



(11) **EP 2 508 887 A1**

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 153(4) EPC

(43) Date of publication:
10.10.2012 Bulletin 2012/41

(21) Application number: **10834639.6**

(22) Date of filing: **03.12.2010**

(51) Int Cl.:
G01N 33/569 (2006.01) **C07K 16/12** (2006.01)
C12N 15/09 (2006.01) **C12Q 1/68** (2006.01)
G01N 33/53 (2006.01)

(86) International application number:
PCT/JP2010/071652

(87) International publication number:
WO 2011/068189 (09.06.2011 Gazette 2011/23)

(84) Designated Contracting States:
**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR**

(30) Priority: **04.12.2009 JP 2009276115**
04.02.2010 JP 2010023102

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(54) **METHOD FOR DETECTING MICROORGANISMS BELONGING TO MYCOPLASMA
PNEUMONIAE AND/OR MYCOPLASMA GENITALIUM**

(57) A detection method and a detection kit for rapidly and specifically diagnosing *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* infections are provided.

The DnaK of *Mycoplasma pneumoniae* or *Mycoplasma genitalium* is used as an indicator.

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Description

TECHNICAL FIELD

5 **[0001]** The present invention relates to a method and a reagent kit for detecting microorganisms belonging to *Mycoplasma pneumoniae* which is, in general, a pathogenic microorganism for pneumonia, and/or *Mycoplasma genitalium*, using a molecule specific to the detection of the microorganisms as an indicator.

BACKGROUND ART

10 (1) Patient ratio and symptoms of *Mycoplasma pneumoniae* pneumonia

[0002] *Mycoplasma pneumoniae* infections are classified as a community-acquired atypical pneumonia, and it is said that the proportion of *Mycoplasma pneumoniae* infections in community-acquired pneumonia amounts to 30 to 40% in adults and to even 60 to 70% when the adults are limited to young adults aged 15 to 25. The infection route of *Mycoplasma pneumoniae* is a respiratory tract infection, and it is not rare that such infections spread in facilities such as schools, and in families. In addition, in the *Mycoplasma pneumoniae* infections, pneumonia occurs in about 3 to 5% of the infections, and the remainder is bronchitis, upper respiratory tract inflammation, or inapparent infection. Characteristic symptoms include an obstinate cough that is not accompanied by expectoration from an early period of infection, and may be sometimes accompanied by symptoms such as fever, headache, pharyngeal pain, chills, or general malaise.

(2) Current status of screening for *Mycoplasma pneumoniae* infections

25 **[0003]** A screening test of the culture from a pharyngeal swab sample in patients and an antibody screening test using a patient's serum are common as screening tests for *Mycoplasma pneumoniae* infections. Since *Mycoplasma pneumoniae* per se grows only in a special culture medium, the culturing is difficult to execute, and it is necessary to perform a PCR test for the final identification of *Mycoplasma pneumoniae*, the culture screening can be carried out only in limited facilities, and this is a current status of the screening test of the culture. In addition, a screening test to quickly obtain the results has been demanded, because several weeks are needed for the culture.

30 **[0004]** On the other hand, because the antibody screening test is generally easy in the procedure and provides results more quickly compared to the screening test of the culture, such an antibody screening test is a test that has been well used. But there are problems such that it is difficult to determine whether the infection is a previous one or a current one because IgM antibody titers of *Mycoplasma* are long-lasting, and it takes a long time to increase the antibody titers. In order to solve the above problems, the judgment based on rise in the antibody titers between the acute phase of the infection and the convalescent phase of the infection over time is recommended, but since it takes a very long time to perform an antibody testing until the convalescent phase, therapy is delayed, so that its delay may cause prolongation and worsening of symptoms, as well as may cause the adverse effect of infection expansion due to secondary infection.

35 **[0005]** In addition, in order to solve the above problems, antibodies and detection methods for specifically detecting a microorganism belonging to *Mycoplasma pneumoniae*, which are useful for diagnosing *Mycoplasma pneumoniae* infections, have been disclosed.

40 For example, patent literature 1 describes an immunodetection method using a monoclonal antibody against a membrane protein antigen of *Mycoplasma pneumoniae* of about 43 kilodaltons (kDa). Also, patent literature 2 describes that detection of *Mycoplasma pneumoniae* can be performed with high accuracy by using an antibody against ribosomal protein L7/L12. In addition, patent literature 3 describes that a quick and specific diagnosis of *Mycoplasma pneumoniae* infection is possible by using a monoclonal antibody against protein P1 of *Mycoplasma pneumoniae*, the monoclonal antibody having a cross-reactivity of only 1% or less to other species of the genus *Mycoplasma* or other pathogenic species of coexisting flora.

45 **[0006]** However, in order to detect a microorganism belonging to *Mycoplasma pneumoniae* in clinical specimens, the antibody described above and the detection method using the antibody may require a complicated pretreatment of the specimens containing the microorganism, and have a problem such that they are insufficient for a specific diagnosis of *Mycoplasma pneumoniae* because of still low specificity and sensitivity.

(3) *Mycoplasma genitalium* and diseases

55 **[0007]** *Chlamydia trachomatis* is known as the major causative bacteria of nongonococcal urethritis. However, *Chlamydia trachomatis* is detected in about 30 to 40% of patients with nongonococcal urethritis, and, in most cases, it is not clear where their symptoms originate. In addition to *Chlamydia trachomatis*, microorganisms of the genera *Mycoplasma* and *Ureaplasma* have attracted attention, and *Mycoplasma genitalium* in particular is shown as one of the causative

bacteria of nongonococcal urethritis and sexually transmitted disease.

(4) Current status of screening for *Mycoplasma genitalium* infections

5 **[0008]** Reports of *Mycoplasma genitalium* infections by the culture method or the PCR method have been published in papers, but since a quick diagnosis cannot be performed by these methods, a method for detecting quickly and specifically a microorganism belonging to *Mycoplasma genitalium* in clinical specimens has been demanded.

10 CITATION LