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(54) Title: METHODS AND KITS FOR PREDICTING OR ASSESSING THE SEVERITY OF INFECTIONS CAUSED BY STAPHYLOCOCCUS AUREUS

(57) Abstract: The invention relates to methods and kits for predicting or assessing the severity of infections caused by *Staphylococcus aureus*. *Staphylococcus aureus* is a commensal and a pathogen, and uncovering the identifying markers of the 'colonization to disease' transition is very useful. Several S. *aureus* small RNAs regulate virulence. The presence/absence and expression of eight sRNAs were investigated in 83 strains from 42 sepsis or shock patients, and 41 carriers. These isolates were characterized by MLST and *spa* typing and monitored for virulence and resistance. RNAIII expression was lower in strains from shock patients than from colonizing strains. Noteworthy, when RNAIII was associated with SprD, colonizing strains were significantly discriminated from those from patients with bloodstream infections, including sepsis and shock. Isolates associated with colonization may have different sRNA and sRNA target expressions than disease isolates. Monitoring RNAIII and SprD expressions could inform about infection severity. Thus, the invention relates to a method of assessing of predicting or assessing severity of an infection caused by *Staphylococcus aureus* comprising quantifying the RNAIII and/or SprD expression level in bacteria recovered from a culture obtained from the patient.

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METHODS AND KITS FOR PREDICTING OR ASSESSING THE SEVERITY OF INFECTIONS CAUSED BY STAPHYLOCOCCUS AUREUS

FIELD OF THE INVENTION:

The present invention relates to methods and kits for predicting or assessing the severity of infections caused by *Staphylococcus aureus*.

BACKGROUND OF THE INVENTION:

Staphylococcus aureus is one of the top three pathogens that cause community-acquired, healthcare-related nosocomial infections in humans. It lives as a commensal organism, but it can also infect the body at various sites. Diseases greatly differ, from skin lesions to invasive infections. Twenty to thirty percent of the healthy population is colonized with *S. aureus* in the nostrils (*1*), and a substantial percentage of *S. aureus* bacteremia originates from endogenous colonies from the nasal mucosa (*2*, *3*). The clinical expression of sepsis covers a continuum of manifestations, with the most violent form termed "septic shock." In this state, vascular offense and systemic inflammation lead to endangered cardiac function and blood pressure drops that cause impaired oxygen delivery, organ failure, and death. Sepsis-related mortality and the lack of mitigating clinical approaches attest to our limited understanding of the complex host–*S. aureus* interactions.

S. aureus features high transmissibility and elevated antibiotic resistance, and produces many virulence factors (*4*). To coordinate expression of its virulence genes during infection, *S. aureus* uses two-component systems, transcription factors (*5*), and regulatory RNAs (sRNAs) acting as either positive (*6*) or negative (*7*) virulence determinants. There are about 160 sRNAs compiled in the Staphylococcal Regulatory RNA (SRD) database (*8*). Although their functions are not well-explored, some sRNAs are known to regulate virulence factors. Quorum-sensing is mediated by the accessory gene regulator (agr), and RNAIII is the effector (*9*). Staphylococcal infection severity is based on host factors and bacterial pathogenesis (*10*).

SUMMARY OF THE INVENTION:

The present invention relates to methods and kits for predicting or assessing the severity of infections caused by *Staphylococcus aureus*. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

Staphylococcus aureus is a commensal and a pathogen, and uncovering the identifying markers of the 'colonization to disease' transition would be very useful. Several *S. aureus* small RNAs regulate virulence. The presence/absence and expression of eight sRNAs were investigated in 83 strains from 42 sepsis or shock patients, and 41 carriers. These isolates were characterized by MLST and *spa* typing and monitored for virulence and resistance. The *sprB* and *sprC* small RNAs are specific to clades. Six sRNAs had variable expression not correlated with patient clinical status. RNAIII expression, however, was lower in strains from shock patients than from colonizing strains. Noteworthy, when RNAIII was associated with SprD, colonizing strains were significantly discriminated from those from patients with bloodstream infections, including sepsis and shock. Isolates associated with colonization may have different sRNA and sRNA target expressions than disease isolates. Monitoring RNAIII and SprD expressions could inform about infection severity (32).

Accordingly the first object of the present invention relates to a method of assessing of predicting or assessing severity of an infection caused by *Staphylococcus aureus* comprising quantifying the RNAIII expression level in bacteria recovered from a culture obtained from the patient, comparing the expression level quantified at step i) with a predetermined reference value and iii) detecting differential in the expression level quantified at step i) and the predetermined reference value is indicative of the severity of the infection.

As used herein, a "patient" is an animal, preferably a mammal, more preferably a non-human primate, and most preferably a human. The terms "patient", "individual" and "patient" are used interchangeably herein.

The term "*Staphylococcus aureus*" or "*S. aureus*" is understood in the following way. Staphylococcus aureus bacteria are normally found on the skin or in the nose of people and animals. The bacteria are generally harmless, unless they enter the body through a cut or other wound. Typically, infections are minor skin problems in healthy people.

In some embodiments, the method of the present invention is particularly suitable for prediction or assessing a blood stream infection. As used herein, the term "bloodstream infection" refers to a disease wherein the infectious agent is present in the bloodstream of the patient.

The present invention is thus particularly suitable predicting whether a patient is at risk of having sepsis or septic shock. The term "sepsis" as used herein, means potentially life-threatening systemic infection that can arise from infections throughout the body, including

infections in the blood, lungs, abdomen, and urinary tract, etc. It may precede or coincide with infections of the bone (osteomyelitis), central nervous system (meningitis), or other tissues. Sepsis can rapidly lead to shock, adrenal collapse, and disseminated intravascular coagulopathy (a life threatening bleeding condition) and death. Sepsis can begin with spiking fevers and chills, rapid breathing and heart rate, the outward appearance of being seriously ill. These symptoms can rapidly progress to shock with decreased body temperature (hypothermia), decreased blood pressure, confusion or other changes in mental status, and blood-clotting abnormalities. The term "septic shock" as used herein is a consequence of sepsis in which the systemic inflammatory response leads to the failure of vital organs' function (for example of the lungs as in ARDS (acute respiratory distress syndrome)).

The term "culture" as used herein refers to any amount of sample obtained from the patient (e.g. a blood sample or a nostril sample) that has been mixed with culture media allowing growth of *Staphylococcus aureus*. Examples of culture media include Luria-Bertani media. In some embodiments, a blood culture is obtained when a patient has symptoms of a blood infection or bacteremia. Blood is drawn from a patient and put directly into a vessel containing the nutritional culture media.

In some embodiments, the method of the present invention comprises quantifying the SprD expression in the bacteria. In some embodiments, the ratio between RNAIII expression level and SprD expression level indicates the severity of the infection caused by *Staphylococcus aureus*.

As used herein, the term "RNAIII" has its general meaning in the art and refers to a gene of *Staphylococcus aureus* which is an archetype of RNA-mediated regulation of virulence gene. An exemplary nucleic acid sequence of RNAIII is shown by SEQ ID NO:1 or SEQ ID NO:2.

SEQ ID NO:1:

GGCCTAGATCACAGAGATGTGATGGAAAATAGTTGATGAGTTGTTTAATTT
TAAGAATTTTTATCTTAATTAAGGAAGGAGTGATTTCAATGGCACAAGATATCAT
TTCAACAATCGGTGACTTAGTAAAATGGATTATCGACACAGTGAACAAATTCACT
AAAAAATAAGATGAATAATTAATTACTTTCATTGTAAATTTGTTATCTTCGTATAG
TACTAAAAGTATGAGTTATTAAGCCATCCCAACTTAATAACCATGTAAAATTAGC
AAGTGAGTAACATTTGCTAGTAGAGTTAGTTTCCTTGGACTCAGTGCTATGTATTT
TTCTTAATTATCATTACAGATAATTATTTCTAGCATGTAAGCTATCGTAAACAACA
TCGATTTATCATTATTTGATAAATAAAATTTTTTTCATAATTAATAACATCCCCAA
AAATAGATTGAAAAAATAACTGTAAAACATTCCCTTAATAATAAGTATGGTCGTG
AGCCCCTCCCAAGCTCGCGGCCTTTTG

SEQ ID NO: 2:

GGCCUAGAUCACAGAGAUGUGAUGGAAAAUAGUUGAUGAGUUGUUUAA
UUUUAAGAAUUUUUAUCUUAAUUAAGGAAGGAGUGAUUUCAAUGGCACAAGA
UAUCAUUUCAACAAUCGGUGACUUAGUAAAAUGGAUUAUCGACACAGUGAACA
AAUUCACUAAAAAAUAAGAUGAAUAAUUAAUUACUUUCAUUGUAAAUUUGUU
AUCUUCGUAUAGUACUAAAAGUAUGAGUUAUUAAGCCAUCCCAACUUAAUAAC
CAUGUAAAAUUAGCAAGUGAGUAACAUUUGCUAGUAGAGUUAGUUUCCUUGG
ACUCAGUGCUAUGUAUUUUUCUUAAUUAUCAUUACAGAUAAUUAUUUCUAGC
AUGUAAGCUAUCGUAAACAACAUCGAUUUAUCAUUAUUUGAUAAAUAAAAUU
UUUUUCAUAAUUAAUAACAUCCCCAAAAAUAGAUUGAAAAAAUAACUGUAAA
ACAUUCCCUUAAUAAUAAGUAUGGUCGUGAGCCCCUCCCAAGCUCGCGGCCUU
UUG

As used herein the term "SprD" has its general meaning in the art and refers to another gene encoding for a regulatory RNA of *Staphylococcus aureus*. An exemplary nucleic acid sequence of SprD is shown by SEQ ID NO:3 or SEQ ID NO:4.

SEQ ID NO:3:

GGGCGTTTTCAAGGAGCGCCTTTCATTTTTTATGTATTGCTCCCCTTCGGGC
TAGTATATTAAATTTATTTTTGCGCTTTCCAAATCAATGTATATGTGTTATATTGTT
TATGGGAAGTAGGTAAGCATTTCGGTGCTTACCTTT

SEQ ID NO:4:

GGGCGUUUUCAAGGAGCGCCUUUCAUUUUUUAUGUAUUGCUCCCCUUCG
GGCUAGUAUAUUAAAUUUAUUUUUGCGCUUUCCAAAUCAAUGUAUAUGUGUU
AUAUUGUUUAUGGGAAGUAGGUAAGCAUUUCGGUGCUUACCUUU

Typically the RNAIII or SprD expression level is determined by RT-PCR.

Nucleic acids may be extracted from a sample by routine techniques such as those described in Diagnostic Molecular Microbiology: Principles and Applications (Persing et al. (eds), 1993, American Society for Microbiology, Washington D.C.). U.S. Pat. Nos. 4,683,202, 4,683,195, 4,800,159, and 4,965,188 disclose conventional PCR techniques. PCR typically employs two oligonucleotide primers that bind to a selected target nucleic acid sequence. Typically, the cDNA sample is prepared as follows. mRNA contained in the tumor tissue sample is extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. Then cDNA synthesis is performed according to standard methods involving reverse transcriptase. In some embodiments, random hexamer primers (instead of gene specific

primers) are used for the cDNA synthesis. Random hexamers primers are well known in the art and are typically commercially available from FISCHER.

Primers useful in the present invention include oligonucleotides capable of acting as a point of initiation of nucleic acid synthesis within the target nucleic acid sequence. A primer can be purified from a restriction digest by conventional methods, or it can be produced synthetically. If the template nucleic acid is double-stranded (e.g. DNA), it is necessary to separate the two strands before it can be used as a template in PCR. Strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One method of separating the nucleic acid strands involves heating the nucleic acid until it is predominately denatured (e.g., greater than 50%, 60%, 70%, 80%, 90% or 95% denatured). The heating conditions necessary for denaturing template nucleic acid will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the nucleic acids being denatured, but typically range from about 90° C. to about 105° C. for a time depending on features of the reaction such as temperature and the nucleic acid length. Denaturation is typically performed for about 30 sec to 4 min (e.g., 1 min to 2 min 30 sec, or 1.5 min). If the double-stranded template nucleic acid is denatured by heat, the reaction mixture is allowed to cool to a temperature that promotes annealing of each primer to its target sequence on the target nucleic acid sequence. The temperature for annealing is usually from about 35° C. to about 65° C. (e.g., about 40° C. to about 60° C.; about 45° C. to about 50° C.). Annealing times can be from about 10 sec to about 1 min (e.g., about 20 sec to about 50 sec; about 30 sec to about 40 sec). The reaction mixture is then adjusted to a temperature at which the activity of the polymerase is promoted or optimized, i.e., a temperature sufficient for extension to occur from the annealed primer to generate products complementary to the template nucleic acid. The temperature should be sufficient to synthesize an extension product from each primer that is annealed to a nucleic acid template, but should not be so high as to denature an extension product from its complementary template (e.g., the temperature for extension generally ranges from about 40° C. to about 80° C. (e.g., about 50° C. to about 70° C.; about 60° C.). Extension times can be from about 10 sec to about 5 min (e.g., about 30 sec to about 4 min; about 1 min to about 3 min; about 1 min 30 sec to about 2 min).

Typically the primers are as follows:

SprD PCR-Q AS	TATTGCTCCTTTTCGGGCTA (SEQ ID NO :5)
SprD PCR-Q S	ATTGATTTGGAAAGCGCAAA (SEQ ID NO :6)
RNAIII PCR-Q AS	GAAGGAGTGATTTCAATGGCACAAGATAT (SEQ ID NO:7)

RNAIII PCR-Q S	GAATTTTGTTCACTGTGTCGATAATCCATTT (SEQ ID NO:8)

One or more of the nucleotides of the primer can be modified for instance by addition of a methyl group, a biotin or digoxigenin moiety, a fluorescent tag or by using radioactive nucleotides. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5′ end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Primers are typically labelled with a detectable molecule or substance, such as a fluorescent molecule, a radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal. The term "labelled" is intended to encompass direct labelling of the probe and primers by coupling (i.e., physically linking) a detectable substance as well as indirect labeling by reactivity with another reagent that is directly labeled. Examples of detectable substances include but are not limited to radioactive agents or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)).

PCR involves use of a thermostable polymerase. The term “thermostable polymerase” refers to a polymerase enzyme that is heat stable, i.e., the enzyme catalyzes the formation of primer extension products complementary to a template and does not irreversibly denature when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded template nucleic acids. Generally, the synthesis is initiated at the 3′ end of each primer and proceeds in the 5′′ to 3′ direction along the template strand. Thermostable polymerases have been isolated from *Thermus fiavus, T. ruber, T. thermophilus, T. aquaticus, T. lacteus, T. rubens, Bacillus stearothermophilus, and Methanothermus fervidus*. Nonetheless, polymerases that are not thermostable also can be employed in PCR assays provided the enzyme is replenished. Typically, the polymerase is a Taq polymerase (i.e. *Thermus aquaticus polymerase*).

The primers are combined with PCR reagents under reaction conditions that induce primer extension. Typically, chain extension reactions generally include 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 15 mM MgCl2, 0.001% (w/v) gelatin, 0.5-1.0 μg denatured template DNA, 50 pmoles of each oligonucleotide primer, 2.5 U of Taq polymerase, and 10% DMSO). The reactions usually contain 150 to 320 μM each of dATP, dCTP, dTTP, dGTP, or one or more analogs thereof.

The newly synthesized strands form a double-stranded molecule that can be used in the succeeding steps of the reaction. The steps of strand separation, annealing, and elongation can

be repeated as often as needed to produce the desired quantity of amplification products corresponding to the target nucleic acid sequence molecule. The limiting factors in the reaction are the amounts of primers, thermostable enzyme, and nucleoside triphosphates present in the reaction. The cycling steps (i.e., denaturation, annealing, and extension) are preferably repeated at least once. For use in detection, the number of cycling steps will depend, e.g., on the nature of the sample. If the sample is a complex mixture of nucleic acids, more cycling steps will be required to amplify the target sequence sufficient for detection. Generally, the cycling steps are repeated at least about 20 times, but may be repeated as many as 40, 60, or even 100 times.

Quantitative PCR is typically carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by the excited fluorophore. The thermal cycler is also able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and thermal polymerase.

In order to detect and measure the amount of amplicon (i.e. amplified target nucleic acid sequence) in the sample, a measurable signal has to be generated, which is proportional to the amount of amplified product. All current detection systems use fluorescent technologies. Some of them are non-specific techniques, and consequently only allow the detection of one target at a time. Alternatively, specific detection chemistries can distinguish between non- specific amplification and target amplification. These specific techniques can be used to multiplex the assay, i.e. detecting several different targets in the same assay.

The majority of the thermocyclers on the market now offer similar characteristics. Typically, thermocyclers involve a format of glass capillaries, plastics tubes, 96-well plates or 384-wells plates. The thermocylcer also involve a software analysis.

In some embodiments, the predetermined reference value is a threshold value or a cut-off value. Typically, a "threshold value" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. For example, retrospective measurement of RNAIII expression level in properly banked historical patient samples may be used in establishing the predetermined reference value. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the

RNAIII expression in a group of reference, one can use algorithmic analysis for the statistic treatment of the measured expression levels of the gene(s) in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the area under the curve (AUC), the higher the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When AUC>0.5, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.5 and 0.7, the accuracy is low. When AUC is between 0.7 and 0.9, the accuracy is moderate. When AUC is higher than 0.9, the accuracy is quite high. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

In particular, the lower is the RNAIII expression level, the higher is the severity of the infection. In particular, the lower is the ratio between the RNAIII expression level and the SprD expression, the higher is the severity of the infection.

Once the patient is at risk of having sepsis or septic shock, an antibiotic treatment may be administered. Representative antibiotics that may be useful in the present invention include penicillinase-resistant penicillins, cephalosporins and carbapenems. In some embodiments, the antibiotic is selected from the group consisting of aminoglycosides, beta lactams, quinolones or fluoroquinolones, macrolides, sulfonamides, sulfamethaxozoles, tetracyclines, streptogramins, oxazolidinones (such as linezolid), rifamycins, glycopeptides, polymixins, lipo-peptide antibiotics. Typically beta lactames include 2-(3-alanyl)clavam, 2-hydroxymethylclavam, 7-methoxycephalosporin, epi-thienamycin, acetyl-thienamycin, amoxicillin, apalcillin, aspoxicillin, azidocillin, azlocillin, aztreonam, bacampicillin, blapenem,

carbenicillin, carfecillin, carindacillin, carpetimycin A and B, cefacetril, cefaclor, cefadroxil, cefalexin, cefaloglycin, cefaloridine, cefalotin, cefamandole, cefapirin, cefatrizine, cefazedone, cefazolin, cefbuperazone, cefcapene, cefdinir, cefditoren, cefepime, cefetamet, cefixime, cefinenoxime, cefinetazole, cefminox, cefmolexin, cefodizime, cefonicid, cefoperazone, ceforamide, cefoselis, cefotaxime, cefotetan, cefotiam, cefoxitin, cefozopran, cefpiramide, cefpirome, cefpodoxime, cefprozil, cefquinome, cefradine, cefroxadine, cefsulodin, ceftazidime, cefteram, ceftezole, ceftibuten, ceftizoxime, ceftriaxone, cefuroxime, cephalosporin C, cephamycin A, cephamycin C, cephalothin, chitinovorin A, chitinovorin B, chitinovorin C, ciclacillin, clometocillin, cloxacillin, cycloserine, deoxy pluracidomycin B and C, dicloxacillin, dihydro pluracidomycin C, epicillin, epithienamycin D, E, and F, ertapenem, faropenem, flomoxef, flucloxacillin, hetacillin, imipenem, lenampicillin, loracarbef, mecillinam, meropenem, metampicillin, meticillin (also referred to as methicillin), mezlocillin, moxalactam, nafcillin, northienamycin, oxacillin, panipenem, penamecillin, penicillin G, N, and V, phenethicillin, piperacillin, povampicillin, pivcefalexin, povmecillinam, pivmecillinam, pluracidomycin B, C, and D, propicillin, sarmoxicillin, sulbactam, sultamicillin, talampicillin, temocillin, terconazole, thienamycin, andticarcillin. Typically, quinolones include nalidixic acid, cinoxacin, oxolinic acid, flumequine, pipemidic acid, rosoxacin, norfloxacin, lomefloxacin, ofloxacin, enrofloxacin, ciprofloxacin, enoxacin, amifloxacin, fleroxacin, gatifloxacin, gemifloxacin, clinafloxacin, sitafloxacin, pefloxacin, rufloxacin, sparfloxacin, temafloxacin, tosufloxacin, grepafloxacin, levofloxacin, moxifloxacin, and trovafloxacin. Dosages of these antibiotics are well known in the art. See, e.g., MERCK MANUAL OF DIAGNOSIS AND THERAPY, Section 13, Ch. 157, 100th Ed. (Beers & Berkow, eds., 2004).

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: RNAIII and an 'RNAIII-SprD' combination discriminate the colonizing strains from patients with BSI. Monitoring of RNAIII expression by qPCR at the early exponential growth phase in 61 strains (Table 2, asterisks). The RNAIII expression levels were divided into two sets (carrier *versus* infectious; *A*) or three sets (carrier, sepsis, and shock; *B*). Differing RNAIII expression levels detected in colonized and infected individuals were statistically significant (*A*) ($p = 0.035$ using the Mann-Whitney U test), especially among the strains isolated from colonized individuals and from infected shock patients (*B*) ($p = 0.017$ using the Mann-Whitney U test). Combining the expression of RNAIII and SprD allow

discriminating the carrier versus infectious (*C*) at a *p* value of 0.0065, using the Mann-Whitney U test (The data are considered highly significant for *p* values ≤0.01, **), and also distinguishing carriage from sepsis (*p* value of 0.018) and carriage from shock (*p* value of 0.025). Inset: ROC analysis showing the discrimination of carriage from infectious strains when using RNAIII and SprD. For each data set, medians are represented as horizontal bars. Using the comparative Ct method, the amount of RNAIII was normalized against tmRNA expression and referred to the methicillin-susceptible *S. aureus* colonization control strain L102. The data presented by each point are the means of three independent experiments.

Figure 2: Sbi expression levels in Staphylococcus aureus strains isolated from bloodstream infections and asymptomatic carriers. (A) Sbi protein expression levels in 61 strains monitored during the early exponential growth phase and isolated by Western blots from carriers, patients with sepsis, and patients with shock. The protein sample from strain 19 was loaded on each gel and used as an internal control to prevent intensity variations of the bands between each experiment. The Sbi protein levels were divided into two sets (carrier and infectious strains; B) (p value of 0.057, Mann-Whitney U test) or into three (asymptomatic carriers, sepsis, and shock; C) (p value of 0.04, Mann-Whitney U test). D) SaeR, a positive regulator of the Sbi protein, was monitored by Western blots (p value of 0.04, Mann-Whitney U test). These experiments were performed in triplicate. For each set of data, medians are indicated with horizontal bars. To exclude loading variations between samples, the values were normalized against total protein levels.

