Diese wurden mit einem dafür spezifischen Protein-Mikroarray überprüft, um die optimale Antikörper-Kombination für den Lateral Flow Assay zu finden. Zur Validierung des Lateral Flow Tests wurden PVL-positive *S. aureus* Isolate aus verschiedenen Teilen der Welt, mit verschiedenen Prävalenzen von PVL-MRSA, getestet, mit dem Ergebnis einer 99,7%igen Sensitivität und 98,3%igen Spezifität des Assays.

Autorenbeiträge:

Idee und Planung: SM, RE, BS

Durchführung der Experimente: EM, BS

Datenanalyse: SM, RE

Bereitstellung von Materialien: JB, JR, PEA, DB, RB, GC, GAHA, PH, AK, SMA, BP, LS, BSö

Schreiben des Manuskripts: alle Autoren

Zusätzlich ergänzende Informationen („Full datasets for the individual isolates, including results from lateral flow tests, PCRs, and array hybridization profiles“) zum Manuskript sind online unter folgendem Link zu finden:

http://jcm.asm.org/content/suppl/2013/01/17/JCM.02285-12.DCSupplemental/zjm999092258so1.pdf

Publikation IV

Development and Usage of Protein Microarrays for the Quantitative Measurement of Panton-Valentine Leukocidin

Bettina Stieber (BS), Stefan Monecke (SM), Elke Müller (EM), Vico Baier (VB), Geoffrey W. Coombs (GWC) & Ralf Ehricht (RE)

Molecular and Cellular Probes, 2014, 28(4): 123-32.

(Publiziert)

In dieser Publikation haben wir die Entwicklung eines Assays zur quantitativen Messung von PVL beschrieben. Dafür wurden speziell lukF-PV Protein-Mikroarrays hergestellt und mittels Referenzexperimenten eine Berechnungsgrundlage hinterlegt. Eine breite Auswahl an PVL-positiven *S. aureus* Isolaten wurde getestet. Die resultierenden PVL-Konzentrationen können in Zusammenhang mit dem klonalen Komplex des Stammes gebracht und analysiert werden. Die Technik kann zudem für die Untersuchung von Einflüssen anderer Toxine und Substanzen verwendet werden.

Autorenbeiträge:

Idee und Planung: BS, SM, VB, RE

Durchführung der Experimente: BS, EM

Datenanalyse: BS, SM

Bereitstellung von Materialien: GWC

Bioinformatik, Statistik: VB

Schreiben des Manuskripts: BS, SM, EM, VB, GWC, RE

Publikation V

Staphylococcus aureus In Vitro Secretion of Alpha Toxin (hla) Correlates with the Affiliation to Clonal Complexes

Stefan Monecke (SM), Elke Müller (EM), Joseph Büchler (JB), Bettina Stieber (BS) & Ralf Ehricht (RE)

PLoS One, 2014, 9(6): e100427.

(Publiziert)

In dieser Publikation wird die quantitative Messung des vom *S. aureus* gebildeten Alpha Toxin (Hla) dargestellt. Dafür wurden anti-Hla-Antikörper basierte Mikroarrays hergestellt und eine Vielzahl verschiedener *S. aureus* Isolate getestet. Es wurde deutlich, dass die Alpha Toxin Produktion vom klonalen Komplex des jeweiligen *S. aureus* Isolates abhängig ist.

Autorenbeiträge:

Idee und Planung: RE, SM, BS

Durchführung der Experimente: EM, BS

Datenanalyse: RE, SM

Bereitstellung von Reagenzien, Materialien, Analysetools: JB

Schreiben des Manuskripts: RE, SM, BS, EM, JB

Zusätzlich ergänzende Informationen („Alignment of *hla* sequences"") zum Manuskript sind online unter folgendem Link zu finden:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4062534/bin/pone.0100427.s001.fas

Manuskript I

Direct, Specific and Rapid Detection of Staphylococcal Proteins and Exotoxins using a Multiplex Antibody Microarray

Bettina Stieber (BS), Stefan Monecke (SM), Elke Müller (EM), Joseph Büchler (JB) & Ralf Ehricht (RE)

In Vorbereitung zur Einreichung

In diesem Manuskript ist die Entwicklung eines Multiplex-Protein-Mikroarrays beschrieben. Damit können verschiedene *S. aureus* Toxine (PVL, Hla, TSST-1, Beta-Hämolysin, Enterotoxine A und B und Staphylokinase) sowie ein *S. aureus* Speziesmarker (Spa) und Resistenz-vermittelndes Protein (PBP2a) in einem Experiment phänotypisch gleichzeitig und schnell nachgewiesen werden.

Autorenbeiträge:

Idee und Planung: RE, SM, EM, BS

Durchführung der Experimente: EM, BS

Datenanalyse: BS, RE, SM

Bereitstellung von Reagenzien, Materialien, Analysetools: JB

Schreiben des Manuskripts: BS, SM, RE

Zusätzliche Publikation

Während der Arbeiten an dieser Dissertation habe ich zur Erstellung einer weiteren Veröffentlichung beigetragen. Diese behandelt einen anderen Erreger und wurde daher hier nicht mit eingefügt.

Der Titel und die Autoren dieser Veröffentlichung sind:

Development of a Rapid Microarray-Based DNA Subtyping Assay for the Alleles of Shiga Toxins 1 and 2 of *Escherichia coli*

Lutz Geue, Bettina Stieber, Stefan Monecke, Ines Engelmann, Florian Gunzer, Peter Slickers, Sascha D. Braun & Ralf Ehricht

Journal of Clinical Microbiology, 2014, 52(8): 2898-904.

(Publiziert)

Publikation I

OPEN ACCESS Freely available online

PLOS ONE

Population Structure of *Staphylococcus aureus* from Trinidad & Tobago

Stefan Monecke[1,2]*, Bettina Stieber[1,2], Rashida Roberts[3], Patrick Eberechi Akpaka[3], Peter Slickers[2], Ralf Ehricht[2]

1 Institute for Medical Microbiology and Hygiene, Technical University of Dresden, Dresden, Germany, **2** Alere Technologies GmbH, Jena, Germany, **3** Department of Para-Clinical Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago

Abstract

It has been shown previously that high rates of methicillin- and mupirocin-resistant *Staphylococcus aureus* exist in the Caribbean islands of Trinidad and Tobago, as well as a high prevalence of Panton-Valentine leukocidin-positive *S. aureus*. Beyond these studies, limited typing data have been published. In order to obtain insight into the population structure not only of MRSA but also of methicillin-susceptible *S. aureus*, 294 clinical isolates collected in 2012/2013 were typed by microarray hybridisation. A total of 15.31% of the tested isolates were MRSA and 50.00% were PVL-positive. The most common MSSA strains were PVL-positive CC8-MSSA (20.41% of all isolates tested), PVL-positive CC152-MSSA (9.52%) and PVL-positive CC30-MSSA (8.84%) while the most common MRSA were ST239-MRSA-III&SCC*mer* (9.18%) and ST8-MRSA-IV, "USA300" (5.78%). 2.38% of characterised isolates belonged to distinct strains likely to be related to "*Staphylococcus argenteus*" lineages. The population structure of *S. aureus* isolates suggests an importation of strains from Africa, endemicity of PVL-positive MSSA (mainly CC8) and of ST239-MRSA-III, and a recent emergence of the PVL-positive CC8-MRSA-IV strain "USA300".

Citation: Monecke S, Stieber B, Roberts R, Akpaka PE, Slickers P, et al. (2014) Population Structure of *Staphylococcus aureus* from Trinidad & Tobago. PLoS ONE 9(2): e89120. doi:10.1371/journal.pone.0089120

Editor: Paul J. Planet, Columbia University, United States of America

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Competing Interests: S. Monecke, B. Stieber, and R. Ehricht are employees of Alere, the company that manufactures the tests described herein. This does not alter the authors' adherence to all the PLOS policies on sharing data and materials. The other authors have declared that no competing interests exist.

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Introduction

In Trinidad and Tobago, methicillin-resistant *Staphylococcus aureus* (MRSA) poses a public health risk [1,2,3,4]. MRSA prevalence is high and there is no strict isolation of patients with suspected MRSA-infection, as practised in hospitals elsewhere. A limited number of studies have been conducted on *S. aureus* for which the main focus has generally been MRSA. Swanston [5] performed a study in the Port of Spain General Hospital between June 1995 and May 1996 where the prevalence of MRSA was found to be 4.6%. Akpaka *et al.* [2] conducted a study of 1912 *S. aureus* isolates collected from major hospitals in the country between 2000 and 2001 and found that 12.8% of the isolates were methicillin-/oxacillin-resistant. In another study by Orrett [4] isolates were collected from various hospital and communal facilities in Trinidad between January 1, 1999 and December 31, 2004. It was found that the prevalence of MRSA from surgical/burn wounds, urine and pus/abscess were 60.1%, 15.5% and 6.6%, respectively. The major sources of MSSA were surgical/burn wounds, pus/abscess samples and upper respiratory tract specimens with rates of 32.9%, 17.1% and 14.3%, respectively. The vast majority of MRSA isolates from Trinidad and Tobago belong to the hospital-associated strain ST239-MRSA-III. Other strains were rare although the emerging "community-acquired" MRSA (CA-MRSA) strain "USA300" (ST8-MRSA-IV [PVL+/ACME+]) was also found [3]. Of special interest with regard to infection control is a high rate of mupirocin resistance among MRSA isolates [3,6].

Another issue is the frequent carriage of Panton-Valentine leukocidin genes, lukF-PV and lukS-PV, in S. aureus isolates from Trinidad and Tobago. Following a case of a child who died hours after admission despite being managed in the best intensive care unit of the country [7], further studies [8] revealed a high prevalence of PVL genes in isolates from skin and soft tissue infections, as well as a high proportion of PVL-positive, methicillin-susceptible S. aureus (MSSA) belonging to clonal complex (CC) 8.

In order to obtain an overview not only on MRSA but also on MSSA, clinical isolates from Trinidad and Tobago were collected in 2012/2013 and characterised by microarray hybridisation and, partially, by multi locus sequence typing (MLST).

Materials and Methods

Study sites

There are five main regional hospitals in Trinidad and Tobago, namely, Port of Spain General Hospital, Eric Williams Medical Sciences Complex (EWMSC), San Fernando General Hospital (SFGH), Sangre Grande Hospital and Scarborough General Hospital (SGH). Additionally, there are nine district health facilities and 97 health centres throughout the country besides several privately run hospitals. For the purpose of this study, isolates were collected at three major hospitals. Two of them

(EWMSC and SFGH) are located on the island of Trinidad, while the third one (SGH) is situated on Tobago.

The population served by EWMSC is about 600,000 and 19,029 admissions were recorded for the year 2011. Approximately 450 *S. aureus* strains were isolated, 20% of which were MRSA. The population served by the SFGH is also about 600,000. For 2011, there were 52,137 admissions. Over 700 *S. aureus* strains were isolated, 35% of which were MRSA.

SGH is Tobago's major health facility, being the only hospital located on the island during the period of the study. Supplemented by 18 health centres it serves a population of 55,000. During the study period, the hospital was still located on Fort Street. SGH was then a 100 bed facility that provided a variety of critical facilities such as Accident and Emergency, Medical and Surgical procedures. It has since been relocated to Signal Hill where a state of the art health care facility has been constructed.

Isolates and origin

S. aureus isolates for this study were recovered from routine clinical samples processed at the hospitals. The isolates were included in the study after being initially identified at the medical microbiological laboratories of the participating hospitals. Prior to shipping the isolates to Dresden, further tests were carried out at the laboratory of the Department of Para-Clinical Sciences to confirm the species of the isolates and determine their antibiotic susceptibilities. Standard laboratory methods (including the automated Microscan walkaway 96 system (Siemens, USA)) were used to identify these bacterial isolates according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

Microarray procedures

The Alere StaphyType DNA microarray (Alere Technologies GmbH, Jena, Germany) was used for the characterisation of the isolates. The array allows the detection of 333 genes including species markers, typing markers, resistance genes and exotoxins encoding sequences as well as an assignment to clonal complexes and strains. The protocols and procedures were described in detail in previous work [9,10].

In short, bacteria were grown on Columbia blood agar and incubated overnight at 37°C. The harvested staphylococci were suspended to enzymatic lysis by lysostaphin, lysozyme and RNAse A and digested using Proteinase K. Then, the extracted DNA was purified using an automated system (EZ1, Qiagen, Hilden, Germany).

The purified DNA was used as template for an iterated linear primer elongation using one primer per target. During linear amplification, all targets were labelled by internal incorporation of biotin-16-dUTP. Labelled and amplified single-stranded DNA was hybridised to the array, followed by washing and blocking. Horseradish-peroxidase-streptavidin was added which bound to biotin-labelled DNA. Positive reactions were visualised by the local precipitation of a dye by the peroxidase. An image of the microarray was taken and automatically analysed using dedicated reader and software (ArrayMate and Iconoclust, Alere Technologies).

MLST

Multilocus sequence typing (MLST) was performed by amplification and sequencing of housekeeping genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* using primers and reaction conditions that have been described previously [11]. However, alternative primers were used for the *aroE* gene in order to better fit a formerly published sequence (GenBank FR821777) of "*Staphylococcus argenteus*"-like strains [12]. These primer sequences were aro_fw_agt: GTCCAATTGAGCATTCTTTATCA and aro_rv_agt: CATACCTGCCGGAGTAGT. Resulting sequences were analysed using the software tools on the MLST website (http://saureus.mlst.net/).

Tree reconstruction

In order to visualise similarities (although not necessarily true phylogenetic relationships) between the hybridisation profiles, a network tree (Figure 1) was constructed using SplitsTree software [13]. Array hybridisation profiles of the tested isolates (see File S1) were converted into a series of 'sequences'. Each position in this 'sequence', *i.e.*, each target gene or allele, could have a value of 'positive' ('C'), 'negative' ('G') or 'ambiguous' ('A'). These 'sequences' were used with SplitsTree version 4.11.3 on default settings (characters transformation: uncorrected P/ignore ambiguous states, distance transformation: Neighbour-Net, and variance: ordinary least squares).

Results

294 clinical isolates collected in 2012/2013 were typed by microarray hybridisation. Demographic data as well as full array hybridisation profiles are provided as File S1.

Resistance markers

An overview on abundances of resistance genes is given in Table 1.

A total of 15.31% of the tested isolates proved to be *mecA*-positive. The occurrence of *mecA* across the different lineages is shown in Figure 1. SCC*mec* III was the most common type of SCC*mec* element, found in 27 isolates (all belonging to ST239). All these isolates were also positive for *merA/B*, indicating the presence of a composite SCC*mec* III/SCC*mer* element as previously described [14]. Two of these isolates harboured additional *ccrA/B-4* genes. SCC*mec* IV was detected in 15 isolates that were assigned to the "USA300" strain. Two additional isolates were identical except for a lack of *ccr* genes. SCC*mec* V was found only once in a CC5 strain.

Other common resistance markers were the beta-lactamase operon (*blaZ/I/R*; in 86.39% of isolates), *erm*(A) (in 9.86%, mostly ST239-MRSA-III), *msr*(A)/*mph*(C) (in 8.16% and 7.14%, respectively; mostly associated with "USA300") and *aphA3/sat* (in 15.31%, largely associated with ST239-MRSA-III and "USA300"). The gentamicin/tobramycin resistance gene *aacA-aphD* occurred in 9.52% of isolates that all belonged to ST239-MRSA-III or "USA300". A gene associated with mupirocin resistance, *mupA*, was detected in 5.78% of isolates. Among MSSA, the rate of *mupA*-positives was 3.61% (9 out of 249 isolates), while for MRSA it was 17.78% (8 out of 45). Other resistance markers were rare; *vanA* (vancomycin resistance) and *cfr* (linezolid resistance) were not found.

Virulence genes

An overview on abundances of virulence-associated genes is given in Table 2. Most notably, half (50.00%) of the tested isolates were PVL-positive (Figure 1).

Typing data

An overview on typing data is provided in Table 3 and in Figure 1. Therefore, clonal complexes and strains as well as their abundances are only discussed shortly here.

Clonal Complex 5 Strains. Twenty-three isolates belonged to CC5. Most (n = 14) were PVL-positive MSSA; one was an ACME-positive MRSA harbouring a SCC*mec* V element.

Clonal Complex 8 Strains. CC8 was the most common lineage and it largely contributed to the high prevalence of PVL in

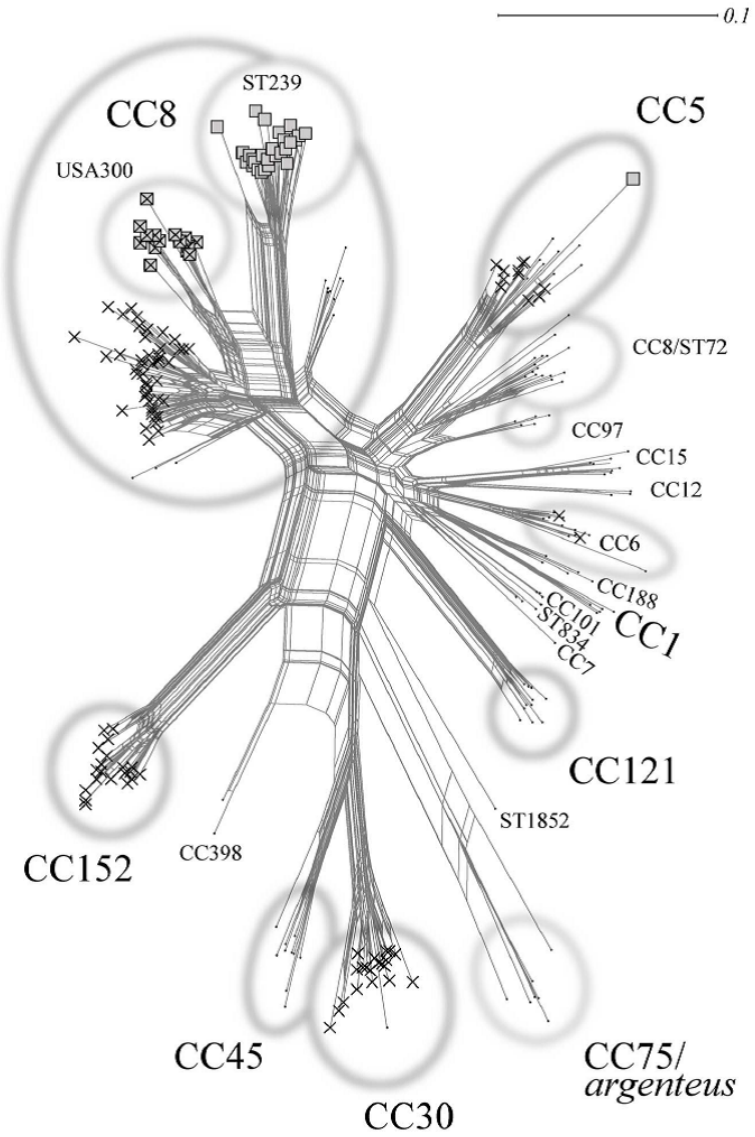

Figure 1. SplitsTree graph visualising strain assignments and similarities between isolates. Isolates were numbered as in file S1. *mecA* positives are indicated with squares, PVL-positives with crosses.
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the study group. 133 (45.24%; ST72 and ST239 included) of tested isolates belonged to this clonal complex and 20.41% of all isolates tested were PVL-positive CC8-MSSA. These isolates were virtually identical to each other. Out of the 60 isolates, 44 were indistinguishable using the current array, others differed only in some genes known to be localised on mobile elements (*e.g.*, *sed/j/r*, beta-lactamase operon and other resistance markers, genes associated with haemolysin beta-converting phages). Another 15 isolates (5.10%) were assigned to the "USA300" strain (ST8-MRSA-IV [PVL+/ACME+]). Two isolates (0.68%) were closely related to this strain but lacked *ccrA/B-2* genes. 9.18% of isolates belonged to ST239-MRSA-III, or, respectively, to a variant that additionally carried *ccrA/B-4* recombinase genes. The remaining CC8 isolates were either PVL-negative CC8 MSSA *sensu strictu*, or belonged to ST72. ST72 is a distinct lineage that differs from other CC8 strains in several traits such as in the presence of the *egc* enterotoxin gene cluster. All ST72 strains were MSSA although one harboured genes associated with a "SCC*fus*" element (*ccrA/B-1*, *fusC*), whilst another contained these genes plus the SCC-associated *kdp* (potassium translocating) locus.

Clonal complex 152. Roughly one-tenth of the collection, 28 isolates, was assigned to this CC. All were PVL-positive, and all were MSSA. Three of them carried enterotoxin genes *sek* and *seq*, which seems to be an unusual feature in CC152 that has not been noted in previously characterised isolates [8,9,15,16].

Table 1. Genes associated with antimicrobial resistance.

Gene	Gene product/explanation	All isolates	MSSA only	MRSA only
mecA	Alternate penicillin binding protein 2, defining MRSA	45 (15.31%)	0	45 (100%)
SCCmec I	Cassette chromosome elements SCC*mec*	0	0	0
SCCmec II		0	0	0
SCCmec III		25 (8.5%)	0	25 (55.56%)
SCCmec III+ccrA/B-4		2 (0.68%)	0	2 (4.44%)
SCCmec IV		15 (5.10%)	0	15 (33.33%)
SCCmec IV (trunc.)		2 (0.68%)	0	2 (4.44%)
SCCmec V		1 (0.34%)	0	1 (2.22%)
merA/B	Mercury resistance operon	31 (10.54%)	4 (1.61%)	27 (60%)
blaZ/I/R	Beta-lactamase operon	254 (86.39%)	211 (84.74%)	43 (95.56%)
erm(A)	Methyltransferases, erythromycin/clindamycin resistance	29 (9.86%)	2 (0.80%)	27 (60%)
erm(B)		0	0	0
erm(C)		0	0	0
lnu(A)	Lincosamid-nucleotidyltransferase	1 (0.34%)	1 (0.4%)	0
mef(A)	Macrolide efflux protein A	0	0	0
mph(C)	Probable lysylphosphatidylglycerol synthetase	21 (7.14%)	4 (1.61%)	17 (37.78%)
msr(A)	Energy dependent efflux of erythromycin	24 (8.16%)	7 (2.81%)	17 (37.78%)
aacA-aphD	Bifunctional enzyme Aac/Aph, gentamicin resistance	28 (9.52%)	0	28 (62.22%)
aadD	Aminoglycoside adenyltransferase, tobramycin resistance	12 (4.08%)	7 (2.81%)	5 (11.11%)
aphA3	Aminoglycoside phosphotransferase, neo-/kanamycin resist.	45 (15.31%)	2 (0.80%)	43 (95.56%)
fusB	Fusidic acid resistance gene (= *far1*)	1 (0.34%)	1 (0.4%)	0
fusC	Fusidic acid resistance gene from "SCC*fus*" elements	2 (0.68%)	2 (0.8%)	0
mupA	Mupirocin resistance protein	17 (5.78%)	9 (3.61%)	8 (17.78%)
tet(K)	Tetracycline resistance	29 (9.86%)	6 (2.41%)	23 (51.11%)
tet(M)	Tetracycline resistance	30 (10.20%)	3 (1.20%)	27 (60%)
cat	Chloramphenicol acetyltransferase	2 (0.68%)	1 (0.40%)	1 (2.22%)
fexA	Chloramphenicol/florfenicol exporter	0	0	0
cfr	Linezolid resistance	0	0	0
dfrS1	Dihydrofolate reductase type 1	1 (0.34%)	1 (0.4%)	0
qacA	Quaternary ammonium compound resistance protein A	22 (7.48%)	3 (1.2%)	19 (42.22%)
qacC	Quaternary ammonium compound resistance protein C	9 (3.06%)	6 (2.41%)	3 (6.67%)
sat	Streptothricine-acetyltransferase	45 (15.31%)	2 (0.80%)	43 (95.56%)
vanA	Vancomycin resistance gene	0	0	0

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"Alien", "Staphylococcus argenteus"-like strains. Seven isolates with deviant hybridisation profiles clustered into three distinct groups that were also confirmed by MLST. One group comprising five isolates yielded MLST sequences as well as hybridisation profiles similar to ST2250/2277. Capsule genes, *agr* genes and the coagulase gene *coA* were not detected. Other species-specific genes such as *spa*, *sbi* or *nuc1* were present as well as some adhesion factors including *sasG*. PVL or enterotoxin genes were absent. All isolates were *mecA*-negative although *blaZ* and *tet*(K) were found occasionally. A further alternate pattern was demonstrated for one isolate that yielded a hybridisation signal with one *agrB* III probe and with probes for *egc* enterotoxin cluster genes. It lacked *sasG* and its hybridisation profile was identical to a previously characterised single locus variant of ST1223 [8]. A third group comprised one isolate displaying hybridisations with probes for *agrB* III and capsule type 5 associated genes. It apparently lacked *coA*, *nuc1*, enterotoxin genes and *sasG*. Its MLST profile was a double locus variant of ST2596 (151-269-20-34-256-261-49).

Discussion

Typing of pathogenic bacteria is a prerequisite to understanding their population structure and dynamics, and thus to controlling them. This study gives a first insight in the population structure not only of MRSA but also of MSSA for a country for which only few typing data are currently available. Limitations are mainly caused by the need to retrospectively use isolates from diagnostic procedures. It was unfortunately not yet possible to collect prospectively and to obtain a collection of isolates from healthy carriers that could serve as a control group in order to analyse the frequencies of certain virulence markers with regard to clinical presentations. It was also not possible to retrieve full clinical and supporting epidemiological information; this was due to the lack of an electronic system to manage laboratory information in this area.

Generally, the population of *S. aureus* on Trinidad and Tobago might fall into four epidemiological categories that likely originate from different epidemic waves in the past.

Table 2. Genes associated with virulence.

Gene	Gene product/explanation	All isolates	MSSA only	MRSA only
agrI	Accessory gene regulator allele I	206 (70.07%)	162 (65.06%)	44 (97.78%)
agrII	Accessory gene regulator allele II	36 (12.24%)	35 (14.06%)	1 (2.22%)
agrIII	Accessory gene regulator allele III	33 (11.22%)	33 (13.25%)	0
agrIV	Accessory gene regulator allele IV	12 (4.08%)	12 (4.82%)	0
agr -	Unidentified *agr* allele or negatives	7 (2.38%)	7 (2.81%)	0
tst1	Toxic shock syndrome toxin 1	5 (1.70%)	5 (2.01%)	0
sea	Enterotoxin A	36 (12.24%)	15 (6.02%)	21 (46.67%)
sea (N315)/sep	Enterotoxin A (=P), allele from N315	14 (4.76%)	14 (5.62%)	0
seb	Enterotoxin B	24 (8.16%)	24 (9.64%)	0
sec/l	Enterotoxin C and L	20 (6.80%)	20 (8.03%)	0
sed	Enterotoxin D	63 (21.43%)	63 (25.30%)	0 (0%)
see	Enterotoxin E	0	0	0
seh	Enterotoxin H	5 (1.70%)	5 (2.01%)	0
sej	Enterotoxin J	64 (21.77%)	64 (25.70%)	0
sek/q	Enterotoxin K and Q	110 (37.41%)	67 (26.91%)	43 (95.56%)
ser	Enterotoxin R	63 (21.43%)	63 (25.30%)	0
egc	*egc* cluster (*seg, sei, selm, seln, selo, selu*)	90 (30.61%)	89 (35.74%)	1 (2.22%)
ORF CM14	Enterotoxin homologue	15 (5.10%)	15 (6.02%)	0
lukF/S-**PV**	Panton-Valentine leukocidin	147 (50%)	130 (52.21%)	17 (37.78%)
sak	Staphylokinase	255 (86.73%)	218 (87.55%)	37 (82.22%)
chp	Chemotaxis inhibiting protein (CHIPS)	158 (53.74%)	142 (57.03%)	16 (35.56%)
scn	Staphyl. complement inhibitor	266 (90.48%)	229 (91.97%)	37 (82.22%)
etA	Exfoliative toxin serotype A	11 (3.74%)	11 (4.42%)	0
etB	Exfoliative toxin serotype B	5 (1.70%)	5 (2.01%)	0
etD	Exfoliative toxin D	0	0	0
edinA	Epidermal cell differentiation inhibitor	13 (4.42%)	13 (5.22%)	0
edinB	Epidermal cell differentiation inhibitor B	27 (9.18%)	27 (10.84%)	0
edinC	Epidermal cell differentiation inhibitor C	4 (1.36%)	4 (1.61%)	0
ACME	ACME locus	18 (6.12%)	0	18 (40%)
cna	Collagen adhesin	136 (46.26%)	109 (43.78%)	27 (60%)
sasG	Staphylococcal protein G	198 (67.35%)	153 (61.45%)	45 (100%)
cap5	Capsule type 8 alleles	166 (56.46%)	148 (59.44%)	18 (40%)
cap8	Capsule type 5 alleles	122 (41.50%)	95 (38.15%)	27 (60%)
cap -	Unidentified capsule type or negatives	6 (2.04%)	6 (2.41%)	0

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First, there are MSSA strains that are related to Western African strains. These include ST72, CC152, and possibly others for which not few data are currently available (such as ST1852), as well as the "alien" or "*S. argenteus*"-like lineages. ST72 has been already found in West Africa [17] and, as MRSA, in the Caribbean [18] and in Latin America [9]. CC152 is known to exist in Gabon [19], Mali [20] and Nigeria [21]. ST1852 has been found in Gabon according to the MLST database (http://saureus.mlst.net/sql/fulldetails.asp?id=3702&send=240). The group of "alien" or "*S. argenteus*"-like lineages were most similar to a cluster of strains observed mainly in Australia that were assigned to MLST lineages ST75, ST883, ST1303 and ST1304. It has been proposed to merge the Australian strains to a new species "*S. argenteus*" [12] based on their lack of the "golden" pigment, or at least to a distinct subspecies. Other notable differences of this lineage compared to "normal" *S. aureus* include the lack of signals for the *agr* regulatory locus used on the array caused by an unknown sequence related to *agr* I and IV alleles [22], although some of the Trinidad isolates yielded *agrB* III signals. Other "alien" or "*S. argenteus*"-like strains have been found in Cambodia, in sporadic (possibly, travel associated) cases from Europe, in French Guayana and, in animals (Straw-Coloured Fruit Bat, *Eidolon helvum*) from Western Africa [23]. Historically, an importation of Western African strains appears plausible given that a large proportion of the population of Trinidad and Tobago has African roots, and that the forced transportation of Africans into the New World during previous centuries also resulted in a transfer of pathogens and diseases, such as malaria, leprosy, smallpox, measles or yellow fever virus [24] into the Americas.

The second group are lineages that are globally spread, *i.e.*, that could have been imported at anytime and from anywhere. Their geographic background and routes of dissemination might in

Table 3. Typing data.

CC	Strain	N (%)
CC1	CC1-MSSA	5 (1.70%)
CC5	CC5-MSSA	8 (2.72%)
	CC5-MSSA [PVL+]	14 (4.76%)
	CC5-MRSA-V [ACME+]	1 (0.34%)
CC6	CC6-MSSA	12 (4.08%)
	CC6-MSSA [PVL+]	2 (0.68%)
CC7	CC7-MSSA	4 (1.36%)
CC8	CC8-MSSA	13 (4.42%)
	CC8-MSSA [PVL+]	60 (20.41%)
	ST8-MRSA-IV [PVL+/ACME+]	15 (5.10%)
	ST8-MRSA-IV [PVL+/ACME+, truncated SCC*mec*]	2 (0.68%)
	ST72-MSSA	14 (4.76%)
	ST72-MSSA-SCC*fus*	1 (0.34%)
	ST72-MSSA-SCC*fus/kdp*	1 (0.34%)
	ST239-MRSA-III&SCC*mer*	25 (8.50%)
	ST239-MRSA-III+*ccrA/B4*	2 (0.68%)
CC9	ST834-MSSA	1 (0.34%)
CC12	CC12-MSSA	3 (1.02%)
CC15	CC15-MSSA	10 (3.40%)
CC20	CC20-MSSA	1 (0.34%)
CC30	CC30-MSSA	1 (0.34%)
	CC30-MSSA [PVL+]	26 (8.84%)
CC45	CC45-MSSA	10 (3.40%)
CC97	CC97-MSSA	5 (1.70%)
CC101	CC101-MSSA	3 (1.02%)
CC121	CC121-MSSA	12 (4.08%)
CC152	CC152-MSSA [PVL+]	28 (9.52%)
CC188	CC188-MSSA	5 (1.70%)
CC398	CC398-MSSA	2 (0.68%)
ST1852	ST1852-MSSA	1 (0.34%)
"Alien"/	CC1223/1594-MSSA	1 (0.34%)
CC75-like strains	ST2250/2277-MSSA	5 (1.70%)
	ST2596dlv-MSSA	1 (0.34%)

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future be elucidated by massive genome sequencing. CC5-, CC8-, CC30- and CC121-MSSA fall in this category; and these lineages have already been observed at Caribbean islands [18]. Most notable is the high prevalence of PVL-positive CC8-MSSA on Trinidad and Tobago. PVL-positive CC8-MSSA were very common on the islands, as noted previously [8], such that the causative agent from a recently reported fatal case of PVL-associated disease [7] was a rather typical specimen. As previously observed, the high prevalence of this strain in the Caribbean and its rarity in other parts of the world might indicate that the pandemic CC8 CA-MRSA strain "USA300" emerged in this region [8,25]. However, it is not yet clear whether the abundance of PVL-positive CC8-MSSA in Trinidad and Tobago is paralleled on other West Indian/Caribbean islands and in Latin America or if it indicates a local outbreak situation. The extremely high homogeneity of all isolates described in this study (see above) as well as in two previous studies [7,8] might support the latter theory.

A third group comprises ST239-MRSA-III and its variants. This is a globally spread healthcare-associated strain. It is very common in Trinidad and Tobago and is, or used to be, common in many other countries. Overall, it appears the MRSA rate has been rather constant in Trinidad and Tobago for the last decade, being 12.8% for isolates collected in 2001/2002 [2] and 15.2% in this study. The abundance of one single strain and its remarkable homogeneity suggests an outbreak scenario, *i.e.*, transmission is sustained following an importation event that may have occurred several years ago. This was, for the same strain, observed also in Ireland where ST239-MRSA-III became the predominant strain in the 1980s [26], after repatriation of a single infected patient [27]. Since this strain is strictly hospital-associated, the implementation of stringent hospital infection control measures could substantially reduce the burden of MRSA to the healthcare systems on Trinidad and Tobago [3].

Finally, a forth group consists of recently emerging, possibly community-acquired, MRSA. In Trinidad and Tobago this is almost exclusively the PVL/ACME-positive ST8-MRSA-IV strain "USA300". Interestingly, on other Caribbean islands, other strains might fulfil this role. A recent study shows the presence of typical "French" CA-MRSA clones on French West Indian islands [28]. This emphasises the importance of travel in the intercontinental transfer of epidemic clones. As mentioned above, it has been suspected that "USA300" emerged in this part of the world. However it is not yet clear where and when the SCC*mec* and the ACME elements were acquired. While ACME-negative PVL-positive ST8-MRSA-IV are often associated with Spain or Latin America, all isolates from Trinidad and Tobago were ACME-positives, *i.e.*, more likely to be linked to the U.S. or other regions of origin. In order to assess the significance of ACME as an epidemiological marker and its possible correlation with geographic background, more data are still needed, especially from other Caribbean countries.

Open questions for further studies are whether CA-MRSA, *i.e.*, "USA300", can replace the related PVL-positive CC8-MSSA in direct competition under community conditions. This could be of high relevance for public health because such a development could render standard treatments ineffective. Another issue for future study is if "USA300" can compete with hospital-associated MRSA strains in hospitals and if it can spread in healthcare settings. In Northern America this was apparently the case, while in Germany or the U.K. one predominant strain (CC22-MRSA-IV, "Barnim Epidemic Strain/UK-EMRSA-15") is by now so firmly rooted in healthcare facilities that it apparently hinders the spread of other strains including "USA300". Currently, the rate of "USA300" in Trinidad and Tobago appears to be increasing. In a previous study, only three out of 80 MRSA isolates (3.75%) belonged to that strain [3]. In the present study, 17 out of the 45 MRSA isolates (37.78%) belonged to "USA300" (or to a *ccrA/B-2*-negative variant thereof). This warrants further studies and on-going surveillance. Future epidemiological studies should also focus on the population outside of healthcare facilities because a high burden of infections with both, MSSA and MRSA, can be expected in community settings in Trinidad and Tobago.

Supporting Information

File S1 Full demographic data and hybridisation profiles.
(PDF)

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Author Contributions

Conceived and designed the experiments: SM PEA RE. Performed the experiments: RR BS. Analyzed the data: SM RE. Contributed reagents/materials/analysis tools: PEA RR. Wrote the paper: SM RR. Bioinformatic work, probe and primer design: PS.

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Publikation II

APMIS ACTA PATHOLOGICA, MICROBIOLOGICA ET IMMUNOLOGICA SCANDINAVICA

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Characterization of PVL-positive MRSA from Norway

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Norway is a country in which the Methicillin-resistant *Staphylococcus aureus* (MRSA) prevalence has been low for the last decades. There are virtually no epidemic, hospital-acquired MRSA because of an emphasis on strict infection control rules and restrictive use of antibiotics. However, community-acquired and/or Panton-Valentine leucocidin (PVL)-positive MRSA need to be monitored as these strains are transmitted outside of healthcare facilities and cannot be contained by healthcare-centred strategies. All 179 non-repetitive isolates of PVL-positive MRSA that were received during 2011 at the regional infection control laboratory at Akershus University Hospital were preserved and *spa* typed. Seventy isolates were further characterized by DNA microarray hybridization. The most common PVL-MRSA lineages were ST8-MRSA-IV and CC30-MRSA-IV. Further common clones were CC80-MRSA-IV and CC5-MRSA-IV. Other clones were found sporadically. These included ST772-MRSA-V and ST834-MRSA-IV, the latter in patients with epidemiological connections to the Philippines. Small-scale family outbreaks affecting at least 49 individuals were noted, with numbers of known cases per outbreak ranging from two to seven. At least 24 cases were related to foreign travel to Eritrea, India, Iraq, Macedonia, Pakistan, the Philippines, Poland, Singapore, Turkey, the USA and Vietnam. These data show that community-acquired/PVL-positive MRSA are not yet a major public health problem in Southern Norway. Our study corroborates the current practice of mandatory screening of patients and staff with travel histories, admissions or employment in healthcare institutions outside the Scandinavian countries or with known MRSA contacts.

Key words: Community-acquired MRSA; DNA microarray; molecular fingerprinting; MRSA; Norway; Panton-Valentine leucocidin.

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Norway, together with the Netherlands and the other Nordic countries, has a restrictive use of antibiotics and an extensive surveillance and infection control program (1). Among blood culture isolates of *Staphylococcus aureus*, the Methicillin-resistant *Staphylococcus aureus* (MRSA) rate is between 0.4% and 1% for the years 2007–2011. In absolute numbers, this amounts to five individual cases nationwide in, e.g., 2011 [http://www.vetinst.no/eng/Publications/Norm-Norm-Vet-Report; (2)]. However, there was a significant increase in the incidence rate for MRSA infections and for all MRSA cases in humans in Norway from 2010 to 2011 (2). This was caused by an increase in community-acquired MRSA (caMRSA) infection or colonization. Currently, nearly 70% of MRSA cases are diagnosed in the community, while only 20% were inpatients and the rest being nursing home residents and healthcare workers (2). The rise in caMRSA and/or PVL-positive MRSA (PVL-MRSA) is of concern as these strains are transmitted outside hospitals and thus cannot be contained by hospital or healthcare-centred strategies. Panton-Valentine leucocidin (PVL) is a phage-encoded, bi-component toxin (3). Its existence was reported as early as 1932 (4), but PVL-MRSA emerged only rather recently (5). Regardless of their resistance phenotype, PVL-positive *S. aureus* are commonly associated with chronic/recurrent skin and soft tissue infections as well as rare but severe necrotizing pneumonia (6), often in previously healthy young people.

To characterize and identify potential major clones, all PVL-positive MRSA isolates received for genotyping at Akershus University Hospital in 2011

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were collected. This yielded 179 isolates, thus representing roughly 17% of all MRSA isolates registered in Norway during that year. Isolates were further genotyped by *spa* sequencing and a representative sample was characterized in detail by DNA microarray hybridization.

MATERIALS AND METHODS

Setting

Akershus University Hospital administers a regional laboratory for infection control and receives MRSA isolates for genotyping from microbiology laboratories situated in Akershus, Oslo and Østfold counties. These laboratories serve a population of approximately 1.5 million inhabitants, which is almost 1/3 of the population in Norway. All 179 PVL-positive MRSA received at Akershus University Hospital in 2011 were included.

spa typing

The *spa* typing is based on the variations in numbers as well as in sequence of short repeating units within the X region of the gene encoding the staphylococcal protein A (*spa*). It was performed according to previously published protocols (7) using the nomenclature as described on the Ridom website (http://spa.ridom.de/). For 109 isolates that were not further characterized by microarray, the affiliation to clonal complexes was determined based on the analysis of the *spa* type.

PCR procedures

A PCR for the detection of PVL was performed on all isolates according to manufacturer's instructions for FAST applications (Applied Biosystems/Life Technologies, Oslo, Norway) using an in-house real-time PCR protocol (8) with the minor groove binder (MGB) probe 5′-6FAM-CAGAATTT ATTGGTGTCCTATC-3′, a previously published forward primer (9) and the reverse primer (5′-TT TTGCAGCG TTTTGTTTTCG-3′).

Another PCR was performed to screen *edinB* positive/*etD*-negative isolates (CC152) for an exfoliative toxin homologue, putatively named '*etD2*' that was found to accompany *edinB* in CC130 genome sequences [GenBank AEUQ01000009.1; bases 161312–162153 and AEUR 01000016.1; 44379–45221 (10)]. PCR primers were 01_etd2_fw (5′-TCAAGACACCACTAGAAGTC-3′) and 02_etd2-rev (5′-CGTTTTCAGCTAATCGTGC-3′), yielding a PCR product of ca. 130 bp. The PCR included an initial denaturation step of 2 min at 94 °C, 35 cycles (30 s at 94 °C, 30 s at 52 °C, 30 s at 72 °C) and a final elongation (5 min at 72 °C). Strains COL (GenBank CP000046) and N315 (GenBank BA000018) were used as negative controls, and the CC130-MRSA-XI strain M10/0061 (11) as a positive control.

Microarray procedures

Seventy isolates were selected for microarray hybridization aiming on coverage of all clonal complexes (CC) and *spa* types. The arrays as well as all related procedures have previously been described in detail (12). In short, a linear primer elongation reaction is used to simultaneously label and amplify 333 target sequences corresponding to about 170 genes. This includes resistance markers, SCC*mec*-associated markers, toxin genes and genes encoding adhesion factors. After array hybridization and scanning, the absence or presence of these genes, the SCC*mec* type and the affiliation to clonal complexes, SCC*mec* types and epidemic strains are determined automatically (12).

RESULTS

A total of 179 PVL-positive MRSA were identified by susceptibility tests and PVL-PCR. All were *spa* typed. Seventy isolates were selected for microarray hybridization aiming on coverage of all clonal complexes (CC) and *spa* types. PVL-positive MRSA were assigned to CC1 (including CC1/ST772), CC5, CC8, CC9 (ST834), CC22, CC30, CC59, CC80, CC88 and CC93. An overview on clinically relevant markers in the different strains is provided in Fig. 1. The complete microarray hybridization data are provided as Table S1.

Clonal complex 1

Clonal complex 1 strains belonged to two distinct groups. One isolate from a dermatological condition

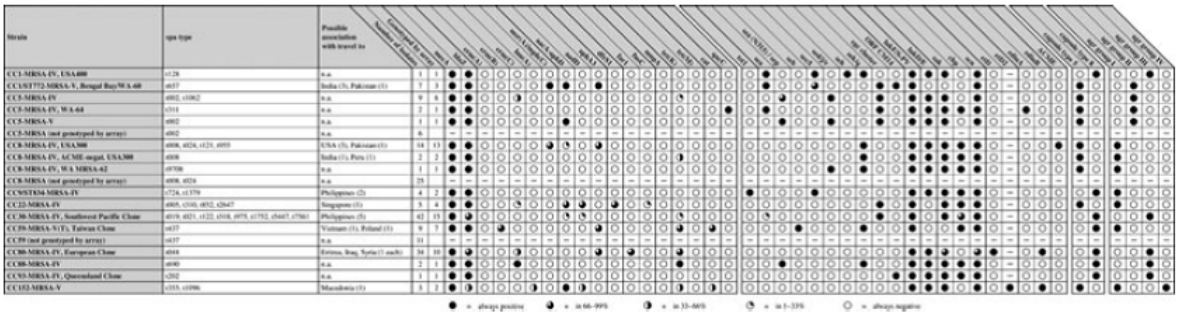

Fig. 1. Overview on detected strains, *spa* types, resistance- and virulence-associated markers.

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with a *spa* type t128 was highly similar to 'MW2-USA400', belonging to *agr* group III and capsule type 8; and carrying a SCC*mec* IV element. Seven isolates with *spa* type t657 were assigned to ST772-MRSA-V [PVL+], 'Bengal Bay Clone/WA MRSA-60'. This lineage differs in many properties from 'normal' CC1 strains, for instance, in belonging to *agr* group II and capsule type 5. Three genotyped isolates were rather multi-resistant harbouring *mecA, blaZ, msr*(A), *mph*(C), *aacA-aphD* and *aphA3*. This strain has been frequently associated with travel to the Indian subcontinent (12). Indeed, one of the seven cases has been hospitalized in Pakistan before, another one reported a putrid insect bite in India. Two isolates originated from one family with a history of childbirth in India.

Clonal complex 5

Eighteen isolates belonged to CC5, and three different strains were distinguished. The most common one was CC5-MRSA-IV, 'PVL-positive Paediatric clone', to which nine isolates were assigned. Seven isolates had *spa* type t002. One case had an unspecified travel history and three belonged to an outbreak affecting one family. Two epidemiologically linked isolates – one of them genotyped by array hybridization – were identified as t1062. Two further isolates from one family had another *spa* type, t311. One was genotyped by array. It was a variant of the 'Paediatric clone' that yielded positive signals for PVL genes as well as for *edinA*, thus being related or identical to the West Australian (WA) strain 'WA MRSA-64' (12, 13). One *spa* t002 isolate was assigned by array to CC5-MRSA-V. Six t002 isolates were not further genotyped.

Clonal complex 8

This was one of the two most abundant lineages. 42 PVL-MRSA isolates were assigned to CC8 because of *spa* types t008, t024, t121, or t9708. Array hybridizations were performed for 17 isolates. Fourteen isolates were ACME-positive and assigned to CC8-MRSA-IV, 'USA300'. One of them was possibly related to a journey to Pakistan and another one to travel to the USA. In the latter case, transmission to a secondary case was noted. Two isolates were ACME-negative, but otherwise identical to the 'USA300' strain. One was related to travel to Peru, the other one to a journey to India. Finally, one of the isolates (*spa* t9708) that was characterized by array hybridization was ACME-negative, but harboured additional enterotoxin genes (*sed/j/r*) thus resembling the West Australian strain CC8-MRSA-IV, 'WA MRSA-62' (12, 13).

Clonal complex 9

Four isolates with SCC*mec* type IV and *spa* types t724 or t1379, of which two were characterized by array hybridization, were assigned to CC 9 and sequence type 834. This lineage differs from other CC9 strains in several traits including the alleles of *agr* (*agr* group I instead of II) and of MSCRAMM genes (*bbp, map, vwb*), capsule type (8 rather than 5), *spa* type and the presence of *sasG*. Three isolates, all *spa* t724, one genotyped by array, originated from one family and the index case reported insect stings during travel to the Philippines. The *spa* t1379 isolate was identical by array hybridization. It was also related to a journey to the Philippines.

Clonal complex 22

Five isolates were found yielding *spa* types t005, t310, t852 and, twice, t2647. One isolate of each *spa* type was genotyped by array. They belonged to CC22-MRSA-IV. Three of these isolates (types t005, t852 and t2647) yielded signals for several resistance markers (*aacA-aphD, aadD. dfrS1*) as previously described for Middle Eastern isolates of that strain (14). One isolate (t310) carried the fusidic acid resistance determinant *fusC*/Q6GD50. For one isolate (t852), a travel history was noted, the presumed place of infection was Singapore.

Clonal complex 30

CC30-MRSA-IV [PVL+], 'Southwest Pacific Clone', was one of the two most common strains. Forty-two isolates were assigned to this complex based on *spa* types t019, t021, t122, t318, t975, t1752, t5447 or t7561, with *spa* type t019 being the most abundant one (33 isolates). Fifteen isolates were characterized by array hybridization being in accordance with previous descriptions of that strain (12). Variable resistance markers were the beta-lactamase operon (in 13 of 15 genotyped isolates) as well as *aacA-aphD* and *tet*(K), in one isolate each. In five cases, patients reported journeys to the Philippines and in one additional case an unspecified travel abroad was noted. Five transmissions within families, affecting two to three persons, were observed. In two additional episodes, family members of patients were found to carry untyped MRSA.

Clonal complex 59

Twenty isolates showed a *spa* type (t437) associated with CC59 in general and with ST59-MRSA-V(T) [PVL+], 'Taiwan Clone', in particular. Seven isolates were characterized by array hybridization. Six of

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them yielded profiles in accordance with that strain (12, 15) being also positive for *ugpQ*, *ccrC*, *'ccrAA'*, *erm*(B), *aphA3*, *sat*, *seb/k/q*. One was atypical, lacking *erm*(B), *aphA3* and *sat*, but was still more similar to the 'Taiwan Clone' than to other CC59-MRSA (12, 15). One case of an infection with CC59 was exposed to health care in Vietnam, three other cases belonged to one family.

Clonal complex 80

A total of 34 isolates belonged to CC80. All had *spa* type t044. Ten isolates were hybridized to microarrays and yielded hybridization patterns in accordance with CC80-MRSA-IV [PVL+], 'European caMRSA Clone'. As previously described, *aphA3*, *sat*, *tet*(K), *far1* as well as *etD* and *edinB* were usually present, while enterotoxin genes and *tst1* were absent. For three patients, travel histories to Iraq, Syria and Eritrea were noted. Two, possibly three, outbreaks were observed; one involved seven persons, another involved two persons. In a possible third transmission, a family member was found positive for MRSA, but no further typing data were retrievable.

Clonal complex 88

Two isolates had *spa* type t690 and thus were assigned to CC88. One was further characterized, and identified as CC88-MRSA-IV.

Clonal complex 93

One isolate belonged to CC93-MRSA-IV [PVL+], 'Queensland Clone', and *spa* type t202.

Clonal complex 152

Three isolates belonged to CC152 having *spa* types t355 or t1096. One isolate of each *spa* type was characterized revealing the presence of SCC*mec* V and *edinB* as well as the absence of enterotoxin genes. Both isolates were positive for '*etD2*', an exfoliative toxin homologue known from CC130, *Staphylococcus hyicus* (where it was named '*shetb*', GenBank AB036767.1) and *Staphylococcus pseudintermedius* ('*exi*' or '*expb*'; GenBank AB489850.1 and AB569087.1). For one isolate, a travel history to Macedonia was known and in another case, an MRSA infection of a relative was reported.

DISCUSSION

These data show that caMRSA/PVL-MRSA are not yet a major public health problem in Norway, given that less than 200 cases were identified in a region with a target population of approximately 1 500 000. The high number of different strains, the frequent association with travel histories and the observation of isolated cases or of small family-scale outbreaks indicate that there is no epidemic transmission of caMRSA/PVL-MRSA in Norway yet. There is no 'Norwegian Epidemic Strain', i.e., no regionally predominant strain such as 'USA300' in the United States or CC93-MRSA-IV [PVL+], 'Queensland Clone' in Australia (16).

Previously identified strains in Norway included PVL-positive ST8-MRSA-IV 'USA300' (17), ST30-MRSA-IV (18), ST80-MRSA-IV, which was the most common MRSA strain around the year 2000 (7) and ST152-MRSA-V (18) as well as PVL-negative ST8-MRSA-IV (12, 17), CC5/ST125-MRSA-IV (7, 17), ST22-MRSA-IV (18), ST45-MRSA-IV (18), ST239-MRSA-III (7) and some other sporadic, mainly SCC*mec* IV, clones (7, 18).

In many cases, travel histories are known suggesting importation. Often, travel histories are not known, but this does not rule out a foreign importation, but just highlights the impression that clinicians commonly do not ask for travel histories when no tropical disease was expected. Associations of some MRSA strains were observed with certain travel destinations (CC80-MRSA with Middle Eastern countries and Eritrea, CC1/ST772 with India, CC152 with the Balkans, CC30- and CC9/ST834-MRSA with the Philippines). While most published works on genotyping of *S. aureus*/MRSA refer to the USA, Western Europe and Australia, there are only few reports from other parts of the world (14, 19–21). Such data indicate that MRSA currently became a public health issue everywhere in the world, warranting more efforts in infection surveillance and control. As long as epidemiological data are lacking for a large part of the world, returning travellers might serve as sentinel population shedding a light on MRSA epidemiology in countries, for which often no concise typing data are available. Thus, our study corroborates the current practice of mandatory screening of patients and hospital staff with travel histories, previous admissions or employment in healthcare institutions outside the Scandinavian countries or with known MRSA contacts.

CONFLICTS OF INTEREST

S. Monecke, B. Stieber and R. Ehricht are employees of Alere Technologies, the company that manufactures some of the assays described herein.

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isolates. We also acknowledge A. Reissig, J. Sachtschal and E. Mueller (Jena) for excellent technical assistance as well as Prof. Dr. E. Jacobs (Dresden) and E. Ermantraut (Jena) for their support.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical and epidemiological data as well as complete microarray hybridization profiles for all isolates included.

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Publikation III

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Rapid Detection of Panton-Valentine Leukocidin in *Staphylococcus aureus* Cultures by Use of a Lateral Flow Assay Based on Monoclonal Antibodies

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Panton-Valentine leukocidin (PVL) is a virulence factor of *Staphylococcus aureus*, which is associated with skin and soft-tissue infections and necrotizing pneumonia. To develop a rapid phenotypic assay, recombinant PVL F component was used to generate monoclonal antibodies by phage display. These antibodies were spotted on protein microarrays and screened using different *lukF-PV* preparations and detection antibodies. This led to the identification of the optimal antibody combination that was then used to establish a lateral flow assay. This test was used to detect PVL in *S. aureus* cultures. The detection limit of the assay with purified native and recombinant antigens was determined to be around 1 ng/ml. Overnight cultures from various solid and liquid media proved suitable for PVL detection. Six hundred strains and clinical isolates from patients from America, Europe, Australia, Africa, and the Middle East were tested. Isolates were genotyped in parallel by DNA microarray hybridization for confirmation of PVL status and assignment to clonal complexes. The sensitivity, specificity, and positive and negative predictive values of the assay in this trial were 99.7, 98.3, 98.4, and 99.7%, respectively. A total of 302 clinical isolates and reference strains were PVL positive and were assigned to 21 different clonal complexes. In summary, the lateral flow test allows rapid and economical detection of PVL in a routine bacteriology laboratory. As the test utilizes cultures from standard media and does not require sophisticated equipment, it can be easily integrated into a laboratory's workflow and might contribute to timely therapy of PVL-associated infections.

Panton-Valentine leukocidin (PVL) is a phage-borne virulence factor of *Staphylococcus aureus*. It consists of two units (S and F components) encoded by two separate, although colocalized and coexpressed, genes (*lukS-PV* and *lukF-PV*; GenBank accession number BA000033.2; open reading frame [ORF] no. MW1378 and MW1379). Polymers of these molecules form pores in human leukocyte membranes, leading to cell death (1, 2) and cytokine release. Alternatively, low concentrations may induce apoptosis in granulocytes (3).

PVL is structurally, and in terms of sequence similarities, related to other leukocidins, such as *lukE–lukD* and *lukM–lukF-P83* in *S. aureus* and *lukF-int–lukS-int* in *S. intermedius*/*pseudintermedius*, and to the *hlgA–lukF–lukS* gamma-hemolysin/leukocidin locus *hlg*.

PVL is frequently detected in *S. aureus* isolates from skin and soft-tissue infections (SSTI) (4, 5) and is associated with chronic/recurrent infections, such as furunculosis, especially in young and previously healthy adults. PVL-positive *S. aureus* can also cause more severe diseases, such as necrotizing pneumonia. This condition is occasionally a complication of other respiratory tract infections, such as influenza virus, and its fatality rate can be as high as 40% (6). In contrast, PVL is rarely isolated in *S. aureus* from healthy carriers (7–9) or from isolates associated with other types of infections, such as bacteremia (10).

Although PVL was described in the 1930s (11), its existence as a potent leukotoxic toxin produced by some *S. aureus* strains was postulated already in the late 19th century (12). In the 1940s and 1960s, worldwide outbreaks of PVL-positive, methicillin-susceptible *S. aureus* were observed, and by the late 1990s, PVL-positive community-acquired methicillin-resistant *S. aureus* (CA-MRSA) had emerged (13, 14).

Because of its clinical relevance, the detection of *S. aureus* strains which carry the PVL genes warrants aggressive therapy and infection control measures. This includes, in addition to incision and drainage, appropriate antibiotic therapy and the use of mupirocin, in analogy to MRSA eradication protocols. In addition, it is recommended that patients with an acute infection due to PVL-

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TABLE 1 Simplified matrix for the identification of the optimal antibody combination[a]

Labeling antibody	Reactivity for capture antibody and antigen											
	1031			1061			1101			1321		
	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923
1031	0	(+)	0	0	0	0	0	+	0	0	(+)	0
1061	0	(+)	0	0	0	0	0	+	0	(+)	++	0
1101	0	+	0	0	(+)	0	0	(+)	(+)	0	++	0
1321	0	+	0	0	(+)	0	(+)	++	0	0	(+)	0
1401	+	++	0	0	+	0	0	+	++	0	+	0
1451	(+)	++	0	0	0	0	++	+++	(+)	+++	+++	0
1631	0	0	0	0	0	0	0	0	0	0	(+)	(+)
1711	0	++	0	0	+	0	(+)	++	0	0	+	0
1771	+	++	0	0	0	0	++	+++	(+)	+++	++++	0
1841	+	+++	0	0	0	0	+++	++++	++	+++	++++	0
1881	0	(+)	(+)	0	0	0	0	(+)	0	0	(+)	0

[a] 0, No reactivity; (+) to +++, weak to strong reactivities, respectively, based on multiple experiments under various conditions.

positive *S. aureus* should not work as health care providers or attend gyms, swimming pools, or saunas (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1218699411960).

To date, PVL detection is primarily achieved using a molecular method that is essentially limited to reference centers and specialized laboratories with equipment and experience to perform such assays. To facilitate the rapid, nonmolecular detection of PVL by routine clinical microbiology laboratories, we have developed a lateral flow test using monoclonal antibodies. We validated the assay against isolates grown in a variety of different culture media and then evaluated the assay using an international collection of *S. aureus* strains recovered from SSTI.

MATERIALS AND METHODS

Recombinant PVL, F component. The PVL F component gene (*lukF-PV*) was amplified using primers designed to include an EcoRI restriction site and a NotI restriction site at the 5′ and 3′ ends (lukF-PV_fw_5Eco, 5′-CCTGAATTCATGAAAAAAATAGTCAAATC-3′; lukF-PV_rev_5Not, 5′-ATAGCGGCCGCTTAGCTCATAGGATTTT-3′). DNA from the fully sequenced ST1-MRSA-IV reference strain MW2 was used as the template. PCR products were cloned into a commercially available vector (TOPO II; Invitrogen, Karlsruhe, Germany) and sequenced. Resulting sequences were compared to the corresponding GenBank entry (BA000033.2; nucleotide coordinates 1529381 to 153035). Confirmed clones were cut with EcoRI and NotI, and the DNA fragments containing the open reading frame were inserted into the pet28a expression vector (Novagen, Darmstadt, Germany). After ligation, the expression vector was transformed into the *Escherichia coli* strain BL21 (ATCC BAA-1025). Expression of recombinant proteins was achieved in 50 ml lysogeny broth (LB) medium (supplemented with kanamycin) after induction with 50 ml isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM). *E. coli* cells were collected by centrifugation and frozen overnight. Expressed recombinant proteins were purified on nickel-nitrilotriacetic acid-agarose (Ni-NTA-agarose) columns (Qiagen, Hilden, Germany) by following the manufacturer's instructions. Aliquots were taken after each step and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the presence of the recombinant protein. Protein concentrations of each sample were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Bonn, Germany).

Phage display procedures and initial testing of antibodies. Overexpressed PVL F component was used to generate monoclonal antibodies via phage display. Following immunization of mice, mRNA from B cells was isolated and amplified. Resulting cDNA, specific for the antigen-binding parts of antibodies, was ligated into bacteriophages and then transformed into *E. coli*. Resulting antibodies were purified and characterized initially by enzyme-linked immunosorbent assay (ELISA), and different dilutions were spotted onto microtiter strip-mounted protein microarrays.

Antibody microarrays were used according to previously described protocols (15–18).

For the selection of the optimal combination of capture and labeling antibodies, five different concentrations of each of the 11 selected antibodies were spotted onto protein microarrays. These arrays were tested with recombinant PVL F component, native PVL (in two different concentrations; from the CC30-MSSA strain ATCC 25923), or bovine leukocidin LukM-LukP83 (from a veterinary CC151/705 isolate) as antigens as well as with biotin-labeled preparations of all 11 antibodies as detection antibodies in order to test all possible combinations. Staining then was performed by streptavidin-horseradish peroxidase conjugate and by peroxidase-triggered dye precipitation.

This approach allowed the determination of the optimal combination of capture and detection antibodies (Table 1).

Principles of the lateral flow assay for PVL. The lateral flow assay to detect PVL from primary cultures of *S. aureus* is an immunochromatographic membrane assay that uses the two highly sensitive phage display recombinant monoclonal antibodies selected by the microarray described above. The two selected antibodies against PVL were used to design a lateral flow test where one of the antibodies is used for antigen capture on the test strip while the second is gold labeled and coated in a reaction tube.

The PVL assay device consisted of a test strip, reaction tube, and dropper bottle containing the extraction buffer. The test strip consists of the PVL capture antibody and control protein immobilized onto a membrane support forming two distinct lines. The addition of sample and absorbent pad makes the test strip complete. The control protein is a recombinant antigen not related to *S. aureus*. The reaction tube contains lyophilized, gold-conjugated labeling monoclonal antibodies for both PVL and the control protein.

