DESCRIPTION

DIAGNOSTIC METHOD

Technical Field

This invention relates to a diagnostic method for performing a genetic diagnosis of a resistant bacterium, especially for specifically detecting at high sensitivity methicillin-resistant *Staphylococcus aureus* (hereinafter referred to as "MRSA") and methicillin-resistant coagulase-negative staphylococci (hereinafter referred to as "MRC-NS"). Namely, the present invention is concerned with a diagnostic method which can promptly detect and identify MRSA and MRC-NS.

Background Art

MRSA and MRC-NS, including *Staphylococcus haemolyticus* and *Staphylococcus epidermidis*, are principal pathogenic bacteria of nosocomial infection at hospitals in all the countries of the world, and have become a serious clinical problem due to the limited availability of effective antibiotics. In clinical activities, their accurate and speedy identification has become an important theme for the diagnosis and treatment of infected patients.

MRSA is *Staphylococcus aureus* which produces PBP(penicillin-binding protein)2' (or PBP2a), that is, a cell-wall synthesizing enzyme PBP having low affinity to all β-lactam antibiotics developed to date led by methicillin. Because of the production of this PBP2', MRSA exhibits resistance to all the conventional β-lactam antibiotics. Since the report of its first clinical strain in England in 1961, it has spread around the whole world and at present, it has become, as a nosocomial infectious bacterium, a serious problem for the present-day medical treatments at hospitals in all the countries of the world.

MRSA produces PBP2' in addition to four PBPs which *Staphylococcus aureus* inherently have. In 1986, the *mec*A gene encoding this PBP was cloned by Matsuhashi et al. and its entire base sequence was determined by them. The *mec*A gene exists on chromosomes of MRSA and MRC-NS, but is not found on methicillin-susceptible *Staphylococcus aureus* (MSSA). Accordingly, the *mec*A gene is considered to be a gene adventitiously acquired on the chromosomes of *Staphylococcus aureus*. Detection of this *mec*A gene on the chromosomal DNA of *Staphylococcus aureus*, generally by PCR (polymerase chain reaction) or hybridization, makes it possible to identify it as MRSA or MRC-NS.

In Japan, a *mec*A identification kit by ED-PCR (enzyme detection PCR) was developed, and subsequent to its approval and the setting of its health insurance price by the Ministry of Health and Welfare, is now frequently used for clinical diagnoses (Ubukata, K., et al. J. Clin. Microbiol. 30, 1728-1733, 1992). However, the identification of MRSA by this method involves at least the following two problems.

1) It can be used only after a bacterium from a patient's sample has been cultured and procedures have then been conducted beforehand for the strain identification of *Staphylococcus aureus*. The above method therefore has a drawback of lack of promptness and creates various problems.

Namely, the *mec*A gene is also distributed widely in other strains of the genus of *Staphylococcus*, for example, wide-spread normal human bacteria having low pathogenicity such as *Staphylococcus (S.) epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. capitis*, *S. warneri*, *S. sciuri* and *S. caprae* (Eiko Suzuki et al. Antimicrb. Agents Chemother. 37, 1219-1226, 1993). In a patient's sample, these *mec*A-containing *Staphylococcus* strains are detected at the same time in many instances. Accordingly, direct detection of the *mec*A gene from a sample cannot be taken as a proof of the existence of MRSA.

This has led to a limitation of the detection method of the *mec*A gene having to be used after a strain has been cultured from a patient's sample and has then been confirmed to be *Staphylococcus aureus* by a conventional strain identification method. Accordingly, there has been no choice other than to rely upon an empiric therapy until an infected strain is identified. Further, administration of vancomycin has been indispensable even if the administration is eventually found to have been unnecessary.

2) Lack of internal control in PCR. Described specifically, false positive or false negative may be determined in PCR operations of nowadays, depending on the working conditions of a thermal cycler. Upon a judgment of positive or negative, it is desirable to have internal controls for negative and positive in each operation. This is however rarely performed in present-day diagnostic methods.

Disclosure of the Invention

With a view to establishing a fast, simple and reliable, specific identification method of MRSA and MRC-NS, the present inventors have analyzed the genes of MRSA, MSSA and MRC-NS in more detail. The present inventors were interested in reports that an additional

DNA of several tens of kilobases (kb) or greater exists in a methicillin-resistant locus (Beck, W.D. et al. J. Bacteriol. **165**, 373-378, 1986; Skinner, S. et al. Mol. Microbiol. **2**, 289-298, 1988).

The present inventors have then proceeded with extensive research to determine if any gene sequence specific to MRSA exists on chromosomes of MRSA and MRC-NS. The research has led to the finding and identification of a specific target DNA fragment, resulting in the completion of the present invention.

Namely, the present invention provides a diagnostic method of an MRSA (methicillin-resistant *Staphylococcus aureus*) or MRC-NS (methicillin-resistant coagulase-negative staphylococci), which comprises performing a reaction with a sample by making combined use of a part of a *mec*DNA, which is an integrated adventitious DNA existing on a chromosome of said MRSA or MRC-NS and carrying a *mec*A gene thereon, and a part of a nucleotide sequence of a chromosomal DNA surrounding said integrated DNA; and also a diagnostic method of an MRSA or MRC-NS by PCR, LCR, hybridization, PT-PCR or NASBA, which comprises performing a reaction with a sample by using a nucleotide sequence of a chromosomal DNA surrounding an integration site of a *mec*DNA in a chromosome of a methicillin-susceptible *Staphylococcus*

aureus or methicillin-susceptible C-NS (MSC-NS), wherein said method makes use of an occurrence of a negative reaction when said sample contains a *mec*DNA integrated therein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram illustrating cloning of a *mec* region DNA of N315 by chromosome walking. H, P and E represent restriction sites by HindIII, PstI and EcoR1, respectively.

FIG. 2 is a diagram illustrating cloning of a *mec* region DNA of NCTC10442 by chromosome walking. The diagram shows restriction maps of clones L02, L05, L07 and L021 obtained from a λ phage library and an integration site of the *mec* region DNA. 1 to 7 indicate probes for the *mec* region DNA. Restriction sites: H, HindIII; E, EcoRI; S, SalI; X, XbaI.

FIG. 3 is a diagram showing a structure of a *mec* region DNA of an MRSA strain, 85/3907 and an integration site on its chromosome. Also shown are restriction maps of clones pSJ9 and pSJ10 obtained from a plasmid library and clones LG12 and LG25 obtained from a λ phage library as well as an integration site of the *mec* region DNA. 1 to 7 indicate probes for the *mec* region DNA. Restriction sites: H, HindIII; E, EcoRI;

S, SalI; X, XbaI; P, PstI.

FIG. 4 is a diagram showing a restriction mapping of LD8325, including a *mec* integration site, and a partial nucleotide sequence thereof. A vertical arrow indicates the *mec* integration site. A horizontal arrow indicates an orientation of the nucleotide sequence (from 5' toward 3'). An underlined part in the nucleotide sequence means an open reading frame of an *intM*. An arrowhead between the nucleotide numbers 1856 and 1857 indicates the *mec* integration site.

FIG. 5 is a diagram showing nucleotide sequences of chromosomal DNAs flanking a *mec* integration site as compared among MSSA strains. Coagulase types produced by the respective strains: ATCC25923, type 4; STP23, Type 4; NCTC8325, type 3; STP43, type 7; STP53, type 5. Compared with 225 nucleotides of NCTC8325pLEC12, ranging from the nucleotide numbers 1708 to 1932, were the corresponding 225 nucleotides of the other strains. Boxed nucleotide are common to all the strains.

FIG. 6 is a diagram showing a comparison in nucleotide sequence between MRSA and methicillin-resistant bacteria. SH 518 and SH JA178 are clinical strains of *S. haemolyticus*. SE G3 is a clinical strain of *S. epidermidis*.

FIG. 7 is a diagram illustrating an outline of an

MRSA identification method. Arrow marks indicate primers for PCR. With a chromosomal DNA of MSSA, the primers set as in A react at the MSSA-side primers but do not react at the *mec*-side primers (B). Further, with a chromosomal DNA of a *Staphylococcus* strain other than *mec*-carrying *S. aureus*, the *mec*-side primers react but the MSSA-side primers do not react (C).

FIG. 8 is a diagram illustrating a concept of an internal control in PCR. When primers are designed in such a way as surrounding an *intM*-containing region of MSSA, they react with the chromosomal DNA of MSSA so that the DNA fragment is amplified by PCR. In the case of the chromosomal DNA of MRSA, on the other hand, no amplification is feasible by usual PCR because of the integration of a *mec* DNA greater than 30 Kb. Further, when primers are synthesized including a *mec* integration site, these primers themselves no longer react in the case of MRSA.

FIG. 9 is a diagram showing homology between nucleotide sequences pLEC12rc and pSJ8-2a. A nucleotide sequence of a subclone pSJ8-2a of N315, including an upstream junction of a *mec* region, (see FIG. 1) (the lower nucleotide sequence) and of a chromosomal DNA clone pLEC12(pLEC1a) of NCTC8325, including an *intM*, (the upper nucleotide sequence) were compared.

The outer nucleotide sequences from the upstream junctions of the *mec* regions are substantially the same. pLEC12rc is reverse complementary in nucleotide sequence to pLEC12.

FIG. 10 is a diagram showing homology in nucleotide sequence between pLEC12rc and pSJ8-2a. Parts continued from FIG. 9 are shown.

FIG. 11 is a diagram showing homology in nucleotide sequence between pLEC12rc and N315J3rc. The nucleotide sequence of a subclone N315J3 of N315, including a downstream junction of the *mec* region, (see FIG. 1) (the lower nucleotide sequence) and the nucleotide sequence of a chromosomal DNA clone pLEC12(pLEC1a) of NCTC8325, including an *intM*, (the upper nucleotide sequence) are compared. The nucleotide sequences of the intM on outer sides of the *mec* region DNA are substantially the same. The nucleotide sequence of N315J3rc is reverse complementary to the nucleotide sequence of N315J3.

FIG. 12 is a diagram comparing the nucleotide sequence of a clone including an upstream junction of a *mec* region of N315 with one of NCTC10442 (N315, the upper nucleotide sequence; NCTC10442, the lower nucleotide sequence). The nucleotide sequences of their *mec* regions are extremely different from each

other except for IRs near the junctions. On the other hand, the nucleotide sequences on outer sides of the junctions are the same.

FIG. 13 is a diagram comparing the nucleotide sequence of a clone including a downstream junction of the *mec* region of N315 with one of NCTC10442 (N315, the upper nucleotide sequence; NCTC10442, the lower nucleotide sequence). The nucleotide sequences of their *mec* regions are extremely different from each other except for IRs. On the other hand, the nucleotide sequences on outer (downstream) sides of the junctions have extremely high homology. The nucleotide sequence of NCTC10442J3 is reverse complementary to the nucleotide sequence of NCTC10442J3.

FIG. 14 is a diagram comparing N315IS-J3rc with J3rc of NCTC10442. The upper nucleotide sequence corresponds to N315IS-J3 (see FIG. 1 and FIG. 17), while the lower nucleotide sequence is a sequence of first 176 nucleotides corresponding to a *mec* region of J3rc of NCTC10442 (see FIG. 13).

FIG. 15 is a diagram comparing N315J3rc with pSJ10-3J3rc. The upper nucleotide sequence corresponds to N315J3rc, while the lower nucleotide sequence corresponds to pSJ10-3J3rc. The nucleotide sequence of pSJ10-3J3rc is reverse complementary to the nucleotide

sequence of pSJ10-3J3.

FIG. 16 is a diagram comparing pSJ8-2a with LG12H2. The left nucleotide sequence corresponds to pSJ8-2a, while the lower nucleotide sequence corresponds to LG12H2.

FIG. 17 is a diagram showing a nucleotide sequence of N315IS-J3. A thin underline indicates a sequence of first 176 nucleotides corresponding to the *mec* region of J3rc of NCTC10442 (see FIG. 14). A thick underline designates a nucleotide sequence corresponding to an *intM* on an outer side of a downstream junction of a *mec* region.

FIG. 18 is a diagram showing a nucleotide sequence of NCTC10442J3rc. An underline indicates a nucleotide sequence corresponding to an *intM* on an outer side of a downstream junction of a *mec* region.

FIG. 19 is a diagram showing a nucleotide sequence of pSJ10-3J3rc. An underline indicates a nucleotide sequence corresponding to an intM on an outer side of a downstream junction of a *mec* region.

Best Mode for Carrying out the Invention

The present inventors determined the nucleotide sequences of the above DNA fragments, namely, the *mec* region DNAs [may hereinafter be referred to as "*mec*";

Keiichi Hiramatsu: Microbiol. Immunol. 39(8), 531-543, 1995] by chromosome walking subsequent to their cloning from MRSA N315 isolated in Japan (type-2 coagulase producing strain; type 2j MRSA), MRSA NCTC10442 isolated in U.K. (type-3 coagulase producing strain; type 3e MRSA) and MRSA 85/3907 isolated in Germany (type-4 coagulase producing strain; type 4e MRSA). These three strains are those chosen, as representative examples of epidemic strains isolated in various countries of the world, on the basis of classification according to ribotyping, the coagulase method and sites and natures of mutations by the determination of nucleotide sequences of *mec*I genes. As a result, as is shown in FIGS. 1, 2 and 3:

1) The *mec* DNAs have been found to be giant DNA regions of 52 Kb in N315, 33 Kb in NCTC10442 and 54 Kb or greater in 85/3907, and to be all composed of DNAs which do not undergo cross hybridization with chromosomal DNAs of many MSSAs studied as controls.

2) Each *mec* contains at opposite ends thereof inverted sequences (inverted repeats; hereinafter called "IR"s) of 20-30 bases (base pairs; bp).

3) These *mec* region DNAs have been found to be integrated in particular orf (open reading frames) (hereinafter called "intM"s) on chromosomal DNAs of the

Staphylococcus aureus strains (FIG. 4);

4) From a comparison among the nucleotide sequences of these three strains, it has been found that they are substantially different from each other on upstream sides of the *mec* region DNAs and that on downstream sides of the *mec* DNAs, N315 and NCTC10442 have the same nucleotide sequence whereas 85/3907 have a sequence different from the other two strains.

5) In each strain, the nucleotide sequence of the chromosomal DNA was well retained in a 5'-side region of the *intM* relative to the *mec* integration site and also in a region upstream from the intM (the left side in FIG. 4), and the nucleotide sequence of the chromosomal DNA in a 3'-side region of the *intM* relative to the *mec* integration site and also in a region downstream from the *intM* (the lower side in FIG. 4) was well retained in N315 and NCTC10442 but was different in 85/3907 (FIG. 16).

From the above facts, an *intM* gene was estimated to be an integration site for a *mec* region DNA. Accordingly, *intM*-containing DNA fragments of five MSSA strains of different origins were cloned, and their nucleotide sequences were determined (FIG. 5). As a result,

6) The intM genes all retained the same

nucleotide sequences on 5'-sides relative to the *mec* integration sites, but their 3'-side nucleotide sequences were different.

7) Concerning the nucleotide sequences outside the *intM*s, the 5'-side nucleotide sequences upstream from the *intM*s were all retained but the 3'-side nucleotide sequences downstream from the *intM*s were different from one strain to another, whereby versatility was observed.

Next, to determine *mec* integration sites in *Staphylococcus* strains other than *Staphylococcus aureus*, DNA fragments containing downstream junctions of *mec* DNAs were cloned from *S. haemolyticus* clinical strains JA178 and 495 and *S. epidermidis* clinical strain G3, all of which contained *mec*A genes, by using probes for downstream ends of the *mec* DNAs. Upon determination of their nucleotide sequences,

8) The nucleotide sequences of all the downstream ends of the *mec* DNAs were homologous with that of the *mec* of N315, but the nucleotide sequences on right extremity to *mec*DNA were considerably different from that of the *intM* in N315 (FIG. 6).

With a view to confirming that a chromosomal DNA of a *Staphylococcus* strain other than *Staphylococcus aureus* does not contain any gene having high homology with the

intM, dot-blot hybridization was then conducted using the *intM* of N315 as primers with respect to the following standard strains of various *Staphylococcus* strains: ATCC25923, NCTC8325 (*S. aureus*), ATCC14990 (*S. epidermidis*), ATCC29970 (*S. haemolyticus*), DSM20672 (*S. arlettae*), CCM3573 (*S. caprae*), ATCC29062 (*S. sciuri*), ATCC33753 (*S. auricularis*), ATCC27840 (*S. capitis*), DSM20501 (*S. carnosus*), ATCC29750 (*S. caseolyticus*), NCTC10530 (*S. chromogenes*), ATCC29974 (*S. cohnii*), DSM20771 (*S. delphini*), DSM20674 (*S. equorum*), JCM7469 (*S. felis*), CCM3572 (*S. gallinarum*), ATCC27844 (*S. hominis*), ATCC29663 (*S. intermedius*), DMS20676 (*S. kloosii*), ATCC29070 (*S. lentus*), ATCC43809 (*S. lugdunensis*), ATCC15305 (*S. saprophyticus*), ATCC43808 (*S. schleiferi subsp. schleiferi*), JCM7470 (*S. schleiferi subsp. coagulans*), ATCC27848 (*S. simulans*), ATCC27836 (*S. warneri*), and ATCC29971 (*S. xylosus*). As a result,

9) The probes of the *intM* did not undergo hybridization with the standard strain of any strain other than *Staphylococcus aureus*.

From the foregoing results, the following conclusions have been obtained:

1) There are at least three types of *mec* DNAs in MRSA strains. They are different from each other in most regions, but two (N315mec and NCTC10442mec) of

them have homologous sequences in downstream ends of their *mec* DNAs (FIG. 12).

2) At the both ends of *mec* there are relatively-retained IRs of 20-30 nucleotides.

3) In all the MRSA strains studied, the integration sites of the *mec* DNAs in the chromosomes of the MRSA strains are the same, that is, are located in the intMs (FIGS. 17, 18, 19).

4) The nucleotide sequences of the *intM*s are substantially the same at least on right sides (in downstream regions) relative to the downstream junctions of the *mec* region DNAs (FIG. 6).

5) The nucleotide sequences of the region upstream from the 5' end of the *intM*s are the same in the three types of MRSA strains (FIGS. 13, 15, 17, 18 and 19).

6) Between the type-2 and type-4 coagulase strains, their nucleotide sequences are different on the 3' sides (left sides) of the *intM*s relative to the *mec* integration sites (FIG. 6).

By using the nucleotide sequences of *mec*, *intM* and a 5'-side region thereof on the basis of these findings, it is possible to conduct identification of MRSA strains by various methods to be described hereinafter. Although a description will hereinafter be made of a

concept of a method in which primers or probes are used surrounding a downstream junction of a *mec*, similar effects can also be obtained from surrounding an upstream junction of the *mec*. However, the latter method which is performed surrounding the upstream junction of the *mec* requires to design a set of primers or probes of at least N315, NCTC10442 and 85/3907 on the basis of the nucleotide sequences of these three strains including the upstream junctions of the *mec* region DNAs. For the selection of the primers or probes in this case, designing and combinations of numerous primers are feasible surrounding the upstream junctions of the *mec* region DNAs from PSJ8-2a (the nucleotide sequences of FIGS. 9 and 10), L02C4 (the nucleotide sequence of FIG. 12), LG12H2 (the nucleotide sequence of FIG. 16) and the nucleotide sequence of FIG. 5.

Identification method of MRSA by setting primers or probes which surround a downstream junction of *mec*

(1) Primers are designed in a *mec* region DNA and at *intM* and its 5'-side, and PCR is used. As is illustrated in FIG. 7, a strain is determined to be an MRSA if a DNA fragment containing a junction between the *mec* and the *intM* is amplified (FIG. 7A). In the case of an MSSA, a negative reaction is shown because the primer on the side of the *mec* region DNA cannot

react (FIG. 7B). Concerning a *mec*regionDNA containing strain other than *Staphylococcus aureus*, a negative reaction is also shown as no reaction is feasible with the primer at the intM and its 5'-side (FIG. 7C).

(2) When LCR (ligase chain reaction) is relied upon, an MRSA can be identified by preparing two DNA fragments, surrounding the junction between the *mec* and the *intM*, and then conducting a reaction.

(3) An MRSA can be identified by synthesizing a single or double-strand DNA probes , including the junction between the *mec* and the *intM*, and then using hybridization.

(4) By the above methods, many MSSA-side primers or probes can be chosen from the nucleotide sequence pLEC12 of the *intM* and its surrounding chromosome region (the nucleotide sequence of FIG. 4) and also from the nucleotide sequences of the *intM* and its 5'-side in the nucleotide sequence of FIG. 5.

(5) Many *mec*-side primers or probes can be chosen from nucleotide sequences corresponding to *mec* region DNAs in the following nucleotide sequences, namely, N315IS-J3 (the nucleotide sequence of FIG. 17), NCTC10442J3rc (the nucleotide sequence of FIG. 18) and pSJ10-3J3rc (the nucleotide sequence of FIG. 19).

(6) An MRSA or MSSA can be identified by extract-

ing an RNA from cells of the MRSA, MSSA or the like, converting it into a DNA with a reverse transcriptase, and conducting PCR, LCR or hybridization while using the above-described primers or probes.

(7) An MRSA or MSSA can also be identified by extracting an RNA from cells and then amplifying it in accordance with nucleic acid sequence-based amplification (NASBA) (Jean Compton: Nature 350, 91-92, 1991) in which three enzymes (RNaseH, AMV-RT, T7RNA polymerase) and two primers are reacted at the same time.

According to these methods, the existence of an MRSA can be directly confirmed without going through culture of the strain from a patient's sample even if a *mec*-containing *Staphylococcus* strain other than *Staphylococcus aureus* is mixed. Accordingly these methods are useful as fast diagnostic methods and have high value in clinical diagnoses.

Further, if as a negative control for an identification method of an MRSA, a primer or probe is designed including a 3'-side region of *intM* relative to a *mec* integration site or the integration site as shown in FIG. 8 and PCR or NASBA is performed, a negative result is obtained if a strain is an MRSA or a positive result is obtained if the strain is an MSSA. The primer or probe therefore makes it possible to conduct

evaluation of diagnosis conditions as an internal negative control. It is also possible to perform identification of an MRSA by making use of the fact that PCR or NASBA gives a negative result in this method. However, a patient's sample with an MSSA mixed therein gives a positive result in these tests. It is therefore desired to perform this method in addition to the above-described method (1), (2), (3), (6) or (7) after isolation and culture of a strain determined as an MRSA. Numerous primers or probes or combinations thereof, which are usable for these purposes, can be chosen from nucleotide sequences corresponding to MSSA chromosomal DNAs containing or surrounding *mec* integration sites of the following nucleotide sequences: pLEC12(pLEC1a) (the nucleotide sequence of FIG. 4), pSJ8-2a (the nucleotide sequence of FIGS. 9 and 10), L02C4 (the nucleotide sequence of FIG. 12) and LG12H2 (the nucleotide sequence of FIG. 16).

When the above methods (1) to (7) were performed with respect to 28 epidemic MRSA strains in 18 countries, including Japan, of the world, positive results were obtained in all the tests. However, negative results were obtained from MSSA strains, standard strains of *Staphylococcus* strains other than *Staphylococcus aureus* and *mec*-containing clinical *Staphylococcus* strains

other than *Staphylococcus aureus*, all of which were employed as controls. This has substantiated that this diagnostic method is extremely effective and useful (Table 1).

Table 1

Specific Identification of MRSAs by PCR, Surrounding *mec-intM* Junction

Strain	Probe (J6+Nmec5+Gmec1045a)	
	Negative	Positive
MSSAs - 11 strains*	11	0
MRSAs - 26 strains**	0	26
Standard *Staphylococcus* strains - 25 strains***	25	0
MR *Staphylococcus* strains including MRC-NS - 20 strains****	20	0

* Japanese clinical strains including the three standard strains, ATCC25923, NCTC8325 and ATCC12600.

** Epidemic strains in 18 countries: England, NCTC10442, 61/6219, 61/3846, 61/4176, 86/4372, 86/560, 86/961, 86/2652, 86/9302; Yugoslavia, 85/1340; Hungary, 85/1762; New Zealand, 85/2082; Norway, 85/2111; Holland, 85/3566; Saudi Arabia, 85/5495; Japan, MR108, N315; South Africa, 84/9580; Germany (West), 85/1836; France, 85/1940; Hong Kong, 85/2147; Austria, 85/3619; Germany

(East), 85/3907; Canada, 85/4231, 85/4670; Israel, 85/4547; U.S.A., 85/2232, 85/2235.

*** Standard *Staphylococcus* strains other than *Staphylococcus aureus*: ATCC14990 (*S. epidermidis*), ATCC29970 (*S. haemolyticus*), DSM20672 (*S. arlettae*), CCM3573 (*S. caprae*), ATCC29062 (*S. sciuri*), ATCC33753 (*S. auricularis*), ATCC27840 (*S. capitis*), DSM20501 (*S. carnosus*), ATCC29750 (*S. caseolyticus*), NCTC10530 (*S. chromogenes*), ATCC29974 (*S. cohnii*), DSM20771 (*S. delphini*), DSM20674 (*S. equorum*), JCM7469 (*S. felis*), CCM3572 (*S. gallinarum*), ATCC27844 (*S. hominis*), ATCC2963 (*S. intermedius*), DMS20676 (*S. kloosii*), ATCC29070 (*S. lentus*), ATCC43809 (*S. lugdunensis*), ATCC15305 (*S. saprophyticus*), ATCC43808 (*S. schleiferi subsp. schleiferi*), JCM7470 (*S. schleiferi subsp. coagulans*), ATCC27848 (*S. simulans*), ATCC27836 (*S. warnerii*), and ATCC29971 (*S. xylosus*).

**** Methicillin-resistant clinical *Staphylococcus* strains other than *Staphylococcus aureus*: *S. haemolyticus*, 10 strains; *S. epidermidis*, 10 strains; *S. sciuri*, 3 strains; *S. caprae*, 2 strains; *S. hominis*, 2 strains; *S. capitis*, 1 strain; *S. warnerii*, 1 strain.

Specific Detection Method of MRC-NS

A *mec* region DNA similar to that integrated in an MRSA is also integrated in an MRC-NS. However, the nucleotide sequence of a chromosomal DNA around the *mec* integration site is different from the corresponding sequence in the MRSA and is a nucleotide sequence specific to the C-NS strain. By using this finding, specific detection of the MRC-NS is possible by combining the nucleotide sequence surrounding the *mec* integration site and the nucleotide sequence of the *mec*. Similarly to the above-described specific detection method of the MRSA, the MRC-NS can also be specifically detected by using a method such as PCR, LCR, hybridization, RT-PCR or NASBA.

The present invention will hereinafter be described in more detail by setting out examples in each of which a DNA fragment containing *mec* and a *mec*-integration site is cloned from an MRSA strain, examples in each of which a DNA fragment containing a *mec* integration site is cloned from an MSSA strain, and the like. It is however to be noted that the present invention shall not be limited only to them.

Example 1

Cloning of DNA fragments (two fragments, i.e., upstream and downstream fragments), which contain a

mec region DNA (mec) and a junction sequence between the *mec* and an MSSA chromosomal DNA, from N315 strain; and cloning of a chromosomal DNA fragment, which contains a *mec* integration site (*int*), from an MSSA standard strain NCTC8325

1) Among Japanese MRSAs in the 1990s, type-2 coagulase and TSST-1 producing strains account for more than 70% throughout Japan [Tae Tanaka et al. J. Infect. Chemother. **1**(1), 40-49, 1995]. An MRSA clinical strain N315 (Keiichi Hiramatsu et al., FEBS Lett. **298**, 133-136, 1991), which is identical in coagulase type and ribotype to these epidemic MRSAs (Keiichi Hiramatsu, Nihon Rinsho **50**, 333-338, 1992), was cultured overnight in L-broth and was then suspended in 10 mM Tris, 1mM EDTA (pH 8.0). After lysed by achromopeptidase treatment, proteinase K treatment was conducted. Extraction was conducted with phenol and then with chloroform, whereby a chromosomal DNA was extracted. Using the chromosomal DNA so extracted, a plasmid library and a λ phage (λ dash II) library were prepared.

Described specifically, the chromosomal DNA was cleaved by a restriction endonuclease Sau3A I. Fragments as large as from 9 Kb to 20 Kb were purified by agarose gel electrophoresis and were then used. In the case of the plasmid library, 0.3 μg of the Sau3A I

fragment and 1.1 μg of a plasmid vector pACYC184, which had been cleaved by a restriction endonuclease BamH I, were subjected to ligation, followed by transformation of an *E. coli* strain MC1061. A target clone was then obtained by colony hybridization. In the case of the phage library, 0.3 μg of the Sau3A I fragment and 1 μg of λdash II, which had been cleaved by BamH I, were subjected to ligation. *In vitro* packaging was then conducted. After infected to an *E. coli* strain XL1-blue MRA(P2), colony hybridization was conducted, whereby a target clone was obtained. Concerning probes to be employed in the hybridization, the *mec*A was used as a first probe. The next probe was then prepared by synthesis of oligonucleotide or by subcloning after determining the nucleotide sequences of DNA fragments of end regions of resultant DNAs. Further, clones containing adjacent DNA regions were successively cloned (chromosome walking) (FIG. 1).

2) In this manner, starting from a clone pSJ1 containing the *mec*A gene, pSJ2, pSJ4, pSJ5, pSJ7 and pSJ8 were obtained. These clones were individually subjected to subcloning, whereby probes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 were obtained. Using these probes, the reactivity of each probe was studied by dot-blot hybridization while employing chromosomal DNAs

extracted from 29 MRSA strains and 9 MSSA strains. Namely, with respect to each of the chromosomal DNA, 200 ng of the chromosomal DNA were subjected to heat treatment at 95°C for 5 minutes and were then spotted on nylon membrane. Subsequent to a reaction with each probe which had been conjugated with digoxigenin, a further reaction was conducted with an anti-digoxigenin antibody conjugated with alkaline phosphatase, followed by the addition of a luminous substrate AMPPD to perform detection. As a result, the probes 1 to 9 did not react at all with the MSSA strains, and it was the probe 10 that reactions with the MSSA strains took place for the first time. From these results, the probe 10 was found to be a DNA fragment containing the upstream junction of the *mec*.

3) In accordance with dideoxy termination, the nucleotide sequence of the probe 10 was determined using an autosequencer (Applied biosystem 373A). Sequence reactions were conducted using a DyeDeoxy Terminator Cycle Sequencing Kit and a Dye Primer Cycle Sequencing Kit.

4) Next, to specify a chromosomal DNA region containing the integration site of the *mec*, cloning of a chromosomal DNA fragment of an MSSA was performed using a DNA probe 11 for a region reactive with the MSSA at

the upstream end of the *mec* (FIG. 1). A chromosomal DNA extracted from NCTC8325 was cleaved by the restriction endonuclease Sau3A I. After 0.3 μg of DNA fragment of from 9 kb to 20 kb, which had been prepared by agarose gel electrophoresis, and 1 μg of λ dash II cleaved by BamH I were subjected to ligation, packaging was performed to prepare a phage library. From plaques showed positive result in plaque hybridization, a recombinant phage was then recovered.

5) By dot-blot hybridization, a DNA clone LD8325 subcloned from the above-described recombinant phage was found to undergo hybridization with the probe 10. Subsequent to preparation of a restriction map, subcloning of an EcoR I-Pst I fragment (pLE1a) containing the integration site of the *mec* was therefore performed to determine its nucleotide sequence.

6) As a result of the nucleotide sequence comparison between the subclone pLE1a (pLEC12 reverse complementary) of LD8325 and the nucleotide sequence of the probe 10 (pSJ8-2a), these nucleotide sequences showed high mutual homology on left sides relative to the *mec* integration sites (FIGS. 9 and 10).

7) Employing as a probe, a DNA fragment (probe N1) amplified by PCR while using primers J4 and a Nif, which was set on a right side relative to the *mec* in-

tegration site in LD8325, a genomic library of N315 was next subjected to screening by plaque hybridization, whereby a DNA clone LN4 containing a downstream junction of N315*mec* was obtained.

8) As a result of determination of the nucleotide sequence of LN4 subclone pLN2 (J3 reverse complementary) containing the *mec* integration site, the nucleotide sequence of the subclone on an outer side relative to the *mec* integration site was found to be substantially the same as that of the *intM* and its upstream region (5' side) (see FIG. 4) of LD8325 (pLEC12 reverse complementary) (FIG. 11). As has been described above, the *mec* of N315 was found to be integrated at a certain particular site on the MSSA chromosome.

Example 2

Using the MRSA clinical strain NCTC10442 reported in England for the first time in the world (Jevons, M.P. Br. Med. J. **1**, 124-125, 1961) as an epidemic MRSA strain isolated outside Japan, a chromosomal DNA containing *mec* and its integration site was cloned. Namely,

1) Using procedures similar to those employed above in the case of N315, clones L02, L05 and L07 containing the *mec* and its upstream junction were cloned

(FIG. 2), and their nucleotide sequences were determined.

2) As a result of a sequence comparison between the subclone L02C4 containing the upstream junction of the *mec* and the pSJ8-2a of N315, a region corresponding to the outside of the junction on the MSSA chromosome was found to be homologous with pSJ8-2a although the nucleotide sequence of the upstream end of the *mec* was substantially different (FIG. 12).

3) Using the probe N1, the phage library of NCTC10442 was thus subjected to screening by plaque hybridization, whereby the clone L021 containing the downstream junction of the *mec* was obtained and its nucleotide sequence was determined.

4) As is shown in FIG. 13, a sequence common to NCTC10442 and N315 was observed in a region which corresponded to the downstream from the *mec* junction on MSSA chromosome In their nucleotide sequences of downstream end regions in the *mec* region DNAs, however, no homology was observed except for the sequences of IRs.

5) However, in a region approximately 200 nucleotides preceding toward the upstream side from the *mec* junction in NCTC10442, a nucleotide sequence homologous to the downstream end region of the *mec* in

N315 was found (FIG. 14).

6) From the foregoing, it has been found that the *mec* region DNAs of the two MRSA strains are integrated at the same integration site although they are considerably apart from each other geologically and in time, that is, they were found in Japan and England and in 1982 and 1961, respectively and moreover, they are different in epidemiological markers such as coagulase type; and further that a nucleotide sequence common to both the MRSA strains can be observed although the structures inside their *mec* region DNAs are different.

7) On the other hand, the structures of *mec* region DNAs are however diverse. It is therefore necessary to adequately perform designing of effective primers based on the concept and procedures disclosed in the present invention after determination of the structure and nucleotide sequence of each *mec* with respect to epidemic MRSA strains spread over the world.

Example 3

Concerning epidemic MRSA strains isolated outside Japan, a structural analysis of their *mec* region DNAs was conducted accordingly. Epidemic MRSA strains most widely spread over the various countries of the world are MRSAs of the coagulase type 4 [Keiichi Hiramatsu: Microbiol. Immunol. 39(8), 531-543, 1995]. Using the

German strain 85/3907 which is a typical example of these MRSAs, a chromosomal DNA fragment containing *mec* and its junctions was cloned (FIG. 3).

1) Using the probe 11 and the probe N1, the plasmid library of the chromosomal DNA of 85/3907 was subjected to screening by colony hybridization, whereby reactive two clones pSJ9 and pSJ10 were obtained.

2) The nucleotide sequences of these clones were determined. The clone pSJ10 contained a nucleotide sequence identical to *intM*, and in registration with the *mec* integration sites in N315 and NCTC10442, homologous base sequences were observed in the nucleotide sequences of the downstream end regions of the *mec* region DNAs in these MRSA strains. However, the nucleotide sequence of the downstream end region of the *mec* in 85/3907 was different from those of N315 and NCTC10442 (FIG. 15).

3) The nucleotide sequence of the other clone pSJ9 (FIG. 3) did not contain any *mec* junction site.

4) Using the phage library of 85/3907, a DNA fragment reactive with the clone pSJ9 was therefore subjected to cloning by plaque hybridization. In addition, a clone LG12 was obtained by using the nucleotide sequence of the DNA fragment ends as probe. A similar operation was then repeated to perform chromosome walk-

ing (FIG. 3).

5) None of these reacted with the chromosomal DNA of NCTC 10442, an MSSA strain, by dot-blot hybridization.

6) Accordingly, the chromosomal DNA of ATCC25923, which is a standard MSSA strain of the coagulase type 4, was extracted, and its dot-blot hybridization with the above-described clones was performed. The hybridization gave positive results. Hybridization, however, gave a negative result when the right-side part of the clone LG12 out of the above-described clones was used as a probe.

7) As a result of determination of the nucleotide sequence LG12H2 of LG12, practically no homology was observed both inside and outside the *mec* except for the observation of sequences homologous to the IR nucleotide sequences of the upstream ends of the *mec* region DNAs and approximately 20 bases located adjacent to the junctions and corresponding to the 3' end regions of intMs in N315 and NCTC10442 (FIG. 16).

8) From the foregoing, it has been found that there are MRSA strains of clearly different origins, which occurred as a result of integration of the *mec* into the chromosomes of different MSSAs, and that at least the MSSA chromosome region on the outer side

(left side) of the upstream junction of the *mec* significantly differs among the MRSA strains of different origins.

9) Nonetheless, the MSSA chromosome region, including the nucleotide sequence of the *intM*, was reserved on the outer side (right side) of the downstream junction of the *mec* as far as all the MRSA strains investigated were concerned.

10) Upon designing primers or probes surrounding *mec* junctions for use in PCR, LCR or hybridization, it has been found that PCR requires to design different *mec*-side base sequences, one for N315 and NCTC10442 and another for 85/3907. When LCR or hybridization is relied upon, three primers or probes have to be prepared for N315, NCTC10442 and 85/3907, respectively.

11) As has been described above, it has become apparent that identification of an MRSA by PCR, LCR or hybridization around the *mec*-MSSA chromosome junction cannot be practiced if the initial finding, which teaches merely that the *mec* has the same chromosome integration site on chromosomal DNA of some MRSAs [Keiichi Hiramatsu: Microbiol. Immunol. **39**(8), 531-543, 1995], is solely relied upon.

Example 4

Verification of homology of the *mec* in MRSAs

1) Using as probes DNA clones covering the respective *mec* regions of N315, NCTC10442 and 85/3907, their reactivity with the chromosomal DNAs of 26 epidemic MRSA strains isolated in 18 countries of the world was screened by dot-blot hybridization. After 100 ng/$\mu\ell$ of a chromosomal DNA solution and T10E1 (10 mM Tris, 1 mM EDTA [pH 8.0]) were mixed in equal amounts, the resulting mixture was heated for 95°C for 5 minutes. The mixture was allowed to cool down, to which 20XSSC was added in an equal amount. Then, 4 $\mu\ell$ of the thus-prepared mixture were spotted on nylon membrane. Subsequent to denaturation with 0.5 N NaOH and 1.5 M NaCl, neutralization was conducted with 1.5 M Tris (pH 7.3), 1.5 M NaCl and 1 mM EDTA. Using the probes conjugated with digoxigenin, hybridization was conducted. Detection was performed using an anti-digoxigenin antibody conjugated with alkaline phosphatase.

