

(51) International Patent Classification:
C12Q 1/68 (2006.01)(21) International Application Number:
PCT/EP2010/054570(22) International Filing Date:
7 April 2010 (07.04.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2009/0288 9 April 2009 (09.04.2009) IE(71) Applicant (for all designated States except US): **NATIONAL UNIVERSITY OF IRELAND, GALWAY** [IE/IE]; University Road, Galway (IE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **SMITH, Terry, James** [IE/IE]; 18 Hawthorn Place, Clybaun Road, Galway (IE). **MAHER, Majella** [IE/IE]; Iona, Gortachalla, Moycullen, Co. Galway (IE). **O GRADY, Justin** [IE/IE]; 8 Rockfield Park, Rahoon, Galway (IE). **BARRY, Thomas** [IE/IE]; Dunguaire East, Kinvara, Co. Galway (IE).(74) Agents: **GATES, Marie, Christina, Esther** et al.; Tomkins & Co., 5 Dartmouth Road, Dublin, 6 (IE).

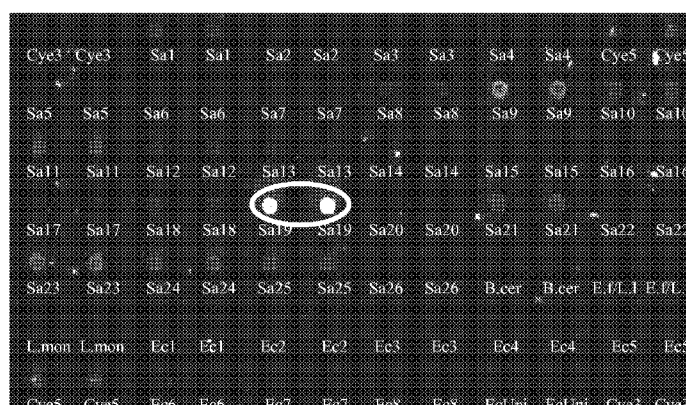
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

— with sequence listing part of description (Rule 5.2(a))

(54) Title: NUCLEIC ACID SEQUENCES BASED ON THE BACTERIAL *SSRA* GENE AND ITS TMRNA TRANSCRIPT FOR THE MOLECULAR DETECTION OF *S.AUREUS*

5 **Figure 1:** *S. aureus* cRNA microarray sandwich assay with Sa20 reporter probe (Sa19 capture probe circled) showing detection of *S. aureus* cRNA.

(57) Abstract: A diagnostic kit for a bacterial species comprising an oligonucleotide probe capable of binding to at least a portion of the *ssrA* gene or its corresponding tRNA. The kit may comprise a primer for amplification of at least a portion of the *ssrA* gene.

Title

Nucleic acid sequences based on the bacterial *ssrA* gene and its tmRNA transcript for the molecular detection of *S. aureus*.

Field of the Invention

5 The invention relates to the identification of *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus* species in mammals, in particular in humans for the diagnosis of infection, and in ruminants for the diagnosis of mastitis. In particular, the invention relates to nucleic acid primers and probes to detect *S. aureus* species. More specifically the invention relates to the *ssrA* gene sequences, the corresponding RNA, specific probes, primers and oligonucleotides
10 related thereto and their use in diagnostic assays to detect and/ or discriminate *S. aureus* and *Staphylococcus* species.

Background to the Invention

S. aureus is an important pathogen in dairy cattle causing mastitis, an inflammation of the mammary gland reducing milk yield and quality. The disease is significant and results in losses
15 of \$200 approximately per cow per case and billions of dollars worldwide. *S. aureus* is a leading cause of contagious mastitis, a form of mastitis that is easily spread between animals which can result in sub-clinical mastitis. Diagnosis of the microbial agent causing mastitis is important to allow effective treatment and control of the infection. Current identification of infecting agent is by a combination of culture and biochemical tests which can take up to 3 days and have variable
20 sensitivity.

Nucleic acid-based tests enable rapid and specific identification of microbial pathogens. PCR-based assays for the identification of pathogens in milk have been described. Cremonesi *et al.* (2006) developed PCR assays based on the 23s rRNA gene for gram positive bacterial causes of mastitis including *S. aureus*. They tested the performance of the PCR assays on 30 bovine and
25 caprine milk samples and achieved 100% correlation with the bacteriology results obtained for these samples. Recently, Lee *et al.* (2008) reported the development of a biochip involving PCR for the identification of 7 microbial causes of mastitis based on detection of RNA and/or other specific genes. All but one of 82 random samples tested correlated with the bacteriology results. Finnzymes Diagnostics recently released the “Pathproof[™] Mastitis PCR” assay which
30 identifies and quantifies important microbial causes of mastitis by real-time PCR.

In humans, *S. aureus* is a major cause of community acquired and nosocomial infections (Stamper *et al.*, 2003). *S. aureus* bacteremia increases the risk of patient mortality particularly when the infecting agent is methicillin resistant *S. aureus* (Wisplinghoff *et al.*, 2004). Nosocomial infections cost the US healthcare system between approximately \$4.5 billion and
35 \$5.7 billion annually. Infections due to MRSA are increasing as are the number of deaths arising

from infection by this pathogen. Surveillance for MRSA is recommended as part of clinical practice in Europe and the US (Verbrugh, 2005).

Traditionally testing for *S. aureus* is by microbiological culture which takes 24 hours while determination of antimicrobial susceptibility requires a further 24 hours. Nucleic acid based tests provide the potential for rapid identification of *S. aureus*. Nucleic acid tests can combine both species identification and determination of resistance to methicillin by including a genetic marker, *MecA* in addition to species-specific genetic marker in the nucleic acid test. Nucleic acid tests can be applied to patient screening, screening prior to admission to hospital and also for environmental monitoring. Rapid identification of MRSA enables the implementation of treatment and targeted control measures to reduce the spread of this pathogen.

A number of nucleic acid tests for MRSA have been described in which the *mecA* gene, a determinant of methicillin resistance is identified in combination with *S. aureus* species-specific genes. Zhang *et al.* (2004) developed a quadriplex PCR assay for discrimination of *S. aureus* from coagulase negative staphylococci (CoNS) based on the thermonuclease *nuc* gene and detection of methicillin and mupirocin resistance. They reported a sensitivity, specificity and accuracy of 100% for the tests applied to cultured *S. aureus*. Real-time PCR assays have been developed for the rapid and sensitive detection of MRSA (Paule *et al.*, 2005; Francois *et al.*, 2003). Hogg *et al.* (2008) developed a biplex real-time PCR assay to detect *S. aureus* and methicillin resistance based on the *nuc* and *mecA* genes respectively within 3 hours from positive blood cultures. Ornskov *et al.* (2008) developed a high-throughput real-time PCR method for screening swabs that were culture enriched overnight enabling colonised individuals to be identified within 24 hours. The analytical sensitivity of the test was 97% and they reported it to be a cost-effective method for screening for *S. aureus*. Deiman *et al.* (2008) recently described a modification of the NASBA method to include a restriction enzyme digestion step that increased the sensitivity of detection of *mecA*. A detection limit of <10 cell equivalents for *S. aureus* was achieved with this method. There are some commercial nucleic acid tests available for identification of MRSA. These include; EVIGENE MRSA detection kit (Statens Serum Institut, Copenhagen, Denmark) which detects and discriminates *S. aureus* and CoNS and identifies methicillin resistance based on hybridisation of probes specific for the *mecA*, *nuc* and 16sRNA genes. The IDI MRSA assay currently marketed as BD GeneOhm MRSA assay (Becton Dickinson) is a rapid PCR based test for identification of MRSA and *S. aureus* by targeting a unique *S. aureus* gene sequence and a region of the SCCmec cassette. The GeneXpert MRSA assay (Cepheid) is run on an automated real-time PCR platform that allows integrated sample preparation and real-time PCR with a result obtained in approximately 1 hour. This invention describes nucleic acid sequences based on the bacterial *ssrA* gene and its tmRNA transcript that can be applied in nucleic acid tests for the specific identification of *S. aureus*. In

particular, the nucleic acid sequences described may be suitable for detection of *S. aureus* in a direct probe hybridisation assay or in *in-vitro* amplification based assays for example real-time PCR or real-time nucleic sequence based amplification (NASBA). NASBA is an isothermal *in vitro* amplification method that uses RNA for its amplification template. The nucleic acid sequences described here for *S. aureus* could be combined with nucleic acid sequences for methicillin resistance in a nucleic acid test to detect MRSA.

One advantage of using sequences based on the *ssrA* gene is that as it is universally present in eubacteria, nucleic acid sequences (primers and probes) for other clinically relevant staphylococci based on the *ssrA* gene can be combined with the sequences for *S. aureus* in a single nucleic acid test. In such a test it may be possible to have a single primer set to amplify all relevant staphylococci species combined with a range of relevant species-specific probes. tmRNA is a labile RNA molecule and may be an indicator of viability in bacteria. Using tmRNA as the nucleic acid target in a nucleic acid based test may provide a more accurate detection of viable bacteria compared to PCR which can amplify DNA from both viable and non-viable organisms. Additionally, tmRNA is present at approximately 1000 copies per cell in *S. aureus* (Glynn et al., 2007). This natural amplification of the target has the potential to enable more sensitive detection of the pathogen in nucleic acid tests involving *in-vitro* amplification or signal amplification direct detection assays.

Definitions

"Synthetic oligonucleotide" refers to molecules of nucleic acid polymers of 2 or more nucleotide bases that are not derived directly from genomic DNA or live organisms. The term synthetic oligonucleotide is intended to encompass DNA, RNA, and DNA/RNA hybrid molecules that have been manufactured chemically, or synthesized enzymatically *in vitro*.

An "oligonucleotide" is a nucleotide polymer having two or more nucleotide subunits covalently joined together. Oligonucleotides are generally about 10 to about 100 nucleotides. The sugar groups of the nucleotide subunits may be ribose, deoxyribose, or modified derivatives thereof such as OMe. The nucleotide subunits may be joined by linkages such as phosphodiester linkages, modified linkages or by non-nucleotide moieties that do not prevent hybridization of the oligonucleotide to its complementary target nucleotide sequence. Modified linkages include those in which a standard phosphodiester linkage is replaced with a different linkage, such as a phosphorothioate linkage, a methylphosphonate linkage, or a neutral peptide linkage.

Nitrogenous base analogs also may be components of oligonucleotides in accordance with the invention.

A "target nucleic acid" is a nucleic acid comprising a target nucleic acid sequence. A "target nucleic acid sequence," "target nucleotide sequence" or "target sequence" is a specific

deoxyribonucleotide or ribonucleotide sequence that can be hybridized to a complementary oligonucleotide.

An "oligonucleotide probe" is an oligonucleotide having a nucleotide sequence sufficiently complementary to its target nucleic acid sequence to be able to form a detectable hybrid probe:target duplex under high stringency hybridization conditions. An oligonucleotide probe is an isolated chemical species and may include additional nucleotides outside of the targeted region as long as such nucleotides do not prevent hybridization under high stringency hybridization conditions. Non-complementary sequences, such as promoter sequences, restriction endonuclease recognition sites, or sequences that confer a desired secondary or tertiary structure such as a catalytic active site can be used to facilitate detection using the invented probes. An oligonucleotide probe optionally may be labelled with a detectable moiety such as a radioisotope, a fluorescent moiety, a chemiluminescent, a nanoparticle moiety, an enzyme or a ligand, which can be used to detect or confirm probe hybridization to its target sequence. Oligonucleotide probes are preferred to be in the size range of from about 10 to about 100 nucleotides in length, although it is possible for probes to be as much as and above about 500 nucleotides in length, or below 10 nucleotides in length.

A "hybrid" or a "duplex" is a complex formed between two single-stranded nucleic acid sequences by Watson-Crick base pairings or non-canonical base pairings between the complementary bases. "Hybridization" is the process by which two complementary strands of nucleic acid combine to form a double-stranded structure ("hybrid" or "duplex").

"Complementarity" is a property conferred by the base sequence of a single strand of DNA or RNA which may form a hybrid or double-stranded DNA:DNA, RNA:RNA or DNA:RNA through hydrogen bonding between Watson-Crick base pairs on the respective strands. Adenine (A) ordinarily complements thymine (T) or uracil (U), while guanine (G) ordinarily complements cytosine (C).

The term "stringency" is used to describe the temperature, ionic strength and solvent composition existing during hybridization and the subsequent processing steps. Those skilled in the art will recognize that "stringency" conditions may be altered by varying those parameters either individually or together. Under high stringency conditions only highly complementary nucleic acid hybrids will form; hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two nucleic acid strands forming a hybrid. Stringency conditions are chosen to maximize the difference in stability between the hybrid formed with the target and the non-target nucleic acid.

With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences (for example,

hybridization under “high stringency” conditions, may occur between homologs with about 85-100% identity, preferably about 70-100% identity). With medium stringency conditions, nucleic acid base pairing will occur between nucleic acids with an intermediate frequency of complementary base sequences (for example, hybridization under “medium stringency” conditions may occur between homologs with about 50-70% identity). Thus, conditions of “weak” or “low” stringency are often required with nucleic acids that are derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually less.

‘High stringency’ conditions are those equivalent to binding or hybridization at 42° C. in a solution consisting of 5xSSPE (43.8g/l NaCl, 6.9 g/l NaH₂PO₄H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5xDenhardt’s reagent and 100µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1xSSPE, 1.0%SDS at 42° C. when a probe of about 500 nucleotides in length is used. For shorter nucleotide probe lengths the hybridisation temperature is determined by the melting temp of the probe-based on its nucleotide composition, so the hybridisation would be carried out at the melting temperature +/- 10°C of the sequence in question.

“Medium stringency” conditions are those equivalent to binding or hybridization at 42° C. in a solution consisting of 5xSSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5xDenhardt’s reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 1.0xSSPE, 1.0% SDS at 42° C, when a probe of about 500 nucleotides in length is used.

‘Low stringency’ conditions are those equivalent to binding or hybridization at 42° C. in a solution consisting of 5xSSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5xDenhardt’s reagent [50xDenhardt’s contains per 500ml: 5g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 5xSSPE, 0.1% SDS at 42° C, when a probe of about 500 nucleotides in length is used.

In the context of nucleic acid *in-vitro* amplification based technologies, “stringency” is achieved by applying temperature conditions and ionic buffer conditions that are particular to that *in-vitro* amplification technology. For example, in the context of PCR and real-time PCR, “stringency” is achieved by applying specific temperatures and ionic buffer strength for hybridisation of the oligonucleotide primers and, with regards to real-time PCR hybridisation of the probe/s, to the target nucleic acid for *in-vitro* amplification of the target nucleic acid.

One skilled in the art will understand that substantially corresponding probes of the invention can vary from the referred-to sequence and still hybridize to the same target nucleic acid sequence. This variation from the nucleic acid may be stated in terms of a percentage of

identical bases within the sequence or the percentage of perfectly complementary bases between the probe and its target sequence. Probes of the present invention substantially correspond to a nucleic acid sequence if these percentages are from about 100% to about 80% or from 0 base mismatches in about 10 nucleotide target sequence to about 2 bases mismatched in an about 10
5 nucleotide target sequence. In preferred embodiments, the percentage is from about 100% to about 85%. In more preferred embodiments, this percentage is from about 90% to about 100%; in other preferred embodiments, this percentage is from about 95% to about 100%

By "sufficiently complementary" or "substantially complementary" is meant nucleic acids having a sufficient amount of contiguous complementary nucleotides to form, under high
10 stringency hybridization conditions, a hybrid that is stable for detection.

By "nucleic acid hybrid" or "probe:target duplex" is meant a structure that is a double-stranded, hydrogen-bonded structure, preferably about 10 to about 100 nucleotides in length, more preferably 14 to 50 nucleotides in length, although this will depend to an extent on the overall length of the oligonucleotide probe. The structure is sufficiently stable to be detected by means
15 such as chemiluminescent or fluorescent light detection, autoradiography, electrochemical analysis or gel electrophoresis. Such hybrids include RNA:RNA, RNA:DNA, or DNA:DNA duplex molecules.

"RNA and DNA equivalents" refer to RNA and DNA molecules having the same complementary base pair hybridization properties. RNA and DNA equivalents have different
20 sugar groups (i.e., ribose versus deoxyribose), and may differ by the presence of uracil in RNA and thymine in DNA. The difference between RNA and DNA equivalents do not contribute to differences in substantially corresponding nucleic acid sequences because the equivalents have the same degree of complementarity to a particular sequence.

By "preferentially hybridize" is meant that under high stringency hybridization conditions
25 oligonucleotide probes can hybridize their target nucleic acids to form stable probe:target hybrids (thereby indicating the presence of the target nucleic acids) without forming stable probe:non-target hybrids (that would indicate the presence of non-target nucleic acids from other organisms). Thus, the probe hybridizes to target nucleic acid to a sufficiently greater extent than to non-target nucleic acid to enable one skilled in the art to accurately detect the
30 presence of (for example *Candida*) and distinguish these species from other organisms.

Preferential hybridization can be measured using techniques known in the art and described herein.

By "theranostics" is meant the use of diagnostic testing to diagnose the disease, choose the correct treatment regime and monitor the patient response to therapy. The theranostics of the
35 invention may be based on the use of an NAD assay of this invention on samples, swabs or specimens collected from the mammal.

Object of the Invention

It is an object of the current invention to provide sequences and/or diagnostic assays to detect and identify one or more *Staphylococcus* species. The current inventors have made use of the *ssrA* gene sequences to design nucleic acid-based tests specific to *S. aureus*.

5 **Summary of the Invention**

The present invention provides for a diagnostic kit for detection and identification of *Staphylococcal* species, comprising an oligonucleotide probe capable of binding to at least a portion of the *ssrA* or its corresponding tmRNA. The oligonucleotide probe may have a sequence substantially homologous to or substantially complementary to a portion of the *ssrA* gene or its corresponding tmRNA. It will thus be capable of binding or hybridizing with a complementary DNA or RNA molecule. The *ssrA* gene may be a bacterial *ssrA* gene. The nucleic acid molecule may be synthetic. The oligonucleotide probe may have a sequence of SEQ ID NO 9 to SEQ ID NO 37, or a sequence substantially homologous to or substantially complementary to those sequences, which can also act as a probe for the *ssrA* gene.

15 The kit may comprise more than one such probe. In particular the kit may comprise a plurality of such probes. In addition, the kit may comprise additional probes for other *Staphylococci*, fungal, yeast species or viruses.

The identified sequences are suitable not only for *in vitro* DNA/RNA amplification based detection systems but also for signal amplification based detection systems. Furthermore, the sequences of the invention identified as suitable targets provide the advantages of having significant intragenic sequence heterogeneity in some regions, which is advantageous and enables aspects of the invention to be directed towards group or species-specific targets, and also having significant sequence homogeneity in some regions, which enables aspects of the invention to be directed towards genus or group-specific primers and probes for use in direct nucleic acid detection technologies, signal amplification nucleic acid detection technologies, and nucleic acid *in vitro* amplification technologies. The sequences allow for multi-test capability and automation in diagnostic assays.

25 The *ssrA* nucleotide sequences, both DNA and RNA can be used with direct detection, signal amplification detection and *in vitro* amplification technologies in diagnostics assays. The *ssrA* sequences allow for multi-test capability and automation in diagnostic assays.

The kit may further comprise a primer for amplification of at least a portion of the *ssrA* gene. Suitably, the kit comprises a forward and a reverse primer for a portion of the *ssrA* gene.

The primer may have a sequence selected from the group SEQ ID NO 1 through to SEQ ID NO 8 or a sequence substantially homologous to or substantially complementary to those sequences, which can also act as a primer for the *ssrA* gene.

The kit may comprise at least one forward *in vitro* amplification primer and at least one reverse *in vitro* amplification primer, the forward amplification primer having a sequence selected from the group consisting of SEQ ID NO 1, 3, 5 and 7, or a sequence being substantially homologous or complementary thereto which can also act as a forward amplification primer for the *ssrA* gene, and the reverse amplification primer having a sequence selected from the group consisting of SEQ ID NO 2, 4, 6 and 8, or a sequence being substantially homologous or complementary thereto which can also act as a reverse amplification primer for the *ssrA* gene.

The diagnostic kit may be based on direct nucleic acid detection technologies, signal amplification nucleic acid detection technologies, and nucleic acid *in vitro* amplification technologies is selected from one or more of Polymerase Chain Reaction (PCR), Ligase Chain Reaction (LCR), Nucleic Acids Sequence Based Amplification (NASBA), Strand Displacement Amplification (SDA), Transcription Mediated Amplification (TMA), Branched DNA technology (bDNA) and Rolling Circle Amplification Technology (RCAT), or other *in vitro* enzymatic amplification technologies.