When performing the test, *S. aureus* isolates or culture supernatants are added to the coated reaction tube. A test strip for the PVL assay is then placed into the reaction tube holding the liquid sample and conjugate. Test results are interpreted after 10 min based on the presence or absence of pink- to purple-colored sample lines. Two bands (PVL line and control line) indicate a valid positive result, and one band (control line) indicates a valid negative result. The absence of a visible control line was interpreted as an invalid test.

Performing the assay. The test was applied to isolates of *S. aureus* from SSTIs (see below) that were also genotyped by microarray hybrid-

TABLE 1 (Continued)

Reactivity for capture antibody and antigen												
1401			1451			1631			1711			
Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923
(+)	+	0	(+)	++	(+)	0	0	0	0	0	(+)
0	+	0	0	(+)	0	0	0	0	0	+	0
0	(+)	+++	+	++	0	0	0	0	0	+	0
0	(+)	0	++	+++	0	0	0	0	0	0	0
0	(+)	++++	++	+++	+	0	(+)	0	0	(+)	0
+	++	++	0	++	0	0	(+)	0	++	+++	0
0	0	0	0	(+)	0	0	0	0	0	0	0
0	+	0	+++	+++	(+)	0	0	0	0	(+)	0
+	+++	++	(+)	++	(+)	0	(+)	0	++	+++	0
++	+++	+++	0	++	0	0	(+)	0	+++	++++	0
0	(+)	0	0	(+)	(+)	0	(+)	(+)	0	(+)	(+)

ization to determine strain and clonal complex affiliation and their PVL status. Specifically, 280 μl of extraction reagent was added to the coated reaction tube containing lyophilized antibody-Au conjugate. An inoculation loop of *S. aureus* colony material (approximately 10 μl) was harvested, placed into the tube, and thoroughly mixed using the inoculation loop until both cells and conjugate pellet were completely dissolved. When using liquid growth media, 200 μl of buffer and 100 μl of overnight liquid culture were added to the reaction tube and mixed. The test strip was then inserted into the reaction tube. After 10 min of incubation at room temperature, the test strip was withdrawn from the tube and read.

Strains and isolates. A total of 600 *S. aureus* strains and isolates were tested for LukF-PV production, including both methicillin-susceptible *S. aureus* (MSSA) and MRSA strains.

PVL-negative reference strains were Sanger MSSA476 (a sequenced ST1-MSSA-SCC*fus*, i.e., with a staphylococcal casette chromosome element harboring *fusC*; GenBank accession number BX571857.1), Mu50 and N315 (both sequenced ST5-MRSA-II strains; GenBank accession numbers BA000017.4 and BA000018.3), NCTC 8325 (a sequenced ST8-MSSA strain; GenBank accession number CP000253.1), and COL (a sequenced CC8/ST250-MRSA-I isolate; GenBank accession number CP000046.1), as well as West Australian (WA) MRSA-8 (ST75-MRSA-IV 03-17848 [19]) and WA-MRSA-59 (a CC12-MRSA strain with an atypical staphylococcal cassette chromosome *mec* element [SCC*mec*] [20]).

PVL-positive reference strains were MW2-USA400 (a sequenced ST1-MRSA-IV strain; GenBank accession number BA000033.2), USA300-FPR3757 (a sequenced ST8-MRSA-IV strain; GenBank accession number CP000255.1), ATCC 25923 (a historic ST30-MSSA isolate widely used in diagnostic microbiology for quality control purposes [21]), Queensland CA-MRSA (ST93-MRSA-IV 03-16790 [19]), and WA-MRSA-60/Bengal Bay CA-MRSA (ST772-MRSA-V [20]).

In addition, 588 clinical isolates were included that were collected from patients with SSTI.

The clinical isolates originated from Australia (as part of the country-wide Australian Group for Antimicrobial Resistance *Staphylococcus aureus* Surveillance Programs SAP 2008 and SAP 2010 [http://www.agargroup.org/files/FED%20REPORT%20SAP2008%20MRSA%20final.pdf and http://www.agargroup.org/files/FED%20REPORT%20SAP210%20MRSA%20FINAL%20shrink.pdf, respectively]). Further isolates came from diagnostic laboratories in Germany (University Hospital Dresden), Saudi-Arabia (King Fahad Medical City, Riyadh), Spain (Hospital Universitari Germans, Trias i Pujol, Badalona), Sweden (Oerebro University Hospital), Trinidad and Tobago (from various regional hospitals in the country), Uganda (Medical Research Council in Entebbe), and England (including a hospital in the southwest, Bristol, and the national Staphylococcus Reference Unit, HPA, London). The PVL status of all of the isolates from England and of 17 from other countries (eight from Saudi Arabia, seven from Germany, and three from Australia) was known. These isolates were included to maximize representation of a broad range of clonal complexes, but they were excluded from analysis of PVL rates in the different countries of origin.

In addition, 17 isolates were tested for LukF-P83, including 14 *lukM–lukF-P83*-positive isolates from veterinary sources (cattle and goats) belonging to livestock-associated lineages CC133, CC151/705, and CC479. These isolates were from a previous study (22) or were referred from the Friedrich Loeffler Institute, Jena, Germany (courtesy of K. Schlotter [23]). For control purposes, three *lukM–lukF-P83*-negative isolates were included: two CC133 isolates, one from a mute swan (courtesy of D. Gavier-Widén) and one from a human from Dresden University Hospital, and a CC479 isolate from cattle (courtesy of K. Schlotter). No *lukF-P83*-negative CC151/705 isolates were available for testing.

Full datasets for the individual isolates, including results from lateral flow tests, PCRs, and array hybridization profiles, are available in the supplemental material.

Validation of the lateral flow assay using different culture media. Liquid growth media included glucose bouillon (bouillon by Oxoid, Wesel, Germany, with glucose added), brain-heart infusion (Oxoid), 2× TY (tryptone peptone-yeast extract), and Schaedler bouillon with vitamin K3 (bioMérieux, Nürtingen, Germany). A broth comprising yeast extract, Casamino Acids, sodium glycerophosphate, lactate, citric acid, $FeSO_4$, Na_2HPO_4, KH_2PO_4, $MgSO_4$, and $MnSO_4$ that was described by Noda and Kato (24) as being suitable for the production of PVL was also tested.

The following solid media were used: nutrient agar (Oxoid), Mueller-Hinton agar (Oxoid) with and without blood added, Columbia blood (Oxoid), Columbia blood agar with aztreonam and polymixin (CAP), chocolate agar (agar base and sheep blood [Oxoid] plus hemin [Serva] and NAD [Merck]), and commercially available chromogenic MRSA medium (MRSA ID agar; bioMérieux).

Array procedures. For confirmation of PVL status and for assignment to clonal complexes and strains, all isolates were characterized by DNA microarray hybridization (StaphyType; Alere, Jena, Germany).

Following enzymatic lysis using lysostaphin, lysozyme, and RNase, as well as a Qiagen kit buffer and proteinase K, DNA was prepared using commercially available spin columns (Qiagen, Hilden, Germany) or an automated system (Qiagen EZ1) (25).

The following array procedures were carried out in accordance with the manufacturer's instructions; primers, probes and further details have been described previously (26, 27). Briefly, a multiplex primer elongation was performed that amplified and labeled (by incorporation of biotin-16-dUTP) a total of 333 target sequences corresponding to ca. 170 genes. Single-stranded amplification products were hybridized against microar-

TABLE 1 (Continued)

Reactivity for capture antibody and antigen								
1771			1841			1881		
Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923
+	++	(+)	+	++	(+)	0	(+)	(+)
0	+	(+)	0	+	(+)	0	(+)	(+)
+	++	0	++	++	++	0	(+)	0
++	+++	(+)	+++	+++	0	0	+	0
++	+++	+	+++	+++	+++	0	++	0
(+)	++	(+)	+	+++	(+)	0	+	0
0	(+)	(+)	0	(+)	(+)	0	(+)	(+)
+++	+++	(+)	+++	++++	(+)	+	++	++
+	++	(+)	+	+++	(+)	0	++	(+)
+	++	0	+	+++	+	0	++	0
0	(+)	(+)	0	(+)	(+)	0	(+)	(+)

rays on which the corresponding probes were spotted. Hybridizations were visualized by adding a streptavidin-horseradish peroxidase conjugate that binds to the biotin tags and by a peroxidase-triggered dye precipitation. The resulting pattern of spots on the array was scanned, analyzed, and compared to a reference database of previously typed strains.

MLST. Multilocus sequence typing (MLST) was performed on selected isolates as previously described by Enright et al. (28). In short, fragments of housekeeping genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* were amplified and sequenced using primers and reaction conditions described in reference 28. Resulting sequences were analyzed using the MLST website (http://saureus.mlst.net/) in order to assign a sequence type.

RESULTS

Antibody screening. Based on the screening results summarized in Table 1, a combination of antibody 1401 and antibody 1841 was selected to establish a lateral flow assay capable of detecting PVL (F component) as well as the gene product of *lukF-P83*. The detection limit for the lateral flow test with purified native and recombinant antigen was determined by dilution series to be approximately 1 ng/ml.

Tests of the lateral flow test with different growth media. In the first series of experiments, known strains cultured on different growth media were tested.

Liquid growth media (glucose bouillon, brain-heart infusion, 2× TY, Schaedler broth, and Noda and Kato medium) were tested with PVL-negative Mu50 (ST5-MRSA-II) and NCTC8523 (ST8-MSSA) and known isolates of ST398-MRSA-V and ST8-MSSA, as well as with PVL-positive USA300-FPR3757 (ST8-MRSA-IV, USA300) and isolates of CC30-MSSA and ST93-MRSA-IV (Queensland clone). The PVL-negative ST8-MSSA strain NCTC8325 gave weak but consistent false-positive results in the growth medium described by Noda and Kato (24). This was not observed using genotypically identical clinical isolates of ST8-MSSA. All other results were correct.

Colony material (of PVL-positive ST22-MRSA-IV and of USA300-FPR3757) harvested from plain agar, Mueller-Hinton agar with and without blood, Columbia blood, CAP, and chocolate agar yielded correct positive results. Screening of clinical isolates (see below) was then performed using overnight colonies from Columbia blood agar.

In addition to the aforementioned growth media, a commercially available chromogenic medium for MRSA detection was tested (MRSA ID agar; bioMérieux). The following PVL-positive strains were tested and yielded correct results: CC1-MRSA-IV (MW2 and USA400), CC5-MRSA-IV, ST8-MRSA-IV (USA300-FPR3757), ST22-MRSA-IV, ST30-MRSA-IV (southwest Pacific clone), ST59/ST952-MRSA-V(T) (Taiwan clone), CC80-MRSA-IV (European CA-MRSA clone), CC88-MRSA-IV, and CC152-MRSA-V. PVL-negative strains CC1-MRSA-IV&SCC*fus* (WA-MRSA-1/45), ST22-MRSA-IV (UK-EMRSA-15/Barnim), ST45-MRSA-IV (Berlin EMRSA), ST75-MRSA-IV (WA-MRSA-8), and ST239-MRSA-III (Vienna/Hungarian/Brazilian epidemic strain) and a PVL-negative variant of CC80-MRSA-IV from MRSA ID agar yielded accurate (negative) results.

Detection of LukF-P83. The 14 *lukF-P83*-positive isolates (two CC133, four CC479, and eight CC151) yielded positive results in the lateral flow assay. The three *lukF-P83*-negative isolates (two CC133 and one CC479) were correctly identified as negative.

Screening of clinical isolates using the lateral flow test and the microarray. Compared to the array-based genotyping data, 301 experiments were true positives and 293 were true negatives; there were five false positives (three isolates of CC8, one each of CC15 and CC22) and one false negative (CC30). This corresponds to a sensitivity of 99.7%, a specificity of 98.3%, a positive predictive value (PPV) of 98.4%, and a negative predictive value (NPV) of 99.7%. The six experiments with false results were repeated subsequently and yielded correct results, suggesting operator errors on primary testing.

Overall, 297 test isolates and five reference strains were PVL positive. By array hybridizations, they were assigned to 21 different clonal complexes: CC1 (including ST772), CC5, CC8 (including ST72), CC15, CC22, CC25, CC30, CC45, CC49, CC59, CC80, CC88, CC93, CC96, CC121, CC152, CC188, CC398, ST2479, and ST2482. Table 2 provides further details, such as affiliation with known CA-MRSA strains. The most frequently isolated PVL-positive lineages were CC121 (50 isolates from different regions, all MSSA), CC30 (46 isolates; MSSA and MRSA with SCC*mec* IV elements), CC8 (46 isolates, including MSSA from Trinidad and Tobago as well as USA300 from various regions), and CC93 (42 isolates, MSSA and ST93-MRSA-IV, Queensland CA-MRSA clone, almost exclusively from Australia).

The 287 PVL-negative test isolates and seven reference strains

TABLE 2 Strains tested, their geographic origins, and test results[a]

Clonal complex	Strain	Total no.	No. PVL positive in lateral flow assay	No. PVL negative in lateral flow assay	No. of isolates from:									No. of reference strains
					Australia	Germany	Saudi Arabia	Spain	Sweden	Trinidad and Tobago	Uganda	United Kingdom: London	United Kingdom: Bristol	
CC1	CC1-MSSA	7		7					3	1	2	1		
	CC1-MSSA (PVL$^+$)	6	6		1	1		1	2				1	
	CC1-MSSA-SCC*fus*	8		8	2		1	1				2	1	1
	CC1-MSSA-SCC*fus* (PVL$^+$)	7	7		4							3		
	CC1-MRSA-IV, WA-MRSA-1/57	1		1								1		
	CC1-MRSA-IV (PVL$^+$), USA400	3	3		1								1	1
	CC1-MRSA-IV&SCC*fus*, WA-1/45	1		1			1							
CC1 (ST573/772)	ST573/772-MSSA (PVL$^+$)	2	2		2									
	ST772-MRSA-V (PVL$^+$), Bengal Bay clone/WA-60	4	4									2	1	1
CC5	CC5-MSSA	10		10			1	1	6		2			
	CC5-MSSA (PVL$^+$)	8	8			1			1	5			1	
	CC5-MRSA-II, UK-3/Rhine-Hesse/New York Japan	3		3		1								2
	CC5-MRSA-IV, Pediatric clone	1		1							1			
	CC5-MRSA-IV (*edinA*$^+$), WA-65	1		1	1									
	CC5-MRSA-IV, Pediatric clone (PVL$^+$)	5	5				2					3		
	CC5-MRSA-IVvar, Maltese clone	1		1			1							
	CC5-MRSA-V (PVL$^+$)	1	1									1		
CC6	CC6-MSSA	3		3			1			2				
CC7	CC7-MSSA	9		9		1	1		5	2				
	CC7-MRSA-IV	1		1		1								
CC8	CC8-MSSA	41	3*	38	2	6	1		3	5	21	2		1
	CC8-MSSA (PVL$^+$)	23	23					2	1	18			2	
	CC8-MSSA-SCC*fus*	1		1								1		
	ST250-MRSA-I, Early/Ancestral MRSA	1		1										1
	CC8-MRSA-IV, WA-62	2	2							2				
	ST8-MRSA-IV (PVL$^+$/ACME$^+$), USA300	10	10		2	1				3		3		1
	ST8-MRSA-IV (PVL$^+$/ACME$^-$)	10	10					10						
CC8 (ST72)	ST72-MSSA	13		13	2	1		1		7	2			
	ST72-MSSA (PVL$^+$)	1	1			1								
CC8 (ST239)	ST239-MRSA-III, Vienna/Hungarian/Brazilian clone	13		13			5			6	1	1		
CC9 (ST834)	ST834-MSSA	2		2							2			
	ST834-MRSA-VI	1		1			1							
CC12	CC12-MSSA	8		8					5	1		2		
	CC12-MRSA, WA-59	1		1										1
CC15	CC15-MSSA	29	1*	28	2	1	3	1	18	2	2			
	CC15-MSSA (PVL$^+$)	2	2			1			1					
CC20	CC20-MSSA	3		3					3					
CC22	CC22-MSSA	6	1*	5		3	1		1			1		
	CC22-MSSA (PVL$^+$)	19	19			2	2	5					10	
	CC22-MSSA-SCC*fus* (PVL$^+$)	5	5										5	
	CC22-MRSA-IV, UK-EMRSA-15/Barnim EMRSA	4		4	1	1	1					1		
	CC22-MRSA-IV (PVL$^+$)	5	5			2	1					2		
CC25	CC25-MSSA	10		10			3				6	1		
	CC25-MSSA (PVL$^+$)	2	2		2									
CC30	CC30-MSSA	20		20	1	8	3		7			1		
	CC30-MSSA (PVL$^+$)	35	34	1*	3	4	4	6	4	10	1		2	1

(Continued on following page)

TABLE 2 (Continued)

Clonal complex	Strain	Total no.	No. PVL positive in lateral flow assay	No. PVL negative in lateral flow assay	No. of isolates from:									No. of reference strains
					Australia	Germany	Saudi Arabia	Spain	Sweden	Trinidad and Tobago	Uganda	United Kingdom: London	United Kingdom: Bristol	
	CC30-MRSA-IV (PVL$^+$), Southwest Pacific CA-MRSA clone	11	11		5	1	1					4		
CC30 (ST34)	ST34-MSSA	5		5	1			1	1			2		
CC45	CC45-MSSA	33		33	2	4	1	2	19	2		3		
	CC45-MSSA (PVL$^+$)	2	2		1				1					
	CC45-MRSA-IV, Berlin EMRSA	1		1		1								
CC49	ST49-MSSA (PVL$^+$)	1	1					1						
CC50	CC50-MSSA	3		3					3					
CC59	CC59-MSSA	1		1								1		
	CC59-MSSA (PVL$^+$)	1	1										1	
	CC59-MRSA-V	1		1								1		
	CC59-MRSA-V (PVL$^+$)	1	1									1		
	ST59/ST952-MRSA-V(T) (PVL$^+$), Taiwan CA-MRSA clone	3	3		1							2		
	CC59-MRSA-V&SCC*fus*	2		2						2				
CC75	MSSA, related to ST1223	2		2						2				
	MSSA, related to ST1667	2		2			1			1				
	ST75-MRSA-IV, WA-MRSA-8/79	1		1										1
CC80	CC80-MSSA (PVL$^+$)	5	5				1	1	2		1			
	CC80-MRSA-IV	2		2			2							
	CC80-MRSA-IV (PVL$^+$), European CA-MRSA clone	13	13				11					2		
CC88	CC88-MSSA	2		2	1				1					
	CC88-MSSA (PVL$^+$)	2	2						2					
	CC88-MRSA-IV (PVL$^+$)	2	2				2							
CC93	ST93-MSSA (PVL$^+$)	9	9		9									
	ST93-MRSA-IV (PVL$^+$), Queensland CA-MRSA clone	33	33		30	1						1		1
CC96	CC96/154-MSSA	1		1			1							
	CC96/154-MSSA (PVL$^+$)	1	1			1								
CC97	CC97-MSSA	8		8			1		7					
CC101	CC101-MSSA	3		3		1			1	1				
CC121	CC121-MSSA	16		16				2	8	4	1	1		
	CC121-MSSA (PVL$^+$)	50	50		15	8		4	3		17	2	1	
CC140	CC140-MRSA-IV	2		2							2			
CC152	CC152-MSSA (PVL$^+$)	8	8					2	1	2		1	2	
	CC152-MRSA-V (PVL$^+$)	1	1			1								
CC188	CC188-MSSA	6		6	1			1	2	2				
	CC188-MSSA (PVL$^+$)	2	2		1		1							
CC398	CC398-MSSA	1		1		1								
	CC398-MSSA (PVL$^+$)	1	1			1								
CC398 (ST291/813)	ST291/813-MSSA	1		1			1							
	ST291/813-MSSA (PVL$^+$)	1	1				1							
CC425	ST425-MRSA-XI	1		1								1		
CC509	CC509-MSSA	1		1					1					
CC707	ST707-MSSA	1		1				1						
CC1021	CC1021-MSSA	1		1					1					
CC1290	CC1290/ST2481-MSSA	1		1			1							
ST2479	ST2479-MSSA (PVL$^+$)	1	1					1						
ST2482	ST2482-MSSA (PVL$^+$)	4	4				3		1					
Unidentified	*agr* IV/capsule 5 MSSA	1		1							1			

[a] Strain assignments were based on array hybridization. False results in lateral flow tests are marked with an asterisk.

TABLE 3 Rates of PVL-negative MSSA, PVL-negative MRSA, PVL-positive MSSA, and PVL-positive MRSA by study site[a]

Study site (total no. of isolates)	No. (%) of isolates in each category			
	PVL negative		PVL positive	
	MSSA	MRSA	MSSA	MRSA
Australia ($n = 90$)	14 (15.6)	2 (2.2)	37 (41.1)	37 (41.1)
Germany ($n = 50$)	26 (52.0)	4 (8.0)	17 (34.0)	3 (6.0)
Saudi Arabia ($n = 53$)	21 (39.6)	8 (15.1)	11 (20.8)	13 (24.5)
Spain ($n = 44$)	11 (25.0)	0 (0)	23 (52.3)	10 (22.7)
Sweden ($n = 114$)	95 (83.3)	0 (0)	19 (16.7)	0 (0)
Trinidad and Tobago ($n = 80$)	32 (40.0)	8 (10.0)	35 (43.8)	5 (6.2)
Uganda ($n = 62$)	39 (62.9)	4 (6.5)	19 (30.6)	0 (0)

[a] Three Australian, six German, eight Saudi, and all English isolates, as well as the reference strains, were excluded from this analysis, because their PVL statuses were known already.

were assigned to 31 different clonal complexes: CC1, CC5, CC6, CC7, CC8 (including ST72 and ST239), CC9 (ST834), CC12, CC15, CC20, CC22, CC25, CC30 (including ST34), CC45, CC50, CC59, CC75, CC80, CC88, CC96, CC97, CC101, CC121, CC140, CC188, CC398, CC425, CC509, CC707, CC1021, and CC1290. One isolate could not be allocated to a clonal complex.

Prevalence of PVL-positive *S. aureus* in the different countries. The prevalence of PVL-positive isolates among all SSTI isolates varied widely between the different countries. Rates of PVL positives and negatives for MSSA and MRSA are summarized in Table 3.

The highest rate of PVL positives was observed among the Australian samples, with 82.2% (74 of 90) being PVL positive. Half of the PVL-positive isolates (37 of 74) belonged to CC93, and the majority of them were MRSA (29 of 37 CC93 isolates; 78%), reflecting the burden the so-called Queensland CA-MRSA clone currently causes. The second and third most frequently isolated PVL-positive clones in Australia were CC121-MSSA ($n = 15$) and CC93-MSSA ($n = 8$). Only two isolates of ST8-MRSA-IV (USA300) were identified. PVL-negative *S. aureus* isolates were from multiple CC lineages and included two MRSA clones, ST22-MRSA-IV (UK-EMRSA-15/Barnim EMRSA) and ST5-MRSA-IV (Pediatric clone/WA-MRSA-65).

Among SSTI isolates from Germany, the PVL rate was 40% (20 of 50). The most common strains were CC121-MSSA ($n = 7$) and CC30-MSSA ($n = 4$). One each of ST8-MRSA-IV (USA300) and ST93-MRSA-IV (Queensland CA-MRSA clone) were identified, the latter being associated with travel to Australia. Among the PVL negatives, CC30 and CC8 were the most frequently isolated. Single isolates of PVL-negative CC7-MRSA-IV, CC22-MRSA-IV (UK-EMRSA-15/Barnim EMRSA), ST5/ST225-MRSA-II (UK-EMRSA-3/Rhine-Hesse EMRSA), and CC45-MRSA-IV (Berlin EMRSA) were identified.

Of the isolates from Saudi Arabia, 47.3% (24 of 53) proved to be positive for PVL. Roughly half were MRSA ($n = 13$), with the single most common PVL-positive clone being CC80-MRSA-IV (European CA-MRSA clone; 10 isolates). The most frequently isolated PVL-MSSA clones were CC30-MSSA ($n = 4$) and ST2482-MSSA ($n = 3$). PVL negatives belonged to various clonal complexes (Table 2), and the proportion of MRSA strains was high (8 of 29 PVL negatives), with the most common strain being ST239-MRSA-III (Vienna/Hungarian/Brazilian clone; $n = 4$). Other MRSA strains belonged to a *tst1*-positive variant of CC22-MRSA-IV, a PVL-negative variant of CC80-MRSA-IV, a CC5-MRSA-IV&SCC*fus* strain previously known only from Malta (29), and CC9/ST834-MRSA-VI.

The second highest PVL rate was found in Spain, with 75% (33 of 44) being positive for PVL. Here, the most common clone was an ACME (arginine catabolic mobile element)-negative variant of ST8-MRSA-IV (USA300), to which 10 isolates were assigned. This was followed by CC30-MSSA ($n = 6$) and CC22-MSSA ($n = 5$). The PVL negatives did not include any MRSA strains.

The lowest prevalence for PVL was observed among the Swedish isolates. Only 16.7% (19 of 114) were PVL positive, all MSSA. The most common PVL-positive strains were CC30-MSSA ($n = 4$) and CC121-MSSA ($n = 3$). The most common PVL-negative lineages were CC45 ($n = 19$) and CC15 ($n = 18$). MRSA isolates were not found.

In Trinidad and Tobago, the PVL prevalence was 50% (40 of 80 isolates). The most abundant PVL-positive strain was CC8-MSSA ($n = 18$), which additionally carried enterotoxin genes *sed*, *sej*, *ser*, *sek*, and *seq*. Two CC8-MRSA-IV isolates were identified with the same toxin profile; they lacked ACME and thus resembled WA-MRSA-62. ST8-MRSA-IV (USA300), i.e., carrying the ACME locus and enterotoxin genes *sek* and *seq* only, was identified in three cases. Other frequently isolated PVL-positive strains were CC30-MSSA ($n = 10$) and CC5-MSSA ($n = 5$). PVL-negative isolates included some isolates of unusual strains related to CC75 (ST1223 and ST1667). The PVL-negative MRSA strains were CC59-MRSA-V&SCC*fus* and ST239-MRSA-III (Vienna/Hungarian/Brazilian clone).

In Uganda, 30.6% (19 of 62) were PVL positive, including 17 isolates belonging to CC121-MSSA and single representatives of CC30- and CC80-MSSA. PVL-positive MRSA was not identified. The most common lineage among PVL negatives was CC8 ($n = 22$, plus one isolate each of CC8/ST72 and CC8/ST239). PVL-negative MRSA strains included two ST140-MRSA-IV isolates as well as single isolates of CC5-MRSA-IV (Pediatric clone) and ST239-MRSA-III (Vienna/Hungarian/Brazilian clone).

In contrast to the collections from other countries, the PVL status of the isolates from England were already known, thus the PVL rates cannot be compared to those of the other countries. A variety of different PVL-MRSA strains was identified among the London isolates: CC30-MRSA-IV (Southwest Pacific CA-MRSA clone), CC5-MRSA-IV (Pediatric clone), CC5-MRSA-V, CC80-MRSA-IV (European CA-MRSA clone), ST59/ST952-MRSA-V(T) (Taiwan CA-MRSA clone), ST772-MRSA-V (Bengal Bay CA-MRSA clone/WA-MRSA-60), ST8-MRSA-IV (USA300), and

ST93-MRSA-IV (Queensland CA-MRSA clone). PVL-negative isolates included MRSA strains CC1-MRSA-IV (WA-MRSA-1/57), ST239-MRSA-III (Vienna/Hungarian/Brazilian clone), CC22-MRSA-IV (UK-EMRSA-15/Barnim EMRSA), ST59-MRSA-V, and CC425-MRSA-XI. Another 28 isolates were included from a second center in the southwest of England that were already known to be PVL positive. These were also excluded from the analysis of PVL rates, but their population structure was notable. This group included only two PVL-positive MRSA isolates, ST772-MRSA-V (Bengal Bay CA-MRSA clone/WA-MRSA-60) and CC1-MRSA-IV (USA400). It also included one CC59-MSSA strain that probably was an SCC*mec* deletion mutant of ST59/ST952-MRSA-V(T) (Taiwan CA-MRSA clone). The most common strain in this group was a PVL-positive CC22-MSSA isolate ($n = 10$). Another five PVL-positive CC22 isolates with *spa* type t417 or t1601 carried SCC*fus* elements (*ccrA* and/or *ccrB-1*, Q6GD50, or *fusC*). These isolates originated from patients with an average age of nearly 94 years. This is an unusual finding among PVL positives and suggests a possible association of this clonal complex with care facilities in this region.

DISCUSSION

PVL is a unique virulence marker in *S. aureus*, and it is most commonly associated with clinical symptoms which tend to be either chronic/recurrent or, occasionally, rapidly progressing and life-threatening. A diagnostic test for PVL would be desirable for targeted patient management. The lateral flow assay described in this paper allows the rapid detection of PVL in a routine bacteriological laboratory that is not able to readily perform molecular assays. As it utilizes pure overnight cultures from standard media, such as Columbia blood agar, it can easily be integrated into a routine diagnostic laboratory workflow. Thus, the assay might contribute to timely therapeutic interventions in cases of PVL-associated infections, and it also might help to select isolates for submission for further typing in reference centers.

The amount of PVL released by *S. aureus in vitro* varies widely (30, 31); however, the high concordance between the genotypic and phenotypic assays suggests that *lukS–lukF-PV*-positive strains generally express detectable amounts of PVL using standard culture conditions. In this study, no isolates were identified that harbored PVL genes without producing the toxin *in vitro*. While further media and different formulations still need to be tested, it can be assumed that the probability of false-negative results due to a lack of expression *in vitro* is low. The diversity of PVL- and *lukM–lukF-P83*-positive strains included in this study indicate that possible lineage-specific variations in PVL sequences do not pose an obstacle to PVL detection by the antibodies described here.

Further, the collection of isolates described here provides a snapshot of the molecular epidemiology of *S. aureus* associated with SSTI. Among the PVL-positive methicillin-susceptible *S. aureus* strains, CC121 (50 isolates in total) and CC30 (35 isolates) dominated. PVL-positive CC8-MSSA was abundant in Trinidad and Tobago, although this strain was rare elsewhere. This lends credence to the hypothesis that the USA300 strain emerged in the Caribbean/Latin American region (32).

The study also shows that MRSA strains, PVL positives as well as negatives, pose a serious problem in different parts of the world, being commonly found in SSTI that mostly are community associated. At most study sites, MRSA was isolated frequently, especially in Australia, Saudi Arabia, and Spain. Dominating clones in this study were PVL-positive/ACME-positive ST8-MRSA-IV (USA300), PVL-positive/ACME-negative ST8-MRSA-IV, PVL-positive ST80-MRSA-IV (European CA-MRSA clone), PVL-positive ST93-MRSA-IV (Queensland CA-MRSA clone), and PVL-negative ST239-MRSA-III. In this study, the countries with the lowest MRSA prevalence (or complete absence) were Sweden, which has a very strict policy on MRSA infection control, and Uganda, where the selective pressure on *S. aureus* by the use of antibiotics in health care and veterinary medicine may be more limited than in other countries.

