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(72) Inventeurs/Inventors:
HULETSKY, ANN, CA;
ROSSBACH, VALERY, CA
(73) Propriétaire/Owner:
GENEOHM SCIENCES CANADA INC., US
(74) Agent: GOUDREAU GAGE DUBUC

(54) Titre : SEQUENCES DE DETECTION ET D'IDENTIFICATION DE STAPHYLOCOCCUS AUREUS RES STANT A LA
METHICILLINE
(54) Title: SEQUENCES FOR THE DETECTION AND IDENTIFICATION OF METHICILLIN-RESISTANT
STAPHYLOCOCCUS AUREUS MREJ TYPE IX STRAINS

(57) **Abrégé/Abstract:**

The present invention describes novel SCCmec right extremity junction (MREJ) sequences for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA). The MRSA strain includes an SCCmec element containing a *mecA* gene inserted into chromosomal DNA, thereby generating a polymorphic MREJ type ix sequence that comprises polymorphic sequences from the SCCmec element right extremity and chromosomal DNA adjoining the extremity. The present invention concerns the use of MREJ type ix sequences for diagnostic purposes and describes a method for detecting the presence of at least one MREJ type ix MRSA strain comprising: a) contacting a sample with a first primer and a second primer to specifically generate an amplicon; and b) detecting the presence of the amplicon. The primers hybridize with polymorphic sequences from the SCCmec element right extremity of an MREJ type ix MRSA strain and with a chromosomal sequence of *S. aureus*.



ABSTRACT

The present invention describes novel SCC*mec* right extremity junction (MREJ) sequences for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA). The MRSA strain includes an SCC*mec* element containing a *mecA* gene inserted into chromosomal DNA, thereby generating a polymorphic MREJ type ix sequence that comprises polymorphic sequences from the SCC*mec* element right extremity and chromosomal DNA adjoining the extremity. The present invention concerns the use of MREJ type ix sequences for diagnostic purposes and describes a method for detecting the presence of at least one MREJ type ix MRSA strain comprising: a) contacting a sample with a first primer and a second primer to specifically generate an amplicon; and b) detecting the presence of the amplicon. The primers hybridize with polymorphic sequences from the SCC*mec* element right extremity of an MREJ type ix MRSA strain and with a chromosomal sequence of *S. aureus*.

TITLE OF THE INVENTION

**SEQUENCES FOR THE DETECTION AND IDENTIFICATION OF
METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* MREJ TYPE
IX STRAINS**

BACKGROUND OF THE INVENTION

Clinical significance of *Staphylococcus aureus*

The coagulase-positive species *Staphylococcus aureus* is well documented as a human opportunistic pathogen. Nosocomial infections caused by *S. aureus* are a major cause of morbidity and mortality. Some of the most common infections caused by *S. aureus* involve the skin, and they include furuncles or boils, cellulitis, impetigo, and postoperative wound infections at various sites. Some of the more serious infections produced by *S. aureus* are bacteremia, pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, cerebritis, meningitis, scalded skin syndrome, and various abscesses. Food poisoning mediated by staphylococcal enterotoxins is another important syndrome associated with *S. aureus*. Toxic shock syndrome, a community-acquired disease, has also been attributed to infection or colonization with toxigenic *S. aureus* (Murray *et al.* Eds, 1999, Manual of Clinical Microbiology, 7th Ed., ASM Press, Washington, D.C.).

Methicillin-resistant *S. aureus* (MRSA) emerged in the 1980s as a major clinical and epidemiologic problem in hospitals. MRSA are resistant to all β -lactams including penicillins, cephalosporins, carbapenems, and monobactams, which are the most commonly used antibiotics to cure *S. aureus* infections. MRSA infections can only be treated with more toxic and more costly antibiotics, which are normally used as the last line of defence. Since MRSA can spread easily from patient to patient via personnel, hospitals over the world are confronted with the

problem to control MRSA. Consequently, there is a need to develop rapid and simple screening or diagnostic tests for detection and/or identification of MRSA to reduce its dissemination and improve the diagnosis and treatment of infected patients.

5 Methicillin resistance in *S. aureus* is unique in that it is due to acquisition of DNA from other coagulase-negative staphylococci (CNS), coding for a supernumerary β -lactam-resistant penicillin-binding protein (PBP), which takes over the biosynthetic functions of the normal PBPs when the cell is exposed to β -lactam
0 antibiotics. *S. aureus* normally contains four PBPs, of which PBPs 1, 2 and 3 are essential. The low-affinity PBP in MRSA, termed PBP 2a (or PBP2'), is encoded by the chromosomal *mecA* gene and functions as a β -lactam-resistant transpeptidase. The *mecA* gene is absent from methicillin-sensitive *S. aureus* but is widely distributed among other species of staphylococci and is highly conserved
5 (Ubukata *et al.*, 1990, Antimicrob. Agents Chemother. **34**:170-172).

By nucleotide sequence determination of the DNA region surrounding the *mecA* gene from *S. aureus* strain N315 (isolated in Japan in 1982), Ito & Hiramatsu (Ito & Hiramatsu 1998, Yonsei Med J **39** (6):526-533) have found that the *mecA* gene is carried by a novel
0 genetic element, designated staphylococcal cassette chromosome *mec* (SCC*mec*), inserted into the chromosome. SCC*mec* is a mobile genetic element characterized by the presence of terminal inverted and direct repeats, a set of site-specific recombinase genes (*ccrA* and *ccrB*), and the *mecA* gene complex (Ito *et al.*, 1999, Antimicrob. Agents Chemother. **43**:1449-1458; Katayama *et al.*, 2000, Antimicrob. Agents Chemother.
5 **44**:1549-1555). The element is precisely excised from the chromosome of *S. aureus* strain N315 and integrates into a specific *S. aureus* chromosomal site in the same orientation through the function of a unique set of recombinase genes comprising *ccrA* and *ccrB*. Two novel genetic elements that shared similar structural features of SCC*mec* were found by cloning and sequencing the DNA

region surrounding the *mecA* gene from MRSA strains NCTC 10442 (the first MRSA strain isolated in England in 1961) and 85/2082 (a strain from New Zealand isolated in 1985). The three SCC*mec* have been designated type I (NCTC 10442), type II (N315) and type III (85/2082) based on the year of isolation of the strains (Ito *et al.*, 2001, Antimicrob. Agents Chemother. **45**:1323-1336) (Figure 1). Ma *et al.* (Ma *et al.* 2002, Antimicrob. Agents Chemother. **46**:1147-1152) have found that the SCC*mec* DNAs are integrated at a specific site in the methicillin-sensitive *S. aureus* (MSSA) chromosome. They characterized the nucleotide sequences of the regions around the left and right boundaries of SCC*mec* DNA (i.e. *attL* and *attR*, respectively) as well as those of the regions around the SCC*mec* DNA integration site (i.e. *attB_{scc}* which is the bacterial chromosome attachment site for SCC*mec* DNA). The *attB_{scc}* site was located at the 3' end of a novel open reading frame (ORF), *orfX*. The *orfX* potentially encodes a 159-amino acid polypeptide sharing identity with some previously identified polypeptides, but of unknown function (Ito *et al.*, 1999, Antimicrob. Agents Chemother. **43**:1449-1458). Recently, a new type of SCC*mec* (type IV) has been described by both Ma *et al.* (Ma *et al.*, 2002, Antimicrob. Agents Chemother. **46**:1147-1152) and Oliveira *et al.* (Oliveira *et al.*, 2001, Microb. Drug Resist. **7**:349-360). The sequences of the right extremity of the new type IV SCC*mec* from *S. aureus* strains CA05 and 8/6-3P published by Ma *et al.* (Ma *et al.*, 2002, Antimicrob. Agents Chemother. **46**:1147-1152) were nearly identical over 2000 nucleotides to that of type II SCC*mec* of *S. aureus* strain N315 (Ito *et al.*, 2001, Antimicrob. Agents Chemother. **45**:1323-1336). No sequence at the right extremity of the SCC*mec* type IV is available from the *S. aureus* strains HDE288 and PL72 described by Oliveira *et al.* (Oliveira *et al.*, 2001, Microb. Drug Resist. **7**:349-360).

Previous methods used to detect and identify MRSA (Saito *et al.*, 1995, J. Clin. Microbiol. **33**:2498-2500; Ubukata *et al.*, 1992, J. Clin. Microbiol. **30**:1728-1733; Murakami *et al.*, 1991, J. Clin. Microbiol. **29**:2240-2244; Hiramatsu *et al.*, 1992,

Microbiol. Immunol. **36**:445-453), which are based on the detection of the *mecA* gene and *S. aureus*-specific chromosomal sequences, encountered difficulty in discriminating MRSA from methicillin-resistant coagulase-negative staphylococci (CNS) because the *mecA* gene is widely distributed in both *S. aureus* and CNS species (Suzuki *et al.*, 1992, Antimicrob. Agents. Chemother. **36**:429-434). Hiramatsu *et al.* (US patent 6,156,507) have described a PCR assay specific for MRSA by using primers that can specifically hybridize to the right extremities of the 3 types of SCC*mec* DNAs in combination with a primer specific to the *S. aureus* chromosome, which corresponds to the nucleotide sequence on the right side of the SCC*mec* integration site. Since nucleotide sequences surrounding the SCC*mec* integration site in other staphylococcal species (such as *S. epidermidis* and *S. haemolyticus*) are different from those found in *S. aureus*, this PCR assay was specific for the detection of MRSA. This PCR assay also supplied information for MREP typing (standing for «*mec* right extremity polymorphism») of SCC*mec* DNA (Ito *et al.*, 2001, Antimicrob. Agents Chemother. **45**:1323-1336; Hiramatsu *et al.*, 1996, J. Infect. Chemother. **2**:117-129). This typing method takes advantage of the polymorphism at the right extremity of SCC*mec* DNAs adjacent to the integration site among the three types of SCC*mec*. Type III has a unique nucleotide sequence while type II has an insertion of 102 nucleotides to the right terminus of SCC*mec* type I. The MREP typing method described by Hiramatsu *et al.* (Ito *et al.*, 2001, Antimicrob. Agents Chemother. **45**:1323-1336; Hiramatsu *et al.*, 1996, J. Infect. Chemother. **2**:117-129) defines the SCC*mec* type I as MREP type i, SCC*mec* type II as MREP type ii and SCC*mec* type III as MREP type iii. It should be noted that the MREP typing method cannot differentiate the new SCC*mec* type IV described by Ma *et al.* (Ma *et al.*, 2002, Antimicrob. Agents Chemother. **46**:1147-1152) from SCC*mec* type II because these two SCC*mec* types exhibit the same nucleotide sequence to the right extremity.

The set of primers described by Hiramatsu *et al.* (Hiramatsu *et al.*, 1996 Infect Chem 2: 117-129) as being the optimal primer combination (SEQ ID NOs.: 22, 24, 28 in US patent 6,156,507 corresponding to SEQ ID NOs.: 56, 58 and 60, respectively, in the present invention) have been used in the present invention to test by PCR a variety of MRSA and
 5 MSSA strains (Figure 1 and Table 1). Twenty of the 39 MRSA strains tested were not amplified by the Hiramatsu *et al.* multiplex PCR assay (Tables 2 and 3). Hiramatsu's method indeed was successful in detecting less than 50% of the tested 39 MRSA strains.

This finding demonstrates that some MRSA strains have sequences at the right extremity of SCC*mec*-chromosome right extremity junction different from those identified by
 0 Hiramatsu *et al.* (Hiramatsu *et al.*, 1996 Infect Chem 2: 117-119). Consequently, the system developed by Hiramatsu *et al.* does not allow the detection of all MRSA. The present invention relates to the generation of SCC*mec*-chromosome right extremity junction sequence data required to detect more MRSA strains in order to improve the Hiramatsu *et al.* assay. There is a need for developing more ubiquitous
 5 primers and probes for the detection of most MRSA strains around the world.

SUMMARY OF THE INVENTION

0 It is an object of the present invention to provide a specific, ubiquitous and sensitive method using probes and/or amplification primers for determining the presence and/or amount of nucleic acids from all MRSA strains.

Ubiquity of at least 50% amongst the strains representing MRSA strains types IV
 5 to X is an objective of this invention.

Therefore, in accordance with the present invention is provided a method to detect the presence of a methicillin-resistant *Staphylococcus aureus* (MRSA) strain in a sample, the MRSA strain being resistant because of the presence of an SCC*mec*

insert containing a *mecA* gene, said *SCC_{mec}* being inserted in bacterial nucleic acids thereby generating a polymorphic right extremity junction (MREJ), the method comprising the step of annealing the nucleic acids of the sample with a plurality of probes and/or primers, characterized by:

- 5 (i) the primers and/or probes are specific for MRSA strains and capable of annealing with polymorphic MREJ nucleic acids, the polymorphic MREJ comprising MREJ types i to x; and
- (ii) the primers and/or probes altogether can anneal with at least four MREJ types selected from MREJ types i to x.

0 In a specific embodiment, the primers and/or probes are all chosen to anneal under common annealing conditions, and even more specifically, they are placed altogether in the same physical enclosure.

A specific method has been developed using primers and/or probes having at least 10 nucleotides in length and capable of annealing with MREJ types i to iii, defined
 5 in any one of SEQ ID NOs: 1, 20, 21, 22, 23, 24, 25, 41, 199, 2, 17, 18, 19, 26, 40, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 185, 186, 197, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 104, 184, 198 and with one or more of MREJ/MREP types iv to ix, having SEQ ID NOs: 42, 43, 44, 45, 46, 51 ; 47, 48, 49, 50, 171, 165, 166, 167, 168. To be perfectly ubiquitous with the all the sequenced MREJs,
 0 the primers and/or probes altogether can anneal with said SEQ ID NOs of MREJ types i to ix.

The following specific primers and/or probes having the following sequences have been designed:

66, 100, 101, 105, 52, 53, 54, 55, 5 56, 57, 64, 71, 72, 73, 74, 75, 76, 70, 103, 130, 132, 158, 159, 59, 62, 126, 127, 128, 129, 131, 200, 201, 60, 61, 63 32, 83, 84, 160, 161, 162, 163, 164 3 85, 86, 87, 88, 89	for the detection of MREJ type i
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66, 97, 99, 100, 101, 106, 117, for the detection of MREJ type ii
 118, 124, 125, 52, 53, 54, 55, 56, 57
 64, 71, 72, 73, 74, 75, 76, 70,
 103, 130, 132, 158, 159
 5 59, 62
 126, 127
 128, 129, 131, 200, 201
 60, 61, 63
 32, 83, 84, 160, 161, 162, 163, 164
 10 85, 86, 87, 88, 89

67, 98, 102, 107, 108 for the detection of MREJ type iii
 64, 71, 72, 73, 74, 75, 76, 70,
 103, 130, 132, 158, 159
 (15 58,
 (59, 62
 126, 127
 128, 129, 131, 200, 201
 60, 61, 63
 20 32, 83, 84, 160, 161, 162, 163, 164
 85, 86, 87, 88, 89

79, 77, 145, 147 for the detection of MREJ type iv
 64, 71, 72, 73, 74, 75, 76, 70,
 25 103, 130, 132, 158, 159
 59, 62
 (126, 127
 (128, 129, 131, 200, 201
 60, 61, 63
 30 68
 32, 83, 84, 160, 161, 162, 163, 164
 85, 86, 87, 88, 89

65, 80, 146, 154, 155 for the detection of MREJ type v
 35 64, 71, 72, 73, 74, 75, 76,
 70, 103, 130, 132, 158, 159
 59, 62
 126, 127
 128, 129, 131, 200, 201
 40 60, 61, 63
 32, 83, 84, 160, 161, 162, 163, 164
 85, 86, 87, 88, 89

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202, 203, 204

for the detection of MREJ type vi

64, 71, 72, 73, 74, 75, 76, 70,

103, 130, 132, 158, 159

59, 62

5 126, 127

128, 129, 131, 200, 201

60, 61, 63

32, 83, 84, 160, 161, 162, 163, 164

85, 86, 87, 88, 89

10

112, 113, 114, 119, 120, 121, 122

for the detection of MREJ type vii,

123, 150, 151, 153

64, 71, 72, 73, 74, 75, 76, 70, 103,

130, 132, 158, 159

(15 59, 62

126, 127

128, 129, 131, 200, 201

60, 61, 63

32, 83, 84, 160, 161, 162, 163, 164

20 85, 86, 87, 88, 89

115, 116, 187, 188, 207, 208 for the detection of MREJ type viii

64, 71, 72, 73, 74, 75, 76, 70,

103, 130, 132, 158, 159

25 59, 62

126, 127

128, 129, 131, 200, 201

60, 61, 63

32, 83, 84, 160, 161, 162, 163, 164

30 85, 86, 87, 88, 89

109, 148, 149, 205, 206

for the detection of MREJ type ix.

64, 71, 72, 73, 74, 75, 76

70, 103, 130, 132, 158, 159

35 59, 62

126, 127

128, 129, 131, 200, 201

60, 61, 63

32, 83, 84, 160, 161, 162, 163, 164

40 85, 86, 87, 88, 89

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Amongst these, the following primer pairs having the following sequences are used:

- | | | |
|----|---------------------------------------|-------------------------------------|
| | 64/66, 64/100, 64/101; 59/52, | for the detection of type i MREJ |
| | 59/53, 59/54, 59/55, 59/56, 59/57, | |
| 5 | 60/52, 60/53, 60/54, 60/55, 60/56 | |
| | 60/57, 61/52, 61/53, 61/54, 61/55 | |
| | 61/56, 61/57, 62/52, 62/53, 62/54 | |
| | 62/55, 62/56, 62/57, 63/52, 63/53 | |
| | 63/54, 63/55, 63/56, 63/57 | |
| 10 | | |
| | 64/66, 64/97, 64/99, 64/100, 64/101 | for the detection of type ii MREJ |
| | 59/52, 59/53, 59/54, 59/55, 59/56, | |
| | 59/57, 60/52, 60/53, 60/54, 60/55, | |
| (| 60/56, 60/57, 61/52, 61/53, 61/54, | |
| (| 15 61/55, 61/56, 61/57, 62/52, 62/53, | |
| | 62/54, 62/55, 62/56, 62/57, 63/52 | |
| | 63/53, 63/54, 63/55, 63/56, 63/57 | |
| | | |
| | 64/67, 64/98, 64/102 ; 59/58, | for the detection of type iii MREJ |
| 20 | 60/58, 61/58, 62/58, 63/58 | |
| | | |
| | 64/79 | for the detection of type iv MREJ |
| | 64/80 | for the detection of type v MREJ |
| | 64/204 | for the detection of type vi MREJ |
| (| | |
| 25 | 64/112, 64/113 | for the detection of type vii MREJ |
| | 64/115, 64/116 | for the detection of type viii MREJ |
| | 64/109 | for the detection of type ix MREJ |

As well, amongst these, the following probes having the following sequences are used:

SEQ ID NOs: 32, 83, 84, 160, 161, 162, 163, 164 for the detection of MREJ types i to ix.

In the most preferred embodied method, the following primers and/or probes having the following nucleotide sequences are used together. The preferred combinations make use of:

- 5 i) SEQ ID NOs: 64, 66, 84, 163, 164 for the detection of MREJ type i
- ii) SEQ ID NOs: 64, 66, 84, 163, 164 for the detection of MREJ type ii
- iii) SEQ ID NOs: 64, 67, 84, 163, 164 for the detection of MREJ type iii
- iv) SEQ ID NOs: 64, 79, 84, 163, 164 for the detection of MREJ type iv
- v) SEQ ID NOs: 64, 80, 84, 163, 164 for the detection of MREJ type v
- 0 vi) SEQ ID NOs: 64, 112, 84, 163, 164 for the detection of MREJ type
- vii.