The invention also provides a nucleic acid molecule selected from the group consisting of SEQ ID NO.1 to SEQ ID NO. 48 and sequences substantially homologous thereto, or substantially complementary to a portion thereof and having a function in diagnostics based on the *ssrA* gene. The nucleic acid molecule may comprise an oligonucleotide having a sequence substantially homologous to or substantially complementary to a portion of a nucleic acid molecule of SEQ ID NO.1 to SEQ ID NO. 48. The invention also provides a method of detecting a target organism in a test sample comprising the steps of:

- (i) Mixing the test sample with at least one oligonucleotide probe as defined above under appropriate conditions; and
- (ii) hybridizing under high stringency conditions any nucleic acid that may be present in the test sample with the oligonucleotide to form a probe:target duplex; and
- (iii) determining whether a probe:target duplex is present; the presence of the duplex positively identifying the presence of the target organism in the test sample.

The nucleic acid molecule and kits of the present invention may be used in a diagnostic assay to detect the presence of one or more bacterial species, to measure bacterial titres in a patient or in a method of assessing the efficacy of a treatment regime designed to reduce bacterial titre in a patient or to measure bacterial contamination in an environment. The environment may be a hospital, or it may be a food sample, an environmental sample e.g. water, an industrial sample such as an in-process sample or an end product requiring bioburden or quality assessment. The kits and the nucleic acid molecule of the invention may be used in the identification and/or characterization of one or more disruptive agents that can be used to disrupt the *ssrA* gene

function. The disruptive agent may be selected from the group consisting of antisense RNA, PNA, and siRNA.

In some embodiments of the invention, a nucleic acid molecule comprising a species-specific probe can be used to discriminate between species of the same genus.

5 The oligonucleotides of the invention may be provided in a composition for detecting the nucleic acids of target organisms. Such a composition may also comprise buffers, enzymes, detergents, salts and so on, as appropriate to the intended use of the compositions. It is also envisioned that the compositions, kits and methods of the invention, while described herein as comprising at least one synthetic oligonucleotide, may also comprise natural oligonucleotides
10 with substantially the same sequences as the synthetic nucleotide fragments in place of, or alongside synthetic oligonucleotides.

The invention also provides for an *in vitro* amplification diagnostic kit for a target bacterium comprising at least one forward *in vitro* amplification primer and at least one reverse *in vitro* amplification primer, the forward amplification primer being selected from the group consisting
15 of one or more of a sequence being substantially homologous or complementary thereto which can also act as a forward amplification primer, and the reverse amplification primer being selected from the group consisting of one or more of a sequence being substantially homologous or complementary thereto which can also act as a reverse amplification primer. The invention also provides for a diagnostic kit for detecting the presence of candidate bacterial
20 species, comprising one or more DNA probes comprising a sequence substantially complementary to, or substantially homologous to the sequence of the *ssrA* gene of the candidate species. The present invention also provides for one or more synthetic oligonucleotides having a nucleotide sequence substantially homologous to or substantially complementary to one or more of the group consisting of the *ssrA* gene or tRNA transcripts
25 thereof, one or more of SEQ ID NO 1-SEQ ID NO 48.

The nucleotide may comprise DNA. The nucleotide may comprise RNA. The nucleotide may comprise a mixture of DNA, RNA and PNA. The nucleotide may comprise synthetic nucleotides. The sequences of the invention (and the sequences relating to the methods, kits compositions and assays of the invention) may be selected to be substantially homologous to a
30 portion of the coding region of the *ssrA* gene. The gene may be a gene from a target bacterium. The sequences of the invention are preferably sufficient so as to be able form a probe:target duplex to the portion of the sequence.

The invention also provides for a diagnostic kit for a target bacterium comprising an oligonucleotide probe substantially homologous to or substantially complementary to an
35 oligonucleotide of the invention (which may be synthetic). It will be appreciated that sequences suitable for use as *in vitro* amplification primers may also be suitable for use as oligonucleotide

probes: while it is preferable that amplification primers may have a complementary portion of between about 15 nucleotides and about 30 nucleotides (more preferably about 15-about 23, most preferably about 20 to about 23), oligonucleotide probes of the invention may be any suitable length. The skilled person will appreciate that different hybridization and or annealing conditions will be required depending on the length, nature and structure (eg. Hybridization probe pairs for LightCycler, Taqman 5' exonuclease probes, hairpin loop structures etc. and sequence of the oligonucleotide probe selected.

Kits and assays of the invention may also be provided wherein the oligonucleotide probe is immobilized on a surface. Such a surface may be a bead, a membrane, a column, dipstick, a nanoparticle, the interior surface of a reaction chamber such as the well of a diagnostic plate or inside of a reaction tube, capillary or vessel or the like.

The target bacterium may be selected from the group consisting of *S. aureus*, *S. capitis*, *S. chromogenes*, *S. cohnii*, *S. epidermidis*, *S. lactis*, *S. warneri*, *S. xylosus*. Under these circumstances, the amplification primers and oligonucleotide probes of the invention may be designed to a gene specific or genus specific region so as to be able to identify one or more, or most, or substantially all of the desired organisms of the target organism grouping.

The test sample may comprise cells of the target organism. The method may also comprise a step for releasing nucleic acid from any cells of the target organism that may be present in said test sample. Ideally, the test sample is a lysate of an obtained sample from a patient (such as a swab, or blood, urine, saliva, a bronchial lavage, dental specimen, skin specimen, scalp specimen, transplant organ biopsy, stool, mucus, or discharge sample). The test samples may be a food sample, a milk sample, a water sample, an environmental sample, an end product, end product or in-process industrial sample.

The invention also provides for the use of any one of SEQ ID NO.1 to SEQ ID NO.48 in a diagnostic assay for the presence of one or more bacterial species. The species may be selected from the group consisting of *S. aureus*.

The invention also provides for kits for use in clinical diagnostics, theranostics, food safety diagnostics, industrial microbiology diagnostics, environmental monitoring, veterinary diagnostics, bio-terrorism diagnostics comprising one or more of the synthetic oligonucleotides of the invention. The kits may also comprise one or more articles selected from the group consisting of appropriate sample collecting instruments, reagent containers, buffers, labelling moieties, solutions, detergents and supplementary solutions. The invention also provides for use of the sequences, compositions, nucleotide fragments, assays, and kits of the invention in theranostics, Food safety diagnostics, Industrial microbiology diagnostics, Environmental monitoring, Veterinary diagnostics, Bio-terrorism diagnostics.

The nucleic acid molecules, composition, kits or methods may be used in a diagnostic nucleic acid based assay for the detection of bacterial species.

The nucleic acid molecules, composition, kits or methods may be used in a diagnostic assay to measure bacterial titres in a patient. The titres may be measured *in vitro*.

- 5 The nucleic acid molecules, composition, kits or methods may be used in a method of assessing the efficacy of a treatment regime designed to reduce bacterial titre in a patient comprising assessing the bacterial titre in the patient (by *in vivo* methods or *in vitro* methods) at one or more key stages of the treatment regime. Suitable key stages may include before treatment, during treatment and after treatment. The treatment regime may comprise an antifungal agent, such as a pharmaceutical drug.

The nucleic acid molecules, composition, kits or methods may be used in a diagnostic assay to measure potential bacterial contamination, for example, in a hospital.

- The nucleic acid molecules, composition, kits or methods may be used in the identification and/or characterization of one or more disruptive agents that can be used to disrupt the *ssrA* gene function. Suitable disruptive agents may be selected from the group consisting of antisense RNA, PNA, siRNA.

Brief Description of the Drawings

Figure 1: *S. aureus* cRNA microarray sandwich assay with Sa20 reporter probe (Sa19 capture probe circled) showing the detection of *S. aureus* cRNA.

- 20 **Figure 2:** Real-time NASBA of dilutions of *S. aureus* total RNA (10^{-2} = 100,000 cells; 10^{-4} = 10,000 cells; 10^{-5} = 1,000 cells; 10^{-6} = 100 cells; 10^{-7} = 10 cells; 10^{-9} = <1 cell equivalents). Limit of detection is < 1 cell equivalent.

- Figure 3:** Detection of *S. aureus* in spiked milk samples using the *S. aureus* NASBA assay. Milk samples were spiked with *S. aureus* cells (10^8 , 10^5 -10 cells) and RNA extracted and included in the NASBA assay. Limit of detection is 10 cells/ml of milk. Controls including an *S. aureus* positive control (RNA), a blank broth sample, a UHT milk sample and a NASBA negative control were also included. *S. aureus* was not detected in the blank broth and UHT milk controls.

- Figure 4:** Detection of *S. aureus* strains in a *S. aureus* real-time PCR assay based on the *ssrA* gene. 10 of 10 *S. aureus* strains were detected.

Figure 5: Real-time amplification of *S. aureus* total DNA (10^{-1} = 180,000 cells; 10^{-2} = 18,000 cells; 10^{-3} = 1,800 cells; 10^{-4} = 180 cells; 10^{-5} = 18 cells; 10^{-6} = 1.8 cell equivalents) in the *ssrA* based real-time PCR. Limit of detection is 18 cells.

Detailed Description of the Invention

- 35 **Materials and Methods**

Bacterial species and strains:

S. aureus strains used for DNA sequencing, for probe evaluation and inclusivity testing of the NASBA assay and real-time PCR assay along with other *Staphylococcus* species strains and other bacterial species related to staphylococci or found in milk samples included in this study are listed in table 1.