EXAMPLE:

Material & Methods:

Staphylococcus aureus isolates and sample collection

Clinical isolates were obtained from a prospective study of all patients diagnosed with *S. aureus* bloodstream infection (BSI) in 2006 at the Rennes University Hospital, a tertiary referral hospital in Western France. We selected patients with non-severe sepsis or septic shock (*11*). Patients with severe sepsis (sepsis with organ dysfunction or tissue hypo perfusion improving after fluid therapy and not requiring vasopressors) were not included, because their clinical status might too closely resemble non-severe sepsis or shock. To prevent other confounding factors, immunodeficient patients were excluded: those with HIV, congenital immunodeficiency, malignant hemopathy, organ or stem cell transplant recipients, and anybody under systemic corticosteroid therapy for over three weeks or undergoing another immunosuppressive treatment). Data were extracted from medical records. Nosocomial BSI was defined as either BSI diagnosed in a patient hospitalized for more than 48 hours before

symptom onset, or as BSI in a patient on chronic hemodialysis or peritoneal dialysis. For each patient, the Charlson's co-morbidity index and the Simplified Acute Physiology Score (SAPS II) at admission were calculated (*12*). Also collected were 41 isolates from asymptomatic carriers, including 23 Rennes medical students, 7 healthcare workers sampled during their medical visit at a hospital in Lausanne, Switzerland, and 11 isolates from the National Reference Laboratory for Staphylococci in Lyon, France. The study was validated by the Rennes University Hospital's review board.

Multilocus sequence typing (MLST) and spa typing

spa typing was performed with spa-1113f and spa-1514r. The sequences were determined with a BigDye Terminator v3.1 cycle sequencing kit and a 3730xl DNA analyzer. The *spa* repeats and types were determined using BioNumerics and Ridom *Spa* Server. *spa* types with similar profiles were grouped within similar lineages. MLST was performed according to (*13*). The PCR products were sequenced using a 3730xl DNA analyzer, and the sequence types (STs) were determined using BioNumerics and the MLST database. MecA1 and MecA2 primers were used to amplify a 1102-bp gene fragment to check for the presence of *mecA*. Isolates were screened for *tst* and *pvl* by real-time PCR. PCR was used to detect *sprA1/2, sprB, sprC, sprD, sprX, ssrA, 6srna,* and *rsaE*. All PCR products were analyzed by 2% agarose gel electrophoresis.

Bacterial cultures, RNA isolation, and expression analysis

S. aureus strains were grown in Luria-Bertani media then harvested. The cells were pelleted and dissolved into 33mM sodium acetate, 17mM SDS, 1mM EDTA at pH5.5 together with glass beads and included in a Fast Prep apparatus. RNA extractions were performed by water-saturated phenol pH5. RNAs were precipitated and ethanol washed. Northern blots were done by loading 10µg of total RNA onto 8M urea 8% PAGE gels. The gels were blotted onto nylon membranes at 30V for 1.5h using 0.5x Tris-HCl borate and EDTA. RNA markers were used. Prehybridization and hybridization were performed in 'ExpressHyb' using $^{5'\text{-}\gamma 32P}$DNAs. Signals were detected with a phosphorimager and quantified. *sRNA* expression levels in the strains were monitored by quantitative PCR. cDNAs were produced using a High-Capacity cDNA Reverse Transcription Kit. Using the comparative CT method, the *sRNA count* was normalized against both transfer-messenger RNA *and the L102 reference strain.*

Bacterial protein extracts and Western blots

To prepare the protein extracts, bacteria were grown until the indicated OD at A_{600nm}. The cells were pelleted for 10min at 4°C (8000g) and suspended into a lysis buffer (10mM Tris-HCl pH 7.5, 20mM NaCl, 1mM EDTA, and 5mM $MgCl_2$) in the presence of a protease inhibitor

cocktail tablet containing 0.1 mg/mL lysostaphin. Each pellet was dissolved in 1x Laemmli with 10% ß-mercaptoethanol and heated at 90° C for 5 min. Samples were separated onto 8% SDS–PAGE gels and transferred to polyvinylidene fluoride membranes at 100V for 1h. Membranes were blocked in a tris-buffered saline (TBS) containing 5% milk. The Sbi protein was visualized using anti-Sbi antibodies, as previously described (*14*), and the SaeR protein by anti-SaeR antibodies (gift from Pr T. Bae, Indiana Univ Northwest, USA). Incubation with primary anti-Sbi antibodies (diluted 1:10000) or anti-SaeR (diluted 1/5000) was performed at room temperature for 2h. After incubation for 1h with the anti-rabbit IgG peroxidase-conjugated secondary antibodies, the blots were washed in TBS 0.05% Tween. They were developed in ECL Western blotting detection reagent and exposed on an ImageQuant LAS4000. Quantifications were performed with ImageQuant. Sbi protein or SaeR protein amounts were normalized against the total proteins. All statistical tests and graphical representations were done using GraphPad Prism software. Quantitative values were compared using the Mann-Whitney U test. A p value<0.05 was considered significant (*).

Results:

Characteristics of patients with S. aureus BSI

Forty-two patients with septic shock (n=17) and non-severe sepsis (n=25) were included in this study, and their clinical characteristics are described in Table 1. When compared to patients with septic shock, patients with non-severe sepsis were more likely to suffer from nosocomial BSI (p = 0.02), with a lower SAPS-II score (p=0.01), and a lower mortality (9% vs. 41.2%). Mortality was also significantly higher in patients with septic shock than in patients with non-severe sepsis: 41.2% of septic shock patients died as compared to 8% of non-severe sepsis ones (p=0.01). The clonal distribution of isolates was similar to that reported for France by the European Antimicrobial Resistance Surveillance System (*15*).

Genotyping of strains originating from invasive diseases and from asymptomatic carriers

We used MLST and spa typing to analyze 83 *S. aureus* isolates from blood cultures in BSI patients (n=42) or nasal samples from asymptomatic carriers (n=41). Our strain collection was obtained from healthcare professionals and medical students. Isolates clustered into 17 sequence types (STs), and these are shown on a phylogenetic tree. Of the 83 strains examined, none possessed genes encoding the Panton-Valentine leucocidin, associated with increased virulence of certain strains. Toxic shock syndrome toxin genes were detected in infectious and methicillin-susceptible *S. aureus* (MSSA) colonization strains belonging to ST5 and ST30. Among the 40 MSSA colonization strains, ST398 was the most common (n=7). Only 4 strains

isolated from nasal carriers were methicillin-resistant *S. aureus* (MRSA) strains, and these belonged to ST8 and ST22. Most MRSA isolates were from ST8. Within our isolates, MRSA prevalence in healthy colonized healthcare workers and students was about 10%, compared to about 2% in the general population (*16*). The prevalence of MRSA in the infectious samples (~21%), all were positive for *mecA*, was in-range for overall staphylococcal infections in France (*17*). We detected a predominance of the ST8 MRSA clone, which is the major French pandemic MRSA clone possessing *sea* and *lukED*. As reported (*18*), isolates from both BSI patients and carriers were evenly distributed among the STs. The genetic distances between the eight Group 1 STs (25.2±10.6) were stretched further than those between the nine Group 2 STs (20±7.4), which is in agreement with the earlier emergence of Group 1.

Phylogenetic studies show that selected srna genes are specific to some clades

We used PCR to monitor the presence of a subset of *srnas*, targeting conserved sequences. Nine sRNAs were selected from the core and accessory genomes for examination of their distribution among the strains. They were chosen according to their presence in the accessory genome, since this implies variability in their presence/absence among the strains and their putative roles in virulence. We included the very few sRNAs ubiquitously detected in bacteria. Housekeeping *tmrna* and *6Srna*, both detected in many bacterial species, were uncovered in all strains. All strains also contained *rnaIII*, which is the quorum-sensing effector (*9*). In addition, we uncovered five *srnas* expressed from pathogenicity islands (PIs): *sprA (srn_3580), sprB* (*srn_3600*), *sprC* (*srn_3610*), *sprD* (*srn_3800*), and *sprX* (*srn_3820*) (*8*). Due to the absence of the PIs *phiSa3* and *vsaβ,* the five PI sRNAs were all detected in Group 2 STs, with none in Group 1. *sprA* was mostly absent in ST398 strains. *sprB* was lacking in all Group 1 strains and STs, as was *sprC* except for its systematic presence in ST398. *sprD* was detected in all but five strains from both groups, while *sprX* was detected in all strains except ST398. The fact that *sprD* and *sprX* were detected in most STs from both groups reflects the evolution of *S. aureus,* which has been punctuated by successive acquisitions and losses of genetic elements. Whereas *sprD* and *sprX* expression is meaningless, the presence of *sprB* and *sprC* among *S. aureus* infectious isolates illustrates *S. aureus* phylogeny, and indicates strain clonality. Strain genotyping showed that the sample reflected the diversity of staphylococcal infections at French national level.

PI-encoded RNA expression differs between isolates obtained from BSI and from asymptomatic carriers

Due to the low amounts (10-100 CFU/ml of blood) of bacteria recovered in patients with BSI (*19*). *S. aureus* isolates must be cultured before assessing sRNA expression. We selected

16 strains for subsequent analyses: 5 from nasal carriers, 6 from patients with sepsis, and 5 from patients with shock. Each sample contained strains from the same sequence types (ST5, ST8, and 25). We intentionally included strains belonging to the same ST (ST8), with strains from carriers, from sepsis and from shock patients. Also, strain selection was dictated by their availability in our collection. In these 16 isolates, sRNA expression levels were assessed at OD_{600nm}=2 (early exponential), OD_{600nm}=4 (late exponential) and OD_{600nm}=8 (stationary) growth phases. The growth curves of all isolates are superimposable. The overall sRNA expression levels were compared among the infectious subgroups. In all strains, tmRNA was constitutively expressed, with no difference in expression among the strains. This is consistent with tmRNA's status as a housekeeping gene involved in ribosome rescue (*20*). 6SRNA was also constitutively expressed, with no differences in expression among the strains (not shown). The expression of the five Spr RNAs varies widely among the strains. sRNA even presented different expression profiles within the same ST, illustrating the complexity and variability of sRNA-driven gene regulation in *S. aureus*. SprD expression is heterogeneous in the asymptomatic carriers, but more homogeneous in infected patients. These results were inferred from Northern blots performed on three independent RNA extractions. Afterwards, the set of analyzed strains was nearly quadrupled to 61 isolates, with 21 from carriers, 23 from non-severe sepsis patients, and 17 from shock patients. Since Northern blots showed variations in SprD expression levels between the clinical sets, SprD expression was monitored by qPCR at OD_{600nm}=2. Strains from asymptomatic carriers and sepsis patients expressed SprD heterogeneously, although SprD was expressed at low levels in all strains isolated from patients with septic shock.

RNAIII and RNAIII/SprD expression levels discriminates the asymptomatic from the BSI isolates

Another RNA implicated in *S. aureus* virulence is RNAIII (*9*), an archetype of RNA-mediated regulation of virulence genes. We therefore used qPCR to monitor RNAIII expression levels in the 61 isolates (Figure 1) during E growth phase. Significantly lower RNAIII levels were detected in strains isolated from BSI as compared to those from nasal carriers (p=0.035; Figure 1A). When comparing with commensal isolates, strains isolated from patients with shock displayed significantly lower RNAIII levels (p=0.017; Figure 1B). Average calculated RNAIII expression levels in infectious and asymptomatic individuals revealed a progressive decline, decreasing from carriage to non-severe sepsis to shock. Combining SprD with RNAIII substantially discriminate carriage from infections isolates (p=0.0065; Figure 1C), as well as carriage from sepsis (p=0.018) and carriage from shock (p=0.025; Figure 1D). Receiver

operating characteristic (ROC) analyses were conducted to challenge the capacity of RNAIII and SprD differential expression to predict disease outcome. They support differences in RNAIII/SprD expression levels between colonization and infection (Figure 1C, inset).

Sbi immune evasion protein expression levels distinguished isolates obtained from asymptomatic carriers and from BSI

SprD and RNAIII negatively regulate the expression of the Sbi immune evasion molecule by blocking translation through pairings with the *sbi* mRNA, having a common target (*6, 17*). We did Western blots during the E growth phase using polyclonal antibodies to both intracellular and membrane proteins to monitor Sbi protein levels within the 61 strain isolates (Figure 2A). As reported (*21*), the molecular weight of the Sbi proteins detected from the various isolates was variable, around 50kDs. The amount of Sbi proteins fluctuated among strains (Figure 2A), but individual assessments revealed significantly lower protein levels in isolates originating from BSI than in those from carriers (p=0.04 between carriage and sepsis).

Discussion:

A set of 83 *S. aureus* strains of known genotypes was collected from asymptomatic carriers and from patients with either non-severe sepsis or septic shock. We used this collection for a prospective study of the presence or absence and expression of certain sRNAs located within the core and accessory genome. We also monitored the expression of Sbi, an immune evasion protein whose expression is negatively controlled by the sRNAs SprD and RNAIII (*16*) and SaeR, a positive regulator of Sbi. In clinical and carriage staphylococcal strains, the presence or absence of at least two PI-encoded *srna*s, *sprB* (*srn_3600*) and *sprC* (*srn_3610*), was indicative of the presence/absence of PIs and prophages. These PI-encoded *srna*s, particularly *sprB*, could be used as probes to improve genotyping studies. These sRNAs probably appear during the transition between ST22 and ST25. *sprB* is mostly absent in the Group 1 isolates. In some strains, we cannot rule out the theory that sequence variations among the *srna* genes may hamper their amplification. Molecular typing uncovers the genetic diversity of the strains, required for epidemiological surveillance of infections. Bacterial strain typing methods include DNA banding pattern, sequencing, and hybridization-based technologies. In the genomic era, bacterial genotyping has benefited from the emergence of novel locus-specific typing markers. *srnas* may be convenient probes for genotyping bacteria. This is because their overall content deviates considerably even among closely-related strains. Furthermore, since several *srnas* are encoded within mobile genetic elements (MGE), they reflect the acquisition/loss of MGE-encoded virulence factors and molecules that confer antibiotic

resistance. To summarize, in phylogenetic studies, selected sRNAs located within accessory genomes may shed light on genomic diversity.

The ability of patients to eradicate pathogens is a major determinant of infection outcome, and unfortunately patients with shock are often immunocompromised (*22*). Our data suggests that for staphylococcal BSI, one must also consider the attributes of the invading strains, including at least an immune evasion molecule and sRNAs RNAIII and SprD. Interestingly, the effector of the *agr* quorum-sensing system was expressed at significantly lower levels in strains isolated from patients with BSI, especially those with shock, than in asymptomatic carriers. Therefore, low RNAIII levels might pinpoint the *S. aureus* isolates which are responsible for BSI, even after isolation and culture. A significant percentage of *S. aureus* BSI is caused by *agr*-defective isolates (*23*). Our observations concur with the previous identification of inactivating mutations in the *S. aureus agr* virulence regulator which have been associated with worse outcomes in BSI patients (*3*). Coupling the expression levels of RNAIII and SprD could discriminate colonization from infection and also inform about bloodstream infection severity, but this must be confirmed in a larger set of clinical isolates.

Since both SprD and RNAIII negatively control the expression of the Sbi immune evasion protein (*16*), we also monitored Sbi expression in the isolates. Sbi is an immune evasion factor (*24*) involved in the *S. aureus*-induced inflammatory response (*25*). Cell wall-anchored Sbi proteins act as essential components in *S. aureus* survival in the commensal state (*26*). The detection of more Sbi proteins in strains isolated from asymptomatic carriers than in those from septic patients is consistent with their importance during colonization and their role in immune tolerance. Sbi expression at the transcriptional level is positively regulated by SaeRS, made up of the histidin kinase SaeS and the response regulator SaeR (*27*). Sbi expression is controlled by at least three regulators: negatively by two sRNAs, and positively by a two-component system. This provides an explanation for why RNAIII and SprD levels are not inversely correlated to Sbi levels in the tested isolates. The carriage strains have higher SaeR levels than the sepsis strains, in agreement with the higher Sbi levels in the carriage versus the sepsis strains (Figure 2).

The transition from commensalism to infection in *S. aureus* is an essential but complex question. From a clinical standpoint, most *S. aureus* infections derive from previous colonizers (*28*). When those strains switch to invasiveness, the transition may be related to regulatory network expression changes, including within sRNAs. Sequencing revealed the changes in the regulatory functions of strains recovered from an individual who started as a carrier then progressed to fatal BSI (*29*), suggesting that molecular evolution may be key in this process.

Our results suggest that certain sRNAs from the gene regulatory network in a human pathogen will provide insights into commensal-to-pathogen transitions. They could be used as surrogate markers for the severity of staphylococcal infections, and as biomarkers for prophylaxis and monitoring of *S. aureus* infection. Comparing the frequency and expression of selected sRNAs in isolates which express, show colonization, or show *S. aureus* infection may be a way to uncover associations between sRNA expression and disease patterns.

In vitro expression levels of some *S. aureus* sRNAs may not reflect their *in vivo* levels (*30*). Nevertheless, direct analysis of the expression levels of the bacterial sRNAs directly in BSI patients' blood is technically difficult due to the low bacterial levels collected. We compared sRNA expression from patient's fresh specimens *versus* the same isolates after being thawed from the freezer (maintained three weeks). There are no differences in RNAIII and SprD expression between the samples recovered directly from the patient *versus* those that were freeze. Subsequent investigations will address the functional and clinical relevance of RNAIII's and SprD's expression patterns. Broadening our pioneering investigations to include additional sRNAs may identify biomarkers that predict staphylococcal disease severity in infected patients. In addition to their roles as biomarkers, sRNAs could also be targets for innovative therapeutic approaches.

Table 1: Clinical characteristics of 42 patients (25 with sepsis and 17 with septic shock) admitted to the Rennes University Hospital (France) for *S. aureus* bloodstream infections. MRSA, methicillin-resistant *Staphylococcus aureus;* SAPS II, Simplified Acute Physiology Score; CRP, C Reactive Protein; PNN, polynuclear neutrophils .

Characteristics	Sepsis	Shock	*P values*
% Male	84	84	1.00
Age	62.7 [15-97]	68.1 [33-84]	0.30
% Nosocomial bacteremia	80	41.1	0.02
% MRSA	32	5.8	0.06
% Diabetes mellitus	16	29.4	0.45
% Alcohol abuse	12	35.3	0.12
Charlson score	1.4 [0-5]	2.1 [0-5]	0.06
% Endovascular device	52	29.4	0.21
SAPS II	43.2 [14-61]	60.9 [38-126]	0.01
Delayed antibiotherapy	4.6 [0-42]	3.1 [0-10]	0.42
% Infective endocarditis	12	17.6	0.70

CRP	186.4 [32-427]	250.5 [67-445]	0.17
PNN	14588 [4,700-33,000]	14977 [4,230-26,000]	0.84
% Mortality	8	41.2	0.01

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method of predicting or assessing severity of an infection caused by *Staphylococcus aureus* comprising quantifying the RNAIII expression level in bacteria recovered from a culture obtained from the patient, comparing the expression level quantified at step i) with a predetermined reference value and iii) detecting differential in the expression level quantified at step i) and the predetermined reference value is indicative of the severity of the infection.

2. The method of claim 1 for predicting or assessing severity of a bloodstream infection caused by *Staphylococcus aureus*.

3. The method of claim 1 for predicting whether a patient is at risk of having sepsis or septic shock.

4. The method of claim 1 wherein the RNAIII expression level is determined by RT-PCR.

5. The method of claim 1 which further comprises quantifying the SprD expression level in the blood culture obtained from the patient.

6. The method of claim 1 wherein when the patient is at risk of having sepsis or septic shock, an antibiotic treatment is administered to the patient.

7. A method of treating sepsis or septic shock in a patient at risk of having sepsis or septic shock in need thereof comprising the steps of: i) predicting whether the patient is at risk of having sepsis or septic shock by performing the method of claim 3, and ii) administering to said patient an antibiotic treatment.

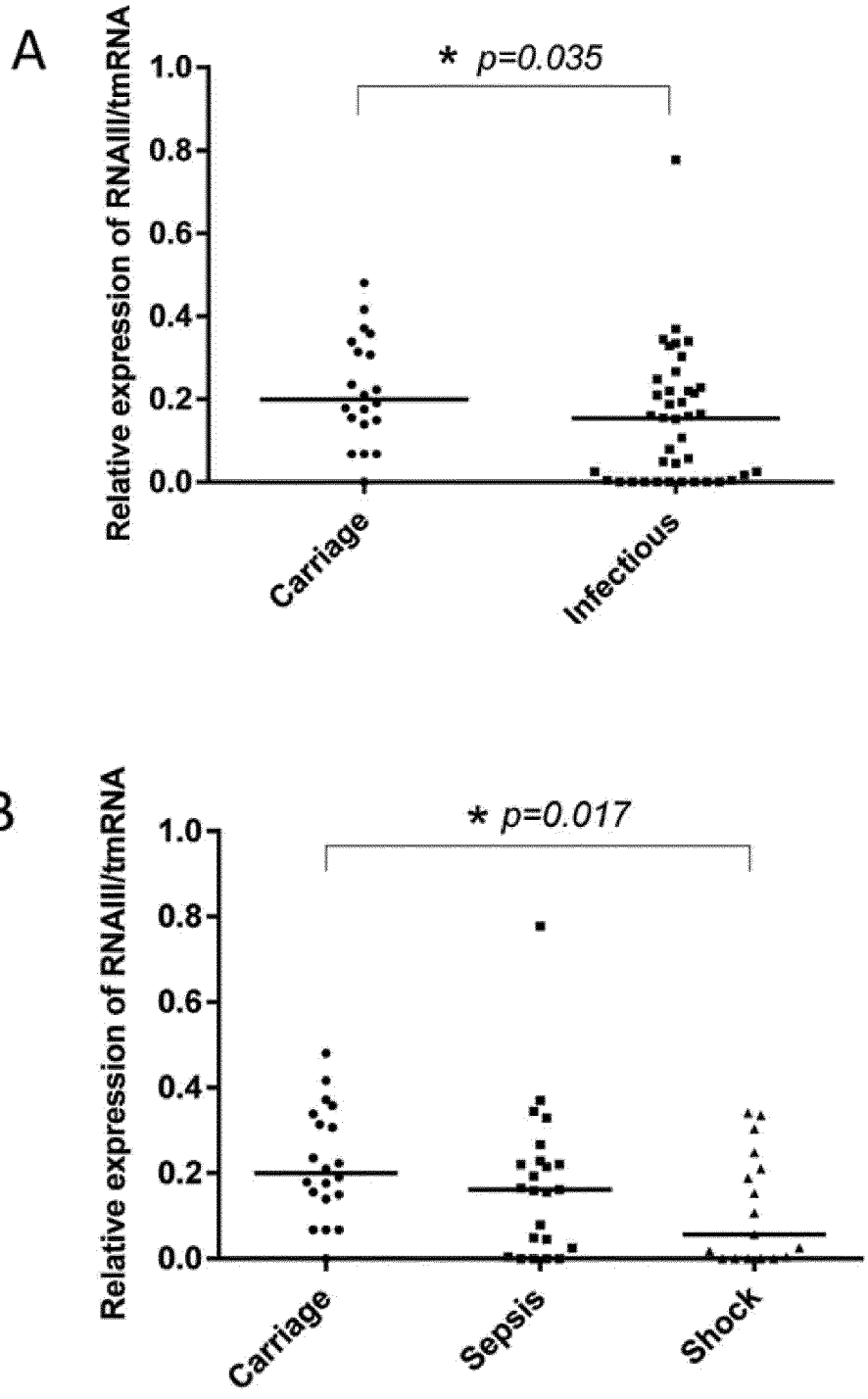


Figure 1 A and B

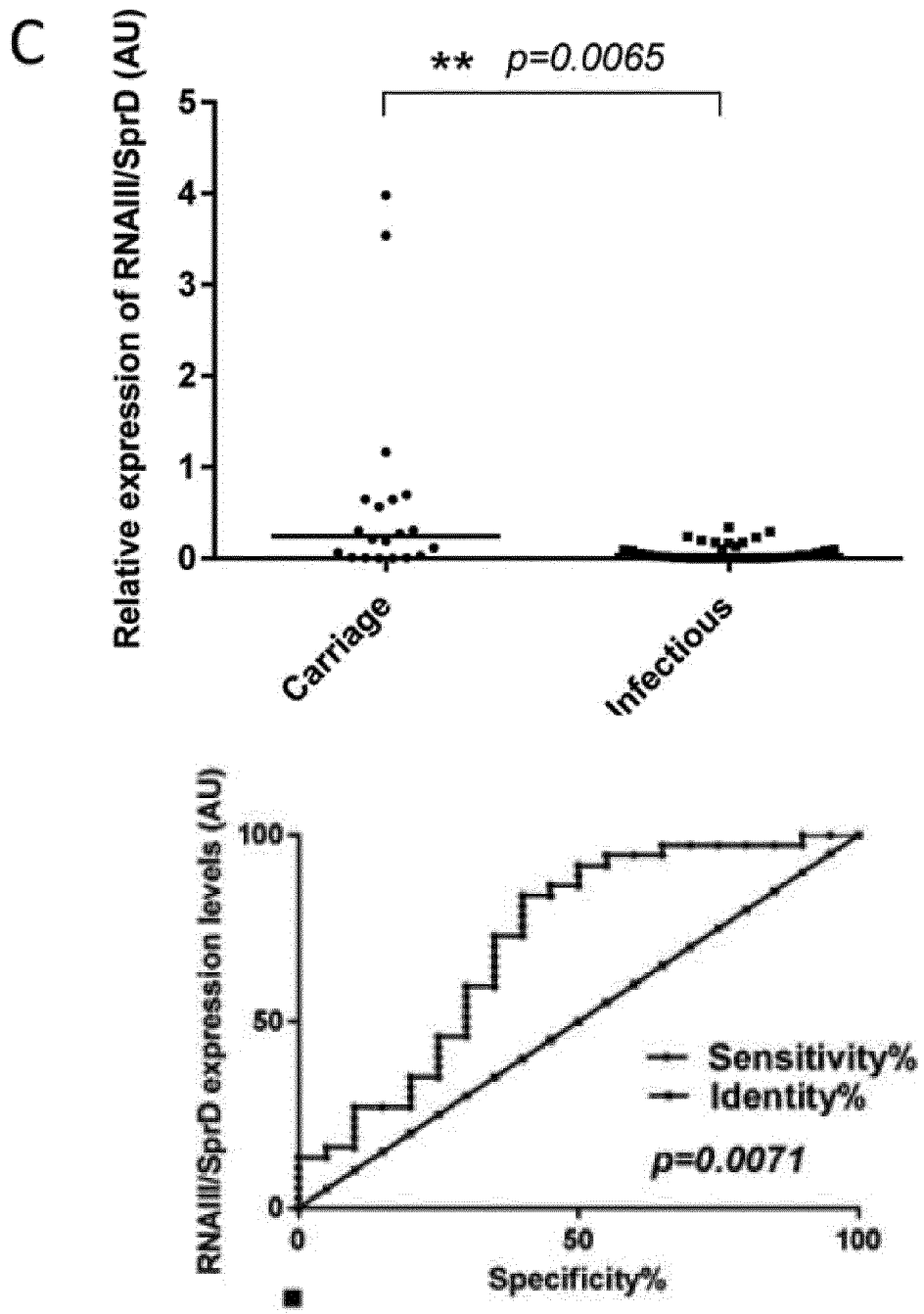

C
** p=0.0065
Relative expression of RNAIII/SprD (AU)
5
4
3
2
1
0
Carriage
Infectious
RNAIII/SprD expression levels (AU)
100
50
0
0
50
100
Specificity%
Sensitivity%
Identity%
p=0.0071

Figure 1C

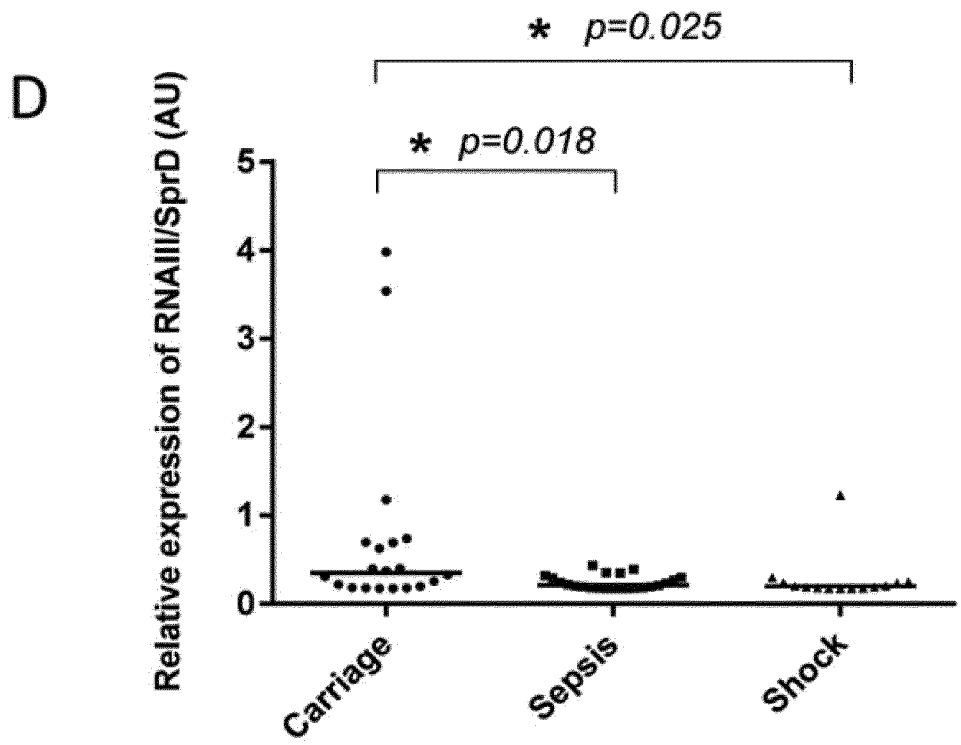
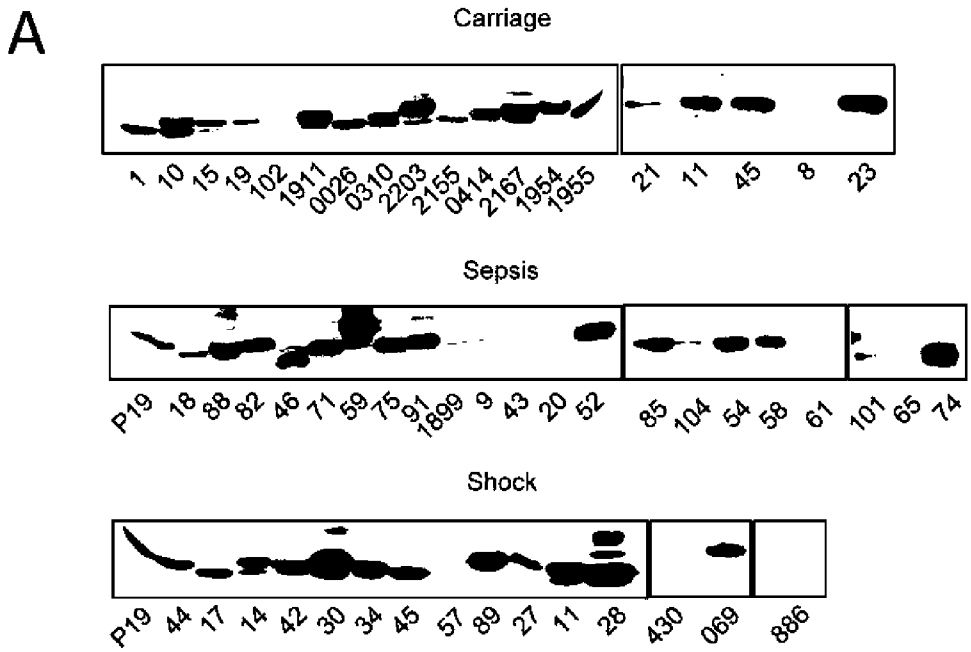

D
* p=0.025
* p=0.018
Relative expression of RNAIII/SprD (AU)
5
4
3
2
1
0
Carriage
Sepsis
Shock