All these probes and primers can even be used together in the same physical enclosure.

- 5 It is another object of this invention to provide a method for typing a MREJ of a MRSA strain, which comprises the steps of: reproducing the above method with primers and/or probes specific for a determined MREJ type, and detecting an annealed probe or primer as an indication of the presence of a determined MREJ type.

- 0 It is further another object of this invention to provide a nucleic acid selected from SEQ ID NOs:

- i) SEQ ID NOs: 43, 44, 45, 46, 51 for sequence of MREJ type iv ;
- ii) SEQ ID NOs: 47, 48, 49, 50 for sequence of MREJ type v ;
- iii) SEQ ID NOs: 171 for sequence of MREJ type vi ;
- 5 iv) SEQ ID NOs: 165, 166 for sequence of MREJ type vii ;
- v) SEQ ID NOs: 167 for sequence of MREJ type viii ;
- vi) SEQ ID NOs: 168 for sequence of MREJ type ix.

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Oligonucleotides of at least 10 nucleotides in length which hybridize with any of these nucleic acids and which hybridize with one or more MREJ of types selected from iv to ix are also objects of this invention. Amongst these, primer pairs (or probes) having the following SEQ ID NOs:

5 64/66, 64/100, 64/101; 59/52, for the detection of type i MREJ

59/53, 59/54, 59/55, 59/56, 59/57,

60/52, 60/53, 60/54, 60/55, 60/56

60/57, 61/52, 61/53, 61/54, 61/55

61/56, 61/57, 62/52, 62/53, 62/54

10 62/55, 62/56, 62/57, 63/52, 63/53

63/54, 63/55, 63/56, 63/57

64/66, 64/97, 64/99, 64/100, 64/101 for the detection of type ii MREJ

59/52, 59/53, 59/54, 59/55, 59/56,

15 59/57, 60/52, 60/53, 60/54, 60/55,

60/56, 60/57, 61/52, 61/53, 61/54,

61/55, 61/56, 61/57, 62/52, 62/53,

62/54, 62/55, 62/56, 62/57, 63/52

63/53, 63/54, 63/55, 63/56, 63/57

20

64/67, 64/98, 64/102 ; 59/58, for the detection of type iii MREJ

60/58, 61/58, 62/58, 63/58

64/79 for the detection of type iv MREJ

25 64/80 for the detection of type v MREJ

64/204 for the detection of type vi MREJ

64/112, 64/113 for the detection of type vii MREJ

64/115, 64/116 for the detection of type viii MREJ

64/109 for the detection of type ix MREJ,

30 are also within the scope of this invention.

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Further, internal probes having nucleotide sequences defined in any one of SEQ ID NOs: 32, 83, 84, 160, 161, 162, 163, 164, are also within the scope of this invention. Compositions of matter comprising the primers and/or probes annealing or hybridizing with one or more MREJ of types selected from iv to ix as well as with
 5 the above nucleic acids, comprising or not primers and/or probes, which hybridize with one or more MREJ of types selected from i to iii, are further objects of this invention. The preferred compositions would comprise the primers having the nucleotide sequences defined in SEQ ID NOs:

- | | | |
|----|-------------------------------------|-------------------------------------|
| | 64/66, 64/100, 64/101; 59/52, | for the detection of type i MREJ |
| 10 | 59/53, 59/54, 59/55, 59/56, 59/57, | |
| | 60/52, 60/53, 60/54, 60/55, 60/56 | |
| | 60/57, 61/52, 61/53, 61/54, 61/55 | |
| | 61/56, 61/57, 62/52, 62/53, 62/54 | |
| | 62/55, 62/56, 62/57, 63/52, 63/53 | |
| 15 | 63/54, 63/55, 63/56, 63/57 | |
| | | |
| | 64/66, 64/97, 64/99, 64/100, 64/101 | for the detection of type ii MREJ |
| | 59/52, 59/53, 59/54, 59/55, 59/56, | |
| | 59/57, 60/52, 60/53, 60/54, 60/55, | |
| 20 | 60/56, 60/57, 61/52, 61/53, 61/54, | |
| | 61/55, 61/56, 61/57, 62/52, 62/53, | |
| | 62/54, 62/55, 62/56, 62/57, 63/52 | |
| | 63/53, 63/54, 63/55, 63/56, 63/57 | |
| | | |
| 25 | 64/67, 64/98, 64/102 ; 59/58, | for the detection of type iii MREJ |
| | 60/58, 61/58, 62/58, 63/58 | |
| | | |
| | 64/79 | for the detection of type iv MREJ |
| | 64/80 | for the detection of type v MREJ |
| 30 | 64/204 | for the detection of type vi MREJ |
| | 64/112, 64/113 | for the detection of type vii MREJ |
| | 64/115, 64/116 | for the detection of type viii MREJ |
| | 64/109 | for the detection of type ix MREJ, |

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or probes, which SEQ ID NOs are: 32, 83, 84, 160, 161, 162, 163, 164, or both.

5

DETAILED DESCRIPTION OF THE INVENTION

Here is particularly provided a method wherein each of MRSA nucleic acids or a variant or part thereof comprises a selected target region hybridizable with said primers or probes developed to be ubiquitous;

10 wherein each of said nucleic acids or a variant or part thereof comprises a
(selected target region hybridizable with said primers or probes ;

 said method comprising the steps of contacting said sample with said probes
or primers and detecting the presence and/or amount of hybridized probes or
amplified products as an indication of the presence and/or amount of MRSA.

15

In the method, sequences from DNA fragments of SCC_{mec}-chromosome right
extremity junction, thereafter named MREJ standing for « *mec* right extremity
junction » including sequences from SCC_{mec} right extremity and chromosomal
DNA to the right of the SCC_{mec} integration site are used as parental sequences
20 from which are derived the primers and/or the probes. MREJ sequences include
our proprietary sequences as well as sequences obtained from public databases and
from US patent 6,156,507 and were selected for their capacity to sensitively,
specifically, ubiquitously and rapidly detect the targeted MRSA nucleic acids.

25 Our proprietary DNA fragments and oligonucleotides (primers and probes) are also
another object of this invention.

Composition of matters such as diagnostic kits comprising amplification primers or
probes for the detection of MRSA are also objects of the present invention.

In the above methods and kits, probes and primers are not limited to nucleic acids and may include, but are not restricted to, analogs of nucleotides. The diagnostic reagents constituted by the probes and the primers may be present in any suitable
 5 form (bound to a solid support, liquid, lyophilized, etc.).

In the above methods and kits, amplification reactions may include but are not restricted to: a) polymerase chain reaction (PCR), b) ligase chain reaction (LCR), c) nucleic acid sequence-based amplification (NASBA), d) self-sustained sequence
 0 replication (3SR), e) strand displacement amplification (SDA), f) branched DNA signal amplification (bDNA), g) transcription-mediated amplification (TMA), h) cycling probe technology (CPT), i) nested PCR, j) multiplex PCR, k) solid phase amplification (SPA), l) nuclease dependent signal amplification (NDSA), m) rolling circle amplification technology (RCA), n) Anchored strand displacement
 5 amplification, o) Solid-phase (immobilized) rolling circle amplification.

In the above methods and kits, detection of the nucleic acids of target genes may include real-time or post-amplification technologies. These detection technologies can include, but are not limited to fluorescence resonance energy transfer (FRET)-
 0 based methods such as adjacent hybridization of probes (including probe-probe and probe-primer methods), *TaqMan* probe, molecular beacon probe, Scorpion probe, nanoparticle probe and Amplifluor™ probe. Other detection methods include target gene nucleic acids detection via immunological methods, solid phase hybridization methods on filters, chips or any other solid support. In these systems,
 5 the hybridization can be monitored by fluorescence, chemiluminescence, potentiometry, mass spectrometry, plasmon resonance, polarimetry, colorimetry, flow cytometry or scanometry. Nucleotide sequencing, including sequencing by dideoxy termination or sequencing by hybridization (e.g. sequencing using a DNA

chip) represents another method to detect and characterize the nucleic acids of target genes.

In a preferred embodiment, a PCR protocol is used for nucleic acid amplification.

5

A method for detection of a plurality of potential MRSA strains having different MREJ types may be conducted in separate reactions and physical enclosures, one type at the time. Alternatively, it could be conducted simultaneously for different types in separate physical enclosures, or in the same physical enclosures. In the latter scenario a multiplex PCR reaction could be conducted which would require that the oligonucleotides are all capable of annealing with a target region under common conditions. Since many probes or primers are specific for a determined MREJ/MREP type, typing a MRSA strain is a possible embodiment. When a mixture of oligonucleotides annealing together with more than one type is used in a single physical enclosure or container, different labels would be used to distinguish one type from another.

We aim at developing a DNA-based test or kit to detect and identify MRSA. Although the sequences from *orfX* genes and some *SCCmec* DNA fragments are available from public databases and have been used to develop DNA-based tests for detection of MRSA, new sequence data allowing to improve MRSA detection and identification which are object of the present invention have either never been characterized previously or were known but not shown to be located at the right extremity of *SCCmec* adjacent to the integration site (Table 4). These novel sequences could not have been predicted nor detected by the MRSA-specific PCR assay developed by Hiramatsu *et al.* (US patent 6,156,507). These sequences will allow to improve current DNA-based tests for the diagnosis of MRSA because they allow the design of ubiquitous primers and probes for the detection and

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identification of more MRSA strains including all the major epidemic clones from around the world.

The diagnostic kits, primers and probes mentioned above can be used to detect
 5 and/or identify MRSA, whether said diagnostic kits, primers and probes are used for *in vitro* or *in situ* applications. The said samples may include but are not limited to: any clinical sample, any environmental sample, any microbial culture, any microbial colony, any tissue, and any cell line.

(10 It is also an object of the present invention that said diagnostic kits, primers and
 (probes can be used alone or in combination with any other assay suitable to detect and/or identify microorganisms, including but not limited to: any assay based on nucleic acids detection, any immunoassay, any enzymatic assay, any biochemical assay, any lysotypic assay, any serological assay, any differential culture medium,
 15 any enrichment culture medium, any selective culture medium, any specific assay medium, any identification culture medium, any enumeration culture medium, any cellular stain, any culture on specific cell lines, and any infectivity assay on animals.

(20 In the methods and kits described herein below, the oligonucleotide probes and
 (amplification primers have been derived from larger sequences (i.e. DNA fragments of at least 100 base pairs). All DNA sequences have been obtained either from our proprietary sequences or from public databases (Tables 5, 6, 7, 8 and 9).

25

It is clear to the individual skilled in the art that oligonucleotide sequences other than those described in the present invention and which are appropriate for detection and/or identification of MRSA may also be derived from the proprietary fragment sequences or selected public database sequences. For example, the

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oligonucleotide primers or probes may be shorter but of a length of at least 10 nucleotides or longer than the ones chosen; they may also be selected anywhere else in the proprietary DNA fragments or in the sequences selected from public databases; they may also be variants of the same oligonucleotide. If the target DNA or a variant thereof hybridizes to a given oligonucleotide, or if the target DNA or a variant thereof can be amplified by a given oligonucleotide PCR primer pair, the converse is also true; a given target DNA may hybridize to a variant oligonucleotide probe or be amplified by a variant oligonucleotide PCR primer. Alternatively, the oligonucleotides may be designed from said DNA fragment sequences for use in amplification methods other than PCR. Consequently, the core of this invention is the detection and/or identification of MRSA by targeting genomic DNA sequences which are used as a source of specific and ubiquitous oligonucleotide probes and/or amplification primers. Although the selection and evaluation of oligonucleotides suitable for diagnostic purposes require much effort, it is quite possible for the individual skilled in the art to derive, from the selected DNA fragments, oligonucleotides other than the ones listed in Tables 5, 6, 7, 8 and 9 which are suitable for diagnostic purposes. When a proprietary fragment or a public database sequence is selected for its specificity and ubiquity, it increases the probability that subsets thereof will also be specific and ubiquitous.

20

The proprietary DNA fragments have been obtained as a repertory of sequences created by amplifying MRSA nucleic acids with new primers. These primers and the repertory of nucleic acids as well as the repertory of nucleotide sequences are further objects of this invention (Tables 4, 5, 6, 7, 8 and 9).

25

Claims therefore are in accordance with the present invention.

SEQUENCES FOR DETECTION AND IDENTIFICATION OF MRSA

In the description of this invention, the terms «nucleic acids» and «sequences» might be used interchangeably. However, «nucleic acids» are chemical entities while «sequences» are the pieces of information encoded by these «nucleic acids». Both nucleic acids and sequences are equivalently valuable sources of information for the matter pertaining to this invention.

0 Oligonucleotide primers and probes design and synthesis

As part of the design rules, all oligonucleotides (probes for hybridization and primers for DNA amplification by PCR) were evaluated for their suitability for hybridization or PCR amplification by computer analysis using standard programs (i.e. the GCG Wisconsin package programs, the primer analysis software Oligo™ 6 and MFOLD 3.0). The potential suitability of the PCR primer pairs was also evaluated prior to their synthesis by verifying the absence of unwanted features such as long stretches of one nucleotide and a high proportion of G or C residues at the 3' end (Persing *et al.*, 1993, Diagnostic Molecular Microbiology: Principles and Applications, American Society for Microbiology, Washington, D.C.). Oligonucleotide amplification primers were synthesized using an automated DNA synthesizer (Applied Biosystems). Molecular beacon designs were evaluated using criteria established by Tyagi and Kramer (Tyagi & Kramer 1996, Nat Biotechnol **14**:303-308).

5 The oligonucleotide sequence of primers or probes may be derived from either strand of the duplex DNA. The primers or probes may consist of the bases A, G, C, or T or analogs and they may be degenerated at one or more chosen nucleotide position(s) (Nichols *et al.*, 1994, Nature **369**:492-493). Primers and probes may also consist of nucleotide analogs such as Locked Nucleic Acids (LNA) (Koskin *et*

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al., 1998, Tetrahedron **54**:3607-3630), and Peptide Nucleic Acids (PNA) (Egholm *et al.*, 1993, Nature **365**:566-568). The primers or probes may be of any suitable length and may be selected anywhere within the DNA sequences from proprietary fragments, or from selected database sequences which are suitable for the detection
5 of MRSA.

Variants for a given target microbial gene are naturally occurring and are attributable to sequence variation within that gene during evolution (Watson *et al.*, 1987, Molecular Biology of the Gene, 4th ed., The Benjamin/Cummings Publishing
10 Company, Menlo Park, CA; Lewin, 1989, Genes IV, John Wiley & Sons, New York, NY). For example, different strains of the same microbial species may have a single or more nucleotide variation(s) at the oligonucleotide hybridization site. The person skilled in the art is well aware of the existence of variant nucleic acids and/or sequences for a specific gene and that the frequency of sequence variations
15 depends on the selective pressure during evolution on a given gene product. The detection of a variant sequence for a region between two PCR primers may be demonstrated by sequencing the amplification product. In order to show the presence of sequence variations at the primer hybridization site, one has to amplify a larger DNA target with PCR primers outside that hybridization site. Sequencing
20 of this larger fragment will allow the detection of sequence variation at this primer hybridization site. A similar strategy may be applied to show variations at the hybridization site of a probe. Insofar as the divergence of the target nucleic acids and/or sequences or a part thereof does not affect significantly the sensitivity and/or specificity and/or ubiquity of the amplification primers or probes, variant
25 microbial DNA is under the scope of this invention. Variants of the selected primers or probes may also be used to amplify or hybridize to a variant target DNA.

DNA amplification

For DNA amplification by the widely used PCR method, primer pairs were derived from our proprietary DNA fragments or from public database sequences.

5

During DNA amplification by PCR, two oligonucleotide primers binding respectively to each strand of the heat-denatured target DNA from the microbial genome are used to amplify exponentially *in vitro* the target DNA by successive thermal cycles allowing denaturation of the DNA, annealing of the primers and
0 synthesis of new targets at each cycle (Persing *et al*, 1993, Diagnostic Molecular Microbiology: Principles and Applications, American Society for Microbiology, Washington, D.C.).

Briefly, the PCR protocols on a standard thermocycler (PTC-200 from MJ
5 Research Inc., Watertown, MA) were as follows: Treated standardized bacterial suspensions or genomic DNA prepared from bacterial cultures or clinical specimens were amplified in a 20 µl PCR reaction mixture. Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2.5 mM MgCl₂, 0.4 µM of each primer, 200 µM of each of the four dNTPs (Pharmacia Biotech), 3.3 µg/µl bovine
0 serum albumin (BSA) (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) and 0.5 unit of *Taq* DNA polymerase (Promega Corp., Madison, WI) combined with the *TaqStart*TM antibody (BD Biosciences, Palo Alto, CA). The *TaqStart*TM antibody, which is a neutralizing monoclonal antibody to *Taq* DNA polymerase, was added to all PCR reactions to enhance the specificity and the sensitivity of the
5 amplifications (Kellogg *et al.*, 1994, Biotechniques **16**:1134-1137). The treatment of bacterial cultures or of clinical specimens consists in a rapid protocol to lyse the microbial cells and eliminate or neutralize PCR inhibitors (described in application WO 03/008636). For amplification from purified genomic DNA, the samples were added directly to the PCR amplification mixture. An internal control,

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derived from sequences not found in the target MREJ sequences or in the human genome, was used to verify the efficiency of the PCR reaction and the absence of significant PCR inhibition.

5 The number of cycles performed for the PCR assays varies according to the sensitivity level required. For example, the sensitivity level required for microbial detection directly from a clinical specimen is higher than for detection from a microbial culture. Consequently, more sensitive PCR assays having more thermal cycles are probably required for direct detection from clinical specimens.

10

The person skilled in the art of nucleic acid amplification knows the existence of other rapid amplification procedures such as ligase chain reaction (LCR), reverse transcriptase PCR (RT-PCR), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence-based amplification
 15 (NASBA), strand displacement amplification (SDA), branched DNA (bDNA), cycling probe technology (CPT), solid phase amplification (SPA), rolling circle amplification technology (RCA), solid phase RCA, anchored SDA and nuclease dependent signal amplification (NDSA) (Lee *et al.*, 1997, Nucleic Acid Amplification Technologies: Application to Disease Diagnosis, Eaton Publishing,
 20 Boston, MA; Persing *et al.*, 1993, Diagnostic Molecular Microbiology: Principles and Applications, American Society for Microbiology, Washington, D.C.; Westin *et al.*, 2000, Nat. Biotechnol. 18:199-204). The scope of this invention is not limited to the use of amplification by PCR, but rather includes the use of any nucleic acid amplification method or any other procedure which may be used to
 25 increase the sensitivity and/or the rapidity of nucleic acid-based diagnostic tests. The scope of the present invention also covers the use of any nucleic acids amplification and detection technology including real-time or post-amplification detection technologies, any amplification technology combined with detection, any hybridization nucleic acid chips or array technologies, any amplification chips or

combination of amplification and hybridization chip technologies. Detection and identification by any nucleotide sequencing method is also under the scope of the present invention.

- 5 Any oligonucleotide derived from the *S. aureus* MREJ DNA sequences and used with any nucleic acid amplification and/or hybridization technologies are also under the scope of this invention.

Evaluation of the MRSA detection method developed by Hiramatsu *et al.*

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- According to Ito *et al.* (Ito *et al.*, 1999, Antimicrob. Agents Chemother. **43**:1449-1458; Katayama *et al.*, 2000, Antimicrob. Agents Chemother. **44**:1549-1555; Ito *et al.*, 2001, Antimicrob. Agents Chemother. **45**:1323-1336, Ma *et al.*, 2002, Antimicrob. Agents Chemother. **46**:1147-1152), four types of SCC*mec* DNA are
- 5 found among MRSA strains. They have found that SCC*mec* DNAs are integrated at a specific site of the MSSA chromosome (named *orfX*). They developed a MRSA-specific multiplex PCR assay including primers that can hybridize to the right extremity of SCC*mec* types I, II and III (SEQ ID NOs.: 18, 19, 20, 21, 22, 23, 24 in US patent 6,156,507 corresponding to SEQ ID NOs.: 52, 53, 54, 55, 56, 57,
 - 0 58, respectively, in the present invention) as well as primers specific to the *S. aureus* chromosome to the right of the SCC*mec* integration site (SEQ ID NO.: 25, 28, 27, 26, 29 in US patent 6,156,507 corresponding to SEQ ID NOs.: 59, 60, 61, 62, 63, respectively, in the present invention) (Table 1 and Figure 1). The set of primers described by Hiramatsu *et al.* (Hiramatsu *et al.*, 1996, J Infect Chemother **2**:117-
 - 5 129) as being the optimal primer combination (SEQ ID NOs.: 22, 24 and 28 in US patent 6,156,507 corresponding to SEQ ID NOs.: 56, 58 and 60 in the present invention) was used in the present invention to test by PCR a variety of MRSA, MSSA, methicillin-resistant CNS (MRCNS) and methicillin-sensitive CNS (MSCNS) strains (Table 2). A PCR assay performed using a standard thermocycler (PTC-200 from MJ Research Inc.) was

used to test the ubiquity, the specificity and the sensitivity of these primers using the following protocol: one μ l of a treated standardized bacterial suspension or of a genomic DNA preparation purified from bacteria were amplified in a 20 μ l PCR reaction mixture. Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% TritonTM X-100, 2.5 mM MgCl₂, 0.4 μ M of each of the SCCmec- and *S. aureus* chromosome-specific primers (SEQ ID NOs.: 22, 24 and 28 in US patent 6,156,507 corresponding to SEQ ID NOs.: 56, 58 and 60 in the present invention), 200 μ M of each of the four dNTPs (Pharmacia Biotech), 3.3 μ g/ μ l BSA (Sigma), and 0.5 U *Taq* polymerase (Promega) coupled with *TaqStart*TM Antibody (BD Biosciences).

PCR reactions were then subjected to thermal cycling 3 min at 94°C followed by 40 cycles of 60 seconds at 95°C for the denaturation step, 60 seconds at 55°C for the annealing step, and 60 seconds at 72°C for the extension step, then followed by a terminal extension of 7 minutes at 72°C using a standard thermocycler (PTC-200 from MJ Research Inc.). Detection of the PCR products was made by electrophoresis in agarose gels (2 %) containing 0.25 μ g/ml of ethidium bromide. Twenty of the 39 MRSA strains tested were not amplified with the PCR assay developed by Hiramatsu *et al.* (Example 1, Tables 2 and 3).

With a view of establishing a rapid diagnostic test for MRSA, the present inventors developed new sets of primers specific to the right extremity of SCCmec types I and II (SEQ ID NOs.: 66, 100 and 101) (Annex 1), SCCmec type II (SEQ ID NOs.: 97 and 99), SCCmec type III (SEQ ID NOs.: 67, 98 and 102) and in the *S. aureus* chromosome to the right of the SCCmec integration site (SEQ ID NOs.: 64, 70, 71, 72, 73, 74, 75 and 76) (Table 5). These primers, amplifying short amplicons (171 to 278 bp), are compatible for use in rapid PCR assays (Table 7). The design of these primers was based on analysis of multiple sequence alignments of *orfX* and SCCmec sequences described by Hiramatsu *et al.* (US patent

6,156,507) or available from GenBank (Table 10, Annex I). These different sets of primers were used to test by PCR a variety of MRSA, MSSA, MRCNS and MSCNS strains. Several amplification primers were developed to detect all three SCCmec types (SEQ ID NOs.: 97 and 99 for SCCmec type II, SEQ ID NOs.: 66, 100 and 101 for SCCmec types I and II and SEQ ID NOs.: 67, 98 and 102 for SCCmec type III). Primers were chosen according to their specificity for MRSA strains, their analytical sensitivity in PCR and the length of the PCR product. A set of two primers was chosen for the SCCmec right extremity region (SEQ ID NO.: 66 specific to SCCmec types I and II; SEQ ID NO.: 67 specific to SCCmec type III). Of the 8 different primers designed to anneal on the *S. aureus* chromosome to the right of the SCCmec integration site (targeting *orfX* gene) (SEQ ID NOs.: 64, 70, 71, 72, 73, 74, 75 and 76), only one (SEQ ID.: 64) was found to be specific for MRSA based on testing with a variety of MRSA, MSSA, MRCNS and MSCNS strains (Table 12). Consequently, a PCR assay using the optimal set of primers (SEQ ID NOs.: 64, 66 and 67) which could amplify specifically MRSA strains containing SCCmec types I, II and III was developed (Figure 2, Annex I). While the PCR assay developed with this novel set of primers was highly sensitive (i.e. allowed the detection of 2 to 5 copies of genome for all three SCCmec types) (Table 11), it had the same shortcomings (i.e. lack of ubiquity) of the test developed by Hiramatsu et al. The 20 MRSA strains which were not amplified by the Hiramatsu et al. primers were also not detected by the set of primers comprising SEQ ID NOs.: 64, 66 and 67 (Tables 3 and 12). Clearly, diagnostic tools for achieving at least 50% ubiquity amongst the tested strains are needed.

With a view to establish a more ubiquitous (i.e. ability to detect all or most MRSA strains) detection and identification method for MRSA, we determined the sequence of the MREJ/MREP present in these 20 MRSA strains which were not amplified. This research has led to the discovery and identification of seven novel distinct MREJ/MREP target sequences which can be used for diagnostic purposes.

These seven new MREJ/MREP sequences could not have been predicted nor detected with the system described in US patent 6,156,507 by Hiramatsu *et al.* Namely, the present invention represents an improved method for the detection and identification of MRSA because it provides a more ubiquitous diagnostic method which allows for the detection of all major epidemic MRSA clones from around the world.

Sequencing of MREJ nucleotide sequences from MRSA strains not amplifiable with primers specific to SCCmec types I, II and III

Since DNA from twenty MRSA strains were not amplified with the set of primers developed by Hiramatsu *et al.* (SEQ ID NOs.: 22, 24 and 28 in US patent 6,156,507 corresponding to SEQ ID NOs.: 56, 58 and 60 in the present invention) (Tables 2 and 3) nor with the set of primers developed in the present invention based on the same three SCCmec types (I, II and III) sequences (SEQ ID NOs.: 64, 66 and 67) (Table 12), the nucleotide sequence of the MREJ/MREP was determined for sixteen of these twenty MRSA strains.

Transposase of IS431 is often associated with the insertion of resistance genes within the *mec* locus. The gene encoding this transposase has been described frequently in one or more copies within the right segment of SCCmec (Oliveira *et al.*, 2000, Antimicrob. Agents Chemother. **44**:1906-1910; Ito *et al.*, 2001, Antimicrob. Agents Chemother. **45**:1323-36). Therefore, in a first attempt to sequence the novel MREJ/MREP for 16 of the 20 MRSA strains described in Table 3, a primer was designed in the sequence of the gene coding for the transposase of IS431 (SEQ ID NO.: 68) and combined with an *orfX*-specific primer to the right of the SCCmec integration site (SEQ ID NO.: 70) (Tables 5 and 8). The strategy used to select these primers is illustrated in Figure 3.

The MREJ/MREP fragments to be sequenced were amplified using the following amplification protocol: one μL of treated cell suspension (or of a purified genomic DNA preparation) was transferred directly into 4 tubes containing 39 μL of a PCR reaction mixture. Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% TritonTM X-100, 2.5 mM MgCl_2 , 1 μM of each of the 2 primers (SEQ ID NOs.: 68 and 70), 200 μM of each of the four dNTPs, 3.3 $\mu\text{g}/\mu\text{L}$ of BSA (Sigma-Aldrich Canada Ltd) and 0.5 unit of *Taq* DNA polymerase (Promega) coupled with the *TaqStart*TM Antibody (BD Biosciences). PCR reactions were submitted to cycling using a standard thermocycler (PTC-200 from MJ Research Inc.) as follows: 3 min at 94 °C followed by 40 cycles of 5 sec at 95 °C for the denaturation step, 30 sec at 55 °C for the annealing step and 2 min at 72 °C for the extension step.

Subsequently, the four PCR-amplified mixtures were pooled and 10 μL of the mixture were resolved by electrophoresis in a 1.2% agarose gel containing 0.25 $\mu\text{g}/\text{mL}$ of ethidium bromide. The amplicons were then visualized with an Alpha-Imager (Alpha Innotech Corporation, San Leandro, CA) by exposing to UV light at 254 nm. Amplicon size was estimated by comparison with a 1 kb molecular weight ladder (Life Technologies, Burlington, Ontario, Canada). The remaining PCR-amplified mixture (150 μL , total) was also resolved by electrophoresis in a 1.2% agarose gel. The amplicons were then visualized by staining with methylene blue (Flores *et al.*, 1992, *Biotechniques*, **13**:203-205). Amplicon size was once again estimated by comparison with a 1 kb molecular weight ladder. Of the sixteen strains selected from the twenty described in Table 3, six were amplified using SEQ ID NOs.: 68 and 70 as primers (CCRI-178, CCRI-8895, CCRI-8903, CCRI-1324, CCRI-1331 and CCRI-9504). For these six MRSA strains, an amplification product of 1.2 kb was obtained. The band corresponding to this specific amplification product was excised from the agarose gel and purified using the QIAquickTM gel extraction kit (QIAGEN Inc., Chatsworth, CA). The gel-

purified DNA fragment was then used directly in the sequencing protocol. Both strands of the MREJ amplification products were sequenced by the dideoxynucleotide chain termination sequencing method by using an Applied Biosystems automated DNA sequencer (model 377) with their Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The sequencing reactions were performed by using the same primers (SEQ ID NOs.: 68 and 70) and 10 ng/100 bp per reaction of the gel-purified amplicons. Sequencing of MREJ from the six MRSA strains (CCRI-178, CCRI-8895, CCRI-8903, CCRI-1324, CCRI-1331 and CCRI-9504) described in Table 3 yielded SEQ ID NOs.: 42, 43, 44, 45, 46 and 51, respectively (Table 4). Once sequenced, SEQ ID NO.: 42 was however shown not to include the *orfX* region and thus only comprises the polymorphic right extremity of the SCC*mec*.

In order to ensure that the determined sequence did not contain errors attributable to the sequencing of PCR artefacts, we have sequenced two preparations of the gel-purified MREJ amplification products originating from two independent PCR amplifications. For most target fragments, the sequences determined for both amplicon preparations were identical. Furthermore, the sequences of both strands were 100% complementary thereby confirming the high accuracy of the determined sequence. The MREJ sequences determined using the above strategy are described in the Sequence Listing and in Table 4.

In order to sequence MREJ in strains for which no amplicon had been obtained using the strategy including primers specific to the transposase gene of IS431 and *orfX*, another strategy using primers targeting *mecA* and *orfX* sequences was used to amplify longer genomic fragments. A new PCR primer targeting *mecA* (SEQ ID NO.: 69) (Table 8) to be used in combination with the same primer in the *orfX* sequence (SEQ ID NO.: 70). The strategy used to select these primers is illustrated in Figure 3.

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The following amplification protocol was used: Purified genomic DNA (300 ng) was transferred to a final volume of 50 μ l of a PCR reaction mixture. Each PCR reaction contained 1X *Herculase* buffer (Stratagene, La Jolla, CA), 0.8 μ M of each of the 2 primers (SEQ ID NOs.: 69 and 70), 0.56 mM of each of the four dNTPs and 5 units of *Herculase* (Stratagene). PCR reactions were subjected to cycling using a standard thermal cycler (PTC-200 from MJ Research Inc.) as follows: 2 min at 92 °C followed by 35 or 40 cycles of 10 sec at 92 °C for the denaturation step, 30 sec at 55 °C for the annealing step and 30 min at 68 °C for the extension step.

10

Subsequently, 10 μ L of the PCR-amplified mixture were resolved by electrophoresis in a 0.7% agarose gel containing 0.25 μ g/mL of ethidium bromide. The amplicons were then visualized as described above. Amplicon size was estimated by comparison with a 1 kb molecular weight ladder (Life Technologies).

15 A reamplification reaction was then performed in 2 to 5 tubes using the same protocol with 3 μ l of the first PCR reaction used as test sample for the second amplification. The PCR-reamplified mixtures were pooled and also resolved by electrophoresis in a 0.7% agarose gel. The amplicons were then visualized by staining with methylene blue as described above. An amplification product of

20 approximately 12 kb was obtained using this amplification strategy for all strains tested. The band corresponding to the specific amplification product was excised from the agarose gel and purified as described above. The gel-purified DNA fragment was then used directly in the sequencing protocol as described above. The sequencing reactions were performed by using the same amplification primers

25 (SEQ ID NOs.: 69 and 70) and 425-495 ng of the gel-purified amplicons per reaction. Subsequently, internal sequencing primers (SEQ ID NOs.: 65, 77 and 96) (Table 8) were used to obtain sequence data on both strands for a larger portion of the amplicon. Five of the 20 MRSA strains (CCRI-1331, CCRI-1263, CCRI-1377, CCRI-1311 and CCRI-2025) described in Table 3 were sequenced using this

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strategy, yielding SEQ ID NOs.: 46, 47, 48, 49 and 50, respectively (Table 4). Sequence within *mecA* gene was also obtained from the generated amplicons yielding SEQ ID NOs: 27, 28, 29, 30 and 31 from strains CCRI-2025, CCRI-1263, CCRI-1311, CCRI-1331 and CCRI-1377, respectively (Table 4). Longer
 5 sequences within the *mecA* gene and from downstream regions were also obtained for strains CCRI-2025, CCRI-1331, and CCRI-1377 as described below.

In order to obtain longer sequences of the *orfX* gene, two other strategies using primers targeting *mecA* and *orfX* sequences (at the start codon) was used to amplify
 10 longer chromosome fragments. A new PCR primer was designed in *orfX* (SEQ ID NO.: 132) to be used in combination with the same primer in the *mecA* gene (SEQ ID NO.: 69). The strategy used to select these primers is illustrated in Figure 3. Eight *S. aureus* strains were amplified using primers SEQ ID NOs.: 69 and 132 (CCRI-9860, CCRI-9208, CCRI-9504, CCRI-1331, CCRI-9583, CCRI-9681,
 15 CCRI-2025 and CCRI-1377). The strategy used to select these primers is illustrated in Figure 3.

The following amplification protocol was used: Purified genomic DNA (350 to 500 ng) was transferred to a 50 µl PCR reaction mixture. Each PCR reaction
 20 contained 1X Herculase buffer (Stratagene), 0.8 µM of each of the set of 2 primers (SEQ ID NOs.: 69 and 132), 0.56 mM of each of the four dNTPs and 7.5 units of *Herculase* (Stratagene) with 1 mM MgCl₂. PCR reactions were subjected to thermocycling as described above.

25 Subsequently, 5 µL of the PCR-amplified mixture were resolved by electrophoresis in a 0.8% agarose gel containing 0.25 µg/mL of ethidium bromide. The amplicons were then visualized as described above. For one *S. aureus* strain (CCRI-9583), a reamplification was then performed by using primers SEQ ID NOs.: 96 and 158 (Figure 3) in 4 tubes, using the same PCR protocol, with 2 µl of

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the first PCR reaction as test sample for the second amplification. The PCR-reamplified mixtures were pooled and also resolved by electrophoresis in a 0.8% agarose gel. The amplicons were then visualized by staining with methylene blue as described above. A band of approximately 12 to 20 kb was obtained using this

5 amplification strategy depending on the strains tested. The band corresponding to the specific amplification product was excised from the agarose gel and purified using the QIAquick™ gel extraction kit or QIAEX II gel extraction kit (QIAGEN Inc.). Two strains, CCRI-9583 and CCRI-9589, were also amplified with primers SEQ ID NOs.: 132 and 150, generating an amplification product of 1.5 kb. Long

10 amplicons (12-20 kb) were sequenced using 0.6 to 1 µg per reaction, while short amplicons (1.5 kb) were sequenced using 150 ng per reaction. Sequencing reactions were performed using different sets of primers for each *S. aureus* strain:

1) SEQ ID NOs.: 68, 70, 132, 145, 146, 147, 156, 157 and 158 for strain CCRI-9504; 2) SEQ ID NOs.: 70, 132, 154 and 155 for strain CCRI-2025; 3) SEQ ID

15 NOs.: 70, 132, 148, 149, 158 and 159 for strain CCRI-9681; 4) SEQ ID NOs.: 70, 132, 187, and 188 for strain CCRI-9860; 5) SEQ ID NOs.: 70, 132, 150 and 159 for strain CCRI-9589, 6) SEQ ID NOs.: 114, 123, 132, 150 and 158 for strain CCRI-9583; 7) SEQ ID NOs.: 70, 132, 154 and 155 for strain CCRI-1377, 8) SEQ ID NOs.: 70, 132, 158 and 159 for strain CCRI-9208; 9) SEQ ID NOs.: 68, 70, 132,

20 145, 146, 147 and 158 for strain CCRI-1331; and 10) SEQ ID NOs.: 126 and 127 for strain CCRI-9770.

In one strain (CCRI-9770), the *orfX* and *orfSA0022* genes were shown to be totally or partially deleted based on amplification using primers specific to these genes

25 (SEQ ID NOs.: 132 and 159 and SEQ ID NOs.: 128 and 129, respectively) (Table 8). Subsequently, a new PCR primer was designed in *orfSA0021* (SEQ ID NO.: 126) to be used in combination with the same primer in the *mecA* gene (SEQ ID NO.: 69). An amplification product of 4.5 kb was obtained with this primer set.

Amplification, purification of amplicons and sequencing of amplicons were performed as described above.

To obtain the sequence of the *SSC_{mec}* region containing *mecA* for ten of the 20 MRSA strains described in Table 3 (CCRI-9504, CCRI-2025, CCRI-9208, CCRI-1331, CCRI-9681, CCRI-9860, CCRI-9770, CCRI-9589, CCRI-9583 and CCRI-1377), the primer described above designed in *mecA* (SEQ ID NO.: 69) was used in combination with a primer designed in the downstream region of *mecA* (SEQ ID NO.: 118) (Table 8). An amplification product of 2 kb was obtained for all the strains tested. For one strain, CCRI-9583, a re-amplification with primers SEQ ID NOs.: 96 and 118 was performed with the amplicon generated with primers SEQ ID NOs.: 69 and 132 described above. The amplification, re-amplification, purification of amplicons and sequencing reactions were performed as described above. Sequencing reactions were performed with amplicons generated with SEQ ID NOs.: 69 and 132 described above or SEQ ID NOs.: 69 and 118. Different sets of sequencing primers were used for each *S. aureus* strain: 1) SEQ ID NOs.: 69, 96, 117, 118, 120, 151, 152 for strains CCRI-9504, CCRI-2025, CCRI-1331, CCRI-9770 and CCRI-1377; 2) SEQ ID NOs.: 69, 96, 118 and 120 for strains CCRI-9208, CCRI-9681 and CCRI-9589; 3) SEQ ID NOs.: 69, 96, 117, 118, 120 and 152 for strain CCRI-9860; and 4) SEQ ID NOs.: 96, 117, 118, 119, 120, 151 and 152 for strain CCRI-9583.

The sequences obtained for 16 of the 20 strains non-amplifiable by the Hiramatsu assay (Table 4) were then compared to the sequences available from public databases. In all cases, portions of the sequence had an identity close to 100% to publicly available sequences for *orfX* (SEQ ID NOs.: 43-51, 165-168 and 171) or *mecA* and downstream region (SEQ ID NOs.: 27-31, 189-193, 195, 197-199 and 225). However, while the *orfX* portion of the fragments (SEQ ID NOs.: 43-51, 165-168 and 171) shared nearly 100% identity with the *orfX* gene of MSSA strain

NCTC 8325 described by Hiramatsu *et al.* (SEQ ID NO.: 3), the DNA sequence within the right extremity of SCC*mec* itself was shown to be very different from those of types I, II, III and IV described by Hiramatsu *et al.* (Table 13, Figure 4).

Six different novel sequence types were obtained. As for SEQ ID NO.: 42 (MREP type iv), the sequence was too short to include any portion of the *orfX*.

It should be noted that Hiramatsu *et al.* demonstrated that SCC*mec* type I could be associated with MREP type i, SCC*mec* types II and IV are associated with MREP type ii, and SCC*mec* type III is associated with MREP type iii. Our MREJ sequencing data from various MRSA strains led to the discovery of 6 novel MREP/MREJ types designated types iv, v, vi, vii, viii, and ix. The MREJ comprising distinct MREP types were named according to the MREP numbering scheme. Hence, MREP type i is comprised within MREJ type i, MREP type ii is comprised within MREJ type ii and so on up to MREP type ix.

The sequences within the right extremity of SCC*mec* obtained from strains CCRI-178, CCRI-8895, CCRI-8903, CCRI-1324, CCRI-1331 and CCRI-9504 (SEQ ID NOs.: 42, 43, 44, 45, 46 and 51) were nearly identical to each other and exhibited nearly 100% identity with IS431 (GenBank accession numbers AF422691, ABO37671, AF411934). However, our sequence data revealed for the first time the location of this IS431 sequence at the right extremity of SCC*mec* adjacent to the integration site. Therefore, as the sequences at the right extremity of SCC*mec* from these 6 MRSA strains were different from those of SCC*mec* type I from strain NCTC 10442, SCC*mec* type II from strain N315, SCC*mec* type III from strain 85/2082 and SCC*mec* type IV from strains CA05 and 8/6-3P described by Hiramatsu *et al.* (Ito *et al.*, 2001, Antimicrob. Agents Chemother. **45**:1323-1336; Ma *et al.*, 2002, Antimicrob. Agents Chemother. **46**:1147-1152), these new sequences were designated as MREJ type iv (SEQ ID NOs.: 43-46 and 51). A BLAST search with the SCC*mec* portion of MREP type iv sequences produced significant alignments with sequences coding for portions of a variety of known transposases. For example, when compared to Genbank accession no. AB037671,

MREP type iv from SEQ ID NO. 51 shared 98% identity with the putative transposase of IS431 and its downstream region; two gaps of 7 nucleotides each were also present in the alignment.

Sequences obtained from strains CCRI-1263, CCRI-1377, CCRI-1311 and CCRI-
 5 2025 (SEQ ID NOs.: 47-50) were nearly identical to each other and different from all three SCC*mec* types and MREP type iv and, consequently, were designated as MREP/MREJ type v. When compared with Genbank sequences using BLAST, MREP/MREJ type v sequences did not share any significant homology with any published sequence, except for the first 28 nucleotides. That short stretch
 0 corresponded to the last 11 coding nucleotides of *orfX*, followed by the 17 nucleotides downstream, including the right inverted repeat (IR-R) of SCC*mec*.

Sequence obtained from strain CCRI-9208 was also different from all three SCC*mec* types and MREP/MREJ types iv and v and, consequently, was designated as MREP/MREJ type vi (SEQ ID NO.: 171). Upon a BLAST search,
 5 MREP/MREJ type vi was shown to be unique, exhibiting no significant homology to any published sequence.

Sequences obtained from strains CCRI-9583 and CCRI-9589 were also different from all three SCC*mec* types and MREP/MREJ types iv to vi and were therefore designated as MREP/MREJ type vii (SEQ ID NOs.: 165 and 166). Upon a BLAST
 0 search, MREP type vii was also shown to be unique, exhibiting no significant homology to any published sequence.

Sequence obtained from strain CCRI-9860 was also different from all three SCC*mec* types and MREP/MREJ types iv to vii and was therefore designated as MREP/MREJ type viii (SEQ ID NO.: 167). Sequence obtained from strain CCRI-
 5 9681 was also different from all three SCC*mec* types and MREP/MREJ types iv to viii and was therefore designated as MREP/MREJ type ix (SEQ ID NO.: 168). BLAST searches with the SCC*mec* portion of MREP/MREJ types viii and ix sequences yielded significant alignments, but only for the first ~150 nucleotides of each MREJ type. For example, the beginning of the MREJ type viii sequence had

88% identity with a portion of Genbank accession no. AB063173, but no significant homology with any published sequence was found for the rest of the sequence. In the same manner, the first ~150 nucleotides of MREJ type ix had 97% identity with the same portion of AB063173, with the rest of the sequence being
 5 unique. The short homologous portion of MREJ types viii and ix corresponds in AB063173 to the last 14 coding nucleotides of *orfX*, the IR-R of *SCCmec*, and a portion of *orfCM009*. Although sharing resemblances, MREJ types viii and ix are very different from one another; as shown in Table 13, there is only 55.2% identity between both types for the first 500 nucleotides of the *SCCmec* portion.

0 Finally, we did not obtain any sequence within *SSCmec* from strain CCRI-9770. However, as described in the section "Sequencing of MREJ nucleotide sequences from MRSA strains not amplifiable with primers specific to *SCCmec* types I, II and III", this strain has apparently a partial or total deletion of the *orfX* and *orfSA0022* genes in the chromosomal DNA to the right of the *SCCmec* integration
 5 site and this would represent a new right extremity junction. We therefore designated this novel sequence as MREP/MREJ type x (SEQ ID NO.: 172). Future sequencing should reveal whether this so called MREJ type x contains a novel MREP type x or if the lack of amplification is indeed caused by variation in the chromosomal part of the MREJ.

0 The sequences of the first 500-nucleotide portion of the right extremity of all *SCCmec* obtained in the present invention were compared to those of *SCCmec* types I, II and III using GCG programs Pileup and Gap. Table 13 depicts the identities at the nucleotide level between *SCCmec* right extremities of the six novel
 5 sequences with those of *SCCmec* types I, II and III using the GCG program Gap. While *SCCmec* types I and II showed nearly 79.2% identity (differing only by a 102 bp insertion present in *SCCmec* type II) (Figures 1, 2 and 4), all other MREP types showed identities varying from 40.9 to 57.1%. This explains why the right

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extremities of the novel MREP types iv to ix disclosed in the present invention could not have been predicted nor detected with the system described by Hiramatsu *et al.*

5 Four strains (CCRI-1312, CCRI-1325, CCRI-9773 and CCRI-9774) described in Table 3 were not sequenced but rather characterized using PCR primers. Strains CCRI-1312 and CCRI-1325 were shown to contain MREP type v using specific amplification primers described in Examples 4, 5 and 6 while strains CCRI-9773 and CCRI-9774 were shown to contain MREP type vii using specific amplification
10 primers described in Example 7.

To obtain the complete sequence of the SCCmec present in the MRSA strains described in the present invention, primers targeting the *S. aureus* chromosome to the left (upstream of the *mecA* gene) of the SCCmec integration site were
15 developed. Based on available public database sequences, 5 different primers were designed (SEQ ID NOs.: 85-89) (Table 9). These primers can be used in combination with *S. aureus* chromosome-specific primers in order to sequence the entire SCCmec or, alternatively, used in combination with a *mecA*-specific primer (SEQ ID NO.: 81) in order to sequence the left extremity junction of SCCmec. We
20 have also developed several primers specific to known SCCmec sequences spread along the locus in order to obtain the complete sequence of SCCmec (Table 9). These primers will allow to assign a SCCmec type to the MRSA strains described in the present invention.

25 Selection of amplification primers from SCCmec/orfX sequences

The MREJ sequences determined by the inventors or selected from public databases were used to select PCR primers for detection and identification of

MRSA. The strategy used to select these PCR primers was based on the analysis of multiple sequence alignments of various MREJ sequences.

Upon analysis of the six new MREP/MREJ types iv to ix sequence data described
 5 above, primers specific to each new MREP type sequence (SEQ ID NOs.: 79, 80, 109, 112, 113, 115, 116 and 204) were designed (Figure 2, Table 5, Examples 3, 4, 5, 6, 7 and 8). Primers specific to MREP types iv, v and vii (SEQ ID NOs.: 79, 80 and 112) were used in multiplex with the three primers to detect SCCmec types I, II and III (SEQ ID NOs: 64, 66 and 67) and the primer specific to the *S. aureus*
 0 *orfX* (SEQ ID NO. 64) (Examples 3, 4, 5, 6 and 7). Primers specific to MREP types vi, viii and ix (SEQ ID NOs.: 204, 115, 116 and 109) were also designed and tested against their specific target (Example 8).

Detection of amplification products

5 Classically, the detection of PCR amplification products is performed by standard ethidium bromide-stained agarose gel electrophoresis as described above. It is however clear that other methods for the detection of specific amplification products, which may be faster and more practical for routine diagnosis, may be
 0 used. Examples of such methods are described in patent application WO01/23604 A2.

Amplicon detection may also be performed by solid support or liquid hybridization using species-specific internal DNA probes hybridizing to an amplification
 5 product. Such probes may be generated from any sequence from our repertory and designed to specifically hybridize to DNA amplification products which are objects of the present invention. Alternatively, amplicons can be characterized by sequencing. See patent application WO01/23604 A2 for examples of detection and sequencing methods.

In order to improve nucleic acid amplification efficiency, the composition of the reaction mixture may be modified (Chakrabarti and Schutt, 2002, Biotechniques, 32:866-874; Al-Soud and Radstrom, 2002, J. Clin. Microbiol., 38:4463-4470; Al-Soud and Radstrom, 1998, Appl. Environ. Microbiol., 64:3748-3753; Wilson, 1997, Appl. Environ. Microbiol., 63:3741-3751). Such modifications of the amplification reaction mixture include the use of various polymerases or the addition of nucleic acid amplification facilitators such as betaine, BSA, sulfoxides, protein gp32, detergents, cations, tetramethylammonium chloride and others.

In a preferred embodiment, real-time detection of PCR amplification was monitored using molecular beacon probes in a Smart Cycler[®] apparatus (Cepheid, Sunnyvale, CA). A multiplex PCR assay containing primers specific to MREP types i to v and *orfX* of *S. aureus* (SEQ ID NOs.: 64, 66, 67, 79 and 80), a molecular beacon probe specific to the *orfX* sequence (SEQ ID NO. 84, see Annex II and Figure 2) and an internal control to monitor PCR inhibition was developed. The internal control contains sequences complementary to MREP type iv- and *orfX*-specific primers (SEQ ID NOs. 79 and 64). The assay also contains a molecular beacon probe labeled with tetrachloro-6-carboxyfluorescein (TET) specific to sequence within DNA fragment generated during amplification of the internal control. Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton[™] X-100, 3.45 mM MgCl₂, 0.8 μM of each of the MREP-specific primers (SEQ ID NOs.: 66 and 67) and *orfX*-specific primer (SEQ ID NO.: 64), 0.4 μM of each of the MREP-specific primers (SEQ ID NOs.: 79 and 80), 80 copies of the internal control, 0.2 μM of the TET-labeled molecular beacon probe specific to the internal control, 0.2 μM of the molecular beacon probe (SEQ ID NO.: 84) labeled with 6-carboxyfluorescein (FAM), 330 μM of each of the four dNTPs (Pharmacia Biotech), 3.45 μg/μl of BSA (Sigma), and 0.875 U *Taq* polymerase (Promega) coupled with *TaqStart*[™] Antibody (BD Biosciences). The PCR

amplification on the Smart Cycler[®] was performed as follows: 3 min. at 95°C for initial denaturation, then forty-eight cycles of three steps consisting of 5 seconds at 95°C for the denaturation step, 15 seconds at 60°C for the annealing step and 15 seconds at 72°C for the extension step. Sensitivity tests performed by using
 5 purified genomic DNA from one MRSA strain of each MREP/MREJ type (i to v) showed a detection limit of 2 to 10 genome copies (Example 5). None of the 26 MRCNS or 10 MSCNS tested were positive with this multiplex assay. The eight MRSA strains (CCRI-9208, CCRI-9770, CCRI-9681, CCRI-9860, CCRI-9583, CCRI-9773, CCRI-9774, CCRI-9589) which harbor the new MREP/MREJ types
 0 vi, viii, ix and x sequences described in the present invention remained undetectable (Example 5).

In a preferred embodiment, detection of MRSA using the real-time multiplex PCR assay on the Smart Cycler[®] apparatus (Cepheid, Sunnyvale, CA) directly from
 5 clinical specimens was evaluated. A total of 142 nasal swabs were collected during a MRSA hospital surveillance program at the Montreal General Hospital (Montreal, Quebec, Canada). The swab samples were tested at the Centre de Recherche en Infectiologie de l'Université Laval within 24 hours of collection. Upon receipt, the swabs were plated onto mannitol agar and then the nasal material
 0 from the same swab was prepared with a simple and rapid specimen preparation protocol described in patent application number WO 03/008636. Classical identification of MRSA was performed by standard culture methods.

The PCR assay detected 33 of the 34 samples positive for MRSA based on the
 5 culture method. As compared to culture, the PCR assay detected 8 additional MRSA positive specimens for a sensitivity of 97.1 % and a specificity of 92.6 % (Example 6). This multiplex PCR assay represents a rapid and powerful method for the specific detection of MRSA carriers directly from nasal specimens and can

be used with any types of clinical specimens such as wounds, blood or blood culture, CSF, etc.

In a preferred embodiment, a multiplex PCR assay containing primers specific to MREP types i, ii, iii, iv, v and vi and orfX of *S. aureus* (SEQ ID NOs.: 66, 67, 79, 80 and 112), and three molecular beacons probes specific to orfX sequence which allowed detection of the two sequence polymorphisms identified in this region of the orfX sequence was developed. Four of the strains which were not detected with the multiplex assay for the detection of MREP/MREJ types i to v were now detected with this multiplex assay while the four MRSA strains (CCRI-9208, CCRI-9770, CCRI-9681, CCRI-9860) which harbor the MREP/MREJ types vi, viii, ix and x described in the present invention remained undetectable (Example 7). Primers specific to MREP types vi, viii and ix (SEQ ID NOs.: 204, 115, 116 and 109) were also designed and were shown to detect their specific target strains (Example 8). While the primers and probes derived from the teaching of Hiramatsu *et al.*, permitted the detection of only 48.7% (19 strains out of 39) of the MRSA strains of Table 2, the primers and probes derived from the present invention enable the detection of 97.4 % of the strains (38 strains out of 39) (see examples 7 and 8). Therefore it can be said that our assay has a ubiquity superior to 50% for the MRSA strains listed in Table 2.

Specificity, ubiquity and sensitivity tests for oligonucleotide primers and probes

The specificity of oligonucleotide primers and probes was tested by amplification of DNA or by hybridization with staphylococcal species. All of the staphylococcal species tested were likely to be pathogens associated with infections or potential contaminants which can be isolated from clinical specimens. Each target DNA could be released from microbial cells using standard chemical and/or physical treatments to lyse the cells (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) or alternatively, genomic DNA purified with the GNOME™ DNA kit (Qbiogene, Carlsbad, CA) was used. Subsequently, the DNA was subjected to

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amplification with the set of primers. Specific primers or probes hybridized only to the target DNA.

Oligonucleotides primers found to amplify specifically DNA from the target
5 MRSA were subsequently tested for their ubiquity by amplification (i.e. ubiquitous primers amplified efficiently most or all isolates of MRSA). Finally, the analytical sensitivity of the PCR assays was determined by using 10-fold or 2-fold dilutions of purified genomic DNA from the targeted microorganisms. For most assays, sensitivity levels in the range of 2-10 genome copies were obtained. The
10 specificity, ubiquity and analytical sensitivity of the PCR assays were tested either directly with bacterial cultures or with purified bacterial genomic DNA.

Molecular beacon probes were tested using the Smart Cycler® platform as described above. A molecular beacon probe was considered specific only when it
15 hybridized solely to DNA amplified from the MREJ of *S. aureus*. Molecular beacon probes found to be specific were subsequently tested for their ubiquity (i.e. ubiquitous probes detected efficiently most or all isolates of the MRSA) by hybridization to bacterial DNAs from various MRSA strains.

20 ***Bacterial strains***

The reference strains used to build proprietary *SCCmec*-chromosome right extremity junction sequence data subrepertoires, as well as to test the amplification and hybridization assays, were obtained from (i) the American Type Culture
25 Collection (ATCC), (ii) the Laboratoire de santé publique du Québec (LSPQ) (Ste-Anne de Bellevue, Québec, Canada), (iii) the Centers for Disease Control and Prevention (CDC) (Atlanta, GA), (iv) the Institut Pasteur (Paris, France), and V) the Harmony Collection (London, United Kingdom) (Table 14). Clinical isolates of MRSA, MSSA, MRCNS and MSCNS from various geographical areas were also

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used in this invention (Table 15). The identity of our MRSA strains was confirmed by phenotypic testing and reconfirmed by PCR analysis using *S. aureus*-specific primers and *mecA*-specific primers (SEQ ID NOs.: 69 and 81) (Martineau *et al.*, 2000, Antimicrob. Agents Chemother. 44:231-238).

5

For sake of clarity, below is a list of the Examples, Tables, Figures and Annexes of this invention.

DESCRIPTION OF THE EXAMPLES

10

Example 1: Primers developed by Hiramatsu *et al.* can only detect MRSA strains belonging to MREP types i, ii, and iii while missing prevalent novel MREP types.

Example 2: Detection and identification of MRSA using primers specific to MREP types i, ii and iii sequences developed in the present invention.

15 **Example 3:** Development of a multiplex PCR assay on a standard thermocycler for detection and identification of MRSA based on MREP types i, ii, iii, iv and v sequences.

Example 4: Development of a real-time multiplex PCR assay on the Smart Cycler® for detection and identification of MRSA based on MREP types i, ii, iii, iv
20 and v sequences.

Example 5: Development of a real-time multiplex PCR assay on the Smart Cycler® for detection and identification of MRSA based on MREP types i, ii, iii, iv and v sequences and including an internal control.

Example 6: Detection of MRSA using the real-time multiplex assay on the Smart
25 Cycler® based on MREP types i, ii, iii, iv and v sequences for the detection of MRSA directly from clinical specimens.

Example 7: Development of a real-time multiplex PCR assay on the Smart Cycler® for detection and identification of MRSA based on MREP types i, ii, iii, iv, v, vi and vii sequences.

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Example 8: Developement of real-time PCR assays on the Smart Cycler® for detection and identification of MRSA based on MREP types vi, viii and ix.

DESCRIPTION OF THE TABLES

5

Table 1 provides information about all PCR primers developed by Hiramatsu *et al.* in US patent 6,156,507.

Table 2 is a compilation of results (ubiquity and specificity) for the detection of *SCCmec-orfX* right extremity junction using primers described by Hiramatsu *et al.*

10 in US patent 6,156,507 on a standard thermocycler.