5 Table 1: Strains and species included in the study

	Species Name	Source
1.	<i>Staphylococcus aureus</i>	DSM 346
2.	<i>Staphylococcus aureus</i>	DSM 6236
3.	<i>Staphylococcus aureus</i>	DSM 6571
4.	<i>Staphylococcus aureus</i>	DSM 6732
5.	<i>Staphylococcus aureus</i>	DSM 9144
6.	<i>Staphylococcus aureus</i>	DSM 12463
7.	<i>Staphylococcus aureus</i>	DSM 15676
8.	<i>Staphylococcus aureus</i> 52/02	Raw milk
9.	<i>Staphylococcus aureus</i> 59/01	Raw milk
10.	<i>Staphylococcus aureus</i> 60/01	Raw milk
11.	<i>Staphylococcus aureus</i> 71/01	Raw milk
12.	<i>Staphylococcus aureus</i> 73/01	Raw milk
13.	<i>Staphylococcus aureus</i> 76/03	Raw milk
14.	<i>Staphylococcus aureus</i> 135/01	Raw milk
15.	<i>Staphylococcus aureus</i> 137/01	Raw milk
16.	<i>Staphylococcus aureus</i> 146/01	Raw milk
17.	<i>Staphylococcus aureus</i> 147/01	Raw milk
18.	<i>Staphylococcus aureus</i> 150/03	Raw milk
19.	<i>Staphylococcus aureus</i> 151/03	Raw milk
20.	<i>Staphylococcus aureus</i> 152/02	Raw milk
21.	<i>Staphylococcus aureus</i> 170/01	Raw milk
22.	<i>Staphylococcus aureus</i> 174/01	Raw milk
23.	<i>Staphylococcus aureus</i> 175/01	Raw milk
24.	<i>Staphylococcus aureus</i> 182/02	Raw milk
25.	<i>Staphylococcus aureus</i> 183/02	Raw milk
26.	<i>Staphylococcus aureus</i> 211/06	Raw milk
27.	<i>Staphylococcus aureus</i> 214/01	Raw milk
28.	<i>Staphylococcus aureus</i> 310/01	Raw milk
29.	<i>Staphylococcus aureus</i> 311/01	Raw milk
30.	<i>Staphylococcus aureus</i> 314/18	Raw milk
31.	<i>Staphylococcus aureus</i> 316/05	Raw milk
32.	<i>Staphylococcus aureus</i> 493/02	Raw milk
33.	<i>Staphylococcus aureus</i> 617/02	Raw milk
34.	<i>Staphylococcus aureus</i> 627/03	Raw milk
35.	<i>Staphylococcus aureus</i> 709/01	Raw milk
36.	<i>Staphylococcus aureus</i> 860/02	Raw milk
37.	<i>Staphylococcus aureus</i> 1018/01	Raw milk
38.	<i>Staphylococcus aureus</i> 2393/01	Raw milk
39.	<i>Staphylococcus aureus</i> 2404/02	Raw milk
40.	<i>Staphylococcus aureus</i> 2423/10	Raw milk
41.	<i>Staphylococcus aureus</i> 2440/02	Raw milk
42.	<i>Staphylococcus aureus</i> 2443/01	Raw milk
43.	<i>Staphylococcus aureus</i> 2454/01	Raw milk
44.	<i>Staphylococcus aureus</i> 2456/01	Raw milk
45.	<i>Staphylococcus aureus</i> 2457/01	Raw milk

	Species Name	Source
46.	<i>Staphylococcus aureus</i> 2478/02	Raw milk
47.	<i>Staphylococcus aureus</i> 2487/01	Raw milk
48.	<i>Staphylococcus aureus</i> 2518	Raw milk
49.	<i>Staphylococcus aureus</i> 2524/01	Raw milk
50.	<i>Staphylococcus aureus</i> 2574/01	Raw milk
51.	<i>Staphylococcus aureus</i> 2575/01	Raw milk
52.	<i>Staphylococcus aureus</i> 4301	Raw milk
53.	<i>Staphylococcus aureus</i> 4407	Raw milk
54.	<i>Staphylococcus aureus</i> dv11/95	Cream cheese
55.	<i>Staphylococcus aureus</i> KM walch1/29	Cheese making milk
56.	<i>Staphylococcus aureus</i> KM fueg5/37	Cheese making milk
57.	<i>Staphylococcus aureus</i> KM tref1/40	Cheese making milk
58.	<i>Staphylococcus aureus</i> Kmeri 12/49	Cheese making milk
59.	<i>Staphylococcus aureus</i> KM reutt 9/107	Cheese making milk
60.	<i>Staphylococcus aureus</i> Kmhatz 7/110	Cheese making milk
61.	<i>Staphylococcus aureus</i> Kmkoess 1/111	Cheese making milk
62.	<i>Staphylococcus aureus</i> KM reith 3/119	Cheese making milk
63.	<i>Staphylococcus aureus</i> Erier	Cheese
1.	<i>Staphylococcus capitis</i>	DSM 20326
2.	<i>Macrococcus caseolyticus</i>	DSM 20597
3.	<i>Staphylococcus chromogenes</i> (n=2)	DSM 20454, Shyla
4.	<i>Staphylococcus cohnii</i> (n=2)	DSM 6669, Sco2
5.	<i>Staphylococcus epidermidis</i> (n=4)	DSM 1798, 20044, 20231, NCTC 11047,
6.	<i>Kocuria varians</i> (<i>Staphylococcus lactis</i>)	DSM 20033
7.	<i>Staphylococcus warneri</i>	CCM 2730
8.	<i>Staphylococcus xylosus</i>	DSM 6179
9.	<i>Lactococcus lactis</i>	NCTC 662
10.	<i>Bacillus cereus</i>	NCTC 7464
11.	<i>Enterococcus faecalis</i>	
12.	<i>Enterococcus faecium</i>	DSM 20477
13.	<i>Enterobacter cloacae</i>	NCTC 11933
14.	<i>Listeria monocytogenes</i>	NCTC 11994
15.	<i>Streptococcus. agalactiae</i> (n=3)	DSM 15081, 15086, 15094
16.	<i>Streptococcus bovis</i>	DSM 20480
17.	<i>Klebsiella aerogenes</i>	
18.	<i>Klebsiella oxytoca</i>	ATCC 43086
19.	<i>Escherichia aninodolica</i>	
20.	<i>E. coli</i> (n=2)	DSM 301, 30083
21.	<i>Klebsiella pneumoniae</i>	
22.	<i>Proteus mirabilis</i>	DSM 4479
23.	<i>Citrobacter freundii</i>	NCTC 8090
24.	<i>Aeromonas hydrophila</i>	ATCC 35654
25.	<i>Salmonella dublin</i>	NCTC 9676
26.	<i>Salmonella enteritidis</i>	ATCC 13076
27.	<i>Salmonella typhimurium</i>	NCTC 12416

DNA sequencing:

DNA sequencing of the *ssrA* gene in 6 *S. aureus* strains, *S. epidermidis* (n=2), *S. capitis* (n=1), *S. caseolyticus*(n=1) and *S. chromogenes* (n=1) was performed using DNA extracted using Edge

5 BioSystems 'Bacterial Genomic DNA Extraction Kit' from cultures of these strains according to manufacturers instructions. Conventional PCR amplification of the *ssrA* gene from position 4-

356 bp (353 bp fragment) was performed using primer set *S. aureus* F/R (Table 2). Following PCR amplification DNA sequencing of the *ssrA* gene products from these strains was performed using primer *S. aureus* F following clean-up of the PCR products using ExoSAP-IT by an external sequence service provider (Sequiserve, Germany). DNA sequence information was
5 obtained for all strains sequenced.

PCR, NASBA primer and DNA probe design

ssrA gene DNA sequence information obtained for *S. aureus* and other staphylococci species strains was aligned using Clustal W with available *ssrA* gene sequence information for other closely related species and for species that may be present in the milk sample environment. The
10 sequences were analysed and a selection of candidate primers and probes for the detection of *S. aureus* and staphylococci were designed from the sequence information.

DNA probe Design:

Twenty-six DNA probes (Table 3) were designed and evaluated for their specificity for the detection of *S. aureus* and staphylococci by applying them on a DNA probe microarray (Asper
15 Biotech) and hybridising them with *in-vitro* transcribed cRNA from *S. aureus*, staphylococci species and a range of other bacterial species. The *ssrA* gene was amplified in *S. aureus*, staphylococci and selected other species and cloned into plasmid vector (Invitrogen). Plasmids were sequenced (Sequiserve, Germany) to confirm presence of correct insert and to identify insert orientation. *In vitro* transcription reactions were performed using MAXIscripTM *in vitro*
20 transcription kit (Ambion). Following transcription, the RNA products were purified using NUCaway spin columns (Ambion). Following transcription and purification, *in vitro* transcribed RNA was vacuum dried to a pellet (Eppendorf concentrator 5301) and labelled with cye-3 dye (Amersham) using standard methods (t Hoen *et al.* 2003). Following labelling, RNA was purified using NUCaway columns and vacuum dried as before. Labelled RNA was resuspended
25 in 20 µl microarray hybridisation buffer (6X SSC, 0.1% SDS, 5X Denhardts). Evaluation of probe specificity was performed on microarrays using fluorescently labelled *in vitro* transcribed cRNAs (t Hoen *et al.* 2003). Overnight hybridisations were performed in a Genetix slide chamber (Hampshire, England) at 55°C under glass coverslips. Total hybridisation volume was 20µl. Post-hybridisation washes were 2X SSC, 0.1% SDS for 5 minutes at room temperature
30 followed by 0.2X SSC, 0.1% SDS for 5 minutes also at room temperature. Slides were rinsed once in distilled water and dried by centrifugation using an Eppendorf centrifuge fitted with a microarray slide adaptor. Washed microarray slides were scanned immediately using ScanArray Express Microarray Analysis System (Perkin Elmer).

NASBA assay design:

35 NASBA primers and a molecular beacon probe (Table 4) were designed from this sequence information and evaluated for the detection of *S. aureus* in a real-time NASBA assay.

NASBA reactions were performed using Nuclisens basic kit reagents (bioMerieux, France) according to manufacturer's instructions with the primers and molecular beacon probe included at a final concentration of 0.2 μ M. The assay was optimised as a real-time NASBA assay performed on the LightCycler 1.2. These primers and probe (Table 4) could be modified for application in other *in-vitro* amplification assays for example real-time PCR.

IAC development:

An internal amplification control was designed for inclusion in the *S. aureus* NASBA assay. *Candida albicans* als1 gene was PCR amplified with Composite F/R primers (Table 5) and then with NASBA 6F/6R (Table 5) producing a 239 bp PCR product from which a 217 base cRNA fragment was synthesized to serve as the IAC for the *S. aureus* NASBA. 20,000 copies of IAC cRNA were included in the *S. aureus* real-time NASBA reaction.

Real-time PCR assay design:

Real-time PCR primers and a TaqMan probe (Table 6) were designed from the sequence information and evaluated for the specific detection of *S. aureus*. The assay was configured on the LightCycler® 2.0 thermocycler. Master mix for real-time PCR was prepared according to manufacturers instructions using the Roche LightCycler® FastStart DNA Master HybProbe Kit with primers F2/R2 included at a final concentration of 0.5mM and probe SA included at a final concentration of 0.2mM.

20

Table 2: PCR primers for PCR amplification and sequencing

Name	Sequence 5'-3'
S.aureusF	5'-GACGTTTCATGGATTCGACA-3'
S.aureusR	5'-AGACGGCGGGATTTGAA-3'

Table 3: List of probes designed for *S. aureus* and staphylococci species

OLIGO NAME	PROBE SEQUENCE	TM
Sa1	ACCCTCCGACACG	53.6
Sa2	CTGTTAGGCGATGCA	53.8
Sa3	CACATAGGAAATGCTGTT	53.8
Sa4	CTATTAAGGTTGAATCGC	51.8
Sa5	CTAGTTTGATTAAGTTTCTTCT	53.5
Sa6	GCATCATGAAAAGTGATAA	52.5
Sa7	ACACGCTTAATGAGCT	53.8
Sa8	CTCCGACACGCTTAAT	54.4
Sa9	TGTGTGTTGATGACGA	53.6
Sa10	TTTGCCAGTTATTATAAACTG	53.7
Sa11	ACTGCGAAATTATTGTTTG	53.8
Sa12	TTAGGCAGCTACTGC	52.9
Sa13	AGAGTGCGATTAGGC	53.0
Sa14	AATGCTGTTAGGCGAT	53.8
Sa15	GAAATGCTGTTAGGCG	53.8
Sa16	GAATCGCGTTAACAGC	54.4
Sa17	TAAGGTTGAATCGCGT	53.8
Sa18	TCCTATTAAGGTTGAATCG	53.1
Sa19	ACGGCAGTGTTTAGC	54.5
Sa20	AAGTTTCTTCTAAACAGACT	53.5
Sa21	CCAACATGATGCTAGC	53.0
Sa22	GGTTTCGCATCATGAAA	54.0
Sa23	CGTGTGTAGTTTATCGAA	53.4
Sa24	ACACATCTTTCTACGTGT	54.7
Sa25	AGGTCCTGATACACATC	53.2
Sa26	GGCGGGATTGTAACC	55.4

Table 4: NASBA primers and probes

Name	5'-3' sequence
NASBA6F	5'AATTGTAATACGACTCACTATAGGGAGTTCGTCATCAACACACA-3'
NASBA6R	5'-CAGAGGTCCTGATACACA-3'
NASBA-Beacon	5'FAM- CCAG CAAGTTTCTTCTAAACAGACT GCTGG -3'BHQ1

Note: NASBA probes are molecular beacons and include a stem sequence not part of the target tmRNA sequence (highlighted in bold).

Table 5: Primers and probes for the IAC for the *S. aureus* NASBA.

Name	Sequence 5'-3'
Composite F	5' - TTCGTCATCAACACACAATACCCAAGTTGGAATG - 3'
CompositeR	5' - CAGAGGTCCTGATACACATCTTCACCAGAATAAAATTG - 3'
NASBA6F	5' - AATTGTAATACGACTCACTATAGGGAGTTCGTCATCAACACACA -
NASBA6R	5'-CAGAGGTCCTGATACACA-3'
NASBA-Beacon	5'FAM- CCGAG TGAATGTATCCCCTGGAC TCGG -3'BHQ1

Note: NASBA probes are molecular beacons and include a stem sequence not part of the target tmRNA sequence (highlighted in bold).

Table 6: Primers and probe for the *S. aureus* real-time PCR assay:

Name	Sequence 5'-3'
<i>S. aur</i> F2	5'CTAACAATAACTTAGCAGTAGCT'3
<i>S. aur</i> R2	5'GCAGTGTTTAGCATATCCTAT'3
SA probe	5'TAACAGCATTTCCTATGTGCTGT'3

Results

Selection of *S. aureus*-specific and staphylococci probes:

- 5 DNA probes listed in table 3 were tested for their specificity for *S. aureus* and/or staphylococci species by hybridising *in-vitro* transcribed cRNA from *S. aureus*, staphylococci and selected other species listed in table 1 to the microarray as described. Seven *S. aureus* species specific probes Sa3, Sa4, Sa6, Sa7, Sa18, Sa20 and Sa22 were identified and 4 staphylococci genus probes Sa8, Sa12, Sa24 and Sa25 were identified. The potential to use these probes for the
- 10 detection of *S. aureus* was demonstrated in a sandwich hybridisation assay where unlabelled *S. aureus* cRNA was hybridised to the microarray and detected using Sa20 which was labelled with Cy-3 as a reporter probe. One microgram cRNA plus 20pmol Sa20 reporter in a 20ul volume were hybridised to the microarray at 55C overnight and washed in 2X SSC, 0.1% SDS for 5 minutes at room temperature. Figure 1 demonstrates the microarray sandwich assay.
- 15 Strongest signal was obtained from Sa19 capture probe with the *S. aureus* specific Sa20 reporter probe.

NASBA assay evaluation:

- RNA template for NASBA evaluation was extracted from overnight cultures of the species listed in table 1 using the Ambion Ribopure yeast kit according to manufacturer's instructions.
- 20 The *S. aureus* real-time NASBA assay was tested for sensitivity by performing NASBA amplifications of serial dilutions of *S. aureus* RNA. A detection limit equivalent to 1-10 cells was determined (Figure 2). Evaluation of the specificity was performed using a panel of RNA for species listed in table 1. Sixty-three *S. aureus* strains were all detected in the assay while 35 other strains (27 species) including closely related staphylococci species were not detected. This
- 25 assay was sensitive and specific for the detection of *S. aureus*. The assay has been evaluated for the application to the detection of *S. aureus* in spiked milk samples. Milk samples (1ml) were spiked with known concentrations of *S. aureus* cells (10^8 , 10^5 - 10 cells). Five hundred microlitres of milk clearing solution (EDTA, IGEPAL and latex beads) was mixed with the milk and centrifuged. The milk fat pellet and the supernatant were removed and the remaining cell pellet
- 30 was resuspended and RNA was extracted using the Ambion Ribopure Yeast kit according to manufacturer's instructions. A detection limit of 10 spiked *S. aureus* cells/ml was achieved when the *S. aureus* NASBA assay was applied to the RNA extracted from the spiked milk samples (Figure 3). Additionally, the NASBA assay detected *S. aureus* in a naturally infected

milk samples contaminated with approximately 2000 cells/ml and did not detect *S. aureus* in 20 milk samples which were negative for *S. aureus* by microbiological culture. In samples where the IAC was included it was detected.

Real-Time PCR evaluation:

5 Bacterial genomic DNA for evaluation of the real-time PCR assay for *S. aureus* was extracted according to manufacturers instructions using the EdgeBioSystems Purelute bacterial genomic purification kit. Evaluation of the specificity of the *S. aureus* real-time PCR assay was performed by testing 10 strains of *S. aureus* and 3 closely related Staphylococci species (*S. epidermidis*, *S. chromogenes*, *S. haemolyticus*) and 7 other bacterial species (*K. oxytoca*, *A. hydrophilia*, *C. freundii*, *K. pneumoniae*, *M. caseolyticus*, *S. agalactiae*, *B. cereus*). All *S. aureus* strains (Figure 4) were detected in the assay and none of the other staphylococcal or other species tested were detected. The sensitivity of the real-time PCR assay was established at 18 cells (Figure 5).

15 In so far as any sequence disclosed herein differs from its counterpart in the attached sequence listing in PatentIn3.3 software, the sequences within this body of text are to be considered as the correct version.

The words “comprises/comprising” and the words “having/including” when used herein with reference to the present invention are used to specify the presence of stated features, integers, steps or components but does not preclude the presence or addition of one or more other
20 features, integers, steps, components or groups thereof.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination.

SEQ IDs

Sites of probes, oligonucleotides etc. are shown in bold and underlined.

N or x= any nucleotide; w=a/t, m=a/c, r=a/g, k=g/t, s=c/g, y=c/t, h=a/t/c, v=a/g/c, d=a/g/t, b=g/t/c. In some cases, specific degeneracy options are indicated in parenthesis: e.g.: (a/g) is either A or G.

SEQ ID NO 1:

5'-GACGTTTCATGGATTCGACA-3'

SEQ ID NO 2:

5'-AGACGGCGGGATTTGAA-3'

SEQ ID NO 3:

35 5'-AATTGTAATACGACTCACTATAGGGAGTTCGTCATCAACACACA-3'

SEQ ID NO 4:

5'-CAGAGGTCCTGATACACA-3'

SEQ ID NO 5:

5' - TTCGTCATCAACACACAATACCCAACTTGAATG - 3'

SEQ ID NO 6:

5' – CAGAGGTCCTGATACACATCTTCACCAGAATAAAATTG – 3'

SEQ ID NO 7:

5'CTAACAATAACTTAGCAGTAGCT'3

5 **SEQ ID NO 8:**

5'GCAGTGTTTAGCATATCCTAT'3

SEQ ID NO 9:

ACCCTCCGACACG

SEQ ID NO 10:

10 CTGTTAGGCGATGCA

SEQ ID NO 11:

CACATAGGAAATGCTGTT

SEQ ID NO 12:

CTATTAAGGTTGAATCGC

15 **SEQ ID NO 13:**

CTAGTTTGATTAAGTTTCTTCT

SEQ ID NO 14:

GCATCATGAAAAGTGATAA

SEQ ID NO 15:

20 ACACGCTTAATGAGCT

SEQ ID NO 16:

CTCCGACACGCTTAAT

SEQ ID NO 17:

TGTGTGTTGATGACGA

25 **SEQ ID NO 18:**

TTTGCCAGTTATTATAAACTG

SEQ ID NO 19:

ACTGCGAAATTATTGTTTG

SEQ ID NO 20:

30 TTAGGCAGCTACTGC

SEQ ID NO 21:

AGAGTGCGATTAGGC

SEQ ID NO 22:

AATGCTGTTAGGCGAT

35 **SEQ ID NO 23:**

GAAATGCTGTTAGGCG

SEQ ID NO 24:

GAATCGCGTTAACAGC

SEQ ID NO 25:

40 TAAGGTTGAATCGCGT

SEQ ID NO 26:

TCCTATTAAGGTTGAATCG

SEQ ID NO 27:

ACGGCAGTGTTTAGC

45 **SEQ ID NO 28:**

AAGTTTCTTCTAAACAGACT

SEQ ID NO 29:

CCAACATGATGCTAGC

SEQ ID NO 30:

50 GGTTTCGCATCATGAAA

SEQ ID NO 31:

CGTGTGTAGTTTATCGAA

SEQ ID NO 32:

ACACATCTTTCTACGTGT

SEQ ID NO 33:

AGGTCCTGATACACATC

SEQ ID NO 34:

GGCGGGATTTGAACC

5 **SEQ ID NO 35:**

CCAGCAAGTTTCTTCTAAACAGACTGCTGG-

SEQ ID NO 36:

5' - CCGAGTGAATGTATCCCCTGGACTCGG - 3'

SEQ ID NO 37:

10 5'TAACAGCATTTCCTATGTGCTGT'3

SEQ ID NO 38:>*S. aureus* (DSM346)

GGGGGTCCCSGAGCTCATTAAAGCGTGTCTGGAGGGTTGTCTTCGTCATCAACACACACAGTTTATAATAAC
TGGCAAATCAAACAATAATTTTCGCAGTAGCTGCCTAATCGCACTCTGCATCGCCTAACAGCATTTCCTAT
15 GTGCTGTTAACGCGATTCAACCTTAATAGGATATGCTAAACACTGCCGTTTGAAGTCTGTTTAGAAGAAA
CTTAATCAAGCTAGCATCATGTTGGTTGTTTATCACTTTTCATGATGCGAAACCTTTTCGATAAACTACAC
ACGTAGAAAGATGTGTATCAGGACCTCTGGACGCGGGTTCAAATCCC GCCGTCT

SEQ ID NO 39:>*S. aureus* (DSM6236)

GGGGGTCCCSGAGCTCATTAAAGCGTGTCTGGAGGGTTGTCTTCGTCATCAACACACACAGTTTATAATAAC
20 TGGCAAATCAAACAATAATTTTCGCAGTAGCTGCCTAATCGCACTCTGCATCGCCTAACAGCATTTCCTAT
GTGCTGTTAACGCGATTCAACCTTAATAGGATATGCTAAACACTGCCGTTTGAAGTCTGTTTAGAAGAAA
CTTAATCAAACCTAGCATCATGTTGGTTGTTTATCACTTTTCATGATGCGAAACCTATCGATAAACTACAC
ACGTAGAAAGATGTGTATCAGGACCTTTGGACGCGGGTTCAAATCCC GCCGTCT

25 **SEQ ID NO 40:**>*S. aureus* (DSM6732)

GGGGGTCCCSGAGCTCATTAAAGCGTGTCTGGAGGGTTGTCTTCGTCATCAACACACACAGTTTATAATAAC
TGGCAAATCAAACAATAATTTTCGCAGTAGCTGCCTAATCGCACTCTGCATCGCCTAACAGCATTTCCTAT
GTGCTGTTAACGCGATTCAACCTTAATAGGATATGCTAAACACTGCCGTTTGAAGTCTGTTTAGAAGAAA
30 CTTAATCAAGCTAGCATCATGTTGGTTGTTTATCACTTTTCATGATGCGAAACCTTTTCGATAAACTACAC
ACGTAGAAAGATGTGTATCAGGACCTCTGGACGCGGGTTCAAATCCC GCCGTCT

SEQ ID NO 41:>*S. aureus* (DSM12463)

GGAGGGTTGTCTTCGTCATCAACACACACAGTTTATAATAACTGGCAAAACAAACAATAATTTTCGCAGTA
35 GCTGCCTAATCGCACTCTGCATCGCCTAACAGCATTTCCTATGTGCTGTTAACGCGATTCAACCTTAATA
GGATATGCTAAACACTGCCGTTTGAAGTCTGTTTAGAAGAACTTAATCAAGCTAGCATCATGTTGGTTG
TTTATCACTTTTCATGATGCGAAACCTTTTCGATAAACTACACACGTAGAAAGATGTGTATCAGGACCTCT
GGACGCGGGTTCAAATCCC GCCGTCT

SEQ ID NO 42:40 >*S. aureus* (DSM15676)

GGGGGTCCCSGAGCTCATTAAAGCGTGTCTGGAGGGTTGTCTTCGTCATCAACACACACAGTTTATAATAAC
TGGCAAAACAAACAATAATTTTCGCAGTAGCTGCCTAATCGCACTCTGCATCGCCTAACAGCATTTCCTAT
GTGCTGTTAACGCGATTCAACCTTAATAGGATATGCTAAACACTGCCGTTTGAAGTCTGTTTAGAAGAAA
CTTAATCAAGCTAGCATCATGTTGGTTGTTTATCACTTTTCATGATGCGAAACCTTTTCGATAAACTACAC
45 ACGTAGAAAGATGTGTATCAGGACCTCTGGACGCGGGTTCAAATCCC GCCGTCT

SEQ ID NO 43:>*S. aureus* (DSM20231)

GGGGGTCCCSGAGCTCATTAAAGCGTGTCTGGAGGGTTGTCTTCGTCATCAACACACACAGTTTATAATAAC
TGGCAAATCAAACAATAATTTTCGCAGTAGCTGCCTAATCGCACTCTGCATCGCCTAACAGCATTTCCTAT
50 GTGCTGTTAACGCGATTCAACCTTAATAGGATATGCTAAACACTGCCGTTTGAAGTCTGTTTAGAAGAAA
CTTAATCAAACCTAGCATCATGTTGGTTGTTTATCACTTTTCATGATGCGAAACCTATCGATAAACTACAC
ACGTAGAAAGATGTGTATCAGGACCTTTGGACGCGGGTTCAAATCCC GCCGTCT

SEQ ID NO 44:>*S. capitis* (DSM20326)

55 AAGCGTGTCTGGAGGGTTGTCTCCGATATCAACACATTTTCAGTAAATATAACTGACAAATCAAACAATAAT
TTTCGCAGTAGCTGCGTAATAGCACACTGCATCGCCTAACAGCATCTCCTACGTGCTGTTAACGCGATTCA
ACCCTAGTAGGATATGCTAAACACTGCCGTTTGAAGTCTGTTTAGATGAATACTAATCAAACCTAGCATAA

TACTGGTTGTCTATTGCTTACTATTATGCGAAATGAATCAATAGACTACACACGTAGAAAGGTGTGTATC
AGGACCTCTGGACGCGGGTTCAAATCCCGCCGTCT

SEQ ID NO 45:

>*S. caseolyticus* (DSM20597)

5 CGTGTCTGGAGGGTTGTCTTCGTCATCAACACAACAGTTTATAATAACTGGCAAAACAAACAATTTTCGCAG
TAGCTGCCTAATCGCACTCTGCACCTCTCTCACGCATCGCCTATGTGCCTGATGTAGAGGTTCAACTTAA
GTAGGATACGCTGTACACCTCCGCTTGGGGTCTGTACAGAAGAGATTAATCGAGCTAGTTATTAACCAGG
ATGTCTGTTTTTCAGGTTAATAGCGAAACTTAATAACAGGACTACACACGTAGAAGTATCAGCTGCAGGAT
CCTTGGACSYGGTTTCGAYTCCC

10 SEQ ID NO 46:

>*S. chromogenes* (DSM20454)

GGGGGTCCSAGAGCTTATGAAGCGTGTCTGGAGGGTTGTCTCCGCAAAAACACATCAGTTTATAATAACTG
GCAAAACTAACAATAACTTAGCAGTAGCTGCCTAATCGCGCTCTGCATCACCTAATCGCATCGCCTATGT
GCGATTAACGTGATTCAACTTTAGTAGGATACGCTCGTCACTGCCGTTTGAAGTCTGACGAGAAGAGATG
15 AATCAAACCTCGCATAATGTCGGTTGTCTGTTTCTAAACATTATGCTAAATTTCTAAACAGACTACACACG
TAGAAACATTTGTATCAGGACCTTTGGACGCGGGTTCAAATCCCGCCGTCT

SEQ ID NO 47:

>*S. epidermidis* (DSM1798)

GGGGGTYCCSGAGCTTATTAAGCGTGTCTGGAGGGTTGGCTCCGTCATCAACACATTTTCGGTTAAATATAA
20 CTGACAAATCAAACAATAATTTTCGCAGTAGCTGCGTAATAGCCACTGCATCGCCTAACAGCATCTCCTAC
GTGCTGTTAACGCGATTCAACCCTAGTAGGATATGCTAAACACTGCCGCTTGAAGTCTGTTTAGATGAAA
TATAATCAAGCTAGTATCATGTTGGTTGTTTATTGCTTAGCATGATGCGAAAATTATCAATAAACTACAC
ACGTAGAAAGATTTGTATCAGGAC CTCTGGACGCGGGTTCAAATCCCGCCGTCT

SEQ ID NO 48:

25 >*S. epidermidis* (DSM20044)

GGGGGTCCCSGAGCTTATTAAGCGTGTCTGGAGGGTTGGCTCCGTCATCAACACATTTTCGGTTAAATATAA
CTGACAAATCAAACAATAATTTTCGCAGTAGCTGCGTAATAGCCACTGCATCGCCTAACAGCATCTCCTAC
GTGCTGTTAACGCGATTCAACCCTAGTAGGATATGCTAAACACTGCCGCTTGAAGTCTGTTTAGATGAAA
TATAATCAAGCTAGTATCATGTTGGTTGTTTATTGCTTAGCATGATGCGAAAATTATCAATAAACTACAC
30 ACGTAGAAAGATTTGTATCAGGACCTCTGGACGCGGGTTCAAATCCCGCCGTCT

References:

- T Hoen P. A., F. De Kort, G. J. Van Ommen, and J. T. Den Dunnen, 2003. Fluorescent labelling of cRNA for microarray applications. NAR. 31:e20.
- 5 Ornskov, D., B. Kolmos, P. Bendix Horn, J. Nederby Nielsen, I Brandslund, and P. Schouenborg. 2008. Screening for methicillin resistant *Staphylococcus aureus* in clinical swabs using a high-through-put real-time PCR based method. Clin. Microbiol. Infect. 14: 22-28.
- Hogg, G.M., J. P. McKenna, and G. Ong. 2008. Rapid detection of methicillin-susceptible and methicillin resistant *Staphylococcus aureus* directly from positive BacT/Alert blood culture
10 bottles using real- time polymerase chain reaction: evaluation and comparison of 4 DNA extraction methods. Diagn. Microbiol. Infect. Dis. 61: 446-452.
- Deiman, B., C. Jay, C. Zintilini, S. Vermeer, D. van Strijp, F. Venema, and P. van de Wiel.
15 2008. Efficient amplification with NASBA of hepatitis B virus, herpes simplex virus and methicillin resistant *Staphylococcus aureus* DNA. J. Virol. Methods 151; 283-293.
- T Hoen P. A., F. De Kort, G. J. Van Ommen, and J. T. Den Dunnen, 2003. Fluorescent labelling of cRNA for microarray applications. NAR. 31:e20.
20
- Ornskov, D., B. Kolmos, P. Bendix Horn, J. Nederby Nielsen, I Brandslund, and P. Schouenborg. 2008. Screening for methicillin resistant *Staphylococcus aureus* in clinical swabs using a high-through-put real-time PCR based method. Clin. Microbiol. Infect. 14: 22-28.
- 25 Hogg, G.M., J. P. McKenna, and G. Ong. 2008. Rapid detection of methicillin-susceptible and methicillin resistant *Staphylococcus aureus* directly from positive BacT/Alert blood culture bottles using real- time polymerase chain reaction: evaluation and comparison of 4 DNA extraction methods. Diagn. Microbiol. Infect. Dis. 61: 446-452.
- 30 Deiman, B., C. Jay, C. Zintilini, S. Vermeer, D. van Strijp, F. Venema, and P. van de Wiel. 2008. Efficient amplification with NASBA of hepatitis B virus, herpes simplex virus and methicillin resistant *Staphylococcus aureus* DNA. J. Virol. Methods 151; 283-293.
- Glynn, B., Lacey, K., Reilly, J., Barry, T., Smith, T. J. and Maher, M. (2007). Quantification of Bacterial tmRNA using *in vitro* Transcribed RNA Standards and Two-Step qRT-PCR
35 Res. J. Biol. Sci. 5, 564-570.

Claims

1. A diagnostic kit for a bacterial species comprising an oligonucleotide probe capable of binding to at least a portion of the *ssrA* gene or its corresponding tRNA.
2. A kit as claimed in claim 1 further comprising a primer for amplification of at least
5 a portion of the *ssrA* gene.
3. A kit as claimed in claim 2 comprising a forward and a reverse primer for a portion of the *ssrA* gene.
4. A kit as claimed in any of claims 1 to 3 wherein the portion of the *ssrA* gene is a
10 portion of the region of the gene from base pair position 1 to base pair position 356 of the *ssrA* gene.
5. A kit as claimed in claim 4 wherein the portion of the *ssrA* gene is a portion of the region of the gene from base pair position 4 to base pair position 356 of the *ssrA* gene.
6. A diagnostic kit as claimed in any preceding claim wherein the probe is selected
15 from SEQ IDs NOs 9 to 37, or sequences substantially similar or complementary thereto which can also act as a probe.
7. A kit as claimed in any preceding claim comprising at least one forward *in vitro* amplification primer and at least one reverse *in vitro* amplification primer, the forward amplification primer being selected from the group consisting of SEQ ID
20 NO 1, 3, 5, 7, or sequences substantially similar or complementary thereto which can also act as a forward amplification primer and the reverse amplification primer being selected from the group consisting of SEQ ID NO 2, 4, 6, 8 or sequences substantially similar or complementary thereto which can also act as a reverse amplification primer.
- 25 8. A diagnostic kit as claimed in claim 7, based on direct nucleic acid detection technologies, signal amplification nucleic acid detection technologies, and nucleic acid *in vitro* amplification technologies is selected from one or more of Polymerase Chain Reaction (PCR), Ligase Chain Reaction (LCR), Nucleic Acids Sequence Based Amplification (NASBA), Strand Displacement Amplification (SDA),
30 Transcription Mediated Amplification (TMA), Branched DNA technology (bDNA) and Rolling Circle Amplification Technology (RCAT) or other enzymatic *in vitro* amplification based technologies.

9. A nucleic acid molecule selected from the group consisting of: SEQ ID NO 1 through SEQ ID NO 48 and sequences substantially homologous or substantially complementary thereto or to a portion thereof and having a function in diagnostics based on the *ssrA* gene.
- 5 10. A nucleic acid molecule comprising an oligonucleotide having a sequence substantially homologous to or substantially complementary to a portion of a nucleic acid molecule as claimed in claim 9.
11. A method of detecting a target organism in a test sample comprising the steps of:
 - 10 (i) Mixing the test sample with at least one oligonucleotide probe capable of binding to at least a portion of the *ssrA* gene or its corresponding mRNA under appropriate conditions;
 - (ii) hybridizing under a high stringency conditions any nucleic acid that may be present in the test sample with the oligonucleotide to form a probe:target duplex; and
 - 15 (iii) determining whether a probe:target duplex is present; the presence of the duplex positively identifying the presence of the target organism in the test sample.
12. A method as claimed in claim 11 wherein the probe is selected from the group consisting of SEQ IDs NOs 9 to 37 or sequences substantially homologous or substantially complementary thereto also capable of acting as a probe for the *ssrA* gene.
- 20 13. Use of a nucleic acid molecule as claimed in any one of claims 9 or 10 in a diagnostic assay to detect the presence of one or more staphylococcus species.
14. Use of a kit as claimed in any one of claims 1 to 8 or a nucleic acid molecule as claimed in any one of claims 9 or 10, in a diagnostic assay to measure bacterial titres in a patient.
- 25 15. A method of assessing the efficacy of a treatment regime designed to reduce bacterial titre in a patient comprising use of a kit as claimed in any one of claims 1 to 8 or a nucleic acid molecule as claimed in any one of claims 9 or 10 at one or more stages of the treatment regime.
- 30 16. Use of a kit as claimed in any one of claims 1 to 8 or a nucleic acid molecule as claimed in any one of claims 9 or 10, in a diagnostic assay to measure bacterial contamination in an environment.

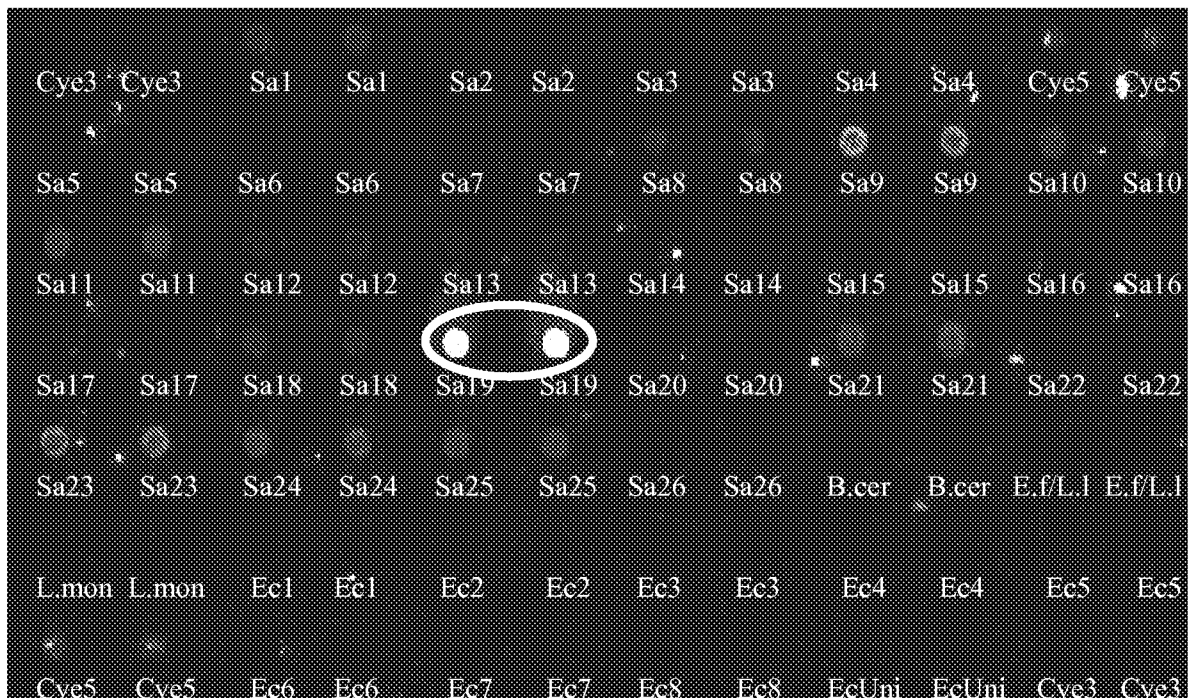
17. Use as claimed in claim 16, wherein the environment is a hospital, a food sample, a milk sample, an environmental sample e.g. water, an industrial sample such as an in-process sample or an end product requiring bioburden or quality assessment.
18. Use of a kit as claimed in any one of claims 1 to 8 or a nucleic acid molecule as
5 claimed in any one of claims 9 or 10, in the identification and/or characterization of one or more disruptive agents that can be used to disrupt the *ssrA* gene function.
19. Use as claimed in claim 18, wherein the disruptive agent is selected from the group consisting of antisense RNA, PNA, siRNA.
20. A kit substantially as described herein with reference to the accompanying figures.
- 10 21. A nucleic acid substantially as described herein with reference to the accompanying figures.
22. A method substantially as described herein with reference to the accompanying figures.

15

20

25

30



5 **Figure 1:** *S. aureus* cRNA microarray sandwich assay with Sa20 reporter probe (Sa19 capture probe circled) showing detection of *S. aureus* cRNA.

Figure 2:

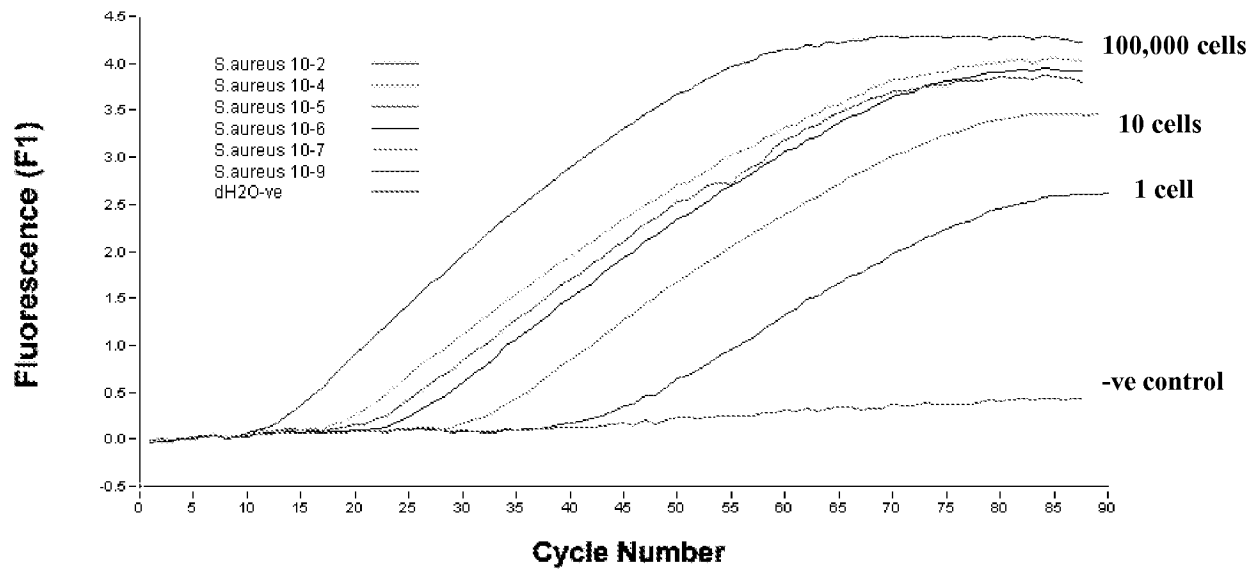


Figure 2: Real-time NASBA of dilutions of *S. aureus* total RNA (10^{-2} = 100,000 cells; 10^{-4} = 10,000 cells; 10^{-5} = 1,000 cells; 10^{-6} = 100 cells; 10^{-7} = 10 cells; 10^{-9} = <1 cell equivalents)