Figure 1D



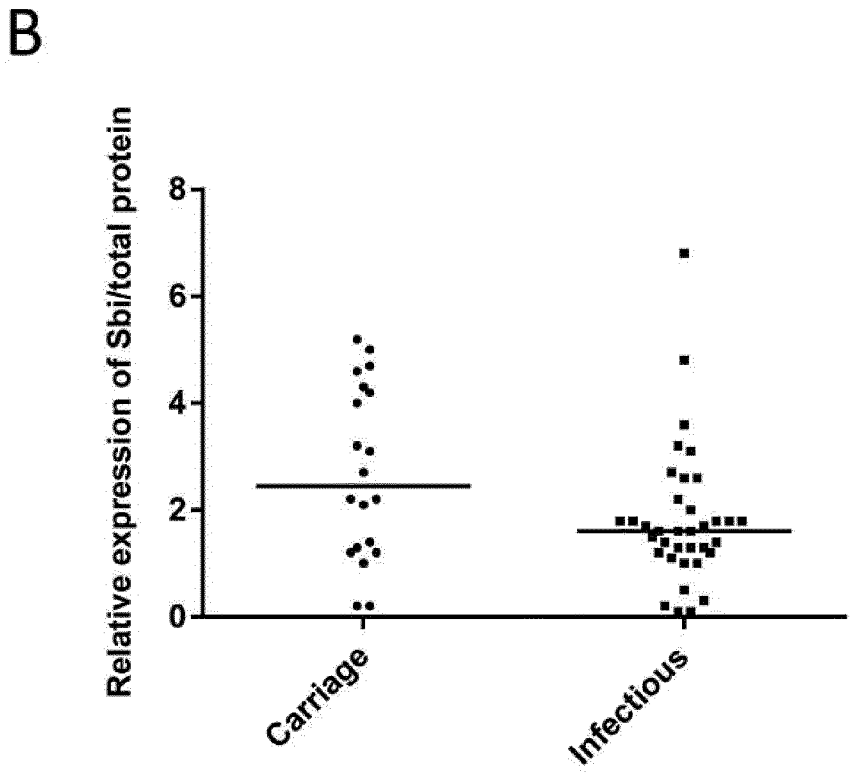


Figure 2 A and B

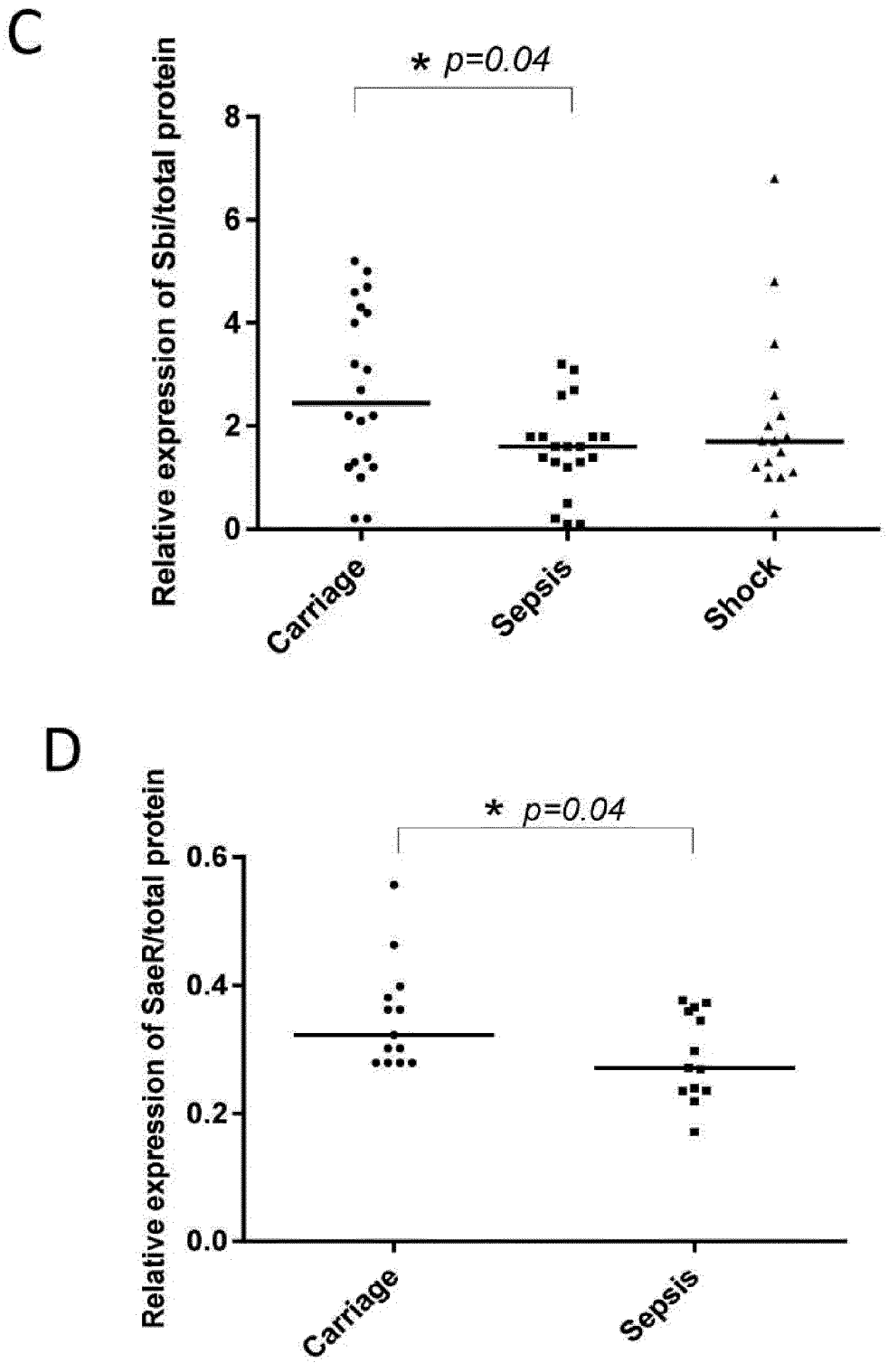


Figure 2 C and D

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/061458

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. M. YARWOOD ET AL: "Repression of the Staphylococcus aureus Accessory Gene Regulator in Serum and In Vivo", JOURNAL OF BACTERIOLOGY, vol. 184, no. 4, 15 February 2002 (2002-02-15), pages 1095-1101, XP055316109, US ISSN: 0021-9193, DOI: 10.1128/jb.184.4.1095-1101.2002	1-4,6,7
A	abstract; figures 1-4 page 1096, paragraph 1 page 1098, column 1 page 1100	5

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[X] Further documents are listed in the continuation of Box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
14 July 2017	25/07/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Tilkorn, A

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	S. CHABELSKAYA ET AL: "Dual RNA regulatory control of a Staphylococcus aureus virulence factor", NUCLEIC ACIDS RESEARCH, vol. 42, no. 8, 1 April 2014 (2014-04-01), pages 4847-4858, XP055315768, GB ISSN: 0305-1048, DOI: 10.1093/nar/gku119 the whole document	1-7
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A	PRAGMAN A A ET AL: "Virulence regulation in Staphylococcus aureus: the need for in vivo analysis of virulence factor regulation", FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY, ELSEVIER SCIENCE B.V., AMSTERDAM, NL, vol. 42, no. 2, 1 October 2004 (2004-10-01), pages 147-154, XP004562484, ISSN: 0928-8244, DOI: 10.1016/J.FEMSIM.2004.05.005 the whole document	1-7

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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	VALÉRIE BORDEAU ET AL: "Staphylococcus aureus Regulatory RNAs as Potential Biomarkers for Bloodstream Infections", EMERGING INFECTIOUS DISEASES, vol. 22, no. 9, 1 September 2016 (2016-09-01), pages 1570-1578, XP055315219, US ISSN: 1080-6040, DOI: 10.3201/eid2209.151801 the whole document -----	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/061458

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2016097099 A1	07-04-2016