2) As is presented in Tables 2, 3 and 4, the above-described three *mec* region DNAs have been found to be practically undetectable by their corresponding probes.

3) It has been suggested that the use of the respective *mec* probes of N315, NCTC10442 and 85/3907 makes it possible to classify the 26 strains into any

one of the above-described three types (Tables 2, 3 and 4).

Table 2

Distribution of the mec of N315 Strain among MRSA strains

Strain[2]	Probe[1]															
	11	10	9	8	7	6	5	4	3	2	1	*mec*I	*mec*R1 PB	*mec*R1 MS	*mec*A	12
II N315	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
III 10443	+	+	-	-	-	-	-	-	+	+	-	-	-	+	+	+
61/6291	+	+	-	-	-	-	-	-	+	±[3]	-	-	-	+	+	NT[4]
64/3846	+	+	-	-	-	-	-	-	+	±	-	-	-	+	+	NT
64/4176	+	+	-	-	-	-	-	-	+	+	±	-	-	+	+	NT
86/4372	+	+	-	-	-	-	-	+	+	+	+	-	-	+	+	NT
IV 86/961	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
86/560	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
85/1340	±	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
85/1762	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
85/2082	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
85/2111	+	-	-	-	-	-	-	-	+	+	±	+	+	+	+	NT
85/1836	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
85/2147	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
86/3907	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
86/2652	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
85/5495	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
85/3619	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
85/3566	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	NT
II 85/2235	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NT
84/9580	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	NT
86/9302	+	+	-	-	-	-	-	+	+	+	±	-	-	+	+	NT
85/1940	+	-	-	-	-	-	+	±	+	+	±	-	-	+	+	NT
IV 85/4231	+	+	+	+	+	+	+	+	+	+	±	+	+	+	+	NT
85/2232	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NT
VII 85/4547	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	NT

1 PB, penicillin-binding domain; MS, membrane-spanning domain.
2 Roman numbers indicate coagulase types.
3 Weak positive signal.
4 Not tested.

Table 3

Distribution of the mec of NCTC10442 Strain among MRSA strains

Strain[2]	Probe[1]										
	7	6	5	4	3	2	1	*mec*I	*mec*R1 PB	*mec*R1 MS	*mec*A
II N315	−	−	−	−	−	−	−	+	+	+	+
III 10442	+	+	+	+	+	+	+	−	−	+	+
61/6291	+	+	+	+	+	+	+	−	−	+	+
64/3846	+	+	+	+	+	+	+	−	−	+	+
64/4176	+	+	+	+	+	+	+	−	−	+	+
86/4372	+	−	−	−	−	−	±[3]	−	−	+	+
IV 86/961	−	−	−	−	−	−	±	+	+	+	+
86/560	−	−	−	−	−	−	−	+	+	+	+
85/1340	−	−	−	−	−	−	−	+	+	+	+
85/1762	−	−	+	−	−	−	+	+	+	+	+
85/2082	−	−	+	−	−	−	+	+	+	+	+
85/2111	−	−	−	−	−	−	−	+	+	+	+
85/1836	−	−	−	−	−	−	−	+	+	+	+
85/2147	−	−	−	−	−	−	−	+	+	+	+
85/3907	−	−	−	−	−	−	−	+	+	+	+
86/2652	−	−	±	−	−	−	±	+	+	+	+
85/5495	−	−	±	−	−	−	−	+	+	+	+
85/3619	±	−	+	−	−	−	±	+	+	+	+
85/3566	−	−	±	−	−	−	−	+	+	+	+
II 85/2235	±	−	+	−	−	−	±	+	+	+	+
84/9580	+	+	+	+	+	+	+	−	−	+	+
86/9302	+	+	+	+	+	+	+	−	−	+	+
85/1940	+	+	+	+	+	+	+	−	−	+	+
IV 85/4231	−	−	−	−	−	−	−	+	+	+	+
85/2232	−	−	−	−	−	−	−	+	+	+	+
VII 85/4547	−	−	+	+	−	−	+	−	−	+	+

1 PB, penicillin-binding domain; MS, membrane-spanning domain.
2 Roman numbers indicate coagulase types.
3 Weak positive signal.

Table 4

Distribution of the mec of 85/3907 Strain among MRSA strains

Strain[2]	Probe[1]										
	7	6	5	4	3	2	1	*mec*I	*mec*R1 PB	*mec*R1 MS	*mec*A
II N315	−	−	−	−	±[3]	−	−	+	+	+	+
III 10442	−	−	−	−	−	−	−	−	−	+	+
61/6291	−	−	±	−	−	−	−	−	−	+	+
64/3846	−	−	±	−	−	−	−	−	−	+	+
64/4176	−	−	−	−	+	+	−	−	−	+	+
86/4372	−	−	−	−	−	−	−	−	−	+	+
IV 86/961	+	±	+	+	+	+	+	+	+	+	+
86/560	+	±	+	+	+	+	+	+	+	+	+
85/1340	+	±	+	+	±	+	+	+	+	+	+
85/1762	+	+	+	+	+	+	+	+	+	+	+
85/2082	+	+	+	+	+	+	±	+	+	+	+
85/2111	+	+	+	+	+	+	+	+	+	+	+
85/1836	+	+	+	+	+	+	+	+	+	+	+
85/2147	+	+	+	+	±	+	+	+	+	+	+
85/3907	+	+	+	+	+	+	+	+	+	+	+
86/2652	+	±	+	+	+	+	+	+	+	+	+
85/5495	+	±	+	−	±	+	±	+	+	+	+
85/3619	+	+	+	+	+	+	+	+	+	+	+
85/3566	+	+	+	+	+	+	+	+	+	+	+
II 85/2235	−	−	+	−	+	+	−	+	+	+	+
84/9580	±	−	±	−	±	+	−	−	−	+	+
86/9302	−	−	−	−	−	−	−	−	−	+	+
85/1940	−	−	−	−	±	−	−	−	−	+	+
IV 85/4231	+	−	±	−	+	+	−	+	+	+	+
85/2232	+	−	±	−	±	+	−	+	+	+	+
VII 85/4547	−	−	+	+	−	−	+	−	−	+	+

1 PB, penicillin-binding domain; MS, membrane-spanning domain.
2 Roman numbers indicate coagulase types.
3 Weak positive signal.

Example 5

Based on the base sequences of the *mec* region DNAs of N315 and NCTC10442, probes for the detection of the *mec* region DNAs of types 2j and 3e were synthesized by a method known *per se* in the art. Nucleotide sequences for the selection of primers were designed based on N315IS-J3 (FIG. 17) and NCTC10442J3rc (FIG. 18), which is a nucleotide sequence of a mec region extending from IS431 (FIG. 1) on the right side of (downstream from) the *mec* to the downstream junction in N315. The followings are primers chosen as desired. Similar primers can be designed in a large number on the basis of the above-described nucleotide sequences. The nucleotide sequences of the primers will be shown next together with their positions as indicated by nucleotide numbers in N315IS-J3 (FIG. 17).

Nmec2	5'-GATAGACTAATTATCTTCATC-3'	229-249
Nmec3	5'-CAGACTGTGGACAAACTGATT-3'	507-527
Nmec4	5'-TGAGATCATCTACATCTTTA-3'	676-695
Nmec4-2	5'-GGATCAAAAGCTACTAAATC-3'	903-922
Nmec5	5'-ATGCTCTTTGTTTTGCAGCA-3'	1504-1523
Nmec6	5'-ATGAAAGACTGCGGAGGCTAACT-3'	2051-2073

Example 6

Based on the nucleotide sequence pSJ10-3J3rc (FIG. 19) of the *mec* of 85/3907, a probe for the detec-

tion of the *mec* of type 4e was synthesized.

Gmec1045 5'-ATATTCTAGATCATCAATAGTTG-3' 10-33

Example 7

Based on the base sequence pLEC12(pLEC1a) (FIG. 4) of the *intM* and its upstream region, primers for the detection of the chromosomal DNAs of MSSAs were synthesized.

J3	5'-AAGAATTGAACCAACGCATGA-3'	1664-1684
*intM*1	5'-AAACGACATGAAAATCACCAT-3'	1389-1409
J7	5'-TCGGGCATAAATGTCAGGAAAAT-3'	1267-1289
J6	5'-GTTCAAGCCCAGAAGCGATGT-3'	949-969
Nif	5'-TTATTAGGTAAACCAGCAGTAAGTGAACAACCA-3'	639-671

Example 8

Preliminary Experiment for the PCR Detection of MRSA by Combination of Primers Constructed on *mec*DNA and Downstream Region to *mec*DNA.

A preliminary experiment for the setting of primers was conducted by combining 6 primers prepared based on the nucleotide sequences of the *mec* region DNAs of N315 and NCTC10443 for the detection of the *mec* region DNAs of types 2j and 3e, 2 primers prepared based on the nucleotide sequence of the *mec* region DNAs of 85/3907 for the detection of the *mec* region DNAs of type 4e, and 6 primers prepared based on the nucleotide sequence of the *intM* and its upstream region for the

detection of the chromosome DNAs of MSSAs. PCR was performed in 20 μℓ to 50 μℓ of reaction mixtures [10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001%(w/v) gelatin, 200 μM dNTPs, 1.0 μM primer, template DNA, 0.01 u/μℓ Taq DNA polymerase). As reaction conditions, a system in which 94°C-30 sec, 55°C-1 min and 72°C-2 min was repeated 30 cycles was used. As PCR, "GeneAmp PCR System 9600" (Parkin Elmer) was used. For the detection of each PCR product, 5 μℓ of the reaction mixture were applied to electrophoresis through a 0.8% agarose gel after completion of the reaction. Subsequent to the electrophoresis, staining was conducted for 30 minutes in a 0.1 M aqueous solution of ethidium bromide, followed by the detection of bands on a transilluminater.