Figure 3:

File: C:\LightCycler3\Users\Watrina Lacey\Data\S.aureus NASBA 8.1.08.ABT Program: Amplification Run By: dna
Run Date: Jan 08, 2008 10:48 Print Date:

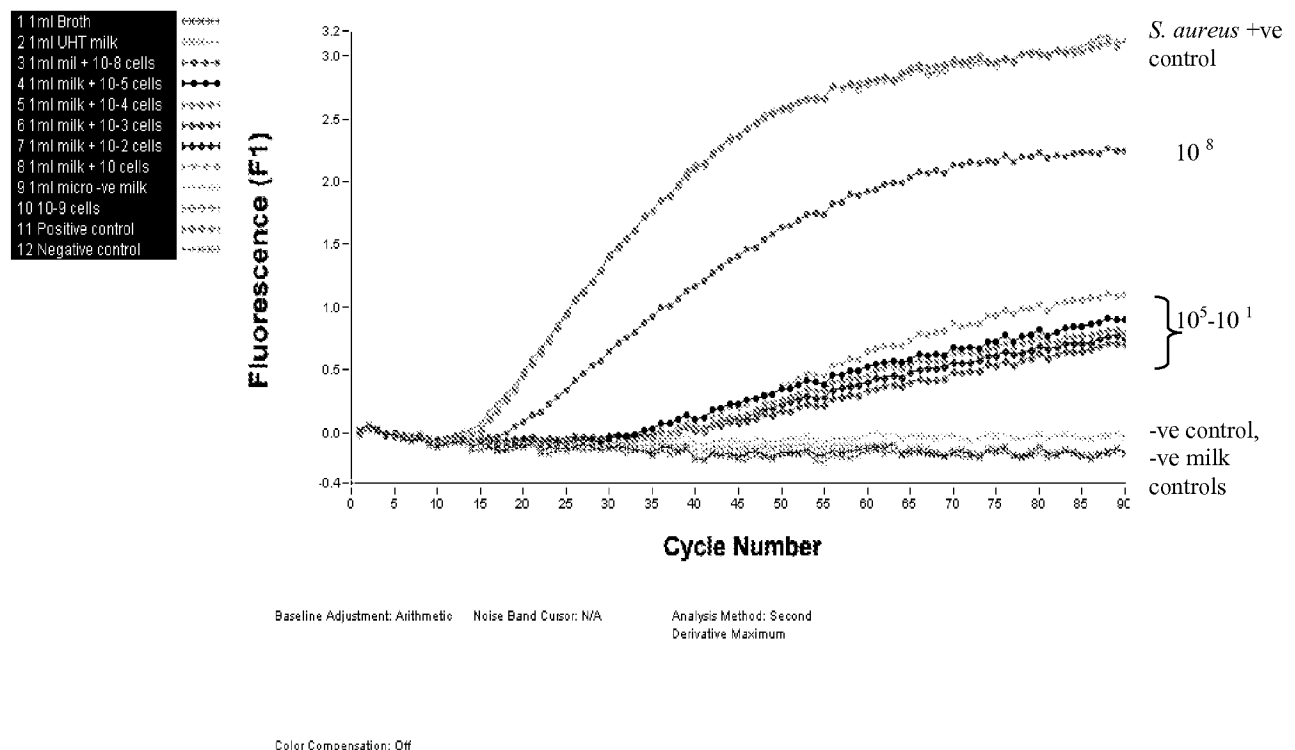


Figure 3: Detection of *S. aureus* in spiked milk samples using the *S. aureus* NASBA assay. Milk samples were spiked with *S. aureus* cells (10^8 , 10^5-10 cells) and RNA extracted and included in the NASBA assay. Limit of detection was 10 *S. aureus* cells/ml. Controls including an *S. aureus* positive control (RNA), a blank broth sample, a UHT milk sample and a NASBA negative control were also included. *S. aureus* was not detected in the blank broth and UHT milk controls.

Figure 4:

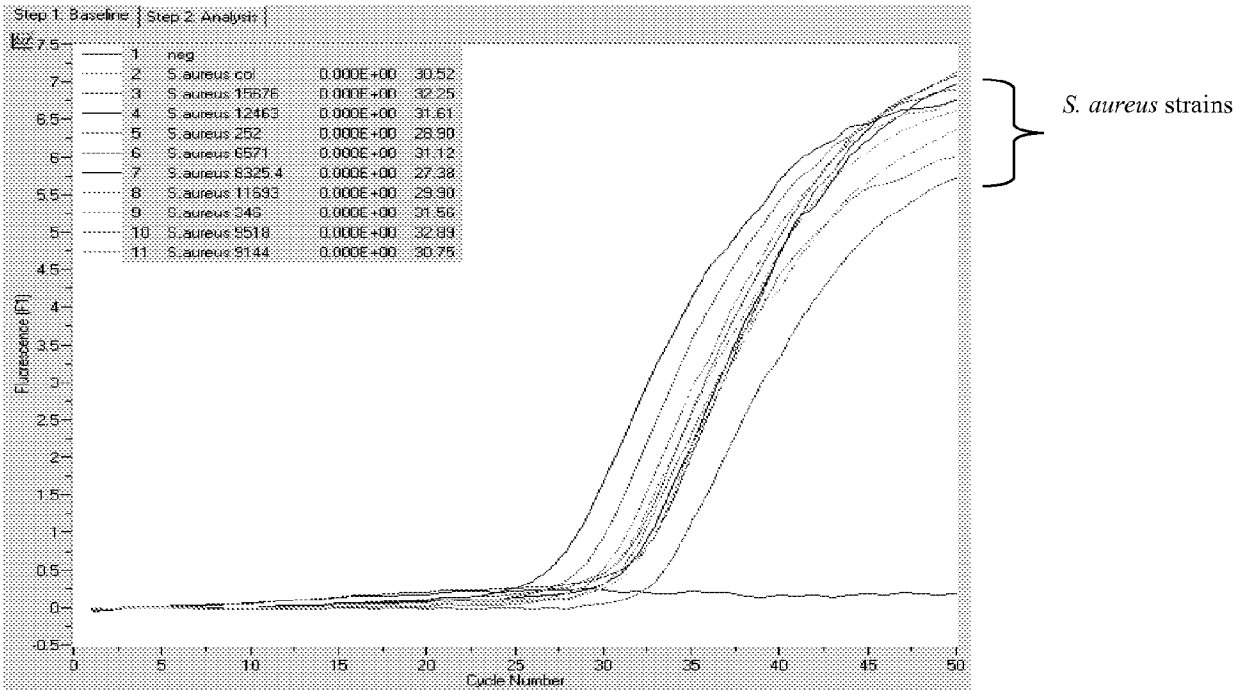


Figure 4: Real-time PCR assay based on the *ssrA* gene specific for *S. aureus* showing detection of 10 *S. aureus* strains.

Figure 5:

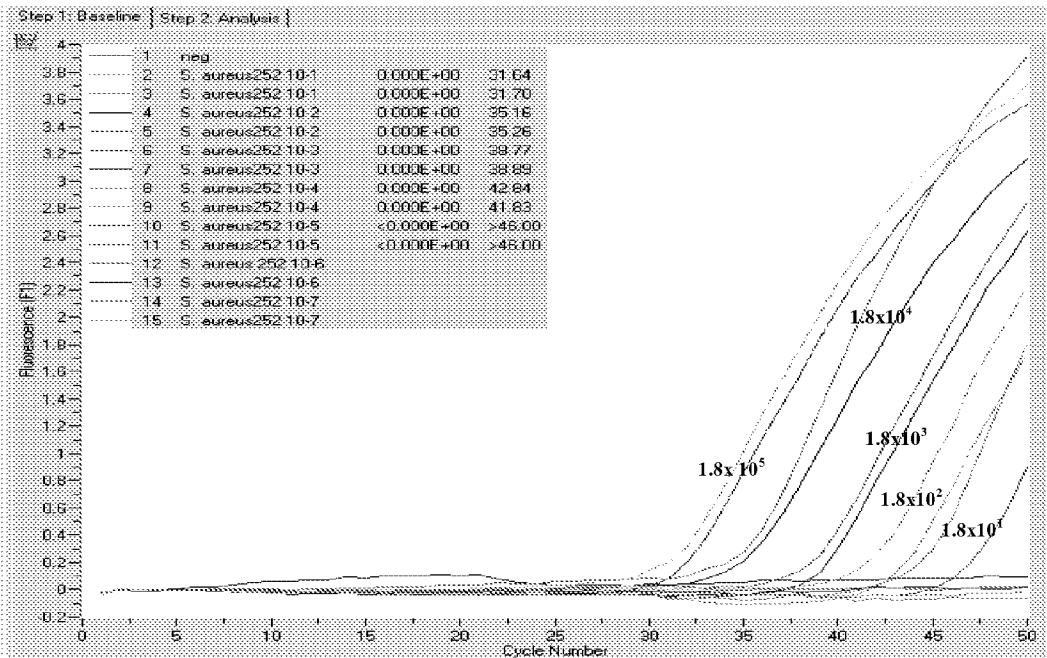


Figure 5: Real-time amplification of *S. aureus* total DNA (10⁻¹ = 180,000 cells; 10⁻² = 18,000 cells; 10⁻³ = 1,800 cells; 10⁻⁴ = 180 cells; 10⁻⁵ = 18 cells; 10⁻⁶ = 1.8 cell equivalents) in the *ssrA* based real-time PCR. Limit of detection is 18 cells. spec2465