Table 3 is a list of MRSA strains not amplifiable using primers targeting types I, II and III of *SCCmec-orfX* right extremity junction sequences.

Table 4 is a list of novel sequences revealed in the present invention.

Table 5 provides information about all primers developed in the present invention.

15 **Table 6** is a list of molecular beacon probes developed in the present invention.

Table 7 shows amplicon sizes of the different primer pairs described by Hiramatsu *et al.* in US patent 6,156,507 or developed in the present invention.

Table 8 provides information about primers developed in the present invention to seequence the *SCCmec*-chromosome right extremity junction.

20 **Table 9** provides information about primers developed in the present invention to obtain sequence of the complete *SCCmec*.

Table 10 is a list of the sequences available from public databases (GenBank, genome projects or US patent 6,156,507) used in the present invention to design primers and probes.

25 **Table 11** gives analytical sensitivity of the PCR assay developed in the present invention using primers targeting types I, II and III of *SCCme-orfX* right extremity junction sequences and performed using a standard thermocycler.

Table 12 is a compilation of results (ubiquity and specificity) for the detection of MRSA using primers developed in the present invention which target types I, II

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and III of *SCCmec-orfX* right extremity junction sequences and performed using a standard thermocycler.

Table 13 shows a comparison of sequence identities between the first 500 nucleotides of *SCCmec* right extremities between 9 types of MREP.

5 **Table 14** provides information about the reference strains of MRSA, MSSA, MRCNS and MSCNS used to validate the PCR assays developed in the present invention.

Table 15 provides information about the origin of clinical strains of MRSA, MSSA, MRCNS and MSCNS used to validate the PCR assays described in the
10 present invention.

Table 16 depicts the analytical sensitivity of the PCR assay developed in the present invention using primers targeting 5 types of MREP sequences and performed on a standard thermocycler.

Table 17 is a compilation of results (ubiquity and specificity) for the PCR assay
15 developed in the present invention using primers targeting 5 types of MREP sequences and performed on a standard thermocycler.

Table 18 depicts the analytical sensitivity of the PCR assay developed in the present invention using the SmartCycler® platform for the detection of 5 types of MREP.

20 **Table 19** is a compilation of results (ubiquity and specificity) for the PCR assay developed in the present invention using primers and a molecular beacon probe targeting 5 types of MREP sequences and performed on the SmartCycler® platform.

Table 20 depicts the analytical sensitivity of the PCR assay developed in the
25 present invention using the SmartCycler® platform for the detection of 6 MREP types.

Table 21 is a compilation of results (ubiquity and specificity) for the PCR assay developed in the present invention using primers and a molecular beacon probe

targeting 6 types of MREP sequences and performed on the Smart Cyclor[®] platform.

DESCRIPTION OF THE FIGURES

5

Figure 1 is a diagram illustrating the position of the primers developed by Hiramatsu *et al.* (US patent 6,156,507) in the SCC*mec*-chromosome right extremity junction for detection and identification of MRSA.

Figure 2 is a diagram illustrating the position of the primers selected in the present invention in the SCC*mec-orfX* right extremity junction for detection and identification of MRSA.

Figure 3 is a diagram illustrating the position of the primers selected in the present invention to sequence new MREP/MREJ types.

Figure 4 illustrates a sequence alignment of nine MREP types.

5

FIGURE LEGENDS

Figure 1. Schematic organization of types I, II and III SCC*mecorfX* right extremity junctions and localization of the primers (SEQ ID NOs: 52-63) described by Hiramatsu *et al.* for the detection and identification of MRSA. Amplicon sizes are depicted in Table 7.

Figure 2. Schematic organization of MREJ types i, ii, iii, iv, v, vi, vii, viii and ix and localization of the primers and molecular beacon targeting all MREJ types (SEQ ID NOs. 20, 64, 66, 67, 79, 80, 84, 112, 115, 116, 84, 163 and 164) which were developed in the present invention. Amplicon sizes are depicted in Table 7.

Figure 3. Schematic organization of the SCC*mec*-chromosome right extremity junctions and localization of the primers (SEQ ID NOs. 65, 68, 69, 70, 77, 96, 118, 126, 132, 150 and 158) developed in the present invention for the sequencing of MREJ types iv, v, vi, vii, viii, ix and x.

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Figure 4. Multiple sequence alignment of representatives of nine MREP types (represented by portions of SEQ ID NOs.: 1, 2, 104, 51, 50, 171, 165, 167 and 168 for types i, ii, iii, iv, v, vi, vii, viii and ix, respectively).

5 DESCRIPTION OF THE ANNEXES

The Annexes show the strategies used for the selection of primers and internal probes:

Annex I illustrates the strategy for the selection of primers from *SCCmec* and *orfX* sequences specific for *SCCmec* types I and II.

Annex II illustrates the strategy for the selection of specific molecular beacon probes for the real-time detection of *SCCmec-orfX* right extremity junctions.

As shown in these Annexes, the selected amplification primers may contain inosines and/or base ambiguities. Inosine is a nucleotide analog able to specifically bind to any of the four nucleotides A, C, G or T. Alternatively, degenerated oligonucleotides which consist of an oligonucleotide mix having two or more of the four nucleotides A, C, G or T at the site of mismatches were used. The inclusion of inosine and/or of degeneracies in the amplification primers allows mismatch tolerance thereby permitting the amplification of a wider array of target nucleotide sequences (Dieffenbach and Dveksler, 1995, PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, New York).

25

EXAMPLES

EXAMPLE 1:

Primers developed by Hiramatsu *et al.* can only detect MRSA strains belonging to MREP types i, ii, and iii while missing prevalent novel MREP types.

As shown in Figure 1, Hiramatsu *et al.* have developed various primers that can specifically hybridize to the right extremities of types I, II and III SCC*mec* DNAs. They combined these primers with primers specific to the *S. aureus* chromosome region located to the right of the SCC*mec* integration site for the detection of MRSA. The primer set (SEQ ID NOs.: 22, 24 and 28 in US patent 6,156,507 corresponding to SEQ ID NOs.: 56, 58 and 60 in the present invention) was shown by Hiramatsu *et al.* to be the most specific and ubiquitous for detection of MRSA. This set of primers gives amplification products of 1.5 kb for SCC*mec* type I, 1.6 kb for SCC*mec* type II and 1.0 kb for SCC*mec* type III (Table 7). The ubiquity and specificity of this multiplex PCR assay was tested on 39 MRSA strains, 41 MSSA strains, 9 MRCNS strains and 11 MSCNS strains (Table 2). One μ L of a treated standardized bacterial suspension or of a bacterial genomic DNA preparation purified from bacteria were amplified in a 20 μ l PCR reaction mixture. Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% TritonTM X-100, 2.5 mM MgCl₂, 0.4 μ M of each of the SCC*mec*- and *orfX*-specific primers (SEQ ID NOs.: 56, 58 and 60), 200 μ M of each of the four dNTPs (Pharmacia Biotech), 3.3 μ g/ μ l of BSA (Sigma), and 0.5 U *Taq* polymerase (Promega) coupled with *TaqStart*TM Antibody (BD Biosciences).

PCR reactions were then subjected to thermal cycling: 3 min at 94°C followed by 40 cycles of 60 seconds at 95°C for the denaturation step, 60 seconds at 55°C for the annealing step, and 60 seconds at 72°C for the extension step, then followed by a terminal extension of 7 minutes at 72°C using a standard thermocycler (PTC-200 from MJ Research Inc.). Detection of the PCR products was made by electrophoresis in agarose gels (2 %) containing 0.25 μ g/ml of ethidium bromide.

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None of the MRCNS or MSCNS strains tested were detected with the set of primers detecting SCCmec types I, II and III. Twenty of the 39 MRSA strains tested were not detected with this multiplex PCR assay (Tables 2 and 3). One of these undetected MRSA strains corresponds to the highly epidemic MRSA Portuguese clone (strain CCRI-9504; De Lencastre *et al.*, 1994. Eur. J. Clin. Microbiol. Infect. Dis. 13:64-73) and another corresponds to the highly epidemic MRSA Canadian clone CMRSA1 (strain CCRI-9589; Simor *et al.* CCDC 1999, 25-12, June 15). These data demonstrate that the primer set developed by Hiramatsu *et al.* (SEQ ID NOs.: 22, 24 and 28 in US patent 6,156,507 corresponding to SEQ ID NOs.: 56, 58 and 60 in the present invention) is not ubiquitous for the detection of MRSA and suggest that some MRSA strains have sequences at the SCCmec right extremity junction which are different from those identified by Hiramatsu *et al.* other types of SCCmec sequences or other sequences at the right extremity of SCCmec (MREP type) are found in MRSA. A limitation of this assay is the non-specific detection of 13 MSSA strains (Table 2).

EXAMPLE 2:

Detection and identification of MRSA using primers specific to MREP types i, ii and iii sequences developed in the present invention. Based on analysis of multiple sequence alignments of *orfX* and SCCmec sequences described by Hiramatsu *et al.* or available from GenBank, a set of primers (SEQ ID NOs: 64, 66, 67) capable of amplifying short segments of types I, II and III of SCCmec-*orfX* right extremity junctions from MRSA strains and discriminating from MRCNS (Annex I and Figure 2) were designed. The chosen set of primers gives amplification products of 176 bp for SCCmec type I, 278 pb for SCCmec type II and 223 bp for SCCmec type III and allows rapid PCR amplification. These primers were used in multiplex PCR to test their ubiquity and specificity using 208 MRSA strains, 252 MSSA strains, 41 MRCNS strains and 21 MRCNS strains

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(Table 12). The PCR amplification and detection was performed as described in Example 1. PCR reactions were then subjected to thermal cycling (3 minutes at 94°C followed by 30 or 40 cycles of 1 second at 95°C for the denaturation step and 30 seconds at 60°C for the annealing-extension step, and then followed by a terminal extension of 2 minutes at 72°C) using a standard thermocycler (PTC-200 from MJ Research Inc.). Detection of the PCR products was made as described in Example 1.

None of the MRCNS or MSCNS strains tested were detected with this set of primers (Table 12). However, the twenty MRSA strains which were not detected with the primer set developed by Hiramatsu *et al.* (SEQ ID NOs: 56, 58 and 60) were also not detected with the primers developed in the present invention (Tables 3 and 12). These data also demonstrate that some MRSA strains have sequences at the SCC*mec*-chromosome right extremity junction which are different from those identified by Hiramatsu *et al.* Again, as observed with the Hiramatsu primers, 13 MSSA strains were also detected non-specifically (Table 12). The clinical significance of this finding remains to be established since these apparent MSSA strains could be the result of a recent deletion in the *mec* locus (Deplano *et al.*, 2000, J. Antimicrob. Chemotherapy, 46:617-619; Inglis *et al.*, 1990, J. Gen. Microbiol., 136:2231-2239; Inglis *et al.*, 1993, J. Infect. Dis., 167:323-328; Lawrence *et al.* 1996, J. Hosp. Infect., 33:49-53; Wada *et al.*, 1991, Biochem. Biophys. Res. Comm., 176:1319-1326).

EXAMPLE 3:

25

Development of a multiplex PCR assay on a standard thermocycler for detection and identification of MRSA based on MREP types i, ii, iii, iv and v sequences. Upon analysis of two of the new MREP types iv and v sequence data described in the present invention, two new primers (SEQ ID NOs.: 79 and 80)

were designed and used in multiplex with the three primers SEQ ID NOs.: 64, 66 and 67 described in Example 2. PCR amplification and detection of the PCR products was performed as described in Example 2. Sensitivity tests performed by using ten-fold or two-fold dilutions of purified genomic DNA from various MRSA strains of each MREP/MREJ type showed a detection limit of 5 to 10 genome copies (Table 16). Specificity tests were performed using 0,1 ng of purified genomic DNA or 1 µl of a standardized bacterial suspension. All MRCNS or MSCNS strains tested were negative with this multiplex assay (Table 17). Twelve of the 20 MRSA strains which were not detected with the multiplex PCR described in Examples 1 and 2 were now detected with this multiplex assay. Again, as observed with the Hiramatsu primers, 13 MSSA strains were also detected non-specifically (Table 12). The eight MRSA strains (CCRI-9208, CCRI-9583, CCRI-9773, CCRI-9774, CCRI-9589, CCRI-9860, CCRI-9681, CCRI-9770) and which harbor the new MREP/MREJ types vi, vii, viii, ix and x sequences described in the present invention remained undetectable.

EXAMPLE 4:

Development of a real-time multiplex PCR assay on the Smart Cycler® for detection and identification of MRSA based on MREP types i, ii, iii, iv and v sequences. The multiplex PCR assay described in Example 3 containing primers (SEQ ID NOs.: 64, 66, 67, 79 and 80) was adapted to the Smart Cycler® platform (Cepheid). A molecular beacon probe specific to the *orfX* sequence was developed (SEQ ID NO. 84, see Annex II). Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton™ X-100, 3.5 mM MgCl₂, 0.4 µM of each of the SCC*mec*- and *orfX*-specific primers (SEQ ID NOs.: 64, 66, 67, 79 and 80), 0.2 µM of the FAM-labeled molecular beacon probe (SEQ ID NO.: 84), 200 µM of each of the four dNTPs, 3.3 µg/µl of BSA, and 0.5 U *Taq* polymerase coupled with *TaqStart*™ Antibody. The PCR amplification on the Smart Cycler® was performed

as follows: 3 min. at 94°C for initial denaturation, then forty-five cycles of three steps consisting of 5 seconds at 95°C for the denaturation step, 15 seconds at 59°C for the annealing step and 10 seconds at 72°C for the extension step. Fluorescence detection was performed at the end of each annealing step. Sensitivity tests performed by using purified genomic DNA from several MRSA strains of each MREP/MREJ type showed a detection limit of 2 to 10 genome copies (Table 18). None of the MRCNS or MSCNS were positive with this multiplex assay (Table 19). Again, as observed with the Hiramatsu primers, 13 MSSA strains were also detected non-specifically. Twelve of the twenty MRSA strains which were not detected with the multiplex PCR described in Examples 1 and 2 were detected by this multiplex assay. As described in Example 3, the eight MRSA strains which harbor the new MREP/MREJ types vi, vii, viii, ix and x sequences described in the present invention remained undetectable.

5 EXAMPLE 5:

Development of a real-time multiplex PCR assay on the Smart Cycler® for detection and identification of MRSA based on MREP types i, ii, iii, iv and v sequences including an internal control. The multiplex PCR assay described in Example 4 containing primers specific to MREP types i to v and *orfX* of *S. aureus* (SEQ ID NOs.: 64, 66, 67, 79 and 80) and a molecular beacon probe specific to the *orfX* sequence (SEQ ID NO. 84, see Annex II) was optimized to include an internal control to monitor PCR inhibition. This internal control contains sequences complementary to MREP type iv- and *orfX*-specific primers (SEQ ID NOs. 79 and 64). The assay also contains a TET-labeled molecular beacon probe specific to sequence within the amplicon generated by amplification of the internal control. Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton™ X-100, 3.45 mM MgCl₂, 0.8 µM of each of the MREP-specific primers (SEQ ID NOs.: 66 and 67) and *orfX*-specific primer (SEQ ID NO.: 64), 0.4 µM of each of

the MREP-specific primers (SEQ ID NOs.: 79 and 80), 80 copies of the internal control, 0.2 μ M of the TET-labeled molecular beacon probe specific to the internal control, 0.2 μ M of the FAM-labeled molecular beacon probe (SEQ ID NO.: 84), 330 μ M of each of the four dNTPs (Pharmacia Biotech), 3.45 μ g/ μ l of BSA (Sigma), and 0.875 U *Taq* polymerase (Promega) coupled with *TaqStart*TM Antibody (BD Biosciences). The PCR amplification on the Smart Cycler[®] was performed as follows: 3 min. at 95°C for initial denaturation, then forty-eight cycles of three steps consisting of 5 seconds at 95°C for the denaturation step, 15 seconds at 60°C for the annealing step and 15 seconds at 72°C for the extension step. Sensitivity tests performed by using purified genomic DNA from one MRSA strain of each MREP/MREJ type (i to v) showed a detection limit of 2 to 10 genome copies. None of the 26 MRCNS or 10 MSCNS were positive with this multiplex assay. Again, as observed with the Hiramatsu primers, 13 MSSA strains were also detected non-specifically. As described in Examples 3 and 4, the eight MRSA strains which harbor the new MREP/MREJ types vi to x sequences described in the present invention remained undetectable.

EXAMPLE 6:

Detection of MRSA using the real-time multiplex assay on the Smart Cycler[®] based on MREP types i, ii, iii, iv and v sequences directly from clinical specimens. The assay described in Example 5 was adapted for detection directly from clinical specimens. A total of 142 nasal swabs collected during a MRSA hospital surveillance program at the Montreal General Hospital (Montreal, Quebec, Canada) were tested. The swab samples were tested at the Centre de Recherche en Infectiologie de l'Université Laval within 24 hours of collection. Upon receipt, the swabs were plated onto mannitol agar and then the nasal material from the same swab was prepared with a simple and rapid specimen preparation protocol

described in patent application number WO 03/008636. Classical identification of MRSA was performed by standard culture methods.

The PCR assay described in Example 5 detected 33 of the 34 samples positive for MRSA based on the culture method. As compared to culture, the PCR assay detected 8 additional MRSA positive specimens for a sensitivity of 97.1 % and a specificity of 92.6 %. This multiplex PCR assay represents a rapid and powerful method for the specific detection of MRSA carriers directly from nasal specimens and can be used with any type of clinical specimens such as wounds, blood or blood culture, CSF, etc.

EXAMPLE 7:

Development of a real-time multiplex PCR assay on the Smart Cycler[®] for detection and identification of MRSA based on MREP types i, ii, iii, iv, v and vii sequences. Upon analysis of the new MREJ type vii sequence data described in the present invention (SEQ ID NOs.: 165 and 166), two new primers (SEQ ID NOs.: 112 and 113 specific for the MREP type vii) were designed and tested in multiplex with the three primers SEQ ID NOs.: 64, 66 and 67 described in Example 2. Primer SEQ ID NO.: 112 was selected for use in the multiplex based on its sensitivity. Three molecular beacon probes specific to the *orfX* sequence which allowed detection of two sequence polymorphisms identified in this region of the *orfX* sequence, based on analysis of SEQ ID NOs.: 173-186, were also used in the multiplex (SEQ ID NOs.: 84, 163 and 164). Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton[™] X-100, 3.45 mM MgCl₂, 0.8 μM of each of the SCC*mec*-specific primers (SEQ ID NOs.: 66 and 67) and *orfX*-specific primer (SEQ ID NO.: 64), 0.4 μM of each of the SCC*mec*-specific primers (SEQ ID NOs.: 79 and 80), 0.2 μM of the FAM-labeled molecular beacon probe (SEQ ID NO.: 84), 330 μM of each of the four dNTPs (Pharmacia Biotech), 3.45

5 $\mu\text{g}/\mu\text{l}$ of BSA (Sigma), and 0.875 U of *Taq* polymerase (Promega) coupled with *TaqStart*TM Antibody (BD Biosciences). The PCR amplification on the Smart Cycler[®] was performed as follows: 3 min. at 95°C for initial denaturation, then forty-eight cycles of three steps consisting of 5 seconds at 95°C for the denaturation step, 15 seconds at 60°C for the annealing step and 15 seconds at 72°C for the extension step. The detection of fluorescence was done at the end of each annealing step. Sensitivity tests performed by using purified genomic DNA from several MRSA strains of each MREP/MREJ type showed a detection limit of 2 genome copies (Table 20). None of the 26 MRCNS or 8 MSCNS were positive with this multiplex assay. Again, as observed with the Hiramatsu primers, 13 MSSA strains were also detected non-specifically (Table 21). Four of the strains which were not detected with the multiplex assay for the detection of MREP/MREJ types i to v were now detected with this multiplex assay while the four MRSA strains (CCRI-9208, CCRI-9770, CCRI-9681, CCRI-9860) which harbor the MREP/MREJ types vi, viii, ix and x described in the present invention remained undetectable.