The possibility of combining the lateral flow assay with a chromogenic MRSA screening medium facilitates a quick screening for emerging strains of PVL-positive CA-MRSA. This could be helpful to arrest their dissemination and further expansion. The high percentage of PVL-positive MRSA (ST93-MRSA-IV, Queensland CA-MRSA clone, and ST8-MRSA-IV) among Australian and Spanish isolates, in addition to high rates of PVL-positive MSSA strains, suggests that an expansion of PVL-positive CA-MRSA does not occur at the expense of the established PVL-positive MSSA populations but in addition to it. Besides limiting the efficacy of beta-lactams as a primary therapeutic option, the emergence of PVL-positive CA-MRSA may result in an increased burden of PVL-associated disease. Although the number of isolates in the present study is not sufficient to unambiguously prove such a trend, it warrants further study with respect to the molecular epidemiology of PVL-positive *S. aureus*.

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Development and usage of protein microarrays for the quantitative measurement of Panton-Valentine leukocidin

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ABSTRACT

Staphylococcus aureus is a human pathogen that can harbour several genes encoding exotoxins including leukocidins. A clinically most relevant factor is Panton-Valentine leukocidin (PVL) because of its association with chronic, recurrent or severe skin and soft tissue infections.

In this study an antibody array was designed and used to obtain an overview about the *in vitro* PVL expression levels of 266 clinical isolates of MRSA as well as of MSSA belonging to a wide variety of clonal complexes. For that purpose, a novel precipitation based method was used. Unknown PVL concentrations were determined by mapping the signal intensities for spotted monoclonal antibodies to calibration curves that resulted from experiments with known concentrations of recombinant LukF-PV.

In most cases, isolates belonging to one clonal complex (CC) showed similar PVL expressions. However, there were also CCs with widely varying PVL concentrations. First analyses, based on *in vitro* PVL measurements, showed low PVL concentrations in isolates from severe and fatal conditions that are not associated with PVL, such as sepsis, while isolates from skin and soft tissue infections yielded higher concentrations. *Agr*-group I and IV isolates generally produced more PVL than isolates from *agr*-groups II and III. The few isolates harbouring the gene encoding toxic shock syndrome toxin (*tst1*) were particularly low level PVL producers. However, these issues warrant further studies.

The method described herein allows rapid quantification of expressed proteins such as PVL in collections of clinical isolates in order to correlate with clinical or genotypic data with a potential for further parallelisation.

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1. Introduction

Staphylococcus aureus is a human pathogen that can harbour several genes encoding exotoxins [1,2]. This includes haemolysins, superantigens, such as the Toxic Shock Syndrome toxin (TSST, encoded by *tst1*), enterotoxins (SEs), and leukocidins such as the Panton-Valentine leukocidin (PVL). PVL is a bi-component exotoxin encoded by two co-localised and co-expressed genes *lukF-PV* and *lukS-PV* that are carried by bacteriophages. The two PVL components LukF-PV and LukS-PV are cytotoxic to human polymorphonuclear neutrophils (PMNs). They build polymers which form pores in leukocyte plasma membranes, trigger interleukin production and lead to cell lysis or apoptosis [2]. Clinically, infections with PVL-positive *S. aureus* are usually chronic or recurrent skin and soft tissue infections (SSTI) such as furunculosis. PVL is also associated with rare, but severe, life-threatening diseases such as necrotizing pneumonia [3]. Because of their association with recurrent or severe disease and with large-scale outbreaks, PVL-positive *S. aureus* warrant surveillance, and aggressive therapeutic and infection-control measures (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1218699411960).

The existence of a "substance leukocidine" was first assumed by Van de Velde in 1894 [4]. PVL was described as a virulence factor in skin and soft tissue infections by Panton and Valentine in 1932 [5]. Pandemics of PVL-positive methicillin sensitive *S. aureus* (MSSA) were observed in the 1940s and 1960s. Since the 1990s, many community-acquired methicillin resistant *S. aureus* (CA-MRSA) strains have been shown to harbour the PVL gene locus [3,6–10].

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Currently, PVL is widely distributed in many different clonal complexes (CCs) and it can be found in both, methicillin-resistant *S. aureus* (MRSA; [6,7,9–13]) as well as in methicillin-sensitive *S. aureus* (MSSA; [14–18]). PVL production is variable [19–22]. This may be due variability of the genetic background of the isolate, *i.e.*, correlating with accessory gene regulation (*agr*) type and clonal complex affiliation. Genetic differences in the bacteriophages harbouring the *lukF/S-PV* genes [20,21] or the integration of other mobile genetic elements such as pathogenicity island or SCC-elements may also interfere with PVL production.

Most molecular biological techniques allow the detection of toxin genes, whereas only a few existing methods reveal information on translated proteins and their interactions. Therefore, protein microarrays were developed and established for the rapid and quantitative measurement of toxin concentrations using precipitation staining. In this study, such an antibody array was designed and used to obtain an overview about the *in vitro* PVL expression levels of clinical isolates of MRSA as well as of MSSA belonging to a wide variety of clonal complexes.

2. Material and methods

2.1. Strains

In this study, 266 PVL positive clinical isolates of MSSA and MRSA were tested. For 169 isolates, clinical information was available. Isolates originated from the German federal state of Saxony and from Western Australia. Some additional isolates were obtained from other centres from Europe and the Middle East (see acknowledgements). Molecular characterisations as well as basic clinical and epidemiological data of these isolates have been described previously in detail [9,17,18,23–30] and can be provided on request.

2.2. Genotypic characterisation

All isolates were genotyped by DNA microarray hybridisation (StaphyType by Alere Technologies, Jena, Germany) as described previously [9,31]. This allowed amongst others the detection of the *lukF/S-PV* genes and the assignment of the isolates to clonal complexes and strains.

Strains with novel hybridisation patterns (ST2479, ST2482) were further characterised by multilocus sequence typing (MLST) as previously described [32].

Strains are defined by their affiliation to clonal complexes and/or sequence types (ST), by the presence of *mecA* and, if present, by the identity of their SCC*mec*-elements. Sequence types cannot always recognised by analysis of array hybridisation profiles. STs that are unambiguously discernible, such as ST567 (CC1), ST573/772 (CC1), ST72 (CC8), ST834 (CC9) or ST291/813 (CC398), they will be treated separately from the parent lineage.

2.3. LukF-PV purification and antibody design

The amplification of *lukF-PV* was performed using designed primers (lukF-PV_fw_5Eco, CCTGAATTCATGAAAAAAATAGTCAA ATC and lukF-PV_rev_5Not, ATAGCGGCCGCTTAGCTCATAGGATTTT) and the template DNA from the sequenced ST1-MRSA-IV reference strain MW2 (Gen Bank entry BA000033.2). Amplified DNA fragments were sequenced and then ligated in the pET28a expression vector (Novagen, Darmstadt, Germany) and subsequently transformed into the *Escherichia coli* strain BL21 (ATCC BAA-1025). For expression of LukF-PV, transformed *E. coli* cells were grown in 500 ml lysogeny broth-medium (LB media, 30 μg/ml kanamycin added) and induced with isopropyl b-D-1-thiogalactopyranoside (IPTG, 1 mM). Broth cultures were centrifuged, and the collected cells were frozen at −20 °C overnight. Purification of the recombinant LukF-PV was performed using nickel-nitrilotriacetic acid-agarose (Ni-NTA-agarose) columns (Qiagen, Hilden, Germany) as per manufacturer's instructions. Resulting aliquots were combined, concentrated and transferred into phosphate buffered saline (PBS) using Vivaspin tubes (Sartorius, Göttingen, Germany). The presence of the recombined protein was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit according to manufacturer's instructions (Pierce, Bonn, Germany).

The resulting LukF-PV was used to generate monoclonal antibodies via phage display. Following preliminary screening, eleven anti-LukF-PV-antibodies were spotted, each in five different dilutions, in microtiterstrip-mounted protein arrays and tested using different PVL preparations, as previously described [18].

The optimal anti-LukF-PV-antibody was further used as secondary detecting antibody in the protein assay [18,22]. The antibody was covalently biotin-labelled using Sulf-NHS-LC-Biotin (Pierce, Bonn, Germany) and purified via Zeba Spin Columns (Thermo Scientific, Bonn, Germany) as per the manufacturer's instructions.

2.4. Culture conditions

Different media and incubation conditions were assessed allowing uniform growing settings to be established (see Results). Media tested included glucose bouillon (bouillon by Oxoid, Wesel, Germany, with glucose added), brain-heart bouillon (Oxoid) and a medium described by Kato and Noda containing yeast extract, casamino acid, sodium glycerophosphate, citric acid, lactate, $FeSO_4$, Na_2HPO_4, KH_2PO_4, $MgSO_4$ and $MnSO_4$ [33].

For measurements, one cryobank bead (Microbank, Pro-Lab, Richmond Hill, Canada) of each preserved strain was incubated in 1.5 ml of liquid medium in PC tubes (Greiner Bio-One, Frickenhausen, Germany) for 18 h at 37 °C and 200 rotations per minute (rpm) on a shaker. Cultures were centrifuged for 5 min at 8000 rpm and three dilutions of the supernatants (1:100, 1:500 and 1:1000) were used in the protein array assays.

2.5. Array hybridisation and detection

The protein microarrays were manufactured by Alere Technologies, Jena, Germany. Each microarray chip sizes 4 mm × 4 mm and was produced spotting antibodies on 3D-epoxy-modified glass.

The arrays were washed with 100 μl washing buffer (1× PBS; 0.005% Tween; FCS; 0.25% Triton X-100) at 37 °C for 5 min at 400 rpm on a shaker. 100 μl of 10% foetal calf serum (FCS) blocking solution was then added and the arrays were incubated at 37 °C to 5 min at 300 rpm. 100 μl of the diluted culture supernatant were added to the arrays and incubated at 37 °C for a further 30 min at 300 rpm.

The specifically bound proteins were detected by the addition of a secondary, biotin-labelled, antibody (1 ng/μl, 100 μl, at 37 °C for 30 min at 300 rpm) and streptavidin-horseradish peroxidase (HRP; 0.5 ng/μl, 100 μl, 15 min at 37 °C and 300 rpm). In a final step, streptavidin-HRP triggered the local precipitation of a dye, tetramethylbenzidine (TMB). TMB was incubated for 10 min, without shaking at room temperature. The precipitation was stopped by removing the substrate.

2.6. Analysis

Array images were taken by the ArrayMate reading device (Alere Technologies, Jena, Germany) and were analysed using IconoClust software and manufacturer's instructions.

3. Results

3.1. Quantitative analysis

Array analysis was based on a set of previous calibration- and reference-experiments. The unknown concentration of an antigen was determined by the usage of a set of calibration curves to the relevant probe signal. To cover a significant measuring range, 11 different anti-LukF-PV-antibodies (addressing different epitopes) were immobilised on the microarray at five different dilutions each. For establishing the calibration curves, recombinant LukF-PV was tested on the arrays using different defined concentrations (0.05 ng/ml, 0.1 ng/ml, 0.3 ng/ml, 0.5 ng/ml, 1.0 ng/ml, 1.2 ng/ml, 1.6 ng/ml, 3.0 ng/ml, 5.0 ng/ml, 8.0 ng/ml and 10.0 ng/ml) on the arrays. Resulting images allowed the correlation of the different antigen concentrations to the signal intensities (Fig. 1) in one calibration curve per single spot, i.e., for each concentration of each antibody. The normalised intensities (NI) of the spots were determined by:

$$NI = 1 - (M/BG)$$

where M is the average intensity of the spot and as BG is the intensity of the local background. Thus, results range between 0 (no signal) and 1 (maximal signal). Signal intensities between 0.1 and 0.7 were considered as valid. Signals above 0.7 were regarded as saturated and not included in the analysis.

A calibration curve with a minimum slope of 0.5 was considered for the calculation of the concentration.

Only if no satisfactory measurement was possible in the first run, the sample was re-tested in a different dilution on a new microarray.

Based on the reference experiments and conditions described above, in the next experiments unknown concentrations were determined by signal intensity of the array spots. The representative concentration of LukF-PV was calculated as the median of all values resulting from all probe dilutions from all microarray experiments.

The concentration was calculated by:

$$\mathbf{C}_{\mathrm{LukF-PV}} = \mathbf{median}(\boldsymbol{c}_{\mathbf{mn}}) \quad : m\varepsilon\{1\ldots\boldsymbol{S}_{\mathbf{D}}\}, \quad n\varepsilon\{1\ldots\boldsymbol{P}_{\mathbf{D}}\}$$

where $\boldsymbol{S}_{\boldsymbol{D}}$ = number of sample dilutions (equal to the number of microarrays)

$\boldsymbol{P}_{\boldsymbol{D}}$ = number of probe dilutions at one microarray
$\mathbf{c}_{\mathbf{mn}}$ is only determined if $\mathbf{signal}_{\mathbf{mn}}$ is between 0.1 and 0.7.

The detection limit for the whole array was determined by serial dilution experiments with recombinant LukF-PV to be 1 ng/ml.

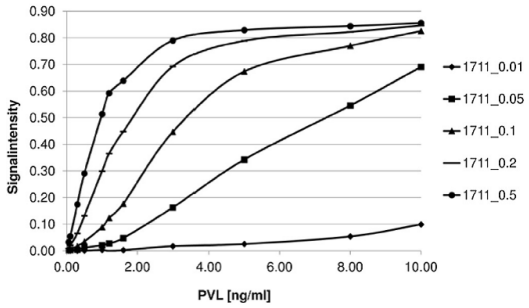

Fig. 1. Calibration curves for one antibody spotted in different concentrations.

3.2. Establishing the test protocol

Different growth media were tested in order to establish reproducible experimental conditions. The strain USA300-FPR3757 (GenBank accession number CP000255.1; [12]) was incubated in 1.5 ml of glucose bouillon, brain-heart bouillon or, respectively, Kato and Noda medium (for 18 h, at 200 rpm and 37 °C, in tubes with cotton plugs to allow oxygenation). In Glucose-bouillon, PVL was not detectable. In brain-heart bouillon, a higher PVL expression was measured (565 ng/ml). In contrast, USA300-FPR3757 yielded a very high PVL level (4500 ng/ml) in Kato and Noda medium and under otherwise identical conditions. Based on these observations, Kato and Noda medium was selected for all subsequent experiments.

PVL concentrations were measured serially after incubation of broth cultures at different time points. This showed increasing PVL levels, depending on the length of incubation with the highest levels being observed after about 24 h of incubation (Fig. 2).

Additionally, two negative reference strains, COL (Gen Bank CP000046) and N315 (Gen Bank BA000018) were tested under identical conditions as controls and no PVL was detectable.

Based on these results, all experiments were performed under the following uniform conditions. As inoculums, one cryobank bead of preserved culture was given into 1.5 ml Kato & Noda medium. Cultures were incubated for 18 h on a shaker at 200 rpm and at 37 °C in tubes with cotton plugs.

3.3. Reproducibility of the method

As described above, the method generates a multitude of redundant measurements that are analysed together. To further check for the reproducibility of the results one high level producer (USA300-FPR3757) and one low level producer (MW2) eight or nine different cultures were grown separately and repeatedly measured. The average PVL concentration of 24 experiments was 5535 ng/ml, with a median of 5150 ng/ml and a standard deviation of 1377 ng/ml (Fig. 3A). The average PVL concentration for 25 experiments with MW2 was 207 ng/ml, with a median of 125 ng/ml and a standard deviation of 232 ng/ml (Fig. 3B).

3.4. Overview on clonal complexes (CCs) and their PVL expression

An overview on clonal complexes and strains (as defined by presence and type of SCC*mec* elements) together with genotypic

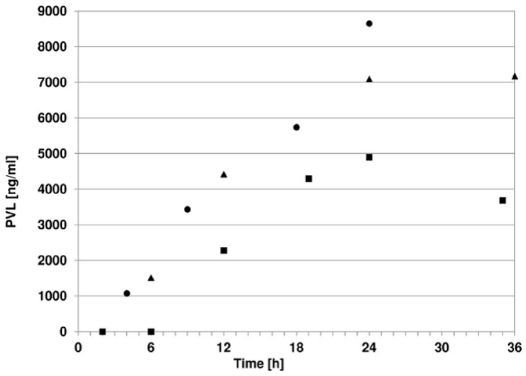

Fig. 2. PVL levels as function of time (from three independent experiments).

characterisations is provided in Table 1. Details on PVL measurements are shown in Table 2.

3.4.1. Clonal complex 1

CC1 *sensu strictu* strains (MLST profile 1-1-1-1-1-1-1 and variants thereof) comprise different strains that can be differentiated based on the presence or absence of SCC*mec* IV and V elements and of a SCC element that includes a fusidic acid resistance gene *fusC*. The measured PVL expression was uniformly low in these CC1 strains.

ST567 (MLST profile 10-1-1-1-1-1-1) and ST573/772 (MLST profile 1-1-1-1-12/22-1-1) belong, according to the MLST database, to CC1, too. However, they differ from other CC1 in hybridisation profiles with regard to several core genomic markers (see Table 1). While ST567 appears to be very rare, with only one isolate being identified for this study, ST573/772 comprises the emerging CA-MRSA clone, ST772-MRSA-V "Bengal Bay Clone" or "WA-MRSA-60". ST567 and ST573/772 yielded higher PVL levels than CC1 *sensu strictu* strains.

3.4.2. Clonal complex 5

The CC5 lineage comprises PVL-positive strains that can be differentiated based on absence or presence of *mecA*, and carriage of SCC*mec* elements of types IV and V. Isolates uniformly carry the *egc* cluster, and additional enterotoxins (namely *sed, sej, ser*) are common. All tested CC5 isolates yielded low PVL concentrations.

3.4.3. Clonal complex 6

Not a single PVL-positive MRSA from this lineage was found and only one PVL-positive CC6-MSSA isolate was identified. This isolate originated from Trinidad and Tobago. It yielded a very high PVL concentration but it still needs to be clarified whether this was typical for that lineage.

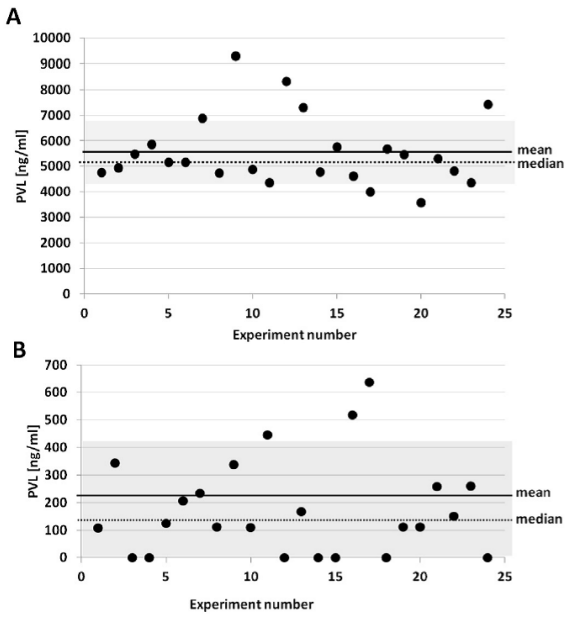

Fig. 3. A. Variability of PVL yields in 24 experiments with USA300-FPR3757. B. Variability of PVL yields in 25 experiments with USA400-MW2.

3.4.4. Clonal complex 8

CC8 is a pandemic lineage which includes several strains that can be differentiated based on the presence of *mecA* and accessory enterotoxin genes. ST72 is discussed separately because of significant differences in gene content.

CC8-MSSA isolates all originated from Trinidad and Tobago, where this strain is common [18,29]. Isolates showed varying, but low PVL concentrations. Furthermore, twenty-one isolates of ST8-MRSA-IV, "USA300" were included. This set comprised clinical isolates from Germany, Australia as well as the reference strain USA300-FPR3757. "USA300" expressed PVL at a high level. Seven isolates of "USA300-like" but ACME negative ST8-MRSA-IV yielded lower PVL levels than "USA300". Finally, another ACME-negative CC8-MRSA-IV strain with a distinct toxin profile [PVL(+), *sed/j/k/q*/r(+)], that has been previously been described from West Australia (WA) as WA MRSA-62, was included. Isolates of this strain yielded comparatively high PVL levels with mean and median values of *ca.* 4000 ng/ml.

While ST72 (MLST profile 1-4-1-8-4-4-3) belongs to CC8, it yields a distinct hybridisation profile (see Table 1). PVL-positives from ST72 appear to be uncommon. A single MSSA isolate was found in a patient with an abscess from Saxony and ST72-MRSA-IV were described from Australia ("WA-MRSA-44") and German travellers to Costa Rica [9]. ST72 strains demonstrated medium to high PVL levels.

3.4.5. Clonal complex 9/sequence type 834

Two PVL-positive MRSA belonged to CC9. Both were assigned to ST834 based on their unique hybridisation profiles that differ from other CC9 strains (ST9: *agr* II, *cap 5, egc*; [9] and ST834: *agr* I, *cap 8, lukD/E, sasG*) although ST834 was assigned to CC9 according to the MLST database (MLST profiles ST9; 3-3-1-1-1-1-10 and ST834; 3-124-1-1-1-1-40). In this study, two isolates of ST834-MRSA-IV was included. These were found in Norwegian SSTI patients with travel histories to the Philippines. They expressed PVL at a low level.

3.4.6. Clonal complex 22

CC22 is another common clonal complex that includes widely distributed MSSA as well as MRSA strains. PVL genes were detected in CC22-MSSA, CC22-MSSA carrying *fusC* and CC22-MRSA-IV. CC22-MSSA-*fusC* were observed exclusively in England, where an epidemiological connection was possible [18]. They yielded higher PVL levels than CC22-MSSA. CC22-MRSA-IV originated from Europe and the Middle East. Their PVL production varied widely, with mean and median values being both *ca.* 1100 ng/ml.

3.4.7. Clonal complex 25

Two clinical MSSA isolates from Germany and the NARSA227 strain (that is also positive for *sec* and *sel*) were checked and the expression of PVL was similar in all three cases (mean and median *ca.* 1500 ng/ml). PVL-positive MRSA from this CC were not found.

3.4.8. Clonal complex 30

CC30-MSSA that harbour *lukF-PV* and *lukS-PV* genes have been known for long time, causing pandemics in the 1940s and 1960s ("phage type 80/81"). Widely used laboratory strains ATCC25923 and "Oxford Staphylococcus" are PVL-positive CC30-MSSA. Clinical MSSA isolates from different geographical regions showed different levels of PVL production ranging from very low up to high concentrations with *tst1* positive isolates yielding less PVL than others (see below). ATCC25923 was in accordance to other CC30-MSSA although an ATCC25923 sub-clone kept and passaged in the laboratory in Dresden produced more PVL than any other strain tested for this study with a mean of 41,000 and a median of 22,000 ng/ml. One MRSA strain from this lineage, CC30-MRSA-IV (also known as

Southwest Pacific Clone, USA1100 or West Samoan Phage Pattern Clone) is widespread, with isolates tested for this study originating from Australia, Saudi Arabia and several European countries. Contrarily, CC30-MRSA-V appears to be very [34] sporadic; a single isolate from the easternmost part of Germany was identified. For both MRSA strains, PVL expressions were at low levels.

3.4.9. Clonal complex 45

CC45 is an important lineage including MSSA as well as several MRSA strains. However, PVL is extremely rare in CC45; only two isolates were found with one of them being additionally positive for *tst1*. In both cases, PVL expression was similar (*ca.* 3000 ng/ml).

3.4.10. Clonal complex 59

To our best knowledge PVL-positive CC59-MSSAs are apparently rare to virtually non-existent; no isolate was identified for this study. There are several PVL-positive MRSA strains that largely differ in SCC*mec* elements and other accessory genes [35]. PVL yields of these strains differed widely.

3.4.11. Clonal complex 80

Most CC80 strains isolates belonged to the widespread "European CA-MRSA clone" CC80-MRSA-IV. CC80-MSSA and CC80-MRSA with other types of SCC*mec* elements as well as CC80 strains without *lukF-PV/lukS-PV* genes are very rare. All CC80 isolates expressed PVL at comparable low levels only.

3.4.12. Clonal complex 88

CC88 appears to be closely related to CC1 and CC80 belonging to the same *agr*-group (III), the same capsule type (8) and to similar *spa* types (such as, *e.g.*, t127, 07-23-21-16-34-33-13 in CC1; t044, 07-23-12-34-34-33-34 in CC80 and t186, 07-12-21-17-13-13-34-34-33-34 in CC88). Tested isolates included CC88-MSSA as well as CC88-MRSA-IV. PVL levels were uniformly relatively low.

3.4.13. Sequence type 93

ST93 are largely restricted to Australia, where ST93-MSSA and ST93-MRSA-IV, "Queensland Clone" [36] are common. Both strains yielded high PVL concentrations.

3.4.14. Clonal complex 96

CC96 is also related to CC1 and CC80, belonging to the same *agr*-group (III) and capsule type 8 as well as to similar *spa* types. There are MSSA, as well as MRSA, the latter being apparently epidemiologically linked to Central Asia [9]. PVL yields within this group differed widely (see Table 2).

3.4.15. Clonal complex 121

CC121 appears to be a pandemic clone of MSSA [14,16,17,37,38], while MRSA from that complex are extremely rare [9]. Fourteen isolates from European countries were found to yield high PVL levels.

3.4.16. Clonal complex 152

CC152-MSSA are rare in Europe but were commonly described from Africa [38,39] and we also found them at Trinidad and Tobago [18]. CC152-MRSA-V are sporadically found in Europe and frequently a travel history to the Balkans is reported [9]. The MSSA isolates tested here showed high PVL levels while CC152-MRSA-V yielded clearly less.

3.4.17. Clonal complex 188

While most CC188 strains including MRSA are PVL-negative, two PVL-positive CC188-MSSA were identified from patients with SSTI from Australia and Saudi-Arabia. They produced very different levels of PVL (see Table 2).

3.4.18. Clonal complex 398

The CC398 lineage consists of PVL-negative strains, both MSSA and MRSA that were recently found in humans and domestic animals from several countries. PVL-positives appear to be restricted to humans and have often an epidemiological connection to Asia [40–42]. For MSSA PVL yields varied widely while CC398-MRSA-V isolates displayed a less variable PVL production of about 1000 ng/ml.

Sequence types 291/813 form a distinct group within CC398 differing in *aroE* and *yqiI* alleles, in lack of signals for the *coa* gene (probably indicating a deviant allele as these isolates were phenotypically positive for plasma coagulase) as well as in the presence of *lukE*, *etD*, *edinB* and of protease genes *splA*, *splB* and *splE*. Two PVL-positive MSSA isolates were tested. In one, no PVL expression was detected, but the second one yielded a high PVL concentration. MRSA from this group were not identified.

3.4.19. Rare sequence types

For ST942, two MSSA isolates identified for this study. Both isolates carried *tst1* besides PVL, and PVL production was either low or entirely absent according to with the described method.

ST2479 is another rare lineage for which also only two MSSA were identified, one from Dresden and another from Spain. Those isolates demonstrated a very low PVL level.

ST2482 has only recently been described from the Middle East and Sweden [18]. Three isolates were obtained from Saudi Arabia. No PVL expression was detectable.

3.5. PVL production and agr-group affiliation

Ninety-five isolates were assigned to *agr*-group I. These isolates yielded a mean of 2791 ng/ml and a median of 1714 ng/ml PVL, respectively. This group included two isolates for which no PVL production was detectable. To *agr*-group II, 49 isolates belonged for that a mean of 986 ng/ml and a median of 574 ng/ml PVL were determined. Three isolates where non-producers. 107 isolates were assigned to *agr*-group III yielding a mean of 981 and a median of 193 ng/ml PVL (with the hyper-producing ATCC25923 from Dresden being excluded, see above). Four isolates did not produce detectable PVL levels. Finally, for 14 *agr* IV isolates (all being PVL-positive) a mean of 2533 ng/ml and a median of 1627 ng/ml PVL were found.

To test the null-hypothesis that the PVL-concentrations are the same in each *agr*-group we performed the Kruskal–Wallis rank sum test [43,44]. Here, in contrast to the analysis of variance (ANOVA), it is not required that the data within the groups are normally distributed and have the same standard deviation (Fig. 4).

The four *agr*-groups have different medians and the results from the Kruskal–Wallis test show a probability below 0.001 for a true null-hypothesis (Chi-Square = 123.55, Degrees of freedom = 3, Probability of a true null-hypothesis < 2.2e-16). Therefore, it is very likely that the PVL concentrations are truly different between the different *agr*-groups.

3.6. PVL production in tst1-positive strains

It was previously described that TSST-1 producing strains yield very low concentrations of other exoproteins [45,46]. Thirteen isolates were analysed that harboured *tst1* and *lukF/S-PV* genes. Only 13 such isolates were found with three of them being obtained subsequently from a single paediatric patient (see below, and [47]). These isolates yielded a mean concentration of 511 and a

Table 1
Overview on clonal complexes, strains and DNA microarray data. All isolates were *lukF/S-PV* positive. (*lukD/E*) in brackets, only one component detected, possibly indicating sequence variations. Pos., always positive; comm, common (in more than 2/3 of tested isolates); var, variable (in 1/3 to 2/3 of tested isolates); rare (in less than 1/3 of tested isolates); –, always absent.