Results 1

As is shown in Table 5, the epidemic MRSA strains in various countries of the world, which were subjected to the test, were all classified into one of two types, one being reactive with Gmec1045 prepared based on the nucleotide sequences of the *mec* region DNAs of N315 and NCTC10442, and the other reactive with Nmec6 prepared based on the nucleotide sequence of the *mec* region DNA of 85/3907. It has therefore been indicated that use of primers prepared based on the nucleotide sequences

of the above-described two types of *mec* region DNAs makes it possible to detect and identify all the MRSAs in the various countries of the world.

Table 5

Strain		Combination of primers J7-Nmec6	J7-Gmec1045
82/N315	Japan	+	-
85/2235	U.S.A.	+	-
84/9580	Germany	+	-
86/9302	U.K.	+	-
85/1940	France	+	-
10442	U.K.	+	-
61/6219	U.K.	+	-
64/3846	U.K.	+	-
986/4372	U.K.	+	-
86/961	U.K.	+	+
86/560	U.K.	+	+
85/1340	Yugoslavia	-	+
85/1762	Hungary	-	+
85/2082	New Zealand	-	+
85/2111	Norway	-	+
85/1836	Germany	-	+
85/3907	Germany	-	+
86/2652	England	-	+
85/5495	Saudi Arabia	-	+
85/3619	Austria	-	+
85/3566	Holland	-	+
85/4231	Canada	+	-
85/2232	U.S.A.	+	-
85/4547	Israel	+	-

Results 2:

Concerning primers, Nmec2, 3, 4, 4-2, 5 and 6 were used as *mec*DNA primers, and J7, Nif and J6 were

employed as downstream primers of the *mec*-integrated regions. In the combinations with the primers of the N*mec* series, J7, J6 and Nif all indicated positive reactions. Strong signals were observed especially when J6 or J7 was used. In an experiment of combinations between G*mec*1045 and MSSA-side primers, a strong signal was obtained from each combination.

Described specifically, in the combinations of J7 and the *mec*DNA primers N*mec*2, 3, 4, 4-2, 5 and 6, bands of 2500, 2300, 2100, 1900, 1300 and 700 bp of PCR products were observed, respectively, on agarose electrophoresis. In the combinations of Nif and the *mec*DNA primers N*mec*2, 3, 4, 4-2, 5 and 6, bands of 3200, 2900, 2700, 2500, 1900 and 1400 bp were observed, respectively. Further, in the combinations of J6 and the *mec*DNA primers N*mec*2, 3, 4, 4-2, 5 and 6, bands of 2800, 2600, 2400, 2200, 1600 and 1000 bp were observed, respectively.

In an experiment in which the DNA of the MRSA strain 85/3907 was used, PCR products were not amplified in any of the above combinations, and PCR products were detected when G*mec*1045 was used as a *mec*DNA primer. Namely, in combinations of G*mec*1045 and Nif, J6, J7, *intM*1 or J3 as downstream primers of the *mec*-integrated region, bands of 1300, 1000, 700, 600

and 300 bp were detected, respectively, by electrophoresis.

Example 9

Experiment for Setting a Primer Combination Suited for Mixing

An investigation was next conducted concerning mixing of two *mec* primers and one MSSA-side primer. Conditions for PCR were similar to those employed in Example 8. To detect all MRSAs by a single operation of PCR, one of J7, Nif and J6 was used as a downstream primer of a *mec*-integrated region, and as *mec*DNA primers, the *mec*DNA primer G*mec*1045 reactive with the *mec*DNA of 85/3907 and one of the *mec*DNA primers, N*mec*2, 3, 4, 4-2, 5 and 6, reactive with *mec*DNAs of the N315 and NCTC10442 type were chosen. These three types of primers were mixed. Using the DNAs of N315 and 85/3907 as templates, amplification was performed by PCR to determine a combination which would lead to detection of a product in a largest quantity.

Results:

When the primers of the N*mec* series were combined with the individual combinations of G*mec*1045 with J7, Nif and J6, the use of G*mec*1045 and J6 gave good signals except that signals became somewhat weaker when N*mec*4, 3 and 2 primers were added. When N*mec*6, 5 and 4-

2 were used in combination with the same combination, signals were rather stronger that those available from the other combinations.

Described specifically, when the DNA of N315 was used as a template, each of the combinations of J7, *Gmec*1045 and (*Nmec*2, 3, 4, 4-2, 5 and 6) led to clear detection of a PCR product upon staining an agarose gel with ethidium bromide. In the combinations of Nif, *Gmec*1045 and (*Nmec*2, 3, 4, 4-2, 5 and 6), on the other hand, strong bands were detected from the use of *Nmec*5 and 6 primers. However, bands were weak in the case of *Nmec*3, 4 and 4-3 primers. No band was detected when *Nmec*2 was used. Further, in the combinations of J6, *Gmec*1045 and (*Nmec*2, 3, 4, 4-2, 5 and 6), a weak band was detected in the case of *Nmec*6, no bands were detected in the case of *Nmec*2, 3 and 4, and strong bands were detected in the case of *Nmec*4-2 and 5. With *Nmec*5 in particular, a strongest band was confirmed out of those obtained from all the combinations. From the foregoing, it has been substantiated that sufficient results cannot be obtained in certain combinations of primers and careful optimization is hence important. From these results, the combination of J6, *Gmec*1045 and *Nmec*5 has been found to be an optimal primer combination.

Example 10

Investigation on Specificity of Detection Method of MRSA by PCR

Based on the results of Example 9, the effectiveness of the primer combination of J6, *Gmec*1045 and *Nmec*5 was investigated in accordance with PCR by using MRSAs, MSSAs and clinical staphylococcus strains other than *Staphylococcus aureus*, said clinical staphylococcus strains including both those containing *mec* and those carrying no *mec*. PCR reactions were performed using as templates the DNAs of 26 MRSA clinical strains, 11 MSSA strains, 25 standard c-NS strains and 20 MRC-NS clinical strains. As a primer, the three primers J6, *Gmec*1045 and *Nmec*5 were used as a mixture. The primer was the mixture of the following three primers, and *Gmec*1045 and *Nmec*5 were used on the *mec* side while J6 was employed on the MSSA side. PCR was performed in 20 $\mu\ell$ to 50$\mu\ell$ of reaction mixtures [10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001%(w/v) gelatin, 200 μM dNTPs, 1.0 μM primer, template DNA, 0.01 u/$\mu\ell$ Taq DNA polymerase]. As reaction conditions, a system in which 94°C-30 sec, 55°C-1 min and 72°C-2 min were repeated 30 cycles was used.

It is Table 1 that summarizes the results of the above investigation. The MRSAs in the various coun-

tries of the world all showed positive signals, whereas the MSSAs all indicated negative results. The staphylococcus strains other than *Staphylococcus aureus* all gave negative results no matter whether or not they had *mec*.

Namely, clear bands were detected from all the MRSA strains. Concerning their sizes, there were bands of 1.6 kb (N315 type) from 8 strains, bands of 1.5 kb (NCTC10442 type) from 5 strains, and bands of 1 kb (85/3907 type) from 13 strains. From these results, it has been indicated that MRSAs can be all detected and can be classified into three types in accordance with the size of amplified DNA fragments. From each of the MSSA strains, C-NS strains and MRC-NS strains, no band was detected at all. Accordingly, MRSA specificity of this detection method has been demonstrated.

Example 11

Specific Detection of MRSA by RT-PCR

Using MRSA, MRC-NS and MSSA clinical strains, the whole RNAs of the individual strains were extracted and then converted into DNAs by RT-PCR, and genes were screened. Employed as primers for detection were N*mec*4-2, N*mec*5 and N*mec*6 in Example 5, G*mec*1045 used in Example 6 and IntM1 in Example 7.

Used as clinical strains were: as MSSA(*mec*-)

strains, ATCC25923 strain and NCTC8325 strain; as MRSA(*mec*+) strains, N315 strain, NCTC10442 strain and 85/3907 strain; and as MRC-NS strains, the *S. haemolyticus* strains SH518 and SH631 and the *S. epidermidis* strain G13.

Each strain was cultured overnight in LB medium. The resultant cells were collected by centrifugation, washed with PBS, suspended in a 1 mℓ RNase-free PK/SDS solution [200 μg/mℓ, Proteinase K, 0.5% SDS, 10 mM Tris-HCl, pH 8.0 1 mM EDTA (pH 8.0), 100 mM NaCl], and then incubated at 55°C for 60 minutes. Extraction was next performed with phenol/chloroform/isoamyl alcohol (50/49/1) and further with chl/IAA (49/1), followed by precipitation in ethanol. The resulting pellet was rinsed with 75% ethanol and, after dried in air, was dissolved with 20 μℓ nuclease-free water. The whole RNAs obtained as described above were treated with DNase (37°C, 60 min). After completion of the reaction, DNase was inactivated. Then, extraction was performed with phl/chl/IAA and then with chl/IAA, followed by precipitation in ethanol. The resulting pellet was dissolved with 10 μℓ of nuclease-free water. Next, the thus-obtained whole RNAs were heated at 95°C for 2 minutes and then quenched on ice. RT buffer (20 μℓ/ sample) was added and reverse transcription was con-

ducted, whereby cDNA was obtained. Subsequent to reaction at 25°C for 10 minutes, at 37°C for 60 minutes, and then at 90°C for 5 minutes in a thermal cycler ("TAKARA MP TP3000"), a PCR reaction was performed in a manner known *per se* in the art.

For the specific detection of MRSA by PCR, it is necessary to combine a primer for N315 and NCTC10442 and a primer for 85/3907 as a forward primer. First, #393(Nmec5) and #347(Gmec1045) were used for the detection of MRSA. Similarly, #210(jun1) and #351(25923-1) were combined as a primer for the detection of MSSA. For the reverse side, #212(jun3) was used out of the primers within the *intM*. Incidentally, the distance from jun3 primer to the *mec* integration site is 193 bp, including the primer (21 mer).

Primer name	Concentration (pmol/$\mu\ell$)	Target (strain)
(1) #210 Junction 1(jun1)	82.1	MSSA(NCTC8325)
(2) #260 10442-J-3-1(N*mec*6)	92.0	MRSA(N315,NCTC10442)
(3) #330 1S431-right 7-2(N*mec*4-2)	138.4	MRSA(N315,NCTC10442)
(4) #347 pSJ10-4-5(G*mec*1045)	67.9	MRSA(85/3907)
(5) #351 25923-1	266.8	MSSA(ATCC25923)
(6) #393 1S431-right 5-new(N*mec*5)	128.6	MRSA(N315,NCTC10442)

Reaction conditions for PCR were as described below, and two forward primers were employed. Concerning the amount of the template, a portion (1 μℓ) of an RT reaction mixture, which is equivalent to 50 ng as calculated from the total RNA amount (1 μg/20 μℓ) at the time of initiation of the RT reaction, was taken and then added to a PCR reaction system of 50 μℓ.

<PCR reaction mixture [MRSA]>

Contents	Volume/sample	Final conc.
Water (nuclease-free, SIGMA)	42.4 μℓ	-
10x PCR buffer (with $MgCl_2$, TAKARA)*	5.0 μℓ	1 x
20 mM dNTPs (TAKARA)	0.5 μℓ	200 μM
Forward primer		
67.9 μM Gmec1045	0.4 μℓ	540 nM
128.6 μM Nmec5	0.2 μℓ	510 nM
84.3 μM reverse primer jun3	0.3 μℓ	510 nM
5 U/μℓ Taq DNA polymerase (TAKARA)	0.2 μℓ	1U/50 μℓ
RT product (cDNA; template)	1.0 μℓ	-
Total	50.0 μℓ	

<PCR reaction mixture [MSSA]>

Contents	Volume/sample	Final conc.
Water (nuclease-free, SIGMA)	42.6 μℓ	-
10x PCR buffer (with $MgCl_2$, TAKARA)*	5.0 μℓ	1 x
20 mM dNTPs (TAKARA)	0.5 μℓ	200 μM
Forward primer		
82.1 μM jun1	0.3 μℓ	490 nM
266.8 μM 25923-1	0.1 μℓ	530 nM
84.3 μM reverse primer jun3	0.3 μℓ	510 nM
5 U/μℓ Taq DNA polymerase (TAKARA)	0.2 μℓ	1U/50 μℓ
RT product (cDNA; template)	1.0 μℓ	-
Total	50.0 μℓ	

* 10x PCR buffer: 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$

After the reaction mixture other than the template was prepared and mixed, it was poured in 49 μℓ portions into 0.5 mℓ PCR tubes in each of which 50 μℓ of light liquid paraffin (TOYOBO) had been placed in advance. Finally, the below-described templates were added in amounts of 1 μℓ, respectively. Each of the resulting mixtures was agitated with tapping, and after being flushed down, the mixture was set in the "Thermal Cycler MP" to initiate a PCR reaction. As a negative control for the PCR, water was used. On the other hand, plasmid pLEC1a DNA (about 500 ng/μℓ) was diluted

100-fold in water, and 1 μℓ (about 5 ng) of the dilute solution was used as a positive control for MSSA-PCR.

	Template	RTase		Template	RTase
(1)	ATCC25923 (MSSA)	-	(7)	NCTC10442 (MRSA)	-
(2)	ATCC25923 (MSSA)	+	(8)	NCTC10442 (MRSA)	+
(3)	NCTC8325 (MSSA)	-	(9)	85/3907 (MRSA)	-
(4)	NCTC8325 (MSSA)	+	(10)	85/3907 (MRSA)	+
(5)	N315 (MRSA)	-	(11)	Water (PCR negative control)	
(6)	N315 (MRSA)	+	(12)	pLEC1a (PCR positive control)	

Denaturation	at 94°C for 30 sec.
Primer annealing	at 50°C for 1 min.
Chain elongation (extension)	at 72°C for 2 min.

After the reaction was repeated 30 cycles at the above reaction temperatures, the amplified product was reserved at 4°C until it was subjected to electrophoresis.

Confirmation of amplified products

With respect to the amplified products obtained by the PCR, their confirmation was performed by electrophoresis in a 2% agarose gel. The gel was prepared by adding 2% of agarose ("SeaKem GTG agarose, FMC) to 0.5x TBE (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA),

dissolving the resultant mixture and then processing the thus-prepared solution in a gel maker of a submarine-type small electrophoresis apparatus (Mupid-COSMOBIO). From the amplified products (50 μℓ), 10 μℓ which were equivalent to one fifth of the amplified products were taken. Subsequent to the addition of 2 μℓ of a dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 1 mM EDTA, 30% glycerol), the amplified products were applied to a well and then subjected to electrophoresis at 100 V for 45 minutes in 0.5x TBE buffer. As a size marker, 100 bp DNA Ladder (100-1500 bp + 2072 bp, 1 μg/μℓ, GIBCO BRL) was used in an amount of 0.5 μℓ. After the electrophoresis, the gel was immersed in TBE buffer containing 0.5 μg/mℓ of ethidium bromide, and was stained at room temperature for 20 minutes. Then, the amplified fragments were confirmed on a UV transilluminater and also photographed by a Polaroid camera (Polaroid MP4/type 667 film).

As a result of electrophoresis of a portion of the amplified products in an 2% agarose gel, no amplified products were confirmed in the case of the RTase-free templates in both of the MRSA and MSSA detection PCRs. mRNA-origin amplified products were confirmed only in the presence of RTase. Accordingly, the thus-obtained amplified products were all confirmed

to be cDNA-origin ones synthesized from mRNA by reverse transcription. In the MRSA detection PCR, no amplified products were confirmed in the case of the two MSSA strains, and those of different lengths [N315(906 bp)>NCTC10442(804 bp) > 85/3907(361 bp) were obtained in the case of the three MRSA strains. In the MSSA detection PCR, on the other hand, no amplified products were confirmed in the case of MRSAs, and amplified products of different lengths (AGTC25923>NCTC8325) were obtained only in the case of the two MSSA strains. Further, in the case of the PCR positive control (pLEC1a), amplified products were observed only in the MSSA detection PCR so that amplified products as large as NCTC8325, the source for pLEC1a, were obtained.

From the above results, it has been confirmed that mRNA of a *mec* region, which is needed for the specific detection of MRSA, is not observed in the case of MSSA but is developed only in the case of MRSA.

Example 12

Specific Detection of MRSA by NASBA

Using MRSA, MRC-NS and MSSA clinical strains, the whole RNAs of the individual strains were extracted. RNAs were then amplified by NASBA. Amplification primers and detection probes were designed as will be described hereinafter.

As forward primers

N315(MRSA), NCTC10442 5'-GAAAGAGGCGGAGGCTAA-3'

85/3907 5'-CATCTAAACATCGTATGA-3'

As reverse primers

As a probe for detecting of the IntM region

3'-AAACGGGAACCCAGTACGCA-promotor-5'

of each strain, reverse primers were designed commonly to the individual strains on the basis of the base sequence of the *intM* region.

Designed were:

For capturing

5'-GCTGAATGATAGTGCGTAGTTAC-3' (A)

For detection

5'-TGAAGACGTCCTTGTGCA-3' (B); or

For capturing

5'-TGAATGATAGTGCGTAGTTACTGCG-3' (C)

For detection

5'-TCATTTGATGTGGGAATGTG-3' (D)

Used as clinical strains were: as MSSA(*mec*-) strains, ATCC25923 strain and NCTC8325 strain; as MRSA(*mec*+) strains, N315 strain, NCTC10442 strain and 85/3907 strain; and as MRC-NS strains, the *S. haemolyticus* strains SH518 and SH631 and the *S. epidermidis* strain G13.

Each strain was cultured overnight in LB medium.

From the resultant cells, the whole RNAs were collected in a similar manner as in Example 11. After samples were prepared by subjecting the RNAs to 10-serial dilution (1 pg/μℓ to 1 μg/μℓ) in water, the following NASBA-dot hybridization was conducted.

1. Elution

    1) Each sample (0.5 mℓ) is placed in an eluent buffer tube and is stirred several times.
    2) The tube is centrifuged (1,500 x g, 15 seconds).

2. Separation

    1) A control solution (system control solution, 20 μℓ) is added to the tube, followed by centrifugation.
    2) A silica suspension (70 μℓ) is added to each tube, is allowed to stand at room temperature (15 to 30°C) for 10 minutes, and is then stirred intervals of 2 minutes.
    3) Each tube is centrifuged (1,500 x g, 2 minutes).
    4) The supernatant is thrown away.
    5) After silica particles are re-suspended, centrifugation is conducted and the supernatant is removed. The residue is washed with a wash buffer, with 70% ethanol (2 twice), and

then with acetone (4 times). Centrifugation is performed.

6) The resulting silica pellet is dried (56°C, 10 minutes).

7) An eluate (100 μℓ) is added for re-suspension. The resultant suspension is stirred (56°C, 10 minutes) to elute nucleic acids.

8) Centrifugation is performed (10,000 x g, 2 minutes).

3. Amplification

1) A primer (15 μℓ) is added to 8 μℓ of the nucleic acid supernatant. After a reaction at 65°C for 5 minutes, the reaction mixture is cooled at 41°C for at least 5 minutes.

2) An enzyme solution (2 μℓ) is added, and a reaction is allowed to proceed (41°C, at least 5 minutes).

3) The tube is moved into a detection compartment. The mixture is gently stirred, followed by a reaction at 41°C for 90 minutes.