EXAMPLE 8:

0 **Development of real-time PCR assays on the Smart Cycler[®] for detection and identification of MRSA based on MREP types vi, viii, ix.** Upon analysis of the new MREP/MREJ types vi, viii and ix sequence data described in the present invention, one new primers specific to MREP type vi (SEQ ID NOs.: 202 and 204), one primer specific to MREP type viii (SEQ ID NO.: 116), a primer specific to MREP type ix (SEQ ID NO.: 109) and a primer specific to both MREP types viii and ix (SEQ ID NO.: 115) were designed. Each PCR primer was used in combination with the *orfX*-specific primer (SEQ ID NO.: 64) and tested against its specific target strain. Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% TritonTM X-100, 3.45 mM MgCl₂, 0.4 μM of each of the SCC*mec*- and *orfX*-specific primers, 200 μM of each of the four dNTPs, 3.4 $\mu\text{g}/\mu\text{l}$ of BSA, and 0.875

U *Taq* polymerase coupled with *TaqStart*[™] Antibody. The PCR amplification was performed as described in Example 7. Sensitivity tests performed by using genomic DNA purified from their respective MRSA target strains showed that the best primer pair combination was SEQ ID NOs.: 64 and 115 for the detection of
 5 MREJ types viii and ix simultaneously. These new *SCCmec*-specific primers may be used in multiplex with primers specific to MREJ types i, ii, ii, iv, v and vii (SEQ ID NOs.: 64, 66, 67, 79 and 80) described in previous examples to provide a more ubiquitous MRSA assay.

) In conclusion, we have improved the ubiquity of detection of MRSA strains. New MREJ types iv to x have been identified. Amongst strains representative of these new types, Hiramitsu's primers and/or probes succeeded in detecting less than 50% thereof. We have therefore amply passed the bar of at least 50% ubiquity, since our primers and probes were designed to detect 100% of the strains tested as
 ; representatives of MREJ types iv to ix. Therefore, although ubiquity depends on the pool of strains and representatives that are under analyse, we know now that close to 100% ubiquity is an attainable goal, when using the sequences of the right junctions (MREJ) to derive probes and primers dealing with polymorphism in this region. Depending on how many unknown types of MREJ exist, we have a margin of manoeuvre going from 50% (higher than Hiramitsu's primers for the tested strains) to 100% if we sequence all the existing MREJs to derive properly the present diagnostic tools and methods, following the above teachings.

The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

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Table 1. PCR amplification primers reported by Hiramatsu et al. in US patent 6,156,507 found in the sequence listing

	SEQ ID NO.: (present invention)	Target	Position ^{a,b}	SEQ ID NO.: (US pat. 6,156,507)
5	52	MREP types i and ii	480	18
	53	MREP types i and ii	758	19
	54	MREP types i and ii	927	20
	55	MREP types i and ii	1154	21
	56	MREP types i and ii	1755	22
	57	MREP types i and ii	2302	23
10	58	MREP type iii	295 ^c	24
	59	orfX	1664	25
15	60	orfSA0022 ^d	3267	28
	61	orfSA0022 ^d	3585	27
	62	orfX	1389	26
	63	orfSA0022 ^d	2957	29

20

^a Position refers to nucleotide position of the 5' end of primer.

^b Numbering for SEQ ID NOs.: 52-57 refers to SEQ ID NO.: 2; numbering for SEQ ID NO.: 58 refers to SEQ ID NO.: 4; numbering for SEQ ID NOs.: 59-63 refers to SEQ ID NO.: 3.

25

^c Primer is reverse-complement of target sequence.

^d orfSA0022 refers to the open reading frame designation from GenBank accession number AP003129 (SEQ ID NO.: 231).

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5 **Table 2. Specificity and ubiquity tests performed on a standard thermocycler using the optimal set of primers described by Hiramatsu et al. (SEQ ID NOs. : 22, 24 and 28 in US patent 6,156,507 corresponding to SEQ ID NOs.: 56, 58 and 60, respectively, in the present invention) for the detection of MRSA**

Strains	PCR results for SCCmec - orfX right extremity junction	
	Positive (%)	Negative (%)
MRSA - 39 strains	19 (48.7)	20 (51.2)
MSSA - 41 strains	13 (31.7)	28 (68.3)
MRCNS - 9 strains*	0 (0%)	9 (100%)
MSCNS - 11 strains*	0 (0%)	11 (100%)

10 * Details regarding CNS strains:

15	MRCNS :	<i>S. caprae</i> (1)
		<i>S. cohnii cohnii</i> (1)
		<i>S. epidermidis</i> (1)
		<i>S. haemolyticus</i> (2)
		<i>S. hominis</i> (1)
		<i>S. sciuri</i> (1)
		<i>S. simulans</i> (1)
		<i>S. warneri</i> (1)
25	MSCNS :	<i>S. cohnii cohnii</i> (1)
		<i>S. epidermidis</i> (1)
		<i>S. equorum</i> (1)
		<i>S. gallinarum</i> (1)
		<i>S. haemolyticus</i> (1)
		<i>S. lentus</i> (1)
		<i>S. lugdunensis</i> (1)
		<i>S. saccharolyticus</i> (1)
		<i>S. saprophyticus</i> (2)
30		<i>S. xylosus</i> (1)

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Table 3. Origin of MRSA strains not amplifiable using primers developed by Hiramatsu et al. (SEQ ID NOs.: 22, 24 and 28 in US patent 6,156,507 corresponding to SEQ ID NOs.: 56, 58 and 60, respectively, in the present invention) as well as primers developed in the present invention targeting MREP types i, ii and iii (SEQ ID NOs.: 64, 66 and 67)

Staphylococcus aureus strain designation: Original CCRI ^a		Origin
ATCC BAA-40 ^b	CCRI-9504	Portugal
ATCC 33592	CCRI-178	USA
R991282	CCRI-2025	Québec, Canada
4508	CCRI-9208	Québec, Canada
19121	CCRI-8895	Denmark
Z109	CCRI-8903	Denmark
45302	CCRI-1263	Ontario, Canada
R655	CCRI-1324	Québec, Canada
MA 50428	CCRI-1311	Québec, Canada
MA 50609	CCRI-1312	Québec, Canada
MA 51363	CCRI-1331	Québec, Canada
MA 51561	CCRI-1325	Québec, Canada
14A0116	CCRI-9681	Poland
23 (CCUG 41787)	CCRI-9860	Sweden
SE26-1	CCRI-9770	Ontario, Canada
SE1-1	CCRI-9583	Ontario, Canada
ID-61880 ^c	CCRI-9589	Ontario, Canada
SE47-1	CCRI-9773	Ontario, Canada
SE49-1	CCRI-9774	Ontario, Canada
39795-2	CCRI-1377	Québec, Canada

^a CCRI stands for "Collection of the Centre de Recherche en Infectiologie".

^b Portuguese clone.

^c Canadian clone EMRSA1.

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Table 4. *Staphylococcus aureus* MREJ nucleotide sequences revealed in the present invention

5	SEQ ID NO.	<i>Staphylococcus aureus</i> strain designation:		Genetic Target
		Original	CCRI ^a	
10	27	R991282	CCRI-2025	<i>mecA</i>
	28	45302	CCRI-1263	<i>mecA</i>
	29	MA 50428	CCRI-1311	<i>mecA</i>
	30	MA 51363	CCRI-1331	<i>mecA</i>
	31	39795-2	CCRI-1377	<i>mecA</i> and 1.5 kb of downstream region
15	42	ATCC 33592	CCRI-178	MREP type iv
	43	19121	CCRI-8895	MREP type iv
	44	Z109	CCRI-8903	MREP type iv
	45	R655	CCRI-1324	MREP type iv
	46	MA 51363	CCRI-1331	MREP type iv
20	47	45302	CCRI-1263	MREP type v
	48	39795-2	CCRI-1377	MREP type v
	49	MA 50428	CCRI-1311	MREP type v
	50	R991282	CCRI-2025	MREP type v
	51	ATCC BAA-40	CCRI-9504	MREP type iv
25	165	SE1-1	CCRI-9583	MREP type vii
	166	ID-61880	CCRI-9589	MREP type vii
	167	23 (CCUG 41787)	CCRI-9860	MREP type viii
	168	14A016	CCRI-9681	MREP type ix
	171	4508	CCRI-9208	MREP type vi
30	172	SE26-1	CCRI-9770	<i>orfSA0021^b</i> and 75 bp of <i>orfSA0022^b</i>
	173	26 (98/10618)	CCRI-9864	MREP type ii
	174	27 (98/26821)	CCRI-9865	MREP type ii
	175	28 (24344)	CCRI-9866	MREP type ii
	176	12 (62305)	CCRI-9867	MREP type ii
35	177	22 (90/14719)	CCRI-9868	MREP type ii
	178	23 (98/14719)	CCRI-9869	MREP type ii
	179	32 (97S99)	CCRI-9871	MREP type ii
	180	33 (97S100)	CCRI-9872	MREP type ii
	181	38 (825/96)	CCRI-9873	MREP type ii
40	182	39 (842/96)	CCRI-9874	MREP type ii
	183	43 (N8-892/99)	CCRI-9875	MREP type ii
	184	46 (9805-0137)	CCRI-9876	MREP type iii
	185	1	CCRI-9882	MREP type ii
	186	29	CCRI-9885	MREP type ii
45	189	SE1-1	CCRI-9583	<i>mecA</i> and 2.2 kb of downstream region, including IS431 <i>mec</i>
	190	ATCC BAA-40	CCRI-9504	<i>mecA</i> and 1.5 kb of downstream region
	191	4508	CCRI-9208	<i>mecA</i> and 0.9 kb of downstream region
	192	ID-61880	CCRI-9589	<i>mecA</i> and 0.9 kb of downstream region
	193	14A016	CCRI-9681	<i>mecA</i> and 0.9 kb of downstream region
50	195	SE26-1	CCRI-9770	<i>mecA</i> and 1.5 kb of downstream region, including IS431 <i>mec</i>
	197	ATCC 43300	CCRI-175	MREP type ii
	198	R522	CCRI-1262	MREP type iii
	199	13370	CCRI-8894	MREP type i
	219	ATCC BAA-40	CCRI-9504	<i>tetK</i>

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Table 4. *Staphylococcus aureus* MREJ nucleotide sequences revealed in the present invention (continued)

5	SEQ ID NO.	<i>Staphylococcus aureus</i> strain designation:		Genetic Target ^a
		Original	CCRI ^b	
	220	MA 51363	CCRI-1331	<i>mecA</i> and 1.5 kb of downstream region
	221	39795-2	CCRI-1377	IS431 <i>mec</i> and 0.6 kb of upstream region
10	222	R991282	CCRI-2025	<i>mecA</i> and 1.5 kb of downstream region
	223	R991282	CCRI-2025	IS431 <i>mec</i> and 0.6 kb of upstream region
	224	23 (CCUG 41787)	CCRI-9860	<i>mecA</i> and 1.5 kb of downstream region
	225	23 (CCUG 41787)	CCRI-9860	IS431 <i>mec</i> and 0.6 kb of upstream region
15	233	14A016	CCRI-9681	MREP type ix

^a CCRI stands for "Collection of the Centre de Recherche en Infectiologie".

^b orfSA0021 and orfSA0022 refer to the open reading frame designation from GenBank accession number AP003129 (SEQ ID NO.: 231).

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Table 5. PCR primers developed in the present invention

		Originating DNA		
SEQ ID NO.	Target	Position ^a	SEQ ID NO.	
5				
	64	orfX	1720	3
	70	orfX	1796	3
	71	orfX	1712	3
	72	orfX	1749	3
0	73	orfX	1758	3
	74	orfX	1794	3
	75	orfX	1797	3
	76	orfX	1798	3
	66	MREP types i and ii	2327	2
5	100	MREP types i and ii	2323	2
	101	MREP types i and ii	2314	2
	97	MREP type ii	2434	2
	99	MREP type ii	2434	2
	67	MREP type iii	207 ^b	4
0	98	MREP type iii	147 ^b	4
	102	MREP type iii	251 ^b	4
	79'	MREP type iv	74 ^b	43
	80	MREP type v	50 ^b	47
	109	MREP type ix	652 ^b	168
5	204	MREP type vi	642 ^b	171
	112	MREP type vii	503 ^b	165
	113	MREP type vii	551 ^b	165
	115	MREP type viii	514 ^b	167
0	116	MREP type viii	601 ^b	167

^a Position refers to nucleotide position of 5' end of primer.

^b Primer is reverse-complement of target sequence.

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Table 6. Molecular beacon probes developed in the present invention

	SEQ ID NO.	Target	Position
5	32	<i>orfX</i>	86 ^a
	83	<i>orfX</i>	86 ^a
	84	<i>orfX</i>	34 ^{a,b}
	160	<i>orfX</i>	55 ^{a,b}
10	161	<i>orfX</i>	34 ^{a,b}
	162	<i>orfX</i>	114 ^a
	163	<i>orfX</i>	34 ^{a,b}
	164	<i>orfX</i>	34 ^{a,b}

15

^a Position refers to nucleotide position of the 5' end of the molecular beacon's loop on SEQ ID NO.: 3.

^b Sequence of molecular beacon's loop is reverse-complement of SEQ ID NO.: 3.

Table 7. Length of amplicons obtained with the different primer pairs which are objects of the present invention

SEQ ID NO.	Target ^d	Amplicon length ^a
5	59/52 ^b <i>orfX</i> /MREP type i and ii	2079 (type i);2181 (type ii)
	59/53 ^b <i>orfX</i> /MREP type i and ii	1801 (type i);1903 (type ii)
	59/54 ^b <i>orfX</i> /MREP type i and ii	1632 (type i);1734 (type ii)
	59/55 ^b <i>orfX</i> /MREP type i and ii	1405 (type i);1507 (type ii)
0	59/56 ^b <i>orfX</i> /MREP type i and ii	804 (type i);906 (type ii)
	59/57 ^b <i>orfX</i> /MREP type i and ii	257 (type i);359 (type ii)
	60/52 ^b <i>orfSA0022</i> /MREP type i and ii	2794 (type i);2896 (type ii)
	60/53 ^b <i>orfSA0022</i> /MREP type i and ii	2516 (type i);2618 (type ii)
	60/54 ^b <i>orfSA0022</i> /MREP type i and ii	2347 (type i);2449 (type ii)
5	60/55 ^b <i>orfSA0022</i> /MREP type i and ii	2120 (type i);2222 (type ii)
	60/56 ^b <i>orfSA0022</i> /MREP type i and ii	1519 (type i);1621 (type ii)
	60/57 ^b <i>orfSA0022</i> /MREP type i and ii	972 (type i);1074 (type ii)
	61/52 ^b <i>orfSA0022</i> /MREP type i and ii	2476 (type i);2578 (type ii)
	61/53 ^b <i>orfSA0022</i> /MREP type i and ii	2198 (type i);2300 (type ii)
0	61/54 ^b <i>orfSA0022</i> /MREP type i and ii	2029 (type i);2131 (type ii)
	61/55 ^b <i>orfSA0022</i> /MREP type i and ii	1802 (type i);1904 (type ii)
	61/56 ^b <i>orfSA0022</i> /MREP type i and ii	1201 (type i);1303 (type ii)
	61/57 ^b <i>orfSA0022</i> /MREP type i and ii	654 (type i);756 (type ii)
5	62/52 ^b <i>orfX</i> /MREP type i and ii	2354 (type i);2456 (type ii)
	62/53 ^b <i>orfX</i> /MREP type i and ii	2076 (type i);2178 (type ii)
	62/54 ^b <i>orfX</i> /MREP type i and ii	1907 (type i);2009 (type ii)
	62/55 ^b <i>orfX</i> /MREP type i and ii	1680 (type i);1782 (type ii)
	62/56 ^b <i>orfX</i> /MREP type i and ii	1079 (type i);1181 (type ii)
	62/57 ^b <i>orfX</i> /MREP type i and ii	532 (type i);634 (type ii)
0	63/52 ^b <i>orfSA0022</i> /MREP type i and ii	3104 (type i);3206 (type ii)
	63/53 ^b <i>orfSA0022</i> /MREP type i and ii	2826 (type i);2928 (type ii)
	63/54 ^b <i>orfSA0022</i> /MREP type i and ii	2657 (type i);2759 (type ii)
	63/55 ^b <i>orfSA0022</i> /MREP type i and ii	2430 (type i);2532 (type ii)
	63/56 ^b <i>orfSA0022</i> /MREP type i and ii	1829 (type i);1931 (type ii)
5	63/57 ^b <i>orfSA0022</i> /MREP type i and ii	1282 (type i);1384 (type ii)
	59/58 ^b <i>orfX</i> /MREP type iii	361
	60/58 ^b <i>orfSA0022</i> /MREP type iii	1076
	61/58 ^b <i>orfSA0022</i> /MREP type iii	758
	62/58 ^b <i>orfX</i> /MREP type iii	656
0	63/58 ^b <i>orfSA0022</i> /MREP type iii	1386
	70/66 <i>orfX</i> /MREP type i and ii	100 (type i);202 (type ii)
	70/67 <i>orfX</i> /MREP type iii	147 (type iii)
	64/66 ^c <i>orfX</i> /MREP type i and ii	176 (type i);278 (type ii)
	64/67 ^c <i>orfX</i> /MREP type iii	223
5	64/79 ^c <i>orfX</i> /MREP type iv	215
	64/80 ^c <i>orfX</i> /MREP type v	196
	64/97 ^c <i>orfX</i> /MREP type ii	171
	64/98 ^c <i>orfX</i> /MREP type iii	163
	64/99 ^c <i>orfX</i> /MREP type ii	171
0	64/100 ^c <i>orfX</i> /MREP types i and ii	180 (type i);282 (type ii)
	64/101 ^c <i>orfX</i> /MREP types i and ii	189 (type i);291 (type ii)
	64/102 ^c <i>orfX</i> /MREP type iii	263
	64/109 ^c <i>orfX</i> /MREP type ix	369
	64/204 ^c <i>orfX</i> /MREP type vi	348
5	64/112 ^c <i>orfX</i> /MREP type vii	214
	64/113 ^c <i>orfX</i> /MREP type vii	263
	64/115 ^c <i>orfX</i> /MREP type viii	227
	64/116 ^c <i>orfX</i> /MREP type viii	318

^a Amplicon length is given in base pairs for MREP types amplified by the set of primers.

^b Set of primers described by Hiramatsu *et al.* in US patent 6,156,507.

^c Set of primers developed in the present invention.

^d *orfSA0022* refers to the open reading frame designation from GenBank accession number AP003129 (SEQ ID NO.: 231).