Clonal complex	CC/ST-specific markers	Strain	Number of isolates	*mecA*	*tst1*	*sea*	*sea (N315)*
CC1	*agr* III, *cap 8, seh, lukD/E, cna, sasG*	**CC1-MSSA**	**3**	–	–	Comm	–
		CC1-MSSA [*tst1*+/PVL+]	**1**	–	Pos	Pos	–
		CC1-MSSA-SCCfus	**3**	–	–	Comm	–
		CC1-MRSA-IV, USA400	**5**	Pos	–	Comm	–
		CC1-MRSA-V	**2**	Pos	–	Pos	–
		CC1-MRSA-V+SCC*fus*	**1**	Pos	–	–	–
CC1 (ST567)	*agr* III, *cap 8*, ORF CM14, *lukD/E, sasG*	**ST567-MSSA**	**1**	–	–	–	–
CC1 (ST573/772)	*agr* II, *cap 5, egc*, ORF CM14, *cna, sasG*	**ST573/772-MSSA**	**1**	–	–	Var	–
		ST772-MRSA-V, Bengal Bay Clone	**24**	Pos	–	Pos	–
CC5	*agr* II, *cap 5, egc, lukD/E, sasG*	**CC5-MSSA**	**6**	–	–	Rare	Var
		CC5-MRSA-IV,	**5**	Pos	–	–	Comm
		CC5-MRSA-IV, WA MRSA-64/121	**2**	Pos	–	Var	–
		CC5-MRSA-V	**3**	Pos	–	–	Comm
CC6	*agr* I, *cap 8, lukD/E, cna, sasG*	**CC6-MSSA**	**1**	–	–	Pos	–
CC8	*agr* I, *cap 5, lukD/E, sasG*	**CC8-MSSA**	**4**	–	–	–	–
		ST8-MRSA-IV [PVL+/ACME+], USA300	**21**	Pos	–	–	–
		ST8-MRSA-IV [PVL+/ACME–]	**7**	Pos	–	–	–
		CC8-MRSA-IV, WA MRSA-62	**3**	Pos	–	–	–
CC8 (ST72)	*agr* I, *cap 5, egc, lukD/E, sasG*	**ST72-MSSA**	**1**	–	–	–	–
		ST72-MRSA-IV, WA MRSA-44	**2**	Pos	–	–	–
CC9 (ST834)	*agr* I, *cap 8, lukD/E, sasG*	**ST834-MRSA-IV [*tst1*+/PVL+]**	**2**	Pos	Pos	–	–
CC15	*agr* II, *cap 8, lukD/E, sasG*	**CC15-MSSA**	**3**	–	–	Comm	–
CC22	*agr* I, *cap 5, egc, cna, sasG*	**CC22-MSSA**	**9**	–	–	–	–
		CC22-MSSA-SCCfus	**2**	–	–	–	–
		CC22-MRSA-IV	**8**	Pos	–	–	–
CC25	*agr* I, *cap 5, egc, lukD/E,*	**CC25-MSSA**	**3**	–	–	–	–
CC30	*agr* III, *cap 8, egc, cna,*	**CC30-MSSA**	**18**	–	–	Rare	–
		CC30-MSSA [*tst1*+/PVL+]	**7**	–	Pos	–	–
		CC30-MRSA-IV, Southwest Pacific Clone	**10**	Pos	–	–	–
		CC30-MRSA-V	**1**	Pos	–	–	–
CC45	*agr* I, *cap 8, egc, cna,*	**CC45-MSSA**	**1**	–	–	–	–
		CC45-MSSA [tst1+/PVL+]	**1**	–	Pos	–	–
CC49	*agr* II, *cap 5, lukD/E, cna, sasG*	**ST49-MSSA**	**1**	–	–	–	–
CC59	*agr* I, *cap 8,* (commonly *sasG*)	**CC59-MRSA-IV, USA1000**	**3**	Pos	–	–	–
		ST59-MRSA-IV, WA MRSA-55/56	**4**	Pos	–	Rare	–
		CC59-MRSA-V	**1**	Pos	–	–	–
		ST59/952-MRSA-V(T), Taiwan Clone	**6**	Pos	–	–	–
CC80	*agr* III, *cap 8, lukD/E, sasG*	**CC80-MSSA**	**3**	–	–	–	–
		CC80-MRSA-IV, European caMRSA	**13**	Pos	–	–	–
		CC80-MRSA-(trunc./atyp. SCC*mec*)	**2**	Pos	–	–	–
CC88	*agr* III, *cap 8, lukD/E, sasG*	**CC88-MSSA**	**6**	–	–	–	Comm
		CC88-MRSA-IV	**5**	Pos	–	–	Comm
ST93	*agr* III, *cap 8*, ORF CM14, *(lukD/E),*	**ST93-MSSA**	**7**	–	–	–	–
		ST93-MRSA-IV, Queensland Clone	**12**	Pos	–	–	–
CC96/154	*agr* III, *cap 8, lukD/E, cna, sasG*	**CC96-MSSA**	**2**	–	–	–	–
		ST154-MRSA-IV	**2**	Pos	–	Rare	–
CC121	*agr* IV, *cap 8, egc*, ORF CM14, *lukD/E, cna*	**CC121-MSSA**	**14**	–	–	Rare	–
CC152	*agr* I, *cap 5, (lukD/E), cna,*	**CC152-MSSA**	**5**	–	–	–	–
		CC152-MRSA-V	**2**	Pos	–	–	–
CC188	*agr* I, *cap 8, lukD/E, cna,*	**CC188-MSSA**	**2**	–	–	–	–
CC398	*agr* I, *cap 5, cna,*	**CC398-MSSA**	**3**	–	–	–	–
		CC398-MRSA-V	**3**	Pos	–	Var	–
CC398 (ST291/813)	*agr* I, *cap 5, (lukD/E)*	**ST291/813-MSSA**	**2**	–	–	–	–
CC942	*agr* III, *cap 5, lukD/E, sasG*	**CC942-MSSA [*tst1*+/PVL+]**	**2**	–	Pos	–	–
ST2479	*agr* III, *cap 8, seh, egc, (lukD/E), cna, sasG*	**ST2479-MSSA**	**2**	–	–	–	–
ST2482	*agr* II, *cap 5, sasG*	**ST2482-MSSA**	**3**	–	–	–	–

median of 70 ng/ml PVL. In contrast, 253 *tst*1-negative strains (with the hyper-producing ATCC25923 variant being excluded) resulted with a mean of 1737 and a median concentration of 650 ng/ml. In order to rule out effects caused by CC affiliation, only the most common lineage, in which *tst1* and PVL co-existed, was analysed. This was CC30-MSSA, with seven isolates that yielded mean and median concentrations of 204 and 70 ng/ml, respectively. For comparison, mean and median for PVL concentrations of 17 *tst1*-negative, PVL-positive CC30-MSSA (again, the hyper-producing ATCC25923 variant was excluded) were 1134 and 498 ng/ml. Further statistics were not performed due to the small number of *tst1*-positives.

3.7. PVL production and severity of disease

Five isolates originated from three cases of lethal infections with *S. aureus*. All these cases were paediatric. One was a child with necrotising fasciitis and sepsis from Trinidad and Tobago, from whom a PVL-positive CC8-MSSA was isolated [29]. Another case was an infant with fulminant pneumonia and multiorgan failure

seb	*sec/l*	*sed/j/r*	*sek/q*	*etD*	*edinA*	*edinB*	ACME
–	–	–	Comm	–	–	–	–
–	–	–	Pos	–	–	–	–
–	–	–	Comm	–	–	–	–
Rare	Var	–	Comm	–	–	–	–
–	–	–	Pos	–	–	–	–
–	–	–	Pos	–	–	–	–
–	–	–	–	–	–	–	–
–	Comm	–	–	–	–	–	–
–	Comm	–	–	–	–	–	–
–	–	Var	–	–	Var	–	–
–	–	Var	–	–	–	–	–
–	–	–	–	–	Pos	–	–
Var	–	Comm	–	–	–	–	–
–	–	–	–	–	–	–	–
–	Rare	Comm	Comm	–	–	–	–
–	–	–	Comm	–	–	–	Pos
–	–	–	Comm	–	–	–	–
–	–	Pos	Pos	–	–	–	–
–	–	–	–	–	–	–	–
–	Pos	–	–	–	–	–	–
–	Pos	–	–	–	–	–	–
–	–	–	–	–	–	–	–
Rare	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–
–	Var	–	–	Pos	–	Pos	–
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–	Comm	–	–	–	–	–	–
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[47] yielding *tst1*- and PVL-positive CC30-MSSA. Finally, the sequenced strain MW2 fell into that category [48]. The mean and mean PVL concentrations for these strains were 175 and 111 ng/ml, respectively.

Eighteen isolates were categorised as originating from severe/systemic infections being cultivated from blood cultures, cerebrospinal fluid or being associated with necrotising fasciitis, pneumonia or shock. Their mean and median PVL concentrations were 1091 and 170 ng/ml.

The vast majority of isolates were non-systemic infections (n = 146; with the hyper-producing ATCC25923 variant being excluded), most of which were abscesses, furuncles or other skin and soft tissue infections. The mean and mean PVL concentrations of these isolates were 1507 or 523 ng/ml, respectively.

Further statistics were not performed due to the small number of fatal and invasive cases.

4. Discussion

We have demonstrated that protein microarrays provide reproducible and valid results and are therefore a suitable technology for quantitative measurement of toxin secretion in bacterial

Table 2
Protein microarray data on PVL expression.

Clonal complex	Strain	Number of isolates	Number of measurements	PVL mean [ng/ml]	PVL median [ng/ml]	PVL range [ng/ml]
CC1	**All strains**	**15**	**57**	**255**	**151**	**0…1658**
	CC1-MSSA	3	7	342	135	0…1658
	CC1-MSSA, [*tst1*+/PVL+]	1	1	933	933	933…933
	CC1-MSSA-SCCfus	3	7	333	331	0…1004
	CC1-MRSA-IV, USA400	5	35	213	150	0…957
	CC1-MRSA-V	2	4	278	174	0…764
	CC1-MRSA-V+SCC*fus*	1	3	110	160	0…169
CC1 (ST567)	**ST567-MSSA**	**1**	**3**	**1604**	**326**	**231…4255**
CC1 (ST772)	**All strains**	**26**	**73**	**1415**	**1278**	**0…8910**
	ST573/772-MSSA	2	5	4733	5193	1283…8910
	ST772-MRSA-V, Bengal Bay Clone	24	68	1171	1270	0…4594
CC5	**All strains**	**16**	**40**	**527**	**326**	**0…2321**
	CC5-MSSA	6	9	948	700	31…2321
	CC5-MRSA-IV,	5	13	530	338	0…1344
	CC5-MRSA-IV, WA MRSA-64/121	2	9	359	0	0…1653
	CC5-MRSA-V	3	9	271	113	0…1083
CC6	**CC6-MSSA**	**1**	**2**	**8018**	**8018**	**7174…8861**
CC8	**All strains**	**35**	**89**	**4336**	**4303**	**0…19,101**
	CC8-MSSA	4	9	1643	225	0…7249
	ST8-MRSA-IV [PVL+/ACME+], USA300	21	68	4806	4766	262…19,101
	ST8-MRSA-IV [PVL+/ACME-]	7	7	3193	3486	814…6873
	CC8-MRSA-IV, WA MRSA-62	3	5	4389	4568	2532…5987
CC8 (ST72)	**All strains**	**3**	**6**	**8639**	**8144**	**2945…18,958**
	ST72-MSSA	1	2	3847	3847	2945…4749
	ST72-MRSA-IV, WA MRSA-44	2	4	11,035	8554	8075…18,958
CC9 (ST834)	**ST834-MRSA-IV, [*tst1*+/PVL+]**	**2**	**5**	**794**	**243**	**0…1848**
CC15	**CC15-MSSA**	**3**	**9**	**669**	**408**	**0…1512**
CC22	**All strains**	**19**	**46**	**1680**	**1433**	**0…5048**
	CC22-MSSA	9	15	2069	1836	369…4487
	CC22-MSSA-SCCfus	2	4	4260	4503	2988…5048
	CC22-MRSA-IV	8	27	1082	1131	0…3365
CC25	**CC25-MSSA**	**3**	**3**	**1575**	**1565**	**1156…2005**
CC30	**All strains (ATCC25923 from Dresden laboratory excluded)**	**36**	**64**	**815**	**336**	**0…8893**
	CC30-MSSA (ATCC25923 excluded)	16	33	1107	472	0…8893
	ATCC25923	1	5	1312	1291	362…2427
	ATCC25923 (from Dresden laboratory)	1	6	41,151	22,175	10,443…150,992
	CC30-MSSA [*tst1*+/PVL+]	7	7	204	70	52…518
	CC30-MRSA-IV, Southwest Pacific Clone	10	16	401	180	0…2770
	CC30-MRSA-V	1	3	404	429	317…467
CC45	**All strains**	**2**	**4**	**2941**	**2996**	**1944…3829**
	CC45-MSSA	1	2	3279	3279	2729…3829
	CC45-MSSA [tst1+/PVL+]	1	2	2603	2603	1944…3262
CC49	**ST49-MSSA**	**1**	**3**	**1227**	**1242**	**339…2099**
CC59	**All strains**	**14**	**53**	**1111**	**678**	**0…6311**
	CC59-MRSA-IV, USA1000	3	12	13	0	0…130
	ST59-MRSA-IV, WA MRSA-55/56	4	6	1500	1590	401…2533
	CC59-MRSA-V	1	1	384	384	384…384
	ST59/952-MRSA-V(T), Taiwan Clone	6	24	1593	1030	281…6311
CC80	**All strains**	**18**	**37**	**234**	**149**	**0…1639**
	CC80-MSSA	3	5	228	170	0…651
	CC80-MRSA-IV, European caMRSA	13	26	283	160	0…1639
	CC80-MRSA-(trunc./atyp. SCC*mec*)	2	6	24	0	0…92
CC88	**All strains**	**11**	**28**	**201**	**0**	**0…2571**
	CC88-MSSA	6	12	186	0	0…1457
	CC88-MRSA-IV	5	16	211	0	0…2571
ST93	**All strains**	**19**	**41**	**3757**	**3346**	**95…12,170**
	ST93-MSSA	7	18	4250	4189	939…10,025
	ST93-MRSA-IV, Queensland Clone	12	23	3372	1039	95…12,170
CC96/154	**All strains**	**4**	**13**	**904**	**0**	**0…7778**
	CC96-MSSA	2	7	84	0	0…374
	ST154-MRSA-IV	2	6	1861	39	0…7778
CC121	**CC121-MSSA**	**14**	**22**	**2533**	**1627**	**251…7649**
CC152	**All strains**	**8**	**14**	**2138**	**990**	**224…7383**
	CC152-MSSA	5	7	3694	3927	330…7383
	CC152-MRSA-V	3	7	583	637	224…1111
CC188	**CC188-MSSA**	**2**	**5**	**1467**	**526**	**182…3399**
CC398	**All strains**	**6**	**13**	**1774**	**1154**	**0…7842**
	CC398-MSSA	3	8	2146	1267	0…7842
	CC398-MRSA-V	3	5	1180	1056	669…1658
CC398 (ST291/813)	**ST291/813-MSSA**	**2**	**8**	**593**	**0**	**0…2957**
CC942	**CC942-MSSA [*tst1*+/PVL+]**	**2**	**8**	**29**	**0**	**0…229**
ST2479	**ST2479-MSSA**	**2**	**6**	**65**	**36**	**0…169**
ST2482	**ST2482-MSSA**	**3**	**11**	**0**	**0**	**0…0**

"All strains" from a lineage is in bold. Individual strains are not in bold.

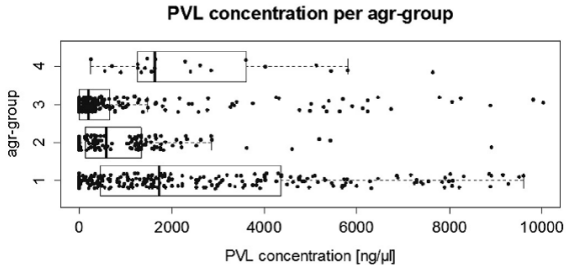

Fig. 4. The diagram shows the measure PVL concentrations for each of the 4 agr-groups as boxplots overlayed by all individual data points (dots). For scaling purposes, two data points above 10,000 ng/µl are not drawn.

cultures. Compared to other immunoassays the protein arrays using precipitation staining, allow fast quantification on previously mapped serial dilutions.

In a few isolates, carrying *lukF-PV* and *lukS-PV* genes, PVL expression could not be measured. Such isolates may produce very low concentrations of PVL, even below the sensitivity of the protein array. Alternatively, these isolates may have *lukF-PV*/*lukS-PV* genes that do not express PVL or, low expression levels due to other factors such as the media. Some of these isolates (such as ST2482) have been shown to produce PVL when cultured on Columbia blood agar [18].

The presence of *tst1* resulted in lower PVL levels. This is in line with previous observations on the effect of *tst1* on other exotoxins. *Agr*-groups I and IV generally produced higher amounts of PVL than *agr*-groups II and III. However, some very high level producers (ST93-MSSA, ST93-MRSA-IV and ATCC25923 from Dresden) belonged to *agr*-group III.

We found no direct correlation of clinical presentation to PVL yield. High and low level PVL producers caused similar skin and soft tissue infections. The severity of infection did not correlate to PVL concentration produced *in vitro*. The isolates from invasive or fatal cases of PVL-positive *S. aureus* infections were even shown to express low levels of PVL. It may be argued that patient outcome could be related to host factors, or to the presence of other toxin genes (*tst1, seh, sed/j/k/r/q,* respectively). Furthermore, it could be argued that isolates from the fatal cases and from the cases categorised as systemic/severe originated from sample types (such as blood cultures) or clinical conditions for which it is already known that PVL is not of major significance [49]. In a clinical condition such as a bloodstream infection, PVL is not required for pathogenesis, and therefore there would be no need for high level expression of PVL, regardless of outcome and severity of the disease. Contrarily, the level of expression would be higher for isolates from the natural "ecological niche" of PVL-positive *S. aureus*, *i.e.*, from skin and soft tissue infections.

High level producers included some recently emerging and highly successful strains (ST8-MRSA-IV, "USA300"; ST93-MSSA and ST93-MRSA-IV, "Queensland Clone"). While it may be tempting to attribute the "success" of the former strains to their high level of PVL production, there are sporadic, rare and localised strains (CC6, ST72) that also express high levels of PVL. Conversely, there are frequent isolated successful strains (CC22, CC30, CC80) that yielded low PVL concentrations.

Assuming there is no direct correlation between PVL expression and the severity of disease or the epidemiological success of a strain, there may be two explanations. PVL may be highly potent, so even very low concentrations may be associated with disease in a susceptible host. Alternatively, the *in vitro* production might be entirely dissociated from the situation *in vivo*. This issue warrants further study [19].

High levels of PVL production were observed in laboratory strains stored for long periods of time (ATCC25923 from Dresden, a phage type 80/81 isolate). This could possibly indicate mutations in regulatory pathways and also warrants further investigation since it has already been observed that old laboratory strains may differ from wild types with regard to regulation of exotoxin production [50].

While typically isolates from the same CC yielded uniform PVL concentrations, it was observed that PVL expression could vary within a CC (Clonal complex 398, Sequence type 291/813). This observation still needs to be explained. Possible reasons for this variability could include mutations in regulatory pathways or possibly the presence of different prophages carrying *lukF-PV* and *lukS-PV* genes and, possibly, different alleles or activities of regulatory genes or promoters.

In conclusion we have shown in a PVL toxin measurement the protein microarray is a reliable method that could be used for rapid quantification of expressed proteins such as PVL in collections of clinical isolates in order to correlate with clinical or genotypic data or to demonstrate the influence environmental factors, such as antimicrobials, have on toxin production. Using several specific antibodies, the method can also be expanded to study simultaneously the expression of different toxins produced by bacteria in one culture.

Conflict of interest

B. Stieber, S. Monecke, E. Müller, V. Baier and R. Ehricht are employees of Alere Technologies.

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Publikation V

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Staphylococcus aureus In Vitro Secretion of Alpha Toxin (hla) Correlates with the Affiliation to Clonal Complexes

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Abstract

The alpha toxin of *Staphylococcus aureus* is a pore forming toxin that penetrates host cell membranes causing osmotic swelling, rupture, lysis and subsequently cell death. Haemolysin alpha is toxic to a wide range of different mammalian cells; i.e., neurotoxic, dermonecrotic, haemolytic, and it can cause lethality in a wide variety of animals. In this study, the *in vitro* alpha toxin production of 648 previously genotyped isolates of *S. aureus* was measured quantitatively using antibody microarrays. Isolates originated from medical and veterinary settings and were selected in order to represent diverse clonal complexes and defined clinical conditions. Generally, the production of alpha toxin *in vitro* is related to the clonal complex affiliation. For clonal complexes CC22, CC30, CC45, CC479, CC705 and others, invariably no alpha toxin production was noted under the given *in vitro* conditions, while others, such as CC1, CC5, CC8, CC15 or CC96 secreted variable or high levels of alpha toxin. There was no correlation between alpha toxin yield and clinical course of the disease, or between alpha toxin yield and host species.

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Introduction

Staphylococcus (S.) aureus is a gram-positive coccus that is an important commensal bacterium and pathogen in both, animals and humans. Some 30% of a healthy human population carries *S. aureus* asymptomatically in the anterior mucosa of their noses. Animals of several species also might be colonized or infected. *S. aureus* also can cause a variety of different infections including localised skin and soft tissue infections, more severe conditions such as osteomyelitis or pneumonia and life-threatening endocarditis or septicaemia. This bacterium can also trigger toxin-mediated diseases such as food intoxication, toxic shock and scalded skin syndromes. It is known to harbour, beside genes associated with drug resistance and adhesion to host tissues etc., a complex array of virulence factors that includes superantigens (toxic shock syndrome toxin, *tst1*, and some 30 enterotoxin genes), exfoliative toxins, proteins that interfere with various functions of the host immune system (complement and chemotaxis inhibitors *etc.*), leukocidins (*i.e.*, toxins that specifically destroy white blood cells by formation of polymeric pores in cell membranes) and different haemolysins. The latter are proteins that lyse amongst others red blood cells, allowing for instance the bacterium to scavenge iron compounds. In *S. aureus*, there are three major, well characterised haemolysins, named alpha, beta, and gamma, as well as additional genes that are assumed to encode for additional haemolysins (such as BA000017.4: locus tag SAV0919; base positions 962,930 to 963,970 or CP000046.1: locus tag SACOL2160; base positions 2,239,231 to 2,239,914).

The alpha toxin (also known as haemolysin alpha) is encoded by the *hla* gene (BA000018.3: locus tag SA1007; base positions 1,140,562 to 1,141,521). To our best knowledge, this gene can be found in all *S. aureus* strains and isolates, and it is always situated at approximately the same localisation within the staphylococcal chromosome, *i.e.*, around base positions 1,110,000 to 1,230,000 in a genome of about 2,743,000 to 3,043,000 base pairs of total length. There is a relatively small sequence variation of *hla* alleles (see File S1) across the different clonal complexes (CC; as defined by Multilocus Sequence Typing, MLST, [1]) of *S. aureus*. Compared to the DNA sequence of the CC5 strain N315 (BA000018.3) as reference there are, for instance, five nucleotide exchanges (out of 960 positions) in the CC8 strain COL (CP000046), 50 nucleotide exchanges in the CC30 strain Sanger MRSA 252 (BX571856) and 137 for the CC75 strain MSHR1132 (FR821777), a strain that is so different from other *S. aureus* that it has been proposed to regard it as species on its own [2]. These nucleotide exchanges result for the three strains in two, three and 36 amino acid exchanges compared to N315, respectively.

The alpha toxin is a pore forming toxin. The pore is a polymeric ring with a diameter of 1–2 nm comprising of seven 33 kDa protein molecules that penetrates in host cell membranes causing, osmotic swelling, rupture, lysis and subsequently cell death. Haemolysin alpha is toxic to a wide range of different mammalian cells; *i.e.*, neurotoxic, dermonecrotic, haemolytic, and it can cause lethality in a wide variety of animals. Its toxic effects include activation of the arachidinic metabolism in endothelial cells due to Ca^{2+} influx, activation of cellular nucleases [3] and resulting apoptosis [4], activation of the autophagic pathway in case of

intracellular presence of *S. aureus* [5], release of procoagulatory factors due to Ca^{2+} influx in platelets, vasoconstriction associated with a liberation of thromboxane A(2) and prostacyclin [6], and an increase of vascular permeability that might lead to pulmonary oedema and adult respiratory distress syndrome. Experimental studies indicated alpha toxin to be an important virulence factor in both rabbit and murine models of keratitis [7] as well as in pneumonia [8,9] and superinfection of influenza [10]. Alpha toxin is regulated by both, the *agr* and *saeR/S* systems as the deletion of *saeRS*, and, to a lesser extent, *agr* resulted in its attenuated expression [11]. Similar observations were also described with regard to *sarA/Z* [12] indicating that alpha toxin, as well as other exotoxins such as Panton-Valentine leukocidin and proteases are up-regulated during the dissemination phase of *S. aureus* infections but down-regulated during a stationary phase when factors prevail that are associated with biofilm formation and adhesion.

The *hla* gene is present essentially in all isolates and lineages of *S. aureus*. Invasive or non-invasive isolates thus do not differ in the mere presence or absence of the gene, and this warrants the study of the expression or regulation of the toxin. Therefore, the aim of the study was to develop, establish and use a simple and robust antibody-based system for the quantitative measurement of alpha toxin in *S. aureus* cultures.

Materials and Methods

Strains

In this study, 648 isolates and reference strains were tested. They originated from medical and veterinary settings and were selected in order to represent diverse clonal complexes and defined clinical conditions. All isolates, were previously genotyped using the StaphyType Kit (Alere Technologies, Jena, Germany) as described in detail before [13,14]. Using this method, relevant genes as typing-, virulence- and resistance marker were determined and isolates were assigned to CCs and strains. Characterisation and hybridisation profiles of all lineages and most isolates have been described previously [13,14,15,16,17,18,19,20,21,22,23,24,25].

Culture Conditions

Strains were cultured on Columbia blood agar (agar basis Oxoid, CM331 and sheep blood OXOID, FSR1055) and incubated for 24 hrs at 37°C. One loop of bacterial material was inoculated into 65 µl 100 mM NaOH, vortexed and incubated for 5 min at room temperature (RT). This procedure yielded slightly better results than a suspension in PBS (data not shown), presumably due to lysis of cells and release of intracellularly stored alpha toxin. Then, 65 µl of phosphate solution (pH 5,5; 1M di-sodium hydrogen phosphate, 1M sodium-di-hydrogen phosphate) was added for neutralisation and vortexed. The mixture was diluted 1:10 in buffer [1x PBS; 0.05% Tween; 1% FCS; 0.25% Triton X-100] for further analysis.

Antibodies

Alpha Toxin (HT101; Toxin Technology, Sarasota, Florida, USA) from the *S. aureus* strain Wood 46 (CC97-MSSA) was used to generate monoclonal antibodies via phage display. Following immunisation of mice, mRNA from their B-cells was isolated and amplified. Resulting cDNA, specific for the antigen-binding parts of antibodies, was ligated into bacteriophages and transformed into *E. coli*. Resulting antibodies were purified and characterized for specificity and sensitivity by ELISA microtiterstrip-mounted protein microarrays. For further experiments, three different alpha toxin antibodies were immobilised on the array at nine different concentrations.

Array Procedures and Detection

First, the protein microarrays were pre-washed and blocked. Arrays were incubated with 150 µl buffer (as above) at 37°C for 5 min at 400 rpm on a shaker, followed by 100 µl blocking solution (10% foetal calf serum) for 5 min at 37°C and 300 rpm. Then, 100 µl of the suspended bacteria was added and incubated at 37°C for 30 min at 300 rpm.

A secondary antibody was labelled with biotin (Sulf-NHS-LC-Biotin; Pierce, Bonn, Germany) and used (*ca.* 0.2 ng/µl, 100 µl, 37°C for 30 min at 300 rpm) to detect bound proteins followed by addition of streptavidin-horseradish peroxidase (HRP; 0,5 ng/µl, 100 µl, 15 min at 37°C and 300 rpm). The latter induced the local precipitation of the added dye, tetramethylbenzidine (TMB; 10 min without shaking at room temperature). The precipitation reaction was stopped by removing the substrate [26,27,28].

Analysis

Array images were taken by the ArrayMate reading device (Alere Technologies, Jena, Germany) and analysed using IconoClust software according to manufacturer's instructions. The quantitative analysis based on a set of previously established calibration- and reference-experiments with three alpha toxin antibodies each spotted at nine different concentrations. For calibration curves, alpha toxin (1 mg/ml; Toxin Technology) was tested at different defined concentrations (0.1 ng/ml, 0.2 ng/ml, 0.4 ng/ml, 0.6 ng/ml, 0.8 ng/ml, 1.0 ng/ml, 2.0 ng/ml, 3.0 ng/ml, 4.0 ng/ml, 6.0 ng/ml, 8.0 ng/ml, 10.0 ng/ml). Resulting signal intensities of each single spot were correlated to the known antigen concentrations. Thus, standard curves were generated for each concentration of each antibody. In the following experiments, unknown concentrations of alpha toxin were determined by mapping the intensity of array spots on the previously established calibration curves. This approach has previously been described in detail for another staphylococcal toxin, PVL [29].

Results

Yield of Alpha Toxin and Affiliation to Clonal Complexes

Generally, the yield of alpha toxin appears to be related to the CC affiliation. For a detailed overview, see Table 1. For clonal complexes CC22, CC30, CC45, CC479, CC705 and others consistently no alpha toxin production, or very low levels thereof, was noted under the given *in vitro* conditions. Isolates from other lineages (such as CC1, CC5, CC8, CC15 or CC96) secreted variable to high levels of alpha toxin. Only few isolates (one or two from CC398, CC59, respectively) yielded positive results despite affiliation to a CC that normally did not produce detectable amounts of alpha toxin; or lacked alpha toxin production while belonging to an otherwise positive ST (one isolate from ST72).

Certain sequence types present with very different DNA array hybridisation patterns (different *agr* group, different capsule type, and other alleles of *ssl* or MSCRAMM genes) than the CC they are assigned to according to sequence based algorithms (BURST; see http://saureus.mlst.net/eburst/). Some of them are known to originate from large scale chromosomal replacements [30]. In some instances, such deviant STs show essentially the same alpha toxin results as the CC they are derived from; examples being CC1 (ST567), CC7 (ST1048), CC8 (ST72), CC30 (ST34/42), CC97 (ST71) or CC188 (ST1774). In a few instances, there were differences to the parental lineage. This included CC398 (ST291/813) strains where the alpha toxin yield was higher than in other

Table 1. Alpha Toxin yields by CC/ST affiliation.

Clonal Complex/ Sequence Type	Tested isolates	Host species	Mean (ng/ml)	Median (ng/ml)	Range (ng/ml)			Standard deviation (ng/ml)
CC1	40	Human	8.2	6.5	0.5	…	31.7	6.7
CC1 (ST567)	1	Human	6.2	6.2	6.3	…	6.2	–
CC1 (ST573/772)	8	Human	0.0	0.0	0.0	…	0.0	0.0
CC5	46	Human	5.3	1.1	0.0	…	32.1	7.9
CC5 (*excluding* **ST228-MRSA-I**)	33	Human	7.6	5.7	0.0	…	32.1	8.5
CC5 (ST228-MRSA-I)	14	Human	0.0	0.0	0.0	…	0.0	0.0
CC6	5	Human (4), camel (1)	12.0	11.3	0.5	…	27.6	11.5
CC7	10	Human	21.1	13.5	4.5	…	78.4	22.2
CC7 (ST1048)	1	Human	8.9	8.9	8.9	…	8.9	–
CC8	70	Human	12.7	11.4	0.0	…	41.3	9.7
CC8 (ST72)	10	Human	8.8	7.9	0.0	…	16.6	5.6
CC8 (ST239)	9	Human	3.3	2.8	0.5	…	6.6	2.2
CC9	8	Human	0.0	0.0	0.0	…	0.0	0.0
CC9 (ST834)	5	Human	9.5	12.3	0.5	…	13.1	5.4
CC10	6	Human	2.1	0.2	0.0	…	6.4	3.1
CC12	11	Human	8.0	4.8	1.1	…	30.8	8.9
CC15	26	Human	17.0	7.2	0.0	…	72.8	22.4
CC20	8	Human (5), cattle (3)	3.0	0.5	0.0	…	12.4	4.6
CC22	34	Human (33), cattle (1)	0.0	0.0	0.0	…	0.0	0.0
CC25	14	Human	6.8	6.0	0.0	…	18.6	6.5
CC30	47	Human	0.2	0.0	0.0	…	2.4	0.5
CC30 (ST34/42)	8	Human	0.0	0.0	0.0	…	0.0	0.0
CC45 (*agr* I)	40	Human	0.0	0.0	0.0	…	0.0	0.0
CC45 (*agr* IV)	2	Human	0.0	0.0	0.0	…	0.0	0.0
CC49	3	Human	0.0	0.0	0.0	…	0.0	0.0
CC50	5	Human	0.0	0.0	0.0	…	0.0	0.0
CC59	15	Human	1.9	0.0	0.0	…	15.9	4.5
CC80	6	Human	10.6	6.2	0.5	…	28.4	11.7
CC88	3	Human (2), cattle (1)	0.0	0.0	0.0	…	0.0	0.0
ST93	4	Human	0.0	0.0	0.0	…	0.0	0.0

Table 1. Cont.