4. Detection

1) The reaction mixture of 3-3) is diluted approximately 10-fold, 1 μℓ of the dilute solution is dropped on a Hybound N+blotting membrane (Amersham), and the membrane is placed for 5 minutes on a piece of

filter paper soaked with 0.6 N NaOH. The membrane is washed and neutralized with a 5x SSC solution. On a piece of filter paper, water was removed. The membrane is heated at 50°C for 15 minutes in a hybridization buffer under shaking, and then placed in a probe solution formed of 1 mℓ of a hybridization buffer and 1 μℓ of an ALP probe added thereto, where the membrane is heated at 50°C for 15 minutes under shaking. The membrane is moved into a 2XSSC/1% SDS solution and heated at 50°C for 15 minutes under shaking. At room temperature, the membrane is heated for 15 minutes in 1XSSC/0.5% Triton X and then for 5 minutes in a 1XSSC solution under shaking. The membrane is then reacted with a staining solution at 37°C for 10 to 60 minutes. After the membrane is washed under shaking in purified water, water is removed on a piece of filter paper and dried to observe staining. The results are showed in Table 6.

Table 6

Staphylococcus aureus	Probe A (for capturing) Probe B (for detection)	Probe B (for capturing) Probe A (for detection)
MSSA		
1) ATCC25923	-	±
2) NCTC8325	-	-
MRSA		
1) N315	+	+
2) NCTC10442	+	+
3) 85/3907	+	+
MRS-NS		
1) *S. haemolyticus* SH518	-	-
2) *S. epidermidis* G3	-	-

+: clear color production,
±: unclear color production, and
-: color production was not detected.

Example 13

Specific Detection of Methicillin-Resistant *S. haemolyticus* by PCR

A primer *intM*h was prepared based on the specific base sequence of the region on the right side of the *mec*-integrated region (the region downstream from the *mec*A) in MR *S. haemolyticus* of FIG. 6.

*intM*h: 5'-GATCAAATGGATTGCATGAGGA-3'

(corresponding to the base numbers 236 to 260 in FIG. 6)

When PCR reactions were conducted by combining this primer with the *mec*-specific primers in Example 5, bands of 2100, 1800, 1700, 1400, 850 and 300 bp were detected, respectively, from the combinations with Nmec2, Nmec3, Nmec4, Nmec4-2, Nmec5 and Nmec6 in the case of 10 methicillin-resistant *S. haemolyticus* strains such as SH518 and SH631. However, bands were not detected at all in the case of MRSAs, MSSAs, standard strains of methicillin-susceptible C-NSs, and clinical strains.

Example 14

Specific Detection of Methicillin-Resistant *S. epidermidis* by PCR

A primer intMe was prepared based on the specific nucleotide sequence of the region on the right side of the *mec*-integrated region (the region downstream from the *mecA*) in MR *S. epidermidis* of FIG. 6.

intMe: 5'-TCTTCAGAAGGACTCGCTAA-3'
(corresponding to the base numbers 318 to 337 in FIG. 6)

When PCR reactions were conducted by combining this primer with the *mec*-specific primers in Example 5, bands of 2200, 1900, 1800, 1600, 900 and 400 bp were detected, respectively, from the combinations with Nmec2, Nmec3, Nmec4, Nmec4-2, Nmec5 and Nmec6 in the case of 10 methicillin-resistant *S. epidermidis* strains such

as G3. However, bands were not detected at all in the case of MRSAs, MSSAs, standard strains of methicillin-susceptible C-NSs, and clinical strains.

From the foregoing, it has been substantiated that, when two primers are synthesized at the downstream end of *mec* and a further primer is synthesized within *intM* or on its 5' side, most of *mec* region DNAs of primary MRSA clinical strains in the world can be detected and also that this method does not give any false positive result even if one or more of wide-spread human staphylococci other than *mec*-carrying *Staphylococcus aureus* are mixed.

Capability of Exploitation in Industry

When conditions for super high-speed identification of MRSA and MRC-NS from a clinical sample are set by using the present method, MRSA can be detected directly from a sample such as sputum, blood, urine or exudate in 30 minutes to 1 hour within the scope of the conventionally-known art. This method makes it possible to perform bedside identification of MRSA from a patient's sample, thereby permitting selection of an effective chemotherapeutic agent and early treatment of MRSA and NRC-NS infectious diseases. Further, this

method is also useful as a test for the prevention of outbreak of nosocomial infection, because it can also be applied for the MRSA carrier screening of normal subjects such as those engaged in medical work.

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CLAIMS

1. A diagnostic method of an MRSA (methicillin-resistant *Staphylococcus aureus*) or MRC-NS (methicillin-resistant coagulase-negative staphylococci), which comprises performing a reaction with a sample by making combined use of a part of a *mec*DNA, which is an integrated adventitious DNA existing on a chromosome of said MRSA or MRC-NS and carrying a *mec*A gene thereon, and a part of a base sequence of a chromosomal DNA surrounding said integrated DNA.

2. A diagnostic method according to claim 1, wherein said MRSA or MRC-NS is identified by amplifying a DNA fragment in said sample, said DNA fragment containing junctions between *mec* and a chromosomal DNA surrounding said *mec*, by a polymerase chain reaction (PCR) while using as a primer said region of said *mec*DNA and said region of said nucleotide sequence of said chromosomal DNA surrounding said *mec*DNA.

3. A diagnostic method according to claim 1, wherein said MRSA or MRC-NS is identified by preparing two DNA fragments, which surround junctions between said region of said *mec*DNA and said chromosomal DNA surrounding said *mec*DNA, and amplifying a DNA in said sample by a ligase chain reaction (LCR).

4. A diagnostic method according to claim 1,

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wherein said MRSA or MRC-NS is identified by preparing as a probe a single or double-strand DNA fragment, which surrounds junctions between said region of said *mec*DNA and said chromosomal DNA surrounding said *mec*DNA, and performing hybridization between said probe and a DNA in said sample.

5. A diagnostic method according to any one of claims 1-4, wherein a nucleotide sequence of an upstream end region of said *mec* is a part of pSJ8-2a (the nucleotide sequence of FIGS. 9 and 10), L02C4 (the nucleotide sequence of FIG. 12) or LG12H2 (the nucleotide sequence of FIG. 16); a nucleotide sequence reverse complementary thereto; or a sequence obtained by replacing one or more of nucleotides thereof by different nucleotides to an extent remaining unchanged in action.

6. A diagnostic method according to any one of claims 1-4, wherein a nucleotide sequence of a downstream end region of said *mec* is a part of N315IS-J3 (the nucleotide sequence of FIG. 17), NCTC10442J3rc (the nucleotide sequence of FIG. 18) or pSJ10-3J3rc (the nucleotide sequence of FIG. 19); a nucleotide sequence reverse complementary thereto; or a sequence obtained by replacing one or more of nucleotides thereof by different bases to an extent remaining unchanged in

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action.

7. A diagnostic method according to any one of claims 1-4, wherein said nucleotide sequence of said chromosomal DNA surrounding said *mec*DNA is a part of the below-described sequence or a sequence reverse complementary thereto; or a sequence obtained by replacing one or more of nucleotides thereof by a like number of different nuleotides to an extent remaining unchanged in action:

pLEC12(pLEC1a) (the nucleotide sequence of FIG. 4),
N315J3rc (the nucleotide sequence of FIG. 11),
NCTC10442J3rc (the nucleotide sequence of FIG. 18),
pSJ10-3J3rc (the nucleotide sequence of FIG. 19),
the nucleotide sequence of FIG. 5,
the nucleotide sequence of FIG. 6,
pSJ8-2a (the nucleotide sequence of FIGS. 9 and 10),
L02C4 (the nucleotide sequence of FIG. 12), or
LG12H2 (the nucleotide sequence of FIG. 16).

8. a diagnostic method of an MRSA or MRC-NS by PCR, LCR or hybridization, which comprises performing a reaction with a sample by using a necleotide sequence of a chromosomal DNA surrounding an integration site of a *mec*DNA in a chromosome of a methicillin-susceptible *Staphylococcus aureus* or methicillin-susceptible C-NS (MSC-NS), wherein said method makes use of an occur-

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rence of a negative reaction when said sample contains said *mec*DNA integrated therein.

9. A diagnostic method according to claim 8, wherein a DNA fragment containing said *mec* integration site or surrounding said *mec* integration site is used as a primer; and said MRSA or MRC-NS is identified by non-amplification of said DNA fragment in PCR because of integration of said *mec* in a DNA of a sample.

10. A diagnostic method according to claim 8, wherein two DNA fragments are prepared containing said *mec*-integrated site or surrounding said *mec*-integrated site; and said MRSA or MRC-NS is identified by non-amplification of said DNA fragments in a ligase chain reaction (LCR) because of integration of said *mec* in a DNA of a sample.

11. A diagnostic method according to claim 8, wherein as a primer, a single or double-strand DNA fragment is prepared containing said *mec*-integrated site, and hybridization is performed between said probe and a DNA in said sample; and said MRSA or MRC-NS is identified by an occurrence of a negative reaction when said *mec* has been integrated in said DNA in said sample.

12. A diagnostic method according to any one of claims 8-11, wherein a DNA fragment containing said

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mec-integration site or surrounding said *mec*-integration site uses a part of the below-described sequence, a sequence reverse complementary thereto or a sequence obtained by replacing one or more of nucleotides thereof by different nucleotides to an extent remaining unchanged in action:

pLEC12(pLEC1a) (the nucleotide sequence of FIG. 4),
pSJ8-2a (the nucleotide sequence of FIGS. 9 and 10),
L02C4 (the nucleotide sequence of FIG. 12),
LG12H2 (the nucleotide sequence of FIG. 16),
the nucleotide sequence of FIG. 5, or
the nucleotide sequence of FIG. 6.

13. A method according to any one of claims 1-12, wherein a DNA in said sample is a DNA obtained by converting an RNA, which has been extracted from a staphylococcus strain, with a reverse transcriptase.

14. A method according to any one of claims 1-12, wherein said sample is an RNA obtained by amplification of an RNA, which has been extracted from a staphylococcus strain, in accordance with NASBA (nucleic acid sequence-based amplification).

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