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Table 8. Other primers developed in the present invention

	SEQ ID NO.	Target	Originating DNA	
			Position ^a	SEQ ID NO.
5	77	MREP type iv	993	43
	65	MREP type v	636	47
	70	<i>orfX</i>	1796	3
	68	<i>IS431</i>	626	92
10	69	<i>mecA</i>	1059	78
	96	<i>mecA</i>	1949	78
	81	<i>mecA</i>	1206	78
	114	MREP type vii	629 ^b	165
15	117	MREP type ii	856	194
	118	MREP type ii	974 ^b	194
	119	MREP type vii	404	189
	120	MREP type vii	477 ^b	189
20	123	MREP type vii	551	165
	124	MREP type ii	584	170
	125	MREP type ii	689 ^b	170
	126	<i>orfSA0021</i>	336	231
25	127	<i>orfSA0021</i>	563	231
	128	<i>orfSA0022^d</i>	2993	231
	129	<i>orfSA0022^d</i>	3467 ^b	231
	132	<i>orfX</i>	3700	231
30	145	MREP type iv	988	51
	146	MREP type v	1386	51
	147	MREP type iv	891 ^b	51
	148	MREP type ix	664	168
35	149	MREP type ix	849 ^b	168
	150	MREP type vii	1117 ^b	165
	151	MREP type vii	1473	189
	152	<i>IS431mec</i>	1592 ^b	189
40	154	MREP type v	996 ^b	50
	155	MREP type v	935	50
	156	<i>tetK</i> from plasmid pT181	1169 ^b	228
	157	<i>tetK</i> from plasmid pT181	136	228
45	158	<i>orfX</i>	2714 ^b	2
	159	<i>orfX</i>	2539	2
	187	MREP type viii	967 ^b	167
	188	MREP type viii	851	167

^a Position refers to nucleotide position of the 5' end of primer.^b Primer is reverse-complement of target sequence.

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Table 9. Amplification and/or sequencing primers developed in the present invention

5	SEQ ID NO.	Target	Originating DNA	
			Position ^a	SEQ ID NO.
10	85	<i>S. aureus</i> chromosome	197 ^b	35
	86	<i>S. aureus</i> chromosome	198 ^b	37
	87	<i>S. aureus</i> chromosome	197 ^b	38
	88	<i>S. aureus</i> chromosome	1265 ^b	39
	89	<i>S. aureus</i> chromosome	1892	3
15	103	<i>orfX</i>	1386	3
	105	MREP type i	2335	2
	106	MREP type ii	2437	2
	107	MREP type iii	153 ^b	4
	108	MREP type iii	153 ^b	4
20	121	MREP type vii	1150	165
	122	MREP type vii	1241 ^b	165
	130	<i>orfX</i>	4029 ^b	231
	131	region between <i>orfSA0022</i> and <i>orfSA0023</i> ^d	3588	231
	133	<i>merB</i> from plasmid pI258	262	226
25	134	<i>merB</i> from plasmid pI258	539 ^b	226
	135	<i>merR</i> from plasmid pI258	564	226
	136	<i>merR</i> from plasmid pI258	444	227
	137	<i>merR</i> from plasmid pI258	529	227
	138	<i>merR</i> from plasmid pI258	530 ^b	227
30	139	<i>rep</i> from plasmid pUB110	796	230
	140	<i>rep</i> from plasmid pUB110	761 ^b	230
	141	<i>rep</i> from plasmid pUB110	600	230
	142	<i>aadD</i> from plasmid pUB110	1320 ^b	229
	143	<i>aadD</i> from plasmid pUB110	759	229
35	144	<i>aadD</i> from plasmid pUB110	646	229
	153	MREP type vii	1030	165
	200	<i>orfSA0022</i> ^d	871 ^c	231
	201	<i>orfSA0022</i> ^d	1006	231
	202	MREP type vi	648	171
40	203	MREP type vi	883 ^b	171
	205	MREP type ix	1180	168
	206	MREP type ix	1311 ^b	233
	207	MREP type viii	1337	167
	208	MREP type viii	1441 ^b	167
45	209	<i>ccrA</i>	184	232
	210	<i>ccrA</i>	385	232
	211	<i>ccrA</i>	643 ^b	232
	212	<i>ccrA</i>	1282 ^b	232
	213	<i>ccrB</i>	1388	232
50	214	<i>ccrB</i>	1601	232
	215	<i>ccrB</i>	2139 ^b	232
	216	<i>ccrB</i>	2199 ^b	232
	217	<i>ccrB</i>	2847 ^b	232
	218	<i>ccrB</i>	2946 ^b	232

^a Position refers to nucleotide position of the 5' end of primer.^b Primer is reverse-complement of target sequence.^c Primer contains two mismatches.^d *orfSA0022* and *orfSA0023* refer to the open reading frame designation from GenBank accession number AP003129 (SEQ ID NO.: 231).

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Table 10. Origin of the nucleic acids and/or sequences available from public databases found in the sequence listing

SEQ ID NO.	Staphylococcal strain	Source	Accession number	Genetic Target ^{a, b}
1	NCTC 10442	Database	AB033763	SCCmec type I MREJ
2	N315	Database	D86934	SCCmec type II MREJ
3	NCTC 8325	Database	AB014440	MSSA chromosome
4	86/560	Database	AB013471	SCCmec type III MREJ
5	86/961	Database	AB013472	SCCmec type III MREJ
6	85/3907	Database	AB013473	SCCmec type III MREJ
7	86/2652	Database	AB013474	SCCmec type III MREJ
8	86/1340	Database	AB013475	SCCmec type III MREJ
9	86/1762	Database	AB013476	SCCmec type III MREJ
10	86/2082	Database	AB013477	SCCmec type III MREJ
11	85/2111	Database	AB013478	SCCmec type III MREJ
12	85/5495	Database	AB013479	SCCmec type III MREJ
13	85/1836	Database	AB013480	SCCmec type III MREJ
14	85/2147	Database	AB013481	SCCmec type III MREJ
15	85/3619	Database	AB013482	SCCmec type III MREJ
16	85/3566	Database	AB013483	SCCmec type III MREJ
17	85/2232	Database	AB014402	SCCmec type II MREJ
18	85/2235	Database	AB014403	SCCmec type II MREJ
19	MR108	Database	AB014404	SCCmec type II MREJ
20	85/9302	Database	AB014430	SCCmec type I MREJ
21	85/9580	Database	AB014431	SCCmec type I MREJ
22	85/1940	Database	AB014432	SCCmec type I MREJ
23	85/6219	Database	AB014433	SCCmec type I MREJ
24	64/4176	Database	AB014434	SCCmec type I MREJ
25	64/3846	Database	AB014435	SCCmec type I MREJ
26	HUC19	Database	AF181950	SCCmec type II MREJ
33	G3	US 6,156,507	SEQ ID NO.: 15	<i>S. epidermidis</i> SCCmec type II MREJ
34	SH 518	US 6,156,507	SEQ ID NO.: 16	<i>S. haemolyticus</i> SCCmec type II MREJ
35	ATCC 25923	US 6,156,507	SEQ ID NO.: 9	<i>S. aureus</i> chromosome
36	STP23	US 6,156,507	SEQ ID NO.: 10	<i>S. aureus</i> chromosome
37	STP43	US 6,156,507	SEQ ID NO.: 12	<i>S. aureus</i> chromosome
38	STP53	US 6,156,507	SEQ ID NO.: 13	<i>S. aureus</i> chromosome
39	476	Genome project ^c		<i>S. aureus</i> chromosome
40	252	Genome project ^c		SCCmec type II MREJ
41	COL	Genome project ^d		SCCmec type I MREJ
78	NCTC 8325	Database	X52593	<i>mecA</i>
82	NCTC 10442	Database	AB033763	<i>mecA</i>
90	N315	Database	D86934	<i>mecA</i>
91	85/2082	Database	AB037671	<i>mecA</i>
92	NCTC 10442	Database	AB033763	IS431
93	N315	Database	D86934	IS431
94	HUC19	Database	AF181950	IS431
95	NCTC 8325	Database	X53818	IS431
104	85/2082	Database	AB037671	SCCmec type III MREJ
226	unknown	Database	L29436	<i>merB</i> on plasmid pI258
227	unknown	Database	L29436	<i>merR</i> on plasmid pI258
228	unknown	Database	S67449	<i>tetK</i> on plasmid pT181
229	HUC19	Database	AF181950	<i>aadD</i> on plasmid pUB110
230	HUC19	Database	AF181950	<i>rep</i> on plasmid pUB110
231	N315	Database	AP003129	<i>orfSA0021</i> , <i>orfSA0022</i> , <i>orfSA0023</i>
232	85/2082	Database	AB037671	<i>ccrA/ccrB</i>

^a MREJ refers to *mec* right extremity junction and includes sequences from SCCmec-right extremity and chromosomal DNA to the right of SCCmec integration site.

^b Unless otherwise specified, all sequences were obtained from *S. aureus* strains.

^c Sanger Institute genome project (<http://www.sanger.ac.uk>).

^d TIGR genome project (<http://www.tigr.org>).

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Table 11. Analytical sensitivity of the MRSA-specific PCR assay targeting MREP types i, ii and iii on a standard thermocycler using the set of primers developed in the present invention (SEQ ID NOs.: 64, 66 and 67)

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Strain designation :		Detection limit
Original	CCRI ^a (MREP type)	(number of genome copies)
13370	CCRI-8894 (I)	5
ATCC 43300	CCRI-175 (II)	2
35290	CCRI-1262 (III)	2

^a CCRI stands for "Collection of the Centre de Recherche en Infectiologie".

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Table 12. Specificity and ubiquity tests performed on a standard thermocycler using the set of primers targeting MREP types i, ii and iii developed in the present invention (SEQ ID NOs.: 64, 66 and 67) for the detection of MRSA

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Strains	PCR results for MREJ	
	Positive (%)	Negative (%)
MRSA - 208 strains	188 (90.4)	20 (9.6)
MSSA - 252 strains	13 (5.2)	239 (94.8)
MRCNS - 41 strains*	0	42 (100)
MSCNS - 21 strains*	0	21 (100)

* Details regarding CNS strains:

10	MRCNS :	<i>S. caprae</i> (2)
		<i>S. cohnii cohnii</i> (3)
		<i>S. cohnii urealyticum</i> (4)
		<i>S. epidermidis</i> (8)
		<i>S. haemolyticus</i> (9)
		<i>S. hominis</i> (4)
15		<i>S. sciuri</i> (4)
		<i>S. sciuri sciuri</i> (1)
		<i>S. simulans</i> (3)
		<i>S. warneri</i> (3)
20	MSCNS :	<i>S. cohnii cohnii</i> (1)
		<i>S. epidermidis</i> (3)
		<i>S. equorum</i> (2)
		<i>S. felis</i> (1)
		<i>S. gallinarum</i> (1)
		<i>S. haemolyticus</i> (1)
25		<i>S. hominis</i> (1)
		<i>S. lentus</i> (1)
		<i>S. lugdunensis</i> (1)
		<i>S. saccharolyticus</i> (1)
30		<i>S. saprophyticus</i> (5)
		<i>S. simulans</i> (1)
		<i>S. warneri</i> (1)
		<i>S. xylosus</i> (1)

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Table 13. Percentage of sequence identity for the first 500 nucleotides of SCCmec right extremities between all 9 types of MREP^{a,b}

MREP type	i	ii	iii	iv	v	vi	vii	viii	ix
i	--	79.2	42.8	42.8	41.2	44.4	44.6	42.3	42.1
ii			43.9	47.5	44.7	41.7	45.0	52.0	57.1
iii				46.8	44.5	42.9	45.0	42.8	45.2
iv					45.8	41.4	44.3	48.0	41.3
v						45.4	43.7	47.5	44.3
vi							45.1	41.1	47.2
vii								42.8	40.9
viii									55.2
ix									--

5

^a "First 500 nucleotides" refers to the 500 nucleotides within the SCCmec right extremity, starting from the integration site of SCCmec in the *Staphylococcus aureus* chromosome as shown on Figure 4.

10 ^b Sequences were extracted from SEQ ID NOs.: 1, 2, 104, 51, 50, 171, 165, 167, and 168 for types i to ix, respectively.

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Table 14. Reference strains used to test sensitivity and/or specificity and/or ubiquity of the MRSA-specific PCR assays targeting MREJ sequences

Staphylococcal species	Strains	Source ^a
MRSA (n = 45)	33591	ATCC
	33592	ATCC
	33593	ATCC
	BAA-38	ATCC
	BAA-39	ATCC
	BAA-40	ATCC
	BAA-41	ATCC
	BAA-42	ATCC
	BAA-43	ATCC
	BAA-44	ATCC
	F182	CDC
	23 (CCUG 41787)	HARMONY Collection
	ID-61880 (EMRSA1)	LSPQ
	MA 8628	LSPQ
	MA 50558	LSPQ
	MA 50428	LSPQ
	MA 50609	LSPQ
	MA 50884	LSPQ
	MA 50892	LSPQ
	MA 50934	LSPQ
	MA 51015	LSPQ
	MA 51056	LSPQ
	MA 51085	LSPQ
	MA 51172	LSPQ
	MA 51222	LSPQ
	MA 51363	LSPQ
	MA 51561	LSPQ
	MA 52034	LSPQ
	MA 52306	LSPQ
	MA 51520	LSPQ
	MA 51363	LSPQ
	98/10618	HARMONY Collection
	98/26821	HARMONY Collection
	24344	HARMONY Collection
	62305	HARMONY Collection
	90/10685	HARMONY Collection
	98/14719	HARMONY Collection
	97S99	HARMONY Collection
	97S100	HARMONY Collection
	825/96	HARMONY Collection
	842/96	HARMONY Collection
	N8-890/99	HARMONY Collection
	9805-01937	HARMONY Collection
	1	Kreiswirth-1
	29	Kreiswirth-1
MRCNS (n = 4)	29060	ATCC
	35983	ATCC
	35984	ATCC
	2514	LSPQ

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Table 14. Reference strains used to test sensitivity and/or specificity and/or ubiquity of the MRSA-specific PCR assays targeting MREJ sequences(continued)

Staphylococcal species	Strains	Source
MSSA (n = 28)	MA 52263	LSPQ
	6538	ATCC
	13301	ATCC
	25923	ATCC
	27660	ATCC
	29213	ATCC
	29247	ATCC
	29737	ATCC
	RN 11	CDC
	RN 3944	CDC
	RN 2442	CDC
	7605060113	CDC
	BM 4611	Institut Pasteur
	BM 3093	Institut Pasteur
	3511	LSPQ
	MA 5091	LSPQ
	MA 8849	LSPQ
	MA 8871	LSPQ
	MA 50607	LSPQ
	MA 50612	LSPQ
	MA 50848	LSPQ
	MA 51237	LSPQ
	MA 51351	LSPQ
	MA 52303	LSPQ
	MA 51828	LSPQ
	MA 51891	LSPQ
	MA 51504	LSPQ
	MA 52535	LSPQ
	MA 52783	LSPQ
MSCNS (n = 17)	12228	ATCC
	14953	ATCC
	14990	ATCC
	15305	ATCC
	27836	ATCC
	27848	ATCC
	29070	ATCC
	29970	ATCC
	29974	ATCC
	35539	ATCC
	35552	ATCC
	35844	ATCC
	35982	ATCC
	43809	ATCC
	43867	ATCC
	43958	ATCC
	49168	ATCC

5

^a ATCC stands for "American Type Culture Collection".

LSPQ stands for "Laboratoire de Santé Publique du Québec".

CDC stands for "Center for Disease Control and Prevention".

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Table 15. Clinical isolates used to test the sensitivity and/or specificity and/or ubiquity of the MRSA-specific PCR assays targeting MREJ sequences

Staphylococcal species	Number of strains	Source
MRSA (n = 177)	150	Canada
	10	China
	10	Denmark
	9	Argentina
	1	Egypt
	1	Sweden
	1	Poland
	3	Japan
	1	France
MSSA (n = 224)	208	Canada
	10	China
	4	Japan
	1	USA
	1	Argentina
MRCNS (n = 38)	32	Canada
	3	China
	1	France
	1	Argentina
	1	USA
MSCNS (n = 17)	14	UK
	3	Canada

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5 **Table 16. Analytical sensitivity of tests performed on a standard thermocycler using the set of primers targeting MREP types i, ii, iii, iv and v (SEQ ID NOs.: 64, 66, 67, 79 and 80) developed in the present invention for the detection and identification of MRSA**

Staphylococcus aureus strain designation:		Detection limit (number of genome copies)
Original	CCRI ^a (MREP type)	
13370	CCRI-8894 (i)	10
ATCC 43300	CCRI-175 (ii)	5
9191	CCRI-2086 (ii)	10
35290	CCRI-1262 (iii)	5
352	CCRI-1266 (iii)	10
19121	CCRI-8895 (iv)	5
ATCC 33592	CCRI-178 (iv)	5
MA 50428	CCRI-1311 (v)	5
R991282	CCRI-2025 (v)	5

^a CCRI stands for "Collection of the Centre de Recherche en Infectiologie".

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Table 17. Specificity and ubiquity tests performed on a standard thermocycler using the set of primers targeting MREP types i, ii, iii, iv and v (SEQ ID NO.: 64, 66, 67, 79 and 80) developed in the present invention for the detection and identification of MRSA

Strains	PCR results for SCCmec - orfX right extremity junction	
	Positive (%)	Negative (%)
MRSA - 35 strains ^a	27 (77.1)	8 (22.9)
MSSA - 44 strains	13 (29.5)	31 (70.5)
MRCNS - 9 strains*	0	9 (100)
MSCNS - 10 strains*	0	10 (100)

^a MRSA strains include the 20 strains listed in Table 3.

*Details regarding CNS strains:

MRCNS : *S. caprae* (1)
S. cohnii cohnii (1)
S. epidermidis (1)
S. haemolyticus (2)
S. hominis (1)
S. sciuri (1)
S. simulans (1)
S. warneri (1)

MSCNS : *S. cohnii* (1)
S. epidermidis (1)
S. equorum (1)
S. haemolyticus (1)
S. lentus (1)
S. lugdunensis (1)
S. saccharolyticus (1)
S. saprophyticus (2)
S. xylosus (1)

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Table 18. Analytical sensitivity of tests performed on the Smart
Cycler® thermocycler using the set of primers targeting
MREP types i, ii, iii, iv and v (SEQ ID NOs.: 64, 66,
67, 79 and 80) and molecular beacon probe (SEQ ID NO.:
84) developed in the present invention for the
detection and identification of MRSA

<i>Staphylococcus aureus</i> strain designation:		Detection limit (number of genome copies)
Original	CCRI ^a (MREP type)	
13370	CCRI-8894 (i)	2
ATCC 43300	CCRI-175 (ii)	2
9191	CCRI-2086 (ii)	10
35290	CCRI-1262 (iii)	2
352	CCRI-1266 (iii)	10
ATCC 33592	CCRI-178 (iv)	2
MA 51363	CCRI-1331 (iv)	5
19121	CCRI-8895 (iv)	10
Z109	CCRI-8903 (iv)	5
45302	CCRI-1263 (v)	10
MA 50428	CCRI-1311 (v)	5
MA 50609	CCRI-1312 (v)	5
MA 51651	CCRI-1325 (v)	10
39795-2	CCRI-1377 (v)	10
R991282	CCRI-2025 (v)	2

^a CCRI stands for "Collection of the Centre de Recherche en Infectiologie".

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5 Table 19. Specificity and ubiquity tests performed on the Smart
Cycler® thermocycler using the set of primers targeting
MREP types i, ii, iii, iv and v (SEQ ID NO. : 64, 66,
67, 79 and 80) and molecular beacon probe (SEQ ID NO. :
84) developed in the present invention for the
detection of MRSA .

Strains	PCR results for MREJ	
	Positive (%)	Negative (%)
MRSA - 29 strains ^a	21 (72.4)	8 (27.6)
MSSA - 35 strains	13 (37.1)	22 (62.9)
MRCNS - 14 strains	0	14 (100)
MSCNS - 10 strains	0	10 (100)

10 ^a MRSA strains include the 20 strains listed in Table 3.