Clonal Complex/ Sequence Type	Tested isolates	Host species	Mean (ng/ml)	Median (ng/ml)	Range (ng/ml)			Standard deviation (ng/ml)
CC96	5	Human	22.9	17.7	0.5	…	64.0	24.8
CC97	12	Human (11), Moose (1)	12.1	11.5	2.0	…	30.6	7.6
CC97 (ST71)	4	Cattle	6.9	7.1	4.8	…	8.7	1.6
CC101	6	Human	0.0	0.0	0.0	…	0.0	0.0
CC121	16	Human	0.30	0.0	0.0	…	1.8	0.5
CC130	2	Human, Hedgehog	0.0	0.0	0.0	…	0.0	0.0
CC133	4	Human (2), Goat (1), Cattle (1)	0.0	0.0	0.0	…	0.0	0.0
ST140	3	Human	0.3	0.5	0.0	…	0.5	0.5
CC152	7	Human	0.0	0.0	0.0	…	0.0	0.0
CC182	4	Human	0.0	0.0	0.0	…	0.0	0.0
CC188	12	Human (6), cattle/water buffalo (6)	10.5	8.6	0.0	…	30.6	10.5
CC188 (ST1774)	4	Human	8.4	9.3	0.5	…	15.6	7.1
ST350	4	Human (1), Cattle (2), Dog (1)	1.5	0.5	0.0	…	4.9	2.3
CC361	5	Human (3), Cattle (2)	0.0	0.0	0.0	…	0.0	0.0
CC395	9	Human	1.7	1.2	0.0	…	5.9	1.8
CC398	19	Human (8), Pig (1), Turkey (2), Cattle (6)	0.32	0.0	0.0	…	6.2	1.4
CC398 (ST291/813)	17	Human (4), cattle/water buffalo (13)	4.3	1.2	0.0	…	23.5	6.5
ST425	6	Human (2), badger (2), cattle (1), Red deer (1),	0.0	0.0	0.0	…	0.0	0.0
CC479	4	Cattle	0.0	0.0	0.0	…	0.0	0.0
CC509	3	Human	0.0	0.0	0.0	…	0.0	0.0
CC522	4	Goat (2), sheep(2)	0.0	0.0	0.0	…	0.0	0.0
CC599	1	Human	0.0	0.0	0.0	…	0.0	–
CC692	4	Common magpie, Green woodpecker, Tawny owl, White-tailed eagle	10.7	5.6	3.8	…	27.8	11.4
CC705	16	Cattle	0.0	0.0	0.0	…	0.0	0.0
CC707	5	Human (4), reindeer (1)	0.0	0.0	0.0	…	0.0	0.0
CC779	3	Human	0.0	0.0	0.0	…	0.0	0.0
ST816	1	Dog	3.4	3.4	3.4	…	3.4	–

Table 1. Cont.

Clonal Complex/ Sequence Type	Tested isolates	Host species	Mean (ng/ml)	Median (ng/ml)	Range (ng/ml)			Standard deviation (ng/ml)
CC913	1	Human	28.3	28.3	28.3	…	28.3	–
CC1021	3	Human	0.3	0.5	0.0	…	0.5	0.3
ST1093	3	Human	1.7	1.7	1.3	…	2.1	0.4
ST1290/2481	1	Human	1.3	1.30	1.3	…	1.3	–
CC1943	1	Human	0.0	0.0	0.0	…	0.0	–
ST2279	2	Lynx, Reindeer (1 each)	0.0	0.0	0.0	…	0.0	0.0
ST2425	2	Brown hare	2.7	2.7	0.5	…	4.9	3.1
ST2691	2	Moose	0.0	0.0	0.0	…	0.0	0.0
***"S. argenteus"* (ST75, ST883)**	7	Human	0.0	0.0	0.0	…	0.0	0.0

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CC398. For the mosaic lineage CC8 (ST239) that comprises genome segments of both, CC8 and CC30 origin [30], less toxin was measured than for CC8 strains but more than for CC30. CC1 (ST573/772) lacked *hla* expression while other CC1 isolates were positive. CC9 (ST834) was a strong alpha toxin producer, while other CC9 isolates invariably were negative.

There was one clearly defined strain that differed in alpha toxin *in vitro* production from other strains belonging to the same clonal complex. 14 isolates of ST228-MRSA-I, "South German Epidemic MRSA", coming from different geographic regions (South-Eastern Germany, Malta, Switzerland and Denmark) were all alpha toxin negative while the parental lineage, CC5, usually is clearly positive.

Alpha Toxin and Agr Group Affiliation

To test the null-hypothesis that the HLA concentrations are the same in each *agr*-group we performed the Kruskal-Wallis rank sum test. Here, in contrast to the analysis of variance (ANOVA), it is not required that the data within the groups are normally distributed and have the same standard deviation. As for the differences between *agr* groups, the Kruskal-Wallis test shows a probability below 0.01 for a true null-hypothesis (Chi-Square = 15.5, Degrees of freedom = 4, Probability of a true null-hypothesis = 0.003777). Therefore, it is likely that the HLA concentrations are truly different between the different *agr* groups. Between *agr* groups I, II and III, there is no major difference in mean/median alpha toxin yields (Table 2); and all these groups harbour clonal complexes that are strong as well as poor producers. The yield of *agr* group IV isolates was clearly lower, there was no strongly producing lineage within this group and isolate numbers also were low compared to the others. The isolates which were not assignable to *agr* groups all belonged to the "*S. argenteus*" lineage (ST75/ST883), and failed to produce detectable alpha toxin.

Alpha Toxin and Host Species

No correlation of alpha toxin production and host species was noted. Bovine isolates ranged from negative to strongly positive, depending not on bovine origin but on CC affiliation with isolates belonging, *e.g.*, to CC398, CC479, CC705 being negative while CC398 (ST291/813) and CC97 (ST71) being positive. For other host species, isolate numbers were low, but apparently the general picture is similar. For instance, two moose (*Alces alces*) isolates belonging to ST2691 were negative while another two assigned to CC97 were positive. CC398 from turkey yielded negative tests, while CC692 from other birds (magpie, tawny owl, green woodpecker, white-tailed eagle; *Pica pica, Strix aluco, Picus viridis, Haliaeetus albicilla*) were positive.

Alpha Toxin, Outcome of Disease and Clinical Syndromes

Animal isolates and isolates without known diagnosis were excluded from this analysis. No correlation between *in vitro* alpha toxin yield and fatal outcome was observed (Table 3).

Discussion

The assay described herein allows to rapidly and quantitatively measuring alpha toxin from staphylococcal cultures. This allows studying expression with regard to strain- or CC affiliations, but also with regard to regulation under different growth conditions in the presence of antibiotics, *etc*. It could also be expanded by spotting additional antibodies for other exotoxins such as PVL [29] on the same array, facilitating simultaneous measurements of several virulence factors.

Table 2. Alpha Toxin yields and *agr* group affiliation.

	Tested isolates	Mean (ng/ml)	Median (ng/ml)	Range (ng/ml)			Standard deviation (ng/ml)
***agr* Group I**	341	5.5	0.5	0.0	…	78.4	8.9
***agr* Group II**	142	5.8	0.5	0.0	…	72.8	12.4
***agr* Group III**	131	4.0	0.5	0.0	…	64.0	8.2
***agr* Group IV**	27	0.4	0.0	0.0	…	4.9	1.0
"*S. argenteus*" (ST75, ST883)	7	0.0	0.0	0.0	…	0.0	0.0

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Table 3. Alpha Toxin yields and clinical outcome (veterinary isolates excluded).

	Tested isolates	Mean (ng/ml)	Median (ng/ml)	Range (ng/ml)			Standard deviation (ng/ml)
All cases	428	5.55	0.5	0.0	…	72.8	10.1
Fatal cases	34	3.01	0.2	0.0	…	19.2	4.8
Surviving; or outcome not recorded	394	5.77	0.5	0.0	…	72.8	10.4

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The most surprising aspect in this work was that the *in vitro* production of alpha haemolysin normally strongly correlates with affiliation to clonal complexes. This can theoretically be explained at least by two different assumptions. One is a possible presence of allelic variants resulting in proteins that might less efficiently be recognised by the antibodies used. The second one could be that expression and/or regulation indeed vary, depending on the affiliation to phylogenetic lineages.

The first possible explanation could be true in CC75/"*S. argenteus*". As mentioned above, a genome sequence from this lineage shows several differences compared to other *S. aureus* sequences. Thus antibodies specific for other alpha toxin variants might false-negative results or give a false impression of low toxin levels. Raising monoclonal antibodies specifically for CC75/"*S. argenteus*" alpha toxin might resolve this issue in future. For other lineages, allelic differences between *hla* sequences of different clonal complexes are rather small (see Introduction and File S1), even less when regarding protein rather than DNA sequences. Allelic variations are so not a likely cause for the different alpha toxin measurements for these lineages. It was observed that CC395 was usually alpha toxin positive while CC22, CC30, CC45 and CC398 are negative in the described test. However, a published CC395 sequence (AGRO01000049:167700 to 168659) is identical to sequences from several CC45 strains and virtually identical to CC22, CC30 and CC398 sequences. The variations within major complexes for which many sequences are available appear to be larger than between complexes (for an example, CC8, see File S1). Besides, the impact of allelic variation is minimised by the use of three monoclonal antibodies.

The second possible explanation is a lineage specific expression. Such a relation of *hla* expression to clonal complex background could also be assumed analysing data from an earlier study [31] that measured toxin expressions in pandemic MRSA strains using an entirely different RNA-based approach, *i.e*, quantitative reverse-transcription PCR. These authors observed a lack of *hla* expression in CC30 strains USA200 and USA1100 (CC30/ST36-MRSA-II and PVL-positive CC30-MRSA-IV). They observed intermediate levels of *hla* expression for "USA400" (CC1), "USA100" (CC5), "USA1100" (CC59) and in a ST72 strain as well as high levels in "USA300" and "USA500" (CC8) as well as in a ST80 strain. This is in quite a good accordance to our observation despite the use of an entirely different method as well as of another growth medium.

The differences in alpha toxin expression are not related to *agr* types since strong as well as poor producers are can both be found within one *agr* group. An exemption might be *agr* group IV, which, however, is genetically much less diverse than *agr* groups I-III comprising only two major complexes (CC50 and CC121). In normally poorly producing lineages (such as CC59), occasionally strong producers can be observed. This could indicate that the variability of alpha toxin levels within one lineage was wider than the relatively few experiments might suggest. It could also imply that a short-term adaption to external selective pressures (immunity in a specific host, exposure to drugs) could modify the modus of regulation and expression.

The absence of detectable alpha toxin production in some CC30 strains (*e.g.*, ST36-MRSA-II, MRSA252, GenBank BX571856) could possibly be attributed to a mutation resulting in a TAG stop codon within the *hla* sequence [32], see File S1 (position nr. 338). There might also be similar mutations in strains for which no sequences are yet known. However, even in CC30 there are strains that do not show this mutation [33,34], see File S1, so that other reasons for absence or low levels of alpha toxin production must also play a role.

Most interestingly, isolates of one epidemic strain (CC5/ST228-MRSA-I, "South German Epidemic Strain") consistently proved to alpha toxin negative although that strain belongs to a strongly producing lineage (CC5). Given that this strain is currently superseded by others [35], one might speculate if this deficiency might play a role in its demise. The reason for an *in vitro* absence of alpha toxin is not yet known. It is likely not related to sequence variations of *hla*, *i.e.*, a possible presence of an epitope altered beyond recognition, as several *hla* sequences (GenBank accession numbers HE579059, HE579061, HE579063, HE579065, HE579067, HE579069, HE579073) of ST228-MRSA-I are identical to N315 and other CC5 sequences. There is one single nucleotide polymorphism in the published *agrC*-II sequences of this strain compared to N315 (A instead of T in position 91), but if this was indeed related to a lack of alpha toxin production warrants further study.

It was also observed that alpha toxin production apparently did not correlate with host species, with alpha toxin production being detected also in isolates from non-human hosts. Although further, more systematic studies on alpha toxin in animal isolates of *S, aureus* are needed, this could indicate a crucial role of this toxin for *S. aureus* as opportunistic pathogen in a wide range of hosts. This observation could also be in accordance to the apparent promiscuity of alpha toxin with regard to target cells.

Finally, the clinical course of staphylococcal disease and the *in vitro* production of alpha toxin did not correlate with isolates from fatal cases and survivors secreting essentially identical amounts of the toxin. This could indicate that i) the *in vitro* and *in vivo* situations cannot be compared, or ii) even small concentrations of alpha toxin are sufficient to fulfil its pathophysiological role so that high level producers just produce in excess, or iii) that the effect of alpha toxin is complemented or superseded by other haemolysins and exotoxins and that it is only one factor among many others. However, its presence in *S. aureus* appears to be crucial for the virulence of that species.

Supporting Information

File S1 Alignment of *hla* sequences.
(FAS)

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Author Contributions

Conceived and designed the experiments: RE SM. Performed the experiments: EM BS. Analyzed the data: RE SM. Contributed reagents/materials/analysis tools: JB. Wrote the paper: RE SM BS.

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Manuskript I

Direct, specific and rapid detection of staphylococcal proteins and exotoxins using a multiplex antibody microarray

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Abstract

S. aureus is a pathogen in humans and animals that harbors a wide variety of virulence factors and resistance genes. This bacterium can cause a wide range of mild to life-threatening diseases. In the latter case, fast diagnostic procedures are important. In routine diagnostic laboratories, several genotypic and phenotypic methods are available to identify *S. aureus* strains and determine their resistances. However, there is a demand for multiplex routine diagnostic tests to detect staphylococcal toxins and proteins directly.

In this study, an antibody microarray based assay was established and validated for the rapid detection of specific staphylococcal markers and relevant exotoxins. The following targets were included: staphylococcal protein A (SPA), penicillin binding protein 2a (PBP2a), alpha toxin (HLA), beta-hemolysin (HLB), Panton Valentine leukocidin (PVL), toxic shock syndrome toxin (TSST), staphylococcal enterotoxins A and B (SEA, SEB) as well as staphylokinase (SAK). All of them can be detected simultaneously within a single experiment, starting from a clonal culture on standard media. The detection of bound proteins was performed using a new fluorescence reading device for microarrays.

110 reference strains and clinical isolates were analyzed using this protein microarray based assay. In addition, all strains and isolates were tested on a DNA microarray for genotypic characterization. The results showed a general high concordance of genotypic and phenotypic data. A sole exception was HLA. *Hla* was present in all *S. aureus* isolates, however its expression in the given experimental setup depended on the clonal complex affiliation of the actual isolate.

The multiplex assay described herein allowed a rapid and reliable detection of clinically relevant staphylococcal toxins as well as resistance- and species-specific markers.

Introduction

Routine laboratories focus on culturing and identifying bacterial species and on obtaining their susceptibility profiles. Some susceptibility test results, such as oxacillin/methicillin resistance in staphylococci, vancomycin resistance in enterococci or III. generation cephalosporin/carbapenem resistance in enterobacteria, require additional assays for confirmation due to their high relevance for the therapy of individual patients and for infection control. This can be done by molecular methods or using antibody-based assays. The former require sophisticated and expensive equipment. Nowadays, antibody-based tests, such as ELISAs, agglutination assays or lateral flow designs, are widely used, e.g. for the confirmation of the modified penicillin binding protein (PBP2a) conferring oxacillin/methicillin resistance in *Staphylococcus aureus*/MRSA.

Staphylococcus aureus is a common opportunistic pathogen. It colonizes approximately 30% of a healthy human population (van Belkum et al., 2009), but also can cause nosocomial or community-acquired infections. Clinically, *S. aureus* is associated with skin and soft tissue infections, food intoxications and life-threatening diseases like pneumonia, endocarditis or septicemia. Carriers of *S. aureus*, in particular hospitalized, dialysis and catheter patients, show an increased risk of invasive infections (von Eiff et al., 2001) but a lower risk of septicemia-related death (Verkaik et al., 2009).

A major problem with *S. aureus* is the high rate of resistance to methicillin and other ß-lactam antibiotics (MRSA), especially in nosocomial settings. Normally, methicillin inhibits the cell wall synthesis of the bacteria by binding to their penicillin-binding proteins (PBPs). The gene *mecA* encodes for a modified penicillin-binding protein (PBP2a). PBP2a performs the function of PBP by synthesizing peptidoglycan, therefore methicillin cannot bind anymore (Fuda et al., 2004). In life-threatening situations it is important to rapidly detect the presence of *mecA* in order to ensure efficient, i.e., non-beta-lactam-based therapy. Additionally, MRSA-positive patients are to be isolated in separate rooms to avoid a transmission to other patients. In routine diagnostics, the ß-lactam resistance, caused by PBP2a, is detected by agar diffusion or micro dilution tests and the presence of *mecA*/PBP2a is then confirmed by either PCR or by agglutination or lateral flow assays.

MRSA has become a global problem – first in hospitals, but later, for approximately the last 25 years, also in non-hospitalized individuals. The so-called community-acquired MRSA (CA-

MRSA) strains often seem to be more virulent than hospital-acquired MRSA (HA-MRSA), sometimes being able to infect even young and otherwise healthy people. Typical properties of these clones are the presence of the smaller sized type IV or V staphylococcal cassette chromosomes *mec* (SCC*mec*) and, in many but not all strains, of the Panton Valentine leukocidin (PVL) (Vandenesch et al., 2003; Boyle-Vavra & Daum, 2007).

Since the emergence of livestock-associated MRSA (la-MRSA), animals such as pigs, calves, turkeys and chickens have become a reservoir for MRSA as well. Especially strains belonging to the clonal complex (CC) 398 have been observed in humans as well as in animals and became a serious issue even in countries where the MRSA prevalence used to be low for decades, such as the Netherlands and Denmark (Graveland et al., 2011).

Besides *mecA*, that encodes the penicillin binding protein 2a (PBP2a), *S. aureus* can harbor *mecA* alleles (*mecA1*) that do not encode resistance to beta-lactam compounds (Monecke et al., 2012). In addition, there is a highly divergent recently identified *mecA* gene homologue, *mecC* (Shore et al., 2011; Garcia-Alvarez et al., 2011). This gene encodes for beta-lactam resistances and due to low homology to *mecA, mecC* caused concern in diagnostics. While routine cultures and susceptibility tests identify methicillin resistance in *mecC* strains, conformational tests frequently do not identify them (Peterson et al., 2013).

Relevant virulence factors in *S. aureus* range from hemolysins and other enzymes that digest host tissues to yield nutrients to proteins that disrupt or manipulate the host immune system. Those are superantigens, such as the toxic shock syndrome toxin or staphylococcal enterotoxins, and leukocidins. Superantigens lead to an antigen-unspecific T-cell activation followed by an immense cytokine release (Foster, 2005). At the moment, the detection of staphylococcal toxins relies largely on molecular methods, i.e., PCR or array hybridization. These approaches are mainly restricted to research and/or reference laboratories. In routine laboratories, the options for detecting staphylococcal toxins are limited since there are no routine diagnostic tests to confirm the presence of multiple staphylococcal toxins. Enzyme-linked Immunosorbent Assays (ELISAs) or Lateral Flow tests are mainly focusing on one target only.

A multiplex test for staphylococcal toxins could be helpful because infections with *S. aureus* producing certain toxins should be treated differently than infections with *S. aureus* lacking those toxins. The presence of PVL mandates special infection control and eradication measures (HPA guideline: https://www.gov.uk/government/collections/panton-valentine-leukocidin-pvl-guidance-data-and-analysis), or a clinical condition related to PVL or TSST1 might be treated with gamma globulin and/or compounds inhibiting toxin biosynthesis

(rifampicin, clindamycin) in addition to the standard regimen (Stevens et al., 2014). This is often hindered or delayed by a lack of fast diagnostic tools.

Therefore, the aim of this study was to develop a new, rapid and economic fluorescence-based assay allowing qualitative or semi-quantitative analysis of expressed proteins, starting with clonal cultures as obtained by routine procedures. A designated new reader and additional software were developed for the analysis of fluorescence microarray images, and an antibody microarray was designed to allow simultaneous detection of PBP2a as well as important secreted virulence proteins (TSST, PVL, SEA, SEB, HLA, HLB, SAK) and a species marker/positive control (staphylococcal protein A).

The toxic shock syndrome toxin 1 (TSST-1) is encoded by the gene *tst1*, that is carried on staphylococcal pathogenicity islands. Clinically, the toxic shock syndrome (TSS) is characterized by high fever, diffuse macular erythroderma, desquamation, shock and a multi-organ involvement (Schlievert et al., 1991; Dinges et al., 2000). However, the toxin can commonly also be detected in isolates from less dramatic clinical presentations or even from healthy carriers (Monecke et al., 2009), suggesting an additional involvement of host factors or disposition in the pathogenesis of the TSS. Carriers of TSST-1-expressing *S. aureus* strains show a higher level of anti-TSST-1 antibodies than non-carriers (Verkaik et al., 2009), and apparently TSS is only observed in persons lacking TSST-1-specific antibodies (Kloppot et al., 2015).

The Panton-Valentine leukocidin (PVL) is a bacteriophage-born exotoxin of *S. aureus*. It consists of two subunits (lukF-PV and lukS-PV) encoded by two co-localized and co-expressed genes, *lukF-PV* and *lukS-PV* (Kaneko & Kamino, 2004). PVL-positive *S. aureus* usually cause chronic or recurrent skin and soft tissue infections (SSTIs) such as furunculosis. Rarely, they also can cause life-threatening diseases such as necrotizing pneumonia or necrotizing fasciitis (Vandenesch et al., 2003). Although PVL has been known for almost a century, it recently became rather prevalent among CA- MRSA lineages. Therefore, it was often, and controversially, discussed to be an important determinant of high virulence in CA- MRSA (Boyle-Vavra & Daum, 2007; Diep et al., 2008). Several reports were published describing local outbreaks of PVL-positive *S aureus* in groups of institutionalized people such as sports teams, prisoners or military personnel and staff of fire departments (Kazakova et al., 2005; Aiellio et al., 2006; Roberts et al., 2011). PVL positive *S. aureus* are rarely observed among healthy carriers (Monecke et al., 2009) or bacteremia patients (Ellington et al., 2007), while they are highly prevalent in patients with SSTI, furunculosis or abscesses. A previous study showed that the *in vitro* expression of PVL varies depending on clonal complex and strain affiliation. A correlation between the *in vitro* expression of PVL and the severity of infections was not found (Stieber et al., 2014). In a recent study, Rasigade et

al. found a correlation between anti-PVL antibody levels in participants and local PVL prevalence, increasing along a north-to-south gradient (Rasigade et al., 2015).
Besides PVL, there is a similar leukocidin, lukF-P83/lukM, that is described to be associated with mastitis in ruminants (Kaneko & Kamino, 2004). The encoding genes, *lukF-P83* and *lukM*, are common in isolates of clonal complexes 151, 479 and 133 and they might be used, at least in some geographic regions, to discern epidemic strains among cattle herds from strains that appear only in isolated, sporadic cases (Schlotter et al., 2012).
Staphylococcal Enterotoxins (SEs) are further clinically relevant exotoxins produced by *S. aureus*. Currently, more than 20 enterotoxins are known. Staphylococcal Enterotoxins A (SEA) and B (SEB) are the best characterized ones. They are encoded by the genes *sea* and *seb*, or *entA*/*entB*, respectively. *Sea* is located on a bacteriophage (Betley & Mekalanos, 1985), whereas *seb* is chromosomally located (Shafer & Iandolo, 1978). SEs are commonly associated with foodborne diseases that present with diarrhea and emesis (Pinchuk et al., 2010), and, rarely, with (posttraumatic/non-menstrual) toxic shock syndrome. The skin of patients with atopic eczema (AE) is often colonized with *S. aureus* strains producing staphylococcal enterotoxins. The toxins are described as increasing the IgE response of patients with AE, indicating that the toxins may act as possible allergens (Wehner & Neuber, 2001).

The alpha toxin (HLA; also known as hemolysin alpha) is a chromosomally encoded exotoxin. The corresponding gene, *hla*, is, as far as currently known, present in all *S. aureus* strains although slightly different alleles can be observed. The *in vitro* gene expression is limited to certain lineages/clonal complexes (Monecke et al., 2014). The toxin is a ß-barrel pore forming toxin that forms cylindrical heptamers into the membrane of target cells, with the ability to lyse erythrocytes as well as other host cells (Valeva et al., 1997). It disrupts host cell membranes leading to osmotic swelling, lysis and consequently to cell death. The alpha toxin plays an important role in *S. aureus* pathogenesis, e.g., for pneumonia, sepsis or arthritis. It also acts dermonecrotically and neurotoxically (Wardenburg et al., 2007; Vandenesch et al., 2012). Antibody levels in patients infected with alpha toxin-positive *S. aureus* isolates are much higher than in alpha toxin-negative isolates (Sharma-Kuinkel et al., 2015).

The beta-hemolysin (HLB; Sphingomyelinase C) is another hemolysin of *S. aureus*. The corresponding gene, *hlb*, is chromosomally located. Beta-hemolysin plays an important role in skin colonization by damaging keratinocytes (Katayama et al., 2013). The toxin is produced mainly by animal isolates. In human strains, phage insertions frequently cause truncations of the *hlb* gene.

Staphylokinase (SAK) is a bacteriophage encoded protein of *S. aureus*, and the corresponding gene, *sak*, is usually situated on phages that truncate *hlb* (Coleman et al., 1991). The protein can be detected in culture supernatants as well on the cell surface. The role as a virulence factor is described as an interaction with host proteins, including α-defensins and plasminogen. The binding to plasminogen facilitates bacterial invasion into host tissues (Bokarewa et al., 2006). SAK expression is associated with skin and mucosal infections and bacterial persistence. The encoding gene was commonly detected in asymptomatic human carriers (71.6%), and it was even more common in patients with invasive infections such as, e.g., infections of bones and/or prosthetic implants (90.8%) (Monecke et al., 2009; Lüdicke et al., 2010). It is less common in animal isolates where *hla* tends to be un-truncated.

Another virulence factor and species marker of *S. aureus* is the cell wall-located protein A. The corresponding *spa* gene belongs to the core genome of *S. aureus* and therefore it is present in virtually all strains and isolates. The *spa* gene includes a variable repeat region. The number as well as the sequence of the repeating units is variable; and this can be used for typing purposes (Frénay et al., 1996; Harmsen et al., 2003). The C-terminus (also called region X) of the protein is linked to the bacterial cell wall. The N-terminus plays an important role in pathogenesis due to its ability to bind IgG, interfering with antibody-mediated opsonization (Foster TJ, 2005). Because of its ubiquitous presence, it was for the present study selected as positive control marker.

Regarding the importance of these staphylococcal toxins and resistance, as described above, it is of interest to detect protein expression.

In parallel to the phenotypical detection using the protein microarrays, the presence of genes and alleles was investigated during this study using DNA microarrays. Therefore, a direct comparison of genotypic and phenotypic data for the targets was possible.

Materials & Methods

Strains

In this study, 110 bacterial strains/isolates were tested. These included 105 *S. aureus*, 2 *S. epidermidis*, 1 *S. sciuri*, 1 *S. capitis* and 1 *E. coli* as negative control. Most of them originated from clinical routine diagnostics, but some well characterized reference strains were also tested (table 2). All of them were genotyped by DNA microarray hybridization (StaphyType Kit, Alere Technologies GmbH, Jena, Germany). This provided information regarding the presence or absence of relevant virulence and resistance genes, including those that encode

PVL, TSST, HLA, HLB, SEA, SEB, SAK, PBP2a and Protein A, as well as on the affiliation to clonal complexes and strains.

Culture conditions

In a previous set of experiments, different growth media were tested for strain culturing and detection of proteins (Stieber et al., 2014; Monecke et al., 2014). Based on these results, strains and isolates were incubated on Columbia Blood agar (Oxoid, Wesel, Germany) at 37°C for 18 – 24 h. One loop of bacterial material was inoculated into 130 µl phosphate buffered saline (1x PBS) or in 65 µl sodium hydroxide (NaOH; 100mM), respectively, and vortexed. To the NaOH suspension, 65 µl of a buffered phosphate buffer (pH 5.5; 1M di-sodium hydrogen phosphate and sodium di-hydrogen phosphate) was added for neutralization (pH 7), followed by further vortexing.

Antibodies

Monoclonal antibodies (AB) for the targets PBP2a, SAK, HLB, HLA, SEB, SEA, TSST and lukF-PV were generated via phage display (Dübel et al., 1993) as previously described (Monecke et al., 2013; Stieber et al., 2014; Monecke et al., 2014). SPA antibodies originated from three polyclonal chicken sera (courtesy of Alere Scarborough/Binax). First, all antibodies were separately screened to find the optimal (i.e., most specific and sensitive) combinations of capture and detection antibodies for each target (Monecke et al., 2013; Stieber et al., 2014; Monecke et al., 2014). Then, all specific antibodies were tested in a mixture to find optimal conditions for usage and functionality of the antibodies. The capture antibodies were spotted onto the microarrays, each 3-4 times redundantly and in two different concentrations, 0.5 mg/ml and 0.05 mg/ml, respectively. The use of different concentrations aimed on minimizing effects of steric interference. In order to reach a total protein concentration of 0.5 mg/ml (which is required for the spotting procedure), bovine serum albumin was added to the antibodies at higher dilutions. For the number of spotted antibodies per target see Figure 1. For each target, a secondary detection antibody was labelled using Sulf-NHS-LC-Biotin (Pierce, Bonn, Germany) according to the manufacturer's instructions. A mixture of all 9 biotin-labelled detection antibodies was prepared. The final concentrations of the different antibodies were anti-lukF-PV, 0.2 ng/µl; anti-PBP2a, 0.02 ng/µl; anti-Protein A, 0.5 ng/µl; anti-TSST, 0.1 ng/µl; anti-SEA, 0.05 ng/µl; anti-SEB, 0.05ng/µl; anti-SAK, 0.1 ng/µl; anti-HLA, 0.2 ng/µl; and anti-HLB, 0.07 ng/µl.

Array procedures

First, 500 µl washing buffer (Protein Binding Kit, Alere Technologies GmbH, Jena, Germany) was added and the arrays incubated at 37 °C and 400 rpm on a shaker for 5 min, followed by 100 µl of blocking solution (Protein Binding Kit, Alere Technologies GmbH) at 37 °C and 300 rpm for 5 min. Meanwhile, culture suspensions in PBS and NaOH (as described above) were prepared. Both suspensions were separately diluted 1:10 in the mixture of biotinylated detection antibodies and subsequently incubated at 37 °C and 400 rpm for 10 min in a separate reaction tube. First, 50 µl of the NaOH-treated sample was incubated at 37 °C and 300 rpm on the array for 5 min, then the sample was removed and 50 µl of the sample aliquot that was treated with PBS were added and incubated at 37 °C and 300 rpm for additional 25 min. Subsequently, the array was washed with 500 µl washing buffer (5 min, 37 °C, 400 rpm). For the detection of specifically bound proteins, 10 µl of purpose-made fluorescent beads (Alere Technologies GmbH), labelled with Cy3 and streptavidin, were incubated at 37 °C and 300 rpm for 30 min. Afterwards, the arrays were washed with 500 µl washing buffer (5 min, 37 °C, 400 rpm). Finally, the washing buffer was replaced by 100 µl fresh washing buffer and the images were taken by a purpose-made reading device, the ATR-Fluo-Reader (Alere Technologies GmbH; http://alere-technologies.com/en/products/lab-solutions/reader-systems/atr-fluo-reader.html; Ehricht et al., 2014).

Analysis

The images were taken by the ATR-Fluo-Reader at exposure times of 500 ms and 1,000 ms, and transferred to a computer for further analysis using the Iconoclust software according to manufacturer's instructions with an assay-specific script. The signals (*S*) of the spots were determined by: $S = M\text{-}BG$ whereby *M* is the average intensity of the spot and *BG* the intensity of the local background. Thus, the signals range between 0 (negative) and 1 (maximum signal). For each target, specific cut-off values were determined (table 1). Resulting signal values were considered positive if previously determined cut-offs were reached or surpassed. A software tool was generated for the automatic analysis of the data resulting from the fluorescent microarrays. This software provides reports giving information on the presence of tested proteins based on the previously determined cut-offs. Additionally, results were manually compared to results of the DNA array based genotyping of the respective isolates.

Results

Assay optimization

The protocol was optimized to facilitate a rapid detection of all 9 targets simultaneously. Starting from clonal cultures, the bacterial material was inoculated into different solutions to find optimal conditions for the detection of all different membrane and secreted proteins within one and the same assay. NaOH and PBS treated cells were initially tested separately and afterwards in combination. This resulted in partially different performances for the single proteins.
After the NaOH treatment, the targets PBP2a, HLA, HLB, SEA, SEB, SAK, protein A and TSST were detected. However, lukF-PV as well as lukF-P83 were not reliably detectable when treating known lukF-PV or lukF-P83 positive strains/isolates with NaOH. In the PBS buffer suspension, all requested targets except PBP2a were detectable.

The protocol was optimized to detect all expressed targets simultaneously as follows: Bacterial culture material was harvested from Columbia blood agar plates (incubated for 18-24h, 37°C) and divided into two aliquots: one in 1x PBS and one in 100 mM NaOH, respectively. Subsequently, a buffer (see Material/Methods) was added to the NaOH suspension for neutralization. Then, both suspensions were added one after the other to the same array for specific binding of all antigens to their corresponding antibodies (see Methods).

Screening of staphylococcal strains using the protein array and the DNA genotyping array

The results of the antibody arrays in comparison to the genotypic characterization are summarized in Table 2. 110 isolates and strains were tested using microarrays, the antibody array for phenotyping and the DNA array for genotyping, in parallel. The results of both arrays showed a general concordance of phenotype and genotype for all 110 tested isolates/strains (table 2). The tested isolates represented diverse clonal complexes (CC1, CC5, CC8, CC9, CC25, CC30, CC 121, CC130, CC133, CC152, CC361, CC479, CC705) from different origins and included MSSA and MRSA.

In addition to the *S. aureus* isolates, an *E. coli* strain (BL21-DE3, National Laboratory New York, USA) and one *S. capitis* isolate (Dublin Trinity College, Dublin Ireland, Courtesy of Joan Geoghegan) were tested on both arrays as negative controls. For these strains, none of the target genes and none of the corresponding proteins were detectable. Two *S. epidermidis* strains (ATCC35984 and ATCC12228) were also tested. The strain ATCC35984 harbored the *mecA* gene. The gene as well as the protein PBP2a were correctly identified. Strain ATCC12228 was negative for all tested targets.

Identification and detection of variants of targets

For three targets, distinct variants are known. These included mecA, PVL and SEA.

In addition to *mecA*, there is "*mecA1*" in various animal staphylococci that does not confer resistance to methicillin, as well as *mecC.* In a "*mecA1*"-positive/*mecA*-negative *S. sciuri* isolate (University of Vienna, Austria) no PBP2a was detected.

2 isolates of CC130-MRSA-XI that carried the *mecC* gene as part of a SCC*mec* XI element did not yield signals with PBP2a antibodies on the present array. That indicates a specific recognition of *mecA* encoded PBP2a by the antibody and no cross-reaction with the gene products of „*mecA1"* or *mecC.*

In *lukF-P83/lukM*-positive *S. aureus* isolates, lukF-P83 was detected by one of the three PVL antibodies.

For SEA, different alleles can be recognized that will be designated here *sea*-FRI100 (GenBank accession number L22565.1 [482…526]), *sea*-320 (GenBank accession number CP001996.1 [1144119…1144898]) and *sea*-N315 (GenBank accession number BA000018.3 [2011380…2012153]). Several *S. aureus* isolates with all of these *sea* alleles were tested. Anti-SEA-antibodies detected SEA corresponding to the alleles *sea*-FRI100 and *sea*-320, whereas SEA of allele *sea*-N315 was not identified.

Discussion

The multiplex protein microarray as described herein is a test for the fast and direct detection of relevant staphylococcal proteins from clonal culture material. These include PBP2a allowing the use of the assay as a confirmatory test for the identification of MRSA after obtaining a doubtful susceptibility test result. In addition, the *S. aureus* species marker SPA and several clinically relevant toxins are included, i.e., PVL, TSST, HLA, HLB and enterotoxins A and B. No simple non-molecular assays such as ELISAs, regardless whether using single- or multiplex assays, are currently available in routine settings.

Currently, there is a set of genotypic methods in bacterial routine diagnostics for strain identification and determination of relevant virulence and resistance markers. Common techniques are, e.g., PCR or DNA microarray hybridization. Most of these assays give information about the presence of species-specific, virulence or resistance genes, but not about their expression. For protein expression and functionality, molecular phenotypic techniques are well established. ELISAs, Lateral Flow (LF) tests for protein detection in general, or automated micro dilution techniques, with regard to resistance markers, are

current methods. A main disadvantage of, e.g., ELISAs or LFs is that only one target can be tested in one experiment. The multiplex protein array developed herein allows a rapid, parallel and economic performance to detect several markers within a single experiment. These are optimal preconditions for integrating the assay into routine laboratories. A pure culture harvested from a blood agar plate can be used to perform the assay.

The fast recognition of several staphylococcal toxins, as described below, might be important for the surveillance of highly virulent strains and adequate treatment of patients.

The array harbors antibodies against epitopes of protein A. This cell wall located protein is used in this assay as species marker for *S. aureus*. All tested *S. aureus* isolates of this study showed positive signals for *spa*/protein A. In contrast, the tested coagulase-negative staphylococci (CoNS), *S. epidermidis, S. sciuri and S. capitis*, as well as another control strain, *E. coli*, were tested negative for this *S. aureus* species-specific marker. Thus, these anti-protein A-antibodies can act as positive controls for tested *S. aureus* isolates in the experiments.

The qualitative detection of proteins using the antibody microarray was verified by checking the presence of corresponding genes using the DNA microarray. Generally, the results described herein showed very good concordance of genotypic and phenotypic results.

The protein microarray allows the detection of PBP2a, which is related to methicillin resistance, in tested isolates. Thus, the protein microarray can be used as a confirmatory assay after methicillin resistance was detected in microdilution or agar diffusion tests. The fast discrimination between MSSA and MRSA plays an important role in hospitals. New patients can be screened and, if necessary (i.e., in case of a MRSA-positive result), be isolated from other patients to avoid transmissions or even outbreaks. In addition, appropriate treatment can be initiated. Recently, a new *mecA* homologue, *mecC*, was discovered. It is located on a novel SCC*mec* element type XI and also confers resistance against methicillin (Shore et al., 2011; Garcia-Alvarez et al., 2011). This *mecC* encoded protein could not be detected using the assay described herein. The antibodies currently used are specific for *mecA*-encoded PBP2a and do not cross-react with the gene product of *mecC*. We did not aim to cover *mecC* at this stage. The reason is a very low prevalence that can be estimated to be in an order of magnitude of about 0.1% to 1% of MRSA isolates only (Petersen et al., 2013; RKI- Epidemiologisches Bulletin Mai 2013 Nr.21). However, in the case of an increasing prevalence, it would be feasible to add an antibody that recognizes the gene product of *mecC*.
S. sciuri harbors "*mecA1*", *i.e.*, one of several deviant *mecA* alleles (Couto et al., 1996; Monecke et al., 2012) that do not confer beta lactam resistance. One *S. sciuri* isolate was

tested which did not yield a signal for PBP2a. This indicates that possible *S. sciuri* contaminants would not interfere with the *mecA*/PBP2a detection.

PVL is a marker for virulent *S. aureus* strains including CA-MRSA. Therefore, a guideline was recently issued in the UK for the management of patients infected with PVL- positive *S. aureus* (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/322363/PVL_LRTI_risk_assessment_protocol.pdf). For the implementation of this guideline, a diagnostic test for the detection of PVL is necessary. Currently, PVL diagnostic is performed in molecular/reference laboratories. The assay described herein provides the opportunity to rapidly detect PVL in routine settings without the use of PCR techniques, and thus to adequately and timely manage patients with PVL-positive *S. aureus*. An increasing prevalence of strains that harbor both, PVL and PBP2a, was observed, and this assay is the first available, non-molecular, test being able to detect both relevant proteins in parallel.

In veterinary medicine, the leukocidin lukF-P83/lukM is associated with veterinary disease such as bovine mastitis. It appears to be a marker for epidemic strains within a herd. The protein microarray might be a useful phenotypic test in agriculture to differentiate between lukF-P83/lukM-positive and –negative strains. Its detection could help to distinguish an isolated, sporadic case, following a transmission from the farmer or secondary to an injury from an infection caused by a virulent strain with potential to epidemic spread within a herd (Schlotter et al., 2012). Isolates of CC479 and CC705, harboring the genes *lukF-P83/lukM*, showed also positive signals for lukF-P83 expression.

The rapid detection of staphylococcal enterotoxins A and B is advisable, e.g., in case of a foodborne intoxication. Their diagnosis is helpful for epidemiological monitoring of such intoxications and might help to elucidate outbreaks. At the current stage, cultured isolates could be screened for the production of enterotoxins A and B. Antibodies to additional enterotoxins could be generated and added at a later stage, and protocols for the direct detection of enterotoxins in suspected food should be developed and evaluated.

A rapid detection of TSST in clinical isolates might also be helpful. For instance, cases of toxic shock syndrome can superficially resemble a Lyell syndrome; and while the former requires aggressive antibiotic chemotherapy, in the latter discontinuation of any unnecessary medication is warranted. A rapid assay for TSST production from *S. aureus* primary cultures could facilitate a rapid decision and thus help with the management of patients with these severe conditions.

The *hla* gene was present in all tested isolates and therefore, it can be regarded as a potential species-specific marker for *S. aureus*. However, for alpha toxin, major

discrepancies between the assays for the gene and the ones for the corresponding protein were detected. Results of a previous study were confirmed that showed that the levels of HLA expression depended on the CC affiliation. Isolates belonging to clonal complexes such as CC1, CC5 and CC8 showed variable to high levels of alpha toxins. Isolates that belonged, e.g., to CC22, CC30, CC45, CC30, CC479 and CC705 did not yield detectable levels of alpha toxin under the given culture conditions despite their proven carriage of the *hla* gene. A reason for this discrepancy is not yet known. It might be related to *in vitro* conditions and/or different gene regulation mechanisms (Monecke et al., 2014).

The *sak*-carrying bacteriophages are usually present in *S. aureus* from humans where they are inserted into the *hlb* coding sequence. Either SAK will be expressed in humans or HLB, mainly in animals (Lüdicke et al., 2010). The results of this study confirm these previous descriptions. The livestock-associated strains of CC398 or CC479 express HLB but do not carry SAK. Human *S. aureus* isolates showed converse expression of both these proteins. The expression of either SAK or HLB can be distinguished by the multiplex array, and is more of a scientific than a diagnostic interest.

The current assay provides for a wide range of future developments and applications. The resulting fluorescent array images showed characteristic expression patterns for several strains. An expanded assay could use these typical “fingerprints” for presumptive strain identification. In detail, several strains are characterized by carrying different resistance and virulence genes. For example, *S. aureus* strain USA300 (ST8-MRSA-IV/PVL+/ACME+) carries the genes *mecA, spa* and *sak*, with additional production of the exotoxins PVL and alpha toxin. In comparison, the Bengal Bay Clone/WA MRSA-60 (ST772-MRSA-V/PVL+) carries the genes *mecA* and *spa,* but not *sak*. Regarding the exotoxins, the Bengal Bay Clones also express PVL and, additionally, enterotoxin A, but under given culture conditions, no alpha toxin. Using these different expression patterns, presumptive strain identification can be done directly based on the fluorescent array images. However, the use for fingerprinting purposes has limitations that require further study. Firstly, some target proteins are located on mobile genetic elements. Thus, their carriage in different isolates of one strain might vary. Secondly, their expression might theoretically vary depending on clinical background including previous medication or on culture conditions. Future studies should therefore aim at an expansion of the target panel and at standardization issues. Additionally, this technique of a multiplex protein microarray can be used for other microorganisms.

The assay was developed for qualitative analyses of protein expression. However a quantitative analysis of the fluorescent arrays might also be possible. Fluorescence allows an easier determination of protein quantities compared to precipitation staining which was used in previously studies.

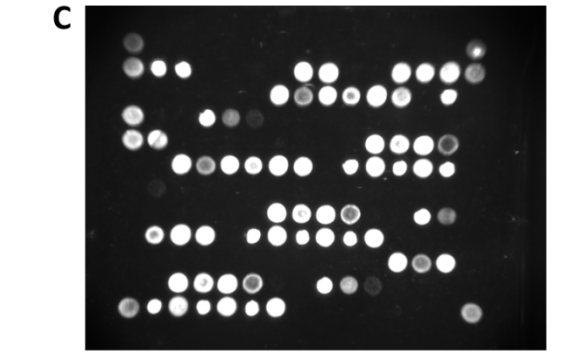
As described, there are quantitative differences between several *S. aureus* strains with regard to the HLA and PVL expression (Monecke et al., 2014; Stieber et al., 2014). Among other factors, the size of the inoculum requires standardization to facilitate a comparison of results from different experiments. The usage of a liquid medium is thus advisable. Further experiments are necessary to establish a suitable medium and a corresponding protocol. For a standardized method, the quantities of individual target proteins need to be normalized on a conserved and constant species marker as an internal standard, referring to the cell count. In future, quantitative measurements could be used to study strain-specific virulence traits and a possible influence of different antibiotics on the virulence factor expression. The newly developed assay as described herein allows the multiplex detection of staphylococcal proteins and contains the option to test the influence of external factors on protein expression. This might be a useful test in routine diagnostics as well as for research issues.

A

1														1
1	4	6	8	10	12	14	16	18	20	36	38	40	42	1
	33	34	35	36	37	38	39	40	41	42	43	44	2	
1	20	21	22	23	24	25	26	27	28	29	30	31	32	
1	7	8	9	10	11	12	13	14	15	16	17	18	19	
	37	38	39	40	41	42	43	44	2	3	4	5	6	
	24	25	26	27	28	29	30	31	32	33	34	35	36	
	11	12	13	14	15	16	17	18	19	20	21	22	23	
	41	42	43	44	2	3	4	5	6	7	8	9	10	
	28	29	30	31	32	33	34	35	36	37	38	39	40	
	15	16	17	18	19	20	21	22	23	24	25	26	27	
1	2	3	4	5	6	7	8	9	10	11	12	13	14	1
1														1

B

Legend of probes	Substance name
1	Positive control
2	Anti-PVL_1_0,5
3	Anti-PVL_1_0,05
4	Anti-PVL_2_0,5
5	Anti-PVL_2_0,05
6	Anti-PVL_3_0,5
7	Anti-PVL_3_0,05
8	Anti-TSST_1_0.5
9	Anti-TSST_1_0.05
10	Anti-TSST_2_0.5
11	Anti-TSST_2_0.05
12	Anti-SEA_1_0.5
13	Anti-SEA_1_0.05
14	Anti-SEA_2_0.5
15	Anti-SEA_2_0.05
16	Anti-SEB_1_0.5
17	Anti-SEB_1_0.05
18	Anti-SEB_2_0.5
19	Anti-SEB_2_0.05
20	Anti-SEB_3_0.5
21	Anti-SEB_3_0.05
22	Anti-SAK_1_0.5
23	Anti-SAK_1_0.05
24	Anti-HLA_1_0.5
25	Anti-HLA_1_0.05
26	Anti-HLB_1_0.48
27	Anti-HLB_1_0.05
28	Anti-HLB_2_0.48
29	Anti-HLB_2_0.05
30	Anti-HLB_3_0.48
31	Anti-HLB_3_0.05
32	Anti-HLB_4_0.5
33	Anti-HLB_4_0.05
34	Anti-HLB_5_0.5
35	Anti-HLB_5_0.05
36	Anti-PBP2A_1_0.5
37	Anti-PBP2A_1_0.05
38	Anti-ProteinA_1_0.5
39	Anti-ProteinA_1_0.05
40	Anti-ProteinA_2_0.5
41	Anti-ProteinA_2_0.05
42	Anti-ProteinA_3_0.5
43	Anti-ProteinA_3_0.05
44	Negative control



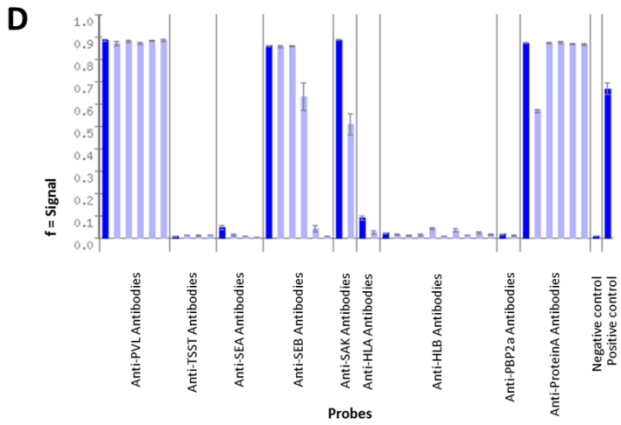


Figure 1: Layout of the protein microarray

A: Positions of each substance on the chip

B: Legend of the probes

C: Picture of a processed fluorescent microarray

D: Bar graph with signal intensities of expressed proteins

Table 1: Specific cut-off values for each target

Target	Cut off
Positive control	> 0.25
Negative control	< 0.1
lukF-P83	Anti-PVL_2 > 0.2 *
lukF-PV	at least 2 anti-PVL antibodies > 0.2
Protein A	at least 1 anti-Protein A antibody > 0.1
PBP2a	at least 1 anti-PBP2a antibody > 0.2
TSST	at least 1 anti-TSST antibody > 0.2
SEA	at least 1 anti-SEA antibody > 0.2
SEB	at least 1 anti-SEB antibody > 0.2
HLA	at least 1 anti-HLA antibody > 0.2
HLB	at least 1 anti-HLB antibody > 0.2
SAK	at least 1 anti-SAK antibody > 0.2

*Anti-PVL_2 Antibody detects exclusive epitopes of lukF-P83. Positive signals for two of the anti-PVL antibodies, 1, 2 or 3, indicate for the target lukF-PV.

Table 2: Comparison of genotyping data and fluorescent protein microarray results for various staphylococcal isolates.

1 = *sea*-FRI100; 2 = *sea*-N315; 3 = *sea*320; 4 = *lukF*-PV+/*lukS*-PV-; A = ambiguous; B = *lukF*-P83/*lukM*; D = DNA; N = negative; P = positive; Pr = Protein; T = truncated; X = one DNA probe out of 2 only

№	ID	typing	Protocol	SPA		mecA/ PBP2a		PVL		TST		SEA		SEB		SAK		HLA		HLB	
				D	Pr	D	Pr	D	Pr	D	Pr	D	Pr	D	Pr	D	Pr	D	Pr	D	Pr
1	160886	CC1-MSSA	NaOH/PBS	P	P	N	N	P	P	P	P	P1	P	N	N	P	P	P	P	T	N
2	199573	CC1-MSSA	NaOH/PBS	P	P	N	N	N	N	N	N	P	P	N	N	P	P	P	P	T	N
3	215392	CC1-MSSA	NaOH/PBS	P	P	N	N	N	N	N	N	P1	N	N	N	P	P	P	N	T	N
4	173633	CC1-MSSA	NaOH	P	P	N	N	N	N	N	N	P1	P	N	N	P	P	P	P	T	N
5	Sanger476	CC1-MSSA-SCCfus	NaOH	P	P	N	N	N	N	N	N	P1	P	N	N	P	P	P	N	P	N
6	231545	CC1-MSSA-SCCfus	NaOH/PBS	P	P	N	N	N	N	N	N	P	P	P	P	P	P	P	P	T	N
7	231549	CC1-MSSA-SCCfus	NaOH/PBS	P	P	N	N	N	N	N	N	P	P	P	P	P	P	P	P	T	N
8	231554	CC1-MSSA-SCCfus	NaOH/PBS	P	P	N	N	N	N	N	N	P	P	P	P	P	P	P	P	T	N
9	MW2	CC1-MRSA-IV [PVL+], USA400	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	N	P	P	P	N	T	N
10	124931	CC1-MRSA-IV+SCCfus [PVL+]	NaOH/PBS	P	P	P	P	P	P	N	N	P1	P	N	N	P	P	P	P	T	N
11	N315	CC5-MRSA-II, New York-Japan Clone	NaOH	P	P	P	P	N	N	P	P	P2	N	N	N	P	P	P	P	T	N
12	ATCC700699	CC5-MRSA-II, New York-Japan Clone	NaOH/PBS	P	P	P	P	N	N	P	P	P1	P	N	N	P	P	P	N	T	N
13	223878	CC5-MSSA with a truncated SCCmec element	NaOH	P	P	N	N	N	N	N	N	P1	P	N	N	P	P	P	P	T	N
14	241318	CC5-MRSA-IV, Paediatric clone [PVL+]	NaOH/PBS	P	P	P	P	P	P	N	N	P2	N	N	N	P	P	P	A	T	N
15	254809	CC5-MRSA-V+SCCfus	NaOH/PBS	P	P	P	P	N	N	N	N	N	N	P	P	P	P	P	A	A	N
16	NCTC8325	CC8-MSSA	NaOH	P	P	N	N	N	N	N	N	N	N	N	N	P	P	P	P	T	N
17	199572	CC8-MSSA	NaOH/PBS	P	P	N	N	N	N	N	N	P	P	N	N	P	P	P	N	T	N
18	FPR3757	CC8-MRSA-IV (PVL+/ACME+), USA300	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	P	T	N
19	200914	CC8-MRSA-IV (PVL+/ACME+), USA300	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	P	T	N
20	200915	CC8-MRSA-IV (PVL+/ACME+), USA300	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	P	T	N
21	200917	CC8-MRSA-IV (PVL+/ACME+), USA300	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	P	T	N
22	205975	CC8-MRSA-IV [PVL+/ACME+], USA300	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	P	T	N
23	227497	CC8-MRSA-IV (PVL+/ACME+), USA300	NaOH	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	P	T	N
24	176042	CC8-MRSA-IV [PVL+/ACME+], USA300	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	P	T	N
25	200916	CC8-MRSA-IV (PVL+/ACME+), USA300	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	N	T	N
26	200918	CC8-MRSA-IV (PVL+/ACME+), USA300	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	N	T	N
27	200919	CC8-MRSA-IV (PVL+/ACME+), USA300	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	N	T	N
29	124407	CC8-MRSA-IV (PVL+/ACME+), USA300	NaOH	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	P	T	N
30	COL	CC8/ST250-MRSA-I, Early/Ancestral MRSA	NaOH/PBS	P	P	P	P	N	N	N	N	N	N	P	P	N	N	P	A	P	N
31	150242	CC9-MRSA-IX	NaOH	P	P	P	P	N	N	N	N	N	N	N	N	N	N	P	N	P	N
32	199027	CC25-MSSA	NaOH/PBS	P	P	N	N	N	N	N	N	N	N	N	N	P	P	P	N	T	N
33	199571	CC30-MSSA	NaOH	P	P	N	N	N	N	P	P	P	P	N	N	P	P	P	N	T	N
34	254033	CC30-MSSA	NaOH/PBS	P	P	N	N	N	N	P	P	P	P	N	N	P	P	P	N	T	N
35	199575	CC30-MSSA	NaOH	P	P	N	N	N	N	P	P	P	P	N	N	P	P	P	N	T	N
36	199576	CC30-MSSA	NaOH	P	P	N	N	N	N	P	P	P[2]	N	N	N	P	P	P	N	T	N
37	252785	CC30-MSSA	NaOH/PBS	P	P	N	N	N	N	P	P	P	P	N	N	P	P	P	N	T	N
38	254801	CC30-MRSA	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	N	P	P	P	N	P	N
39	198848	CC30-MRSA-IV [PVL+]	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	N	P	N
40	234946	CC30-MSSA, [lukF-P83/lukM+]	NaOH/PBS	P	P	N	N	P[b]	N	N	N	N	N	N	N	N	N	P	N	P	N
41	200922	CC30-MRSA-IV-SCCfus [PVL+]	NaOH/PBS	P	P	P	P	P	P	P	P	N	N	N	N	P	P	P	N	T	N
42	200923	CC30-MRSA-IV-SCCfus [PVL+]	NaOH/PBS	P	P	P	P	P	P	P	P	N	N	N	N	N	N	P	N	P	N
43	200924	CC30-MRSA-IV-SCCfus [PVL+]	NaOH/PBS	P	P	P	P	P	P	P	P	N	N	N	N	P	P	P	N	P	N
44	215384	CC30-MRSA-IV	NaOH	P	P	P	P	N	N	P	P	N	N	N	N	P	N	P	N	T	N
45	200920	CC30-MRSA-IV [PVL+], Southwest Pacific Clone	NaOH/PBS	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	N	T	N
46	200921	CC30-MRSA-IV [PVL+], Southwest Pacific Clone	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	N	P	P	P	N	T	N
47	200925	CC30-MRSA-IV [PVL+], Southwest Pacific Clone	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	N	P	P	P	N	P	N
48	Sanger252	ST36/39-MRSA-II, UK-EMRSA-16 ()	NaOH/PBS	P	P	P	P	N	N	N	N	P[1]	P	N	N	P	P	P	N	T	N
49	236783	CC88-MRSA-IV [PVL+], [lukF-PV+, lukS-PV-]	NaOH/PBS	P	P	P	P	P[4]	N	N	N	P[2]	N	N	N	P	P	P	N	T	N
50	238233	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	N	N	P	P	P	N	T	N
51	238234	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	N	N	P	P	P	N	T	N
52	238235	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	N	N	P	P	P	N	T	N

Table 2: Cont.

53	238236	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
54	238237	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	A	P	P	P	N	T	N
55	238238	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
56	238239	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
57	238240	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
58	238241	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
59	238242	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	A	P	P	P	N	T	N
60	238243	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	A	P	P	P	N	T	N
61	238244	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
62	238245	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
63	238246	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	P[2]	N	P	P	P	P	P	N	T	N
64	238247	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
65	238248	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
66	238249	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	N	N	P	P	P	N	T	N
67	238251	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	A	P	P	P	N	T	N
68	238252	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	A	P	P	P	N	T	N
69	238253	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	A	P	P	P	N	T	N
70	253431	CC121-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	P	P	P	P	P	N	T	N
71	238254	CC121-MSSA	NaOH/PBS	P	P	N	N	N	N	N	N	N	N	N	N	P	P	P	N	T	N
72	238255	CC121-MSSA	NaOH/PBS	P	P	N	N	N	N	N	N	N	N	N	N	P	P	P	N	T	N
73	238256	CC121-MSSA	NaOH/PBS	P	P	N	N	N	N	N	N	P[2]	N	N	N	P	P	P	N	T	N
74	224815	CC130-MRSA-XI [mecC +]	NaOH	P	P	N	N	N	N	N	N	N	N	N	N	N	N	N	P	N	P	P
75	225779	CC130-MRSA-XI, [mecC +]	NaOH	P	P	N	N	N	N	N	N	N	N	N	N	N	N	N	P	N	P	P
76	124627	CC133-MSSA [lukF-P83/lukM+]	NaOH/PBS	P	P	N	N	P[b]	P[b]	P	P	P[3]	P	N	N	N	N	P	N	P	P
77	215375	CC133-MSSA, [lukF-P83/lukM+]	NaOH/PBS	P	P	N	N	P[b]	P[b]	P	P	N	N	N	N	N	N	P	N	P	P
78	252784	CC133-MSSA [lukF-P83/lukM+]	NaOH/PBS	P	P	N	N	P[b]	P[b]	P	P	N	N	P[x]	N	N	N	P	N	P	P
79	215373	CC133-MSSA, [lukF-P83/lukM+]	NaOH/PBS	P	P	N	N	P[b]	N	N	N	P[3]	P	N	N	P	P	P	N	P	N
80	238250	CC152-MSSA [PVL+]	NaOH/PBS	P	P	N	N	P	P	N	N	N	N	N	N	P	P	P	N	P	P
81	124474	CC361-MRSA-IV, WA MRSA-29	NaOH/PBS	P	P	P	P	N	N	P	P	N	N	P	P	P	P	P	N	T	N
82	225776	CC398-MRSA-IV	NaOH	P	P	P	P	N	N	N	N	N	N	N	N	N	N	P	N	P	P
83	199873	CC398-MRSA-V	NaOH	P	P	P	P	N	N	N	N	N	N	N	N	N	N	P	N	P	N
84	199874	CC398-MRSA-V	NaOH	P	P	P	P	N	N	N	N	N	N	N	N	N	N	P	N	P	N
85	225778	CC398-MRSA-V	NaOH	P	P	P	P	N	N	N	N	N	N	N	N	N	N	P	N	P	P
86	210067	CC479-MSSA [lukF-P83/lukM+]	NaOH/PBS	P	A	N	N	P[b]	P[b]	N	N	N	N	N	N	N	N	P	N	P	P
87	178400	CC479-MSSA [lukF-P83/lukM+]	NaOH/PBS	P	N	N	N	P[b]	P[b]	N	N	N	N	N	N	N	N	P	N	P	P
88	138830	CC479-MSSA [lukF-P83/lukM+]	NaOH/PBS	P	P	N	N	P[b]	A[b]	N	N	N	N	N	N	N	N	P	N	P	P
89	138831	CC479-MSSA [lukF-P83/lukM+]	NaOH/PBS	P	P	N	N	P[b]	A[b]	N	N	N	N	N	N	N	N	P	N	P	P
90	RF122	CC705-MSSA	NaOH/PBS	P	P	N	N	P[b]	A	P	P	N	N	N	N	N	N	P	N	P	P
91	196911	CC705-MSSA	NaOH/PBS	P	P	N	N	P[b]	P[b]	P	P	N	N	N	N	N	N	P	N	P	P
92	196916	CC705-MSSA	NaOH/PBS	P	P	N	N	P[b]	P[b]	N	N	N	N	N	N	N	N	P	N	P	P
93	119087	CC705-MSSA	NaOH	P	P	N	N	P[b]	P[b]	N	N	N	N	N	N	N	N	P	P	P	P
94	138826	CC705-MSSA	NaOH/PBS	P	A	N	N	P[b]	P[b]	P	P	N	N	N	N	N	N	P	N	P	P
95	101818	ST8-MRSA-IIB, Irish AR13/14	NaOH/PBS	P	P	P	P	N	N	N	N	P[1]	P	N	N	P	P	P	N	T	N
96	124414	ST59-MRSA-IV+V, WA MRSA-15	NaOH/PBS	P	P	P	P	N	N	N	N	P[1]	P	P	P	P	P	P	N	P	P
97	215382	ST80-MRSA-IV, "European Clone"	NaOH	P	P	P	P	P	P	N	N	N	N	N	N	P	P	P	N	T	N
98	224519	ST239-MRSA-III agr/hld deletion variant	NaOH/PBS	P	P	P	P	N	N	N	N	P	P	N	N	P	P	P	N	T	N
99	200926	ST772-MRSA-V [PVL+], Bengal Bay Clone/WA MRSA-60	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	N	N	N	P	N	T	N
100	238824	ST772-MRSA-V [PVL+], Bengal Bay Clone/WA MRSA-60	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	A	N	N	P	N	T	N
101	200930	ST772-MRSA-V [PVL+], Bengal Bay Clone/WA MRSA-60	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	N	N	N	P	N	A	N
102	145330	ST772-MRSA-V [PVL+], Bengal Bay Clone/WA MRSA-60	NaOH/PBS	P	P	P	P	P	P	N	N	P[1]	P	N	N	N	N	P	N	T	N
103	200927	ST772-MRSA-V [PVL+], Bengal Bay Clone/WA MRSA-60	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	N	N	N	P	N	T	N
104	200928	ST772-MRSA-V [PVL+], Bengal Bay Clone/WA MRSA-60	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	N	N	N	P	N	A	N
105	200929	ST772-MRSA-V [PVL+], Bengal Bay Clone/WA MRSA-60	NaOH/PBS	P	P	P	P	P	P	N	N	P	P	N	N	N	N	P	N	A	N
106	200931	ST772-MRSA-V	NaOH/PBS	P	P	P	P	N	N	N	N	N	N	N	N	N	N	P	N	A	N
107	ATCC35984	*S. epidermidis*, [mecA+]	NaOH	N	N	P	P	N	N	N	N	N	N	N	N	N	N	N	N	N	N
108	ATCC12228	*S. epidermidis*	NaOH	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
109	225781	*S. sciuri*	NaOH	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
110	223885	*S. capitis*	NaOH	N	N	N	N	N	N	N	N	N	N	N	N	P[x]	N	N	N	N	N
111	BL21-DE3	*E. coli*	NaOH	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

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Diskussion

S. aureus ist ein weltweit häufig vorkommendes Bakterium, das eine Vielzahl verschiedener Virulenz- und Resistenzfaktoren trägt. Es ist ein Erreger, der ein breites Spektrum an Symptomen verursachen und der durch die weit verbreiteten Resistenzen zum Teil nur noch sehr eingeschränkt behandelt werden kann.

Der Fokus dieser Dissertation lag zum einen auf der genotypischen Analyse von *S. aureus* Stämmen, die zur Aufklärung der Bakterienverbreitung beitragen soll. Dazu wurden in dieser Arbeit zwei *S. aureus* Populationen mittels DNA-Mikroarrays typisiert (Publikationen I und II). Die Isolate stammen von Menschen, die in geografisch verschiedenen Regionen (Trinidad & Tobago und Norwegen) und unter verschiedenen sozioökonomischen Gegebenheiten leben. Zum anderen wurde der Fokus auf den phänotypischen Nachweis relevanter *S. aureus* Toxine gelegt. Es wurde ein Test zum qualitativen Nachweis von exprimiertem PVL entwickelt (Publikation III) sowie ein Protein-Mikroarray-Assay zur Quantifizierung der Toxine PVL und Hla etabliert (Publikation IV und V). Des Weiteren wird die technische Erweiterung zum Multiplexnachweis von Proteinen beschrieben, die die Etablierung eines diagnostischen Tests mit Hilfe eines Protein-Mikroarrays ermöglicht (Manuskript I).

DNA-Typisierung von geografisch verschiedenen *Staphylococcus aureus* Populationen

Die Genotypisierung von Bakterien ist von großer Bedeutung, um das Vorkommen, die Verbreitung und einzelne Populationsstrukturen zu verstehen sowie Infektionsketten nachvollziehen zu können.
Verbreitete Methoden zur Typisierung und Klassifizierung von *S. aureus* sind Spa-Typisierung und MLST. Die Methode der Wahl für den Gennachweis von verschiedenen Resistenzen und Virulenzen ist meist die Polymerase Kettenreaktion (PCR). DNA-Mikroarrays ermöglichen die Typisierung von *S. aureus* (Zuordnung des Isolates zu dem entsprechenden CC) sowie die Detektion vieler relevanter Resistenz- und Virulenzgene des Bakteriums in einem einzigen Experiment. Zusätzlicher Vorteil der DNA-Mikroarrays ist, dass der Assay-Umfang ohne größere Änderungen am Mikroarray und entsprechende Kosten, z. B. um ein neu entdecktes Resistenzgen, erweitert werden kann (Monecke et al., 2003; Monecke und Ehricht, 2005; Monecke et al., 2008).

In vielen Ländern und Regionen, z. B. in den USA, Australien und weiten Teilen Europas, wurden ausführliche Studien zur Verbreitung von *S. aureus* durchgeführt und die auftretenden Stämme typisiert (Munckhof et al., 2003; Stegger et al., 2014; Monecke et al., 2001). Andere Regionen sind dahingehend weniger umfangreich untersucht. So gibt es für Trinidad & Tobago nur einzelne Studien zur Populationsstruktur von MRSA (Akpaka et al., 2006). Wie in Publikation I beschrieben, wurde eine zusätzliche Populationsstudie in Trinidad & Tobago durchgeführt. Dabei wurden das Vorkommen und die relativen Häufigkeiten von MSSA und MRSA Fällen erfasst und deren genetische Typisierung in Hinblick auf Virulenzfaktoren (*tst1*, *sea* – see, *lukF/S-PV, sak, agrI – agrIV* und andere Gene) und Resistenzen (*mecA* und andere Gene) durchgeführt. Die in dieser Studie erfassten *S. aureus* Isolate beinhalten 15,31% MRSA, die sich im Vergleich zu den Daten früherer Erhebungen (Akpaka et al., 2006) dem Trend steigender MRSA Raten in Trinidad & Tobago anschließen. In dieser Population waren die Zahlen PVL-positiver Isolate besonders auffällig. 50% der getesteten Isolate waren für die PVL-Gene *lukF-PV* und *lukS-PV* positiv. Das gehäufte Auftreten von PVL führte zu der Frage, wie sich der prozentuale PVL-Anteil in anderen Regionen verhält und welchen Einfluss PVL auf Krankheitsbilder und deren klinischen Verlauf hat.

In einer zweiten Populationsstudie (Publikation II) wurden nur PVL-positive MRSA in Norwegen genotypisch untersucht. Norwegen unterscheidet sich klimatisch und sozioökonomisch sehr von Trinidad & Tobago. Es ist ein gutes Vorbild im Hinblick auf seine Infektionskontrolle, was sich im unbedeutenden Auftreten von MRSA in dem Land zeigt (Elstrøm et al., 2012). In Norwegen gibt es ein Kontrollprogramm, bei dem ein MRSA Screening von Patienten und medizinischem Personal durchgeführt wird, die einen Aufenthalt oder Kontakt in/zu anderen Ländern, Krankenhäusern oder bekannten MRSA-Fällen hatten. Gehäufte Zahlen von Krankenhauskeimen wie HA-MRSA sind nicht bekannt, allerdings ist ein Anstieg von CA-MRSA inklusive PVL außerhalb medizinischer Einrichtungen zu verzeichnen (Elstrøm et al., 2011). Ein ähnliches Vorkommen von MRSA Fällen wird in Norwegens Nachbarländern Schweden und Dänemark beschrieben (Larsson et al., 2014; Faria et al., 2005). Die Charakterisierung PVL-positiver MRSA in Norwegen zeigt, dass eine Vielzahl genotypisch verschiedener Stämme vorkommt und es keinen vorherrschenden Epidemie-Stamm gibt (Publikation II). Die auftretenden Fälle stehen gehäuft mit vorheriger Reiseaktivität der Betroffenen in Zusammenhang. Diese PVL-positiven CA-MRSA werden kontrolliert und stellen momentan kein großes Gesundheitsproblem in Norwegen dar.

Die beiden oben beschriebenen Studien zeigen verschiedene epidemiologische Situationen. So gibt es Länder mit häufig auftretenden PVL-positiven Stämmen, z. B. afrikanische Länder oder auch Trinidad & Tobago (Breurec et al., 2011; Publikation I). In anderen, z. B.

skandinavischen, Ländern treten PVL-positive Stämme nur sporadisch auf und werden in den meisten Fällen aus anderen „PVL-gefährdeten“ Ländern importiert (Publikation II).

Qualitativer Nachweis vom *Staphylococcus aureus* Toxin PVL

Aufgrund der Assoziationen von PVL mit rezidivierenden Hautinfektionen und zum Teil schwerwiegenden Krankheiten wie nekrotisierenden Pneumonien (Lina et al., 1999) ist eine aggressive Behandlung notwendig (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/322857/Guidance_on_the_diagnosis_and_management_of_PVL_associated_SA_infections_in_England_2_Ed.pdf). Damit PVL entsprechend schnell und richtig erkannt wird, ist ein diagnostischer Test empfehlenswert. Ein einfacher klinischer Test würde das Screening von Verdachtsfällen und potenziell exponierten Personengruppen wie Reiserückkehrern, Flüchtlingen und Militärpersonal erlauben. Ein positives Testergebnis ermöglicht dann eine schnelle, adäquate Behandlung und verhindert damit eine Ausbreitung im medizinischen oder familiären Umfeld.

Der Lateral Flow (LF) Test erfüllt diese Bedingungen und ist zusätzlich ökonomisch und ohne teure Laborausstattung durchführbar. In Publikation III ist die Entwicklung eines solchen phänotypischen Tests zur qualitativen Bestimmung von exprimierten PVL aus Kulturmaterial beschrieben. Dieser immunchromatographische Test basiert auf monoklonalen Antikörpern, die zur Entwicklung des Tests zunächst mittels vorher hergestellter Mikroarrays intensiv und umfangreich analysiert wurden. Protein-Mikroarrays bieten eine optimale Plattform zum Screening von Antikörpern, um die optimale Kombination von Fang- und Detektions-Antikörper in Bezug auf optimale Sensitivität und Spezifität in einer definierten Probenmatrix zu bestimmen. Zur Durchführung des LF Tests ist zunächst die Anzucht des *S. aureus* Isolates auf einem Standardmedium nötig. Die gewachsene Kultur kann direkt als Nachweismaterial eingesetzt werden. In alternativ beschriebenen PVL-Nachweismethoden ist eine ähnliche oder gar umfangreichere Probenaufbereitung nötig. Oishi et al. beschreiben einen Latex-Agglutinationstest zum Nachweis von PVL (Oishi et al., 2008). Dies ist eine einfach zu handhabende Methode, jedoch durch die Probenanzucht mindestens so zeitaufwändig wie der in Publikation III beschriebene LF Assay. Der PVL-Nachweis mittels Matrix-unterstützter Laser-Desorption/Ionisation und Massenspektrometrie mit Flugzeitanalysator (MALDI-TOF-MS) benötigt ebenfalls wie der LF- und Latex-Agglutinationstest eine vorhergehende Probenanzucht sowie zusätzlich noch eine passende Laborausstattung mit sehr teuren Geräten (Bittar et al., 2009). In vielen Laboren erfolgt der PVL-Nachweis mittels PCR (Deurenberg et al., 2004; Francois et al., 2004), die im Vergleich zum LF zeitintensiver ist, technische Erfahrung sowie ebenfalls eine entsprechende

Laborausstattung voraussetzt. In Forschungslaboren sind zusätzlich Enzyme-linked Immunosorbent Assays (ELISA) weit verbreitet (Badiou et al., 2010; Hamilton et al., 2007). Dies sind einfache proteinbasierende Tests, die jedoch deutlich länger als der LF Test dauern. Badiou und Kollegen entwickelten einen PVL-ELISA für den direkten Test von klinischen Proben auf PVL mit einer Sensitivität von 92% (Badiou et al., 2010). Die Sensitivität des *in vitro* LF Tests liegt dagegen bei 99,7%, die Spezifität bei 98,3% und das Detektionslimit bei 1 ng/ml gereinigtem, nativem und rekombinantem Antigen. Alle *in vitro* getesteten *lukF-PV*/*lukS-PV*-positiven Isolate exprimierten nachweisbar PVL. Die Übereinstimmung von genotypischer und phänotypischer PVL-Präsenz mindert die Wahrscheinlichkeit falsch-negativer Ergebnisse, indem PVL *in vitro* nicht exprimiert wird. Momentan ist ein PVL-Schnelltest dieser oder vergleichbarer Art in Routine-Diagnostiklaboren nicht vorhanden, aufgrund der klinischen Relevanz von PVL aber erstrebenswert. Eine mögliche Erweiterung des beschriebenen LF Tests wäre die parallele Detektion von PVL und PBP2a. Damit würden zwei wichtige Marker (Virulenz und Resistenz) im Umgang mit *S. aureus* Infektionen in einem Test zusammengefasst werden.

Quantitative Bestimmung von *Staphylococcus aureus* Toxinen

Protein-Mikroarrays erlauben neben dem qualitativen Toxin-Nachweis auch dessen quantitative Bestimmung. In Publikation IV ist diese technische Entwicklung zur Messung von PVL-Konzentrationen beschrieben. Basierend auf Antigen-Antikörper-Bindungen auf dem Mikroarray werden die Signalstärken der gefärbten Spots gemessen. Über diese lässt sich, im Abgleich mit Referenzexperimenten, die vorhandene Konzentration von PVL bestimmen. Quantitative Messungen sind im Hinblick auf die Korrelation zwischen der Toxinexpression und dem genetischen Hintergrund der Isolate sowie der Schwere einer Erkrankung hilfreich.

Das Screening zahlreicher *S. aureus* Isolate zeigt, dass genetisch ähnliche Stämme, die demselben CC angehören*, in vitro* ähnliche Mengen an PVL exprimieren. Beispielsweise sind von den Isolaten der CCs 8 und 93 hohe Konzentrationen an PVL gemessen worden (im Mittelwert 4300 ng/ml und 3700 ng/ml). Isolate der CCs 1, 5 oder 80 dagegen zeigten deutlich weniger Toxinproduktion (im Mittelwert 260 ng/ml, 520 ng/ml und 230 ng/ml). Ähnlich variable PVL-Produktionen bei verschiedenen CCs wurden auch in anderen Arbeiten beschrieben (Boakes et al., 2011; Badiou et al., 2010).

Da PVL häufig in CA-MRSA vorkommt, die auch schwere Krankheiten wie nekrotisierende Pneumonien und Fasciitis verursachen können (Gillet et al., 2002), wird es als mögliche Ursache dieser Krankheitsbilder und als Marker von CA-MRSA kontrovers diskutiert

(Vandenesch et al., 2003; Boyle-Vavra und Daum, 2007). Die aus Publikation IV resultierenden Ergebnisse zeigen in Isolaten, die von schweren systemischen Infektionen stammten, niedrige PVL-Konzentrationen. Das korreliert mit der Tatsache, dass für die Pathogenese bei einer Sepsis PVL keine Rolle spielt (Shallcross et al., 2013; Ellington et al., 2007). Die PVL-Konzentrationen von Isolaten, die aus Haut- und Weichteilinfektionen stammten, waren *in vitro* teilweise deutlich höher. Nekrotisierende Fasciitis und Pneumonie sind selten, weshalb in dieser Studie keine Messungen der PVL-Produktion solcher Isolate durchgeführt werden konnten. Badiou und Kollegen wiesen die PVL-Expression in Hautabszessen experimentell nach (Badiou et al., 2008). Eine Metaanalyse verfügbarer Publikationen über PVL zeigt ebenso eine konsistente Assoziation von PVL mit Haut- und Weichteilinfektionen, wenngleich es nur selten in Verbindung mit invasiven Erkrankungen gebracht wird (Shallcross et al., 2013; Ellington et al., 2007). Dies führt zur Annahme, dass PVL ggf. nicht der einzig relevante Virulenzfaktor von CA-MRSA ist. Ebenso können regulatorische Faktoren (z. B. *agr*) oder andere, parallel exprimierte Staphylokokken-Toxine die Virulenz der Bakterien mit beeinflussen. Die Ergebnisse dieser Arbeit beruhen jedoch auf *in vitro* Experimenten und die Toxinexpression *in vivo* kann davon abweichen. Eine statistische Auswertung der eigenen Ergebnisse zeigte höhere PVL Konzentrationen von Isolaten mit *agr*-Typen I und IV als mit *agr*-Typen II und III. Anders als in Publikation IV verdeutlicht, konnten Boakes et al. keinen Einfluss der *agr*-Typen auf die PVL-Produktion feststellen (Boakes et al., 2011). Zusätzlich zeigen die Ergebnisse der eigenen Arbeit bei Anwesenheit von *tst1* und *lukF-PV/lukS-PV* in einem Isolat eine niedrigere PVL-Produktion als in *tst1*-negativen und *lukF-PV/lukS-PV*-positiven Isolaten. TSST-1 kann in hohen Konzentrationen gebildet werden, während es die Genexpression anderer Exotoxine hemmt (Vojtov et al., 2002). Somit scheint TSST-1 die Expression, u. a. von PVL, entscheidend beeinflussen zu können. Die Gabe verschiedener Antibiotika hat ebenfalls Einfluss auf die bakterielle Toxinproduktion. So erhöht das auf die bakterielle Zellwand wirkende Oxacillin in subinhibitorischer Konzentration sogar die PVL-Menge, normiert auf die Zellzahl. Antibiotika, die auf die bakterielle Proteinsynthese wirken (z. B. Clindamycin, Linezolid oder Fusidinsäure), verringern die PVL-Konzentration (Dumitrescu et al., 2007).

Bei der Auseinandersetzung mit PVL als Virulenzfaktor ist zu beachten, dass die diskutierten Ergebnisse (Publikation IV; Boakes et al., 2011) auf *in vitro* Messungen basieren. *In vivo* kann die Toxinproduktion der Bakterien anders reguliert sein. Eine Möglichkeit hierfür besteht in einer erhöhten Toxinproduktion unter bestimmten Bedingungen, wie z. B. Stress der Bakterienzelle, ausgelöst durch immunologische Abwehrmechanismen des Körpers oder aber durch den Einsatz von Antibiotika (Dumitrescu et al., 2007). So kann ein *S. aureus* Isolat *in vivo* möglicherweise viel PVL produzieren und damit ein schweres Krankheitsbild hervorrufen, während es in den *in vitro* Versuchen PVL in geringen Konzentrationen gebildet

hat. Weiterhin ist nicht bekannt, ob höhere PVL Konzentrationen überhaupt nötig sind, um klinisch schwere Erkrankungsverläufe zu verursachen.
Bei der Ausprägung PVL-assoziierter Erkrankungen spielt auch die humorale Immunantwort eine entscheidende Rolle. So haben Studien gezeigt, dass hohe anti-PVL-Antikörper-Titer im Serum eines Patienten die Schwere einer PVL-basierten Infektion mindern (Adhikari et al., 2012). Dieser Sachverhalt bietet die Option, zukünftig PVL-Antikörper zur passiven Immunisierung bei schweren Infektionen mit PVL-positiven *S. aureus* einzusetzen, um durch die Neutralisierung des Toxins den Verlauf günstig zu beeinflussen. Die lokal erhöhte Prävalenz von PVL-positiven *S. aureus* in manchen Regionen, wie beispielsweise in Westafrika (Senegal: 60%), korreliert mit einer verstärkten spezifischen anti-PVL Immunantwort der in den betroffenen Gebieten lebenden Menschen. So erkranken etwa im Senegal lebende Menschen seltener an einer lebensbedrohlichen, PVL-vermittelten Pneumonie als solche, die in nördlicheren Ländern, wie Frankreich, leben. Dort tritt PVL vergleichsweise deutlich weniger häufig auf (PVL Prävalenz in Frankreich: ≤5%). Diese Verteilung von PVL haben Rasigade et al. mit einem „Nord-Süd-Gradienten“ beschrieben (Rasigade et al., 2015; Rasigade et al., 2011) und ähnliches wurde im Rahmen dieser Arbeit (Publikation III) ebenfalls beobachtet. Die Seltenheit von nekrotisierenden Pneumonien in südlichen Ländern (wie Senegal - siehe Rasigade et al., 2011) lässt vermuten, dass Patienten, die an PVL-vermittelten, rezidivierenden Haut- und Weichteilinfektionen erkranken, protektive anti-PVL Antikörper bilden. Patienten hingegen, die noch nicht mit PVL in Kontakt gekommen sind, besitzen keine entsprechenden Antikörper. Bei solchen Patienten, wie z.B. auch Kindern, kann eine PVL-positive *S. aureus* Infektion einen klinisch schwereren Verlauf nehmen (Monecke et al., 2008).
Beim Zusammenspiel von Wirt (Immunsystem), der bakteriellen Kolonisierung und anderen bakteriellen Virulenzfaktoren und Regulationsmechanismen mit PVL sind einige Einflüsse noch nicht ausreichend erforscht. Zukünftige Studien sind nötig, um Informationen über diese, die PVL-Expression steuernden Einflüsse zu erlangen. PVL sollte als Virulenzfaktor betrachtet und entsprechende Maßnahmen im Umgang mit PVL-positiven *S. aureus* Infektionen ergriffen werden. In Großbritannien wurde bereits eine entsprechende medizinische Richtlinie eingeführt (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/322857/Guidance_on_the_diagnosis_and_management_of_PVL_associated_SA_infections_in_England_2_Ed.pdf). Um PVL-positive *S. aureus* Infektionen möglichst effizient diagnostisch zu erkennen, bietet ein Lateral Flow (Schnell-) Test, wie in Publikation III beschrieben, optimale Möglichkeiten.

Basierend auf dem in Publikation IV dargestellten Prinzip wurden Protein-Mikroarrays zur quantitativen Messung von exprimiertem Hla entwickelt. Diese bieten die Möglichkeit zu *in vitro* Untersuchungen dieses weiteren *S. aureus* Toxins und können auch für jedes weitere

beliebige Toxin angepasst werden. In Publikation V ist eine Untersuchung verschiedener medizinischer und veterinärmedizinischer *S. aureus* Isolate auf deren Hla-Expression beschrieben. Der Vergleich der Hla-Produktion von *S. aureus* Isolaten menschlicher und verschiedener tierischer Herkunft lässt auf keine Korrelation zwischen Wirtsspezies und Toxinproduktion schließen. Vielmehr scheint eine Korrelation zwischen CC und Hla-Expression zu bestehen. Obwohl alle Isolate den chromosomal gelegenen *hla* Genlocus besitzen, konnte bei verschiedenen Stämmen der CCs 22, 30, 45, 479 oder 705 kein Hla gemessen werden. Bei Isolaten der CCs 1, 5 und 8 hingegen wurden variable Toxinkonzentrationen detektiert. Eine ähnliche Korrelation zwischen der Hla-Produktion und der CC-Zugehörigkeit wurde bereits von Li und Kollegen entdeckt, die die Konzentrationen aber auf molekularem Level mittels quantitativer Reverser Transkriptase (RT)-PCR bestimmten (Li et al., 2010).

Bei den Hla *in vitro* Messungen konnte kein Zusammenhang zwischen Toxinproduktion und Krankheitsverlauf hergestellt werden. Bei der Untersuchung von *S. aureus* Isolaten medizinisch bekannter Fälle zeigten Isolate schwerer Erkrankungen eine ähnliche Hla-Expression (im Mittelwert 3,01 ng/ml) wie Isolate von klinisch weniger schlimmen Fällen (im Mittelwert 5,77 ng/ml). Das lässt vermuten, dass Hla ggf. bereits in geringen Konzentrationen schwere Erkrankungen hervorrufen kann oder die *in vitro* Toxinproduktion nicht der *in vivo* Expression entspricht. Wie bereits für PVL beschrieben, kann auch hier die Toxinexpression *in vivo* anders reguliert sein. So ist es denkbar, dass die Bakterienzelle durch den Angriff des menschlichen Immunsystems mehr Toxin ausschüttet. Dieser Effekt tritt bei *in vitro* Experimenten nicht auf. Das Immunsystem spielt bei Hla-produzierenden *S. aureus* Infektionen eine entscheidende Rolle. Patienten, die eine Infektion mit *in vitro* Hla-exprimierenden *S. aureus* durchlaufen, besitzen höhere Titer an anti-alpha-Toxin-Antikörpern als Patienten, deren *S. aureus* Stamm es nicht exprimiert oder die keinen *S. aureus* tragen (Sharma-Kuinkel et al., 2015). Das zeigt, dass das menschliche Immunsystem das Toxin erkennt und darauf reagiert, indem es neutralisierende Antikörper bildet. Die spezifische humane Antikörper-Antwort des menschlichen Immunsystems auf eine *S. aureus* Infektion ist mit Protein-Mikroarrays detektierbar (Kloppot et al., 2015).

Da Hla genotypisch in *S. aureus* Isolaten weit verbreitet und teilweise an schweren Krankheiten (z. B. Pneumonien) beteiligt sein kann (Bhakdi und Tranum-Jensen, 1991), ist die Untersuchung der Expression und Regulation dieses Toxins von Bedeutung.

Die entwickelten Protein-Mikroarrays bieten die Plattform für solche erweiterten Tests von internen und externen Einflüssen auf die bakterielle Toxinproduktion. Für das *S. aureus* Toxin PVL und das von *Escherichia coli* produzierte Shigatoxin wurde die quantitative Toxinmessung mittels Protein-Mikroarrays bereits für Experimente dieser Art genutzt. Dabei

wurde die jeweilige Toxinexpression unter Einwirkung verschiedener Antibiotika gemessen und erste entscheidende Erkenntnisse für eine medizinische Therapie bei solchen bakteriellen Infektionen gewonnen (zwei Poster-Präsentationen auf der DGHM 2014 - Stieber et al., 2014).

Proteinbestimmung im Multiplexverfahren

S. aureus kann neben PVL und Hla eine Vielzahl weiterer Exotoxine bilden. Dazu zählen unter anderem das Toxische Schock Syndrom Toxin (TSST-1) und verschiedene Enterotoxine (Dinges et al., 2000). Um die parallele Expression mehrerer Exotoxine zu untersuchen, wurde ein Multiplex-Protein-Mikroarray entwickelt (Manuskript I). Zusätzlich zu den bedeutsamen Staphylokokken-Toxinen, wie PVL, Hla, TSST-1, Enterotoxine A und B, Beta-Hämolysin und Staphylokinase, kann der *S. aureus* Speziesmarker Spa und das Methicillin-Resistenz-vermittelnde Protein PBP2a qualitativ nachgewiesen werden. Ein molekularer Test, der mehrere Proteine in einem Experimentansatz nachweisen kann, existiert so noch nicht. Eine häufig verwendete Proteinnachweismethode ist der ELISA (Lequin, 2005). Dieser kann jedoch pro Ansatz nur ein Protein messen. Besonders in der Diagnostik ist ein Multiplex-Nachweistest für Proteine von Relevanz. Der schnelle und parallele Nachweis von Resistenzfaktoren und hoch virulenten Toxinen eines Bakteriums ermöglicht eine schnelle und adäquate Therapie der betroffenen Patienten. Insbesondere bei *S. aureus* mit seiner Vielzahl an Virulenzfaktoren ist eine solche Art Diagnostik sehr nützlich. So können in einem Test z. B. die teils hochvirulenten PVL-positiven MRSA identifiziert und so gezielt behandelt werden.

Ähnlich wie für die Proteinarrays zur quantitativen Toxinmessung (Publikationen IV und V) wurden spezifische Antikörper gegen jedes einzelne Protein auf den Mikroarray gespottet. Die Detektionsmethode wurde von einer Fällungsreaktion auf eine Fluoreszenzdetektion umgestellt (siehe Einleitung). Durch die entsprechende Weiterentwicklung des Assays könnte dieser zukünftig ebenso wie die in den Publikationen IV und V beschriebenen Assays zur quantitativen Messung von Proteinexpressionen eingesetzt werden. Das ermöglicht interessante Perspektiven für die Forschung im Hinblick auf die gegenseitige Regulation der Toxine. So ist beispielsweise bekannt, dass TSST-1 in hohen Konzentrationen gebildet werden kann, während die Expression anderer Exotoxine herunterreguliert wird (Vojtov et al., 2002). Zusätzlich können interne (z. B. Regulationsgene) und externe (z. B. Antibiotika) Einflüsse auf die Produktion und Regulation der Toxine untersucht werden.

Schlussfolgerungen

Diese Arbeit behandelt die Genotypisierung von *S. aureus* und die Analyse der bakteriellen Toxinexpression. Als Plattform für diese Experimente dienten – neben einer Vielzahl von molekularbiologischen und mikrobiologischen Methoden – Mikroarrays, die aufgrund der Möglichkeit von Untersuchungen auf DNA- und Proteinebene mit derselben Technik hierfür eine optimale Grundlage boten.

Die DNA-Mikroarrays ermöglichen durch die Detektion vieler verschiedener Virulenz-, Resistenz- und Speziesmarker in einem Experiment die einfache und schnelle Genotypisierung von *S. aureus* Stämmen. Das lässt die Charakterisierung einzelner Populationen, die Neuentdeckung von Stämmen sowie die Entdeckung und Überwachung von Ausbrüchen in Krankenhäusern zu. Diese Anwendungen sind von großer Bedeutung, da *S. aureus* ein bei Menschen und Tieren weltweit verbreiteter Keim ist, der viele, teilweise lebensbedrohliche, Krankheiten auslöst und aufgrund von Resistenzen nur eingeschränkt behandelt werden kann. Zusätzlich kann die DNA-Mikroarray-Technik auch zur Genotypisierung anderer klinisch relevanten Bakterien angewendet werden. Außerdem wird in der vorliegenden Arbeit die eigene technische Weiterentwicklung von Proteinarrays – vom qualitativen und quantitativen Proteinnachweis bis hin zur Erweiterung zu einem Multiplex-Assays – dargestellt. Diese Entwicklung erlaubt es, die Expression verschiedener *S. aureus* Toxine wie PVL und Hla sowohl einzeln als auch gemeinsam nachzuweisen sowie verschiedene Einflüsse auf die Expression zu untersuchen. Die parallele genotypische Untersuchung von *S. aureus* Isolaten mit DNA-Mikroarrays und der phänotypische Nachweis einzelner Toxine mit Protein-Mikroarrays ermöglicht es, einzelne Virulenzfaktoren auf beiden Ebenen parallel zu untersuchen. Im Fall von Hla konnte z. B. damit gezeigt werden, dass der Genlokus *hla* in allen getesteten Isolaten vorhanden war, das Toxin in verschiedenen CCs jedoch nicht exprimiert wurde.

PVL und Hla zeigen beide *in vitro* eine Korrelation zwischen der exprimierten Toxinkonzentration und dem CC der jeweils getesteten Stämme. Im Gegensatz dazu weisen beide Toxine keine Korrelation zwischen Toxinmenge und der Schwere des klinischen Krankheitsbildes, das ein Stamm ausgelöst hat, auf. Die erhöhte Virulenz von Bakterienstämmen kann möglicherweise schon durch geringe Toxinkonzentrationen hervorgerufen werden, wahrscheinlich verhält sich die Toxinexpression *in vivo* aber auch anders als *in vitro*. Die Menge des jeweiligen gebildeten Toxins kann von vielen inneren und äußeren Einflüssen abhängig sein. Für weitere Untersuchungen dieser Aspekte sowie für die Messung protektiver Antikörper im Blut eines Patienten stellen die Protein-Mikroarrays eine geeignete Untersuchungsmethode dar.

Mit dem Multiplex-Protein-Assay können mehrere Toxine parallel nachgewiesen werden. Methodisch existierte ein solcher Multiplex-Nachweis von Proteinen bisher noch nicht und wäre, insbesondere für die Diagnostik, eine sinnvolle Anwendung, da der phänotypische Nachweis spezieller bakterieller Toxine eine schnelle und zielgerichtete Behandlung der betroffenen Patienten ermöglichen würde.

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Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Dr. med. habil. Stefan Monecke und Dr. rer. nat. Ralf Ehricht.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, 03.07.2015 Unterschrift des Verfassers

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