Details regarding CNS strains:

15 MRCNS : *S. epidermidis* (1)
S. haemolyticus (5)
S. simulans (5)
S. warneri (3)

20 MSCNS : *S. cohnii cohnii* (1)
S. epidermidis (1)
S. gallinarum (1)
S. haemolyticus (1)
S. lentus (1)
S. lugdunensis (1)
S. saccharolyticus (1)
25 *S. saprophyticus* (2)
S. xylosus (1)

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5 **Table 20. Analytical sensitivity of tests performed on the Smart Cyclar® thermocycler using the set of primers targeting MREP types i, ii, iii, iv, v and vii (SEQ ID NOs.: 64, 66, 67, 79 and 80) and molecular beacon probe (SEQ ID NO.: 84) developed in the present invention for the detection and identification of MRSA**

<i>Staphylococcus aureus</i> strain designation:		Detection limit (number of genome copies)
Original	CCRI ^a (MREP type)	
13370	CCRI-8894 (i)	2
ATCC 43300	CCRI-175 (ii)	2
35290	CCRI-1262 (iii)	2
ATCC 33592	CCRI-178 (iv)	2
R991282	CCRI-2025 (v)	2
SE-41-1	CCRI-9771 (vii)	2

10 ^a CCRI stands for "Collection of the Centre de Recherche en Infectiologie".

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5 Table 21. Specificity and ubiquity tests performed on the Smart
Cycler® thermocycler using the set of primers targeting
MREP types i, ii, iii, iv, vi and vii (SEQ ID NOs.: 64,
66, 67, 79 and 80) and molecular beacon probe (SEQ ID
NO.: 84) developed in the present invention for the
detection and identification of MRSA

Strains	PCR results for MREJ	
	Positive (%)	Negative (%)
MRSA - 23 strains ^a	19 (82.6)	4 (17.4)
MSSA - 25 strains	13 (52)	12 (48)
MRCNS - 26 strains	0	26 (100)
MSCNS - 8 strains	0	8 (100)

10 ^a MRSA strains include the 20 strains listed in Table 3.

Details regarding CNS strains:

15	MRCNS :	<i>S. capitis</i> (2)
		<i>S. caprae</i> (1)
		<i>S. cohnii</i> (1)
		<i>S. epidermidis</i> (9)
		<i>S. haemolyticus</i> (5)
		<i>S. hominis</i> (2)
20		<i>S. saprophyticus</i> (1)
		<i>S. sciuri</i> (2)
		<i>S. simulans</i> (1)
		<i>S. warneri</i> (2)
25	MSCNS :	<i>S. cohnii cohnii</i> (1)
		<i>S. epidermidis</i> (1)
		<i>S. haemolyticus</i> (1)
		<i>S. lugdunensis</i> (1)
		<i>S. saccharolyticus</i> (1)
		<i>S. saprophyticus</i> (2)
30		<i>S. xylosus</i> (1)

Annex I: Strategy for the selection of specific amplification primers for types i and ii MREP

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Types i and ii MREP		orfx	
SEQ ID NO.:		2358	2583
2	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	2607
1	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
17 ^a	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
18 ^a	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
19 ^a	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
20 ^a	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
21 ^a	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
22 ^a	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
23 ^a	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
24 ^a	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
25 ^a	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
26	TAT GTCAAAAATC ATGAACCTCA TTACTTATGA TA...	CCT TGTGCAGGCC GTTTGATCCG CC	
33 ^c		CtT gGTGtAaaCC aTTgAGCCa CC	
34 ^c		CCT caTGCaatCC aTTTGATC	

Selected sequence
for type i MREP
and ii primer
(SEQ ID No.: 66)

GTCAAAAATC ATGAACCTCA TTACTTATG

Selected sequence
for orfx primer^b
(SEQ ID NO.: 64)

TGTGCAGGCC GTTTGATCC

The sequence positions refer to SEQ ID NO.: 2.

Nucleotides in capitals are identical to the selected sequences or match those sequences. Mismatches are indicated by lower-case letters. Dots indicate gaps in the displayed sequences.

^a These sequences are the reverse-complements of SEQ ID NOs.: 17-25.
^b This sequence is the reverse-complement of the selected primer.
^c SEQ ID NOs.: 33 and 34 were obtained from CNS species.

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Annex II: Strategy for the selection of a specific molecular beacon probe for the real-time detection of MREJ

SEQ ID NO. :	327	orfx	371
165	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
180	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
181	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
182	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
183	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
184	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
186	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
174	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
175	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
178	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
176	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
173	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
177	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
169	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
199	ACAAG GACGT CTTACAACGC AGTAACTATG	CACTA	CACTA
33 ^{a,b}	ACcAa GACGT CTTACAACGC AGcAACTATG	CttTA	
34 ^{a,b}	AtgAG GACGT CTTACAACGC AGcAACTATG	CACtt	

Selected sequence
for orfx molecular
beacon probes
(SEQ ID NO.:163)^c
(SEQ ID NO.:164)^c
(SEQ ID NO.: 84)^c

GACGT CTTACAACGC AGTAACTATG
GACGT CTTACAACGC AGTAACTATG
GACGT CTTACAACGC AGTAACTATG

Nucleotide discrepancies between the orfx sequences and SEQ ID NO.: 84 are shown in lower-case. Other entries in the sequence listing also present similar variations. The stem of the molecular beacon probes are not shown for sake of clarity. The sequence positions refer to SEQ ID NO.:165.

^a These sequences are the reverse-complements of SEQ ID NOs.: 33 and 34.

^b SEQ ID NOs.: 33 and 34 were obtained from CNS species.

^c The sequences presented are the reverse-complement of the selected molecular beacon probes.

What is claimed is:

1. A method for detecting the presence of at least one MREJ type ix methicillin-resistant *Staphylococcus aureus* (MRSA) strain comprising:

- a) contacting a sample to be analyzed for the presence of said MRSA strain with a first primer and a second primer, said MRSA strain including a Staphylococcal cassette chromosome *mec* (SCC*mec*) element containing a *mecA* gene inserted into chromosomal DNA, thereby generating a polymorphic right extremity junction (MREJ) type ix sequence that comprises polymorphic sequences from the SCC*mec* element right extremity and chromosomal DNA adjoining said polymorphic sequences from the SCC*mec* element right extremity, wherein said first and second primers are at least 10 nucleotides in length, wherein said first primer specifically hybridizes with said polymorphic sequences from the SCC*mec* element right extremity of an MREJ type ix sequence as set forth in SEQ ID NO: 168, SEQ ID NO: 233 or complements thereof, wherein said second primer specifically hybridizes with a chromosomal sequence of *S. aureus* to specifically generate an amplicon which comprises sequences from the SCC*mec* right extremity and chromosomal DNA adjoining said right extremity of said MREJ type ix MRSA strain if such MRSA strain is present in said sample; and wherein said first and second primers are capable of specifically hybridizing with their respective target sequence under PCR conditions comprising 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100™, and 2.5 mM MgCl₂ at 55°C; and
- b) detecting the presence of said amplicon.

2. The method of claim 1, wherein said second primer hybridizes to a chromosomal sequence of *S. aureus* which is *orfX*, SEQ ID NO: 172, SEQ ID NO: 231 or complements thereof.

3. The method of claim 2, wherein said chromosomal sequence of *S. aureus* is *orfX*.

4. The method of any one of claims 1 to 3, wherein said method comprises the use of at least one primer and/or probe specific for an MREJ type ix comprising a sequence as set forth in SEQ ID NO: 109, 148, 149, 205, 206 or complements thereof.
5. The method of any one of claims 1 to 4, wherein said method comprises the use of at least one primer and/or probe specific for the *S. aureus* chromosome and comprising a sequence as set forth in SEQ ID NO: 32, 59, 60, 61, 62, 63, 64, 83, 84, 103, 126, 127, 128, 129, 130, 131, 132, 160, 161, 162, 163, 164, 200, 201 or complements thereof.
6. The method of any one of claims 1 to 3, wherein said method comprises the use of at least one primer or probe as set forth in SEQ ID NO: 109, 148, 149, 205, 206, 64, 71, 72, 73, 74, 75, 76, 70, 103, 130, 132, 158, 159, 59, 62, 126, 127, 128, 129, 131, 200, 201, 60, 61, 63, 32, 83, 84, 160, 161, 162, 163 or 164.
7. The method of any one of claims 1 to 3, comprising the use of a primer pair consisting of SEQ ID NOs: 64 and 109, SEQ ID NOs: 64 and 149 or SEQ ID NOs: 64 and 206.
8. The method of claim 3, wherein said second primer has a sequence as set forth in SEQ ID NO:64.
9. The method of any one of claims 1 to 3, wherein said first primer has a sequence as set forth in SEQ ID NO: 109, 149 or 206.
10. The method of any one of claims 7 to 9, further comprising the use of at least one probe having a sequence as set forth in SEQ ID NO: 32, 83, 84, 160, 161, 162, 163 or 164.
11. The method of claim 1, comprising the use of primers and probes having the following nucleotide sequences: SEQ ID NOs: 64, 109, 84, 163 and 164.

12. The method of any one of claims 1 to 11, further comprising detecting the presence of at least one further methicillin-resistant *Staphylococcus aureus* (MRSA) strain in said sample, said at least one further MRSA strain comprising a *mec* right extremity junction (MREJ) type i, ii, iii, v, vi, or viii nucleic acid sequence, and being resistant because of the presence of an SCC*mec* element containing a *mecA* gene, said SCC*mec* having been inserted into chromosomal DNA, thereby generating a polymorphic right extremity junction (MREJ) sequence that comprises polymorphic sequences from the SCC*mec* element right extremity and chromosomal DNA adjoining said right extremity, wherein said method further comprises contacting said sample with at least one primer and/or probe capable of specifically hybridizing with said polymorphic sequences from the SCC*mec* element right extremity of said at least one further MRSA strain.

13. The method of claim 12, wherein said primer and/or probe has at least 10 nucleotides in length and is capable of specifically hybridizing with said polymorphic sequences from the SCC*mec* element right extremity of at least one of MREJ types i, ii, iii, v, vi, and viii nucleic acid sequences comprising a:

- a) SEQ ID NO: 1, 20, 21, 22, 23, 24, 25, 41 or complements thereof for MREJ type i;
- b) SEQ ID NO: 2, 17, 18, 19, 26, 40, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 185, 186, 197 or complements thereof for MREJ type ii;
- c) SEQ ID NO: 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 104, 184, 198 or complements thereof for MREJ type iii;
- d) SEQ ID NO: 47, 48, 49, 50 or complements thereof for MREJ type v;
- e) SEQ ID NO: 171 or a complement thereof for MREJ type vi; and
- f) SEQ ID NO: 167 or a complement thereof for MREJ type viii;

14. The method of claim 12 or 13, comprising the use of at least one of the primers and/or probes as set forth in SEQ ID NOs:

- a) 66, 100, 101, 105, 52, 53, 54, 55, 56, 57, 64, 71, 72, 73, 74, 75, 76, 70, 103, 130, 132, 158, 159, 59, 62, 126, 127, 128, 129, 131, 200, 201, 60, 61, 63, 32, 83, 84, 160, 161, 162, 163 and 164, for the detection of MREJ type i;

- b) 66, 97, 99, 100, 101, 106, 117, 118, 124, 125, 52, 53, 54, 55, 56, 57, 64, 71, 72, 73, 74, 75, 76, 70, 103, 130, 132, 158, 159, 59, 62, 126, 127, 128, 129, 131, 200, 201, 60, 61, 63, 32, 83, 84, 160, 161, 162, 163 and 164, for the detection of MREJ type ii;
- c) 67, 98, 102, 107, 108, 64, 71, 72, 73, 74, 75, 76, 70, 103, 130, 132, 158, 159, 58, 59, 62, 126, 127, 128, 129, 131, 200, 201, 60, 61, 63, 32, 83, 84, 160, 161, 162, 163 and 164, for the detection of MREJ type iii;
- d) 65, 80, 146, 154, 155, 64, 71, 72, 73, 74, 75, 76, 70, 103, 130, 132, 158, 159, 59, 62, 126, 127, 128, 129, 131, 200, 201, 60, 61, 63, 32, 83, 84, 160, 161, 162, 163 and 164, for the detection of MREJ type v;
- e) 202, 203, 204, 64, 71, 72, 73, 74, 75, 76, 70, 103, 130, 132, 158, 159, 59, 62, 126, 127, 128, 129, 131, 200, 201, 60, 61, 63, 32, 83, 84, 160, 161, 162, 163 and 164, for the detection of MREJ type vi; and
- f) 115, 116, 187, 188, 207, 208, 64, 71, 72, 73, 74, 75, 76, 70, 103, 130, 132, 158, 159, 59, 62, 126, 127, 128, 129, 131, 200, 201, 60, 61, 63, 32, 83, 84, 160, 161, 162, 163 and 164, for the detection of MREJ type viii.

15. The method of any one of claims 12 to 14, comprising the use of at least one of the primer pairs as set forth in SEQ ID NOs:

- a) 64/66, 64/100, 64/101; 59/52, 59/53, 59/54, 59/55, 59/56, 59/57, 60/52, 60/53, 60/54, 60/55, 60/56, 60/57, 61/52, 61/53, 61/54, 61/55, 61/56, 61/57, 62/52, 62/53, 62/54, 62/55, 62/56, 62/57, 63/52, 63/53, 63/54, 63/55, 63/56 and 63/57, for the detection of type i MREJ;
- b) 64/66, 64/97, 64/99, 64/100, 64/101, 59/52, 59/53, 59/54, 59/55, 59/56, 59/57, 60/52, 60/53, 60/54, 60/55, 60/56, 60/57, 61/52, 61/53, 61/54, 61/55, 61/56, 61/57, 62/52, 62/53, 62/54, 62/55, 62/56, 62/57, 63/52, 63/53, 63/54, 63/55, 63/56 and 63/57, for the detection of type ii MREJ;
- c) 64/67, 64/98, 64/102; 59/58, 60/58, 61/58, 62/58 and 63/58, for the detection of type iii MREJ;
- d) 64/80, for the detection of type v MREJ;
- e) 64/204, for the detection of type vi MREJ; and
- f) 64/116, for the detection of type viii MREJ.

16. The method of any one of claims 12 to 15, comprising the use of primers and probes having the following nucleotide sequences:

- a) SEQ ID NOs: 64, 66, 84, 163 and 164 for the detection of MREJ type i or ii;
- b) SEQ ID NOs: 64, 67, 84, 163 and 164 for the detection of MREJ type iii; or
- c) SEQ ID NOs: 64, 80, 84, 163 and 164 for the detection of MREJ type v.

17. The method of any one of claims 1 to 16, wherein multiple primers and/or probes are used together in the same physical enclosure.

18. A method for typing a type ix MREJ of an MRSA strain, and at least one further MRSA strain, wherein said at least one further MRSA strain comprises an MREJ type i, ii, iii, v, vi or viii, said method comprising: reproducing the method of any one of claims 1 to 15 with primers and/or probes specific for said MREJ type ix and specific for said at least one further MRSA strain of MREJ type i, ii, iii, v, vi or viii, and detecting each amplicon distinctively as an indication of the presence of said MREJ type ix and said at least one further MREJ type.

19. The method of any one of claims 1 to 18, wherein a plurality of primers and/or probes is used.

20. The method of claim 19, wherein said plurality of primers and/or probes are all chosen to hybridize under the same hybridization conditions.

21. The method of claim 19, wherein said plurality of primers and/or probes are capable of specifically hybridizing with said MREJ type ix, and at least one further MREJ of type i, type ii, type iii, type v, type vi or type viii.

22. A method for determining the presence of at least four MREJ types of MRSA in a sample comprising:

- a) reproducing the method of any one of claims 9 to 19 with primers and/or probes specific for each of said at least four MREJ types, one of which being MREJ type ix; and

b) detecting each amplicon distinctively,
thereby determining the presence of MREJ types of MRSA in a sample.

23. The method of claim 22, for determining the presence of MREJ type ix, type i, type ii and type iii.

24. The method of claim 22, for determining the presence of MREJ type ix, type i, type ii, type iii, type v, type vi and type viii.

25. The method of claim 22, for determining the presence of MREJ type ix, type i, type ii, type iii and type v.

26. The method of claim 22, for determining the presence of MREJ type ix, type i, type ii, type iii, type v and type vi.

27. The method of any one of claims 1 to 26, wherein real time PCR is used.

28. The method of any one of claims 1 to 26, wherein multiplex PCR is used.

29. A kit for detecting the presence or absence of an MREJ type ix MRSA strain in a sample comprising:

a) a first oligonucleotide which specifically hybridizes with polymorphic sequences from the *SCCmec* element right extremity of an MREJ type ix sequence as set forth in SEQ ID NO: 168, 233 or complements thereof; and

b) a second oligonucleotide which specifically hybridizes with a chromosomal sequence of *S. aureus*;

wherein said oligonucleotides of a) and b) consist of at least 10 nucleotides in length and enable the selective generation of an amplicon which comprises polymorphic sequences from the *SCCmec* element right extremity and chromosomal DNA adjoining said right extremity of said MREJ type ix MRSA strain; and

wherein said oligonucleotides of a) and b) are capable of specifically hybridizing with their respective target sequence under PCR conditions comprising 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100™, and 2.5 mM MgCl₂ at 55°C.

30. The kit of claim 29, wherein said chromosomal sequence of *S. aureus* in b) is *orfX*.

31. The kit of claim 29 or 30, wherein said first oligonucleotide in a) comprises a sequence as set forth in SEQ ID NO: 109, 148, 149, 205, 206 or complements thereof.

32. The kit of any one of claims 29 to 31, wherein said second oligonucleotide in b) comprises a sequence as set forth in SEQ ID NO: 32, 59, 60, 61, 62, 63, 64, 83, 84, 103, 126, 127, 128, 129, 130, 131, 132, 160, 161, 162, 163, 164, 200, 201 or complements thereof.

33. The kit of claim 29 or 30, wherein said kit comprises at least one oligonucleotide primer or probe as set forth in SEQ ID NO: 109, 148, 149, 205, 206, 64, 71, 72, 73, 74, 75, 76, 70, 103, 130, 132, 158, 159, 59, 62, 126, 127, 128, 129, 131, 200, 201, 60, 61, 63, 32, 83, 84, 160, 161, 162, 163 or 164.

34. The kit of claim 29 or 30, comprising a pair of oligonucleotides consisting of SEQ ID NOs: 64 and 109; SEQ ID NOs: 64 and 149 or SEQ ID NOs: 64 and 206.

35. The kit of claim 29 or 30, wherein said second oligonucleotide in b) has a sequence as set forth in SEQ ID NO:64.

36. The kit of claim 29 or 30, wherein said first oligonucleotide in a) has a sequence as set forth in SEQ ID NO: 109, 149 or 206.

37. The kit of any one of claims 34 to 36, comprising at least one probe having a sequence as set forth in SEQ ID NO: 32, 83, 84, 160, 161, 162, 163 or 164.

38. The kit of claim 29, comprising oligonucleotide primers and probes having the following nucleotide sequences: SEQ ID NOs: 64, 109, 84, 163 and 164.

39. The kit of any one of claims 29-38, further comprising at least one oligonucleotide for the detection of at least one additional MRSA strain comprising an MREJ of type i, type ii, type iii, type v, type vi or type viii.

40. Use of the kit as defined in any one of claims 29 to 39, for carrying out the method as defined in any one of claims 1 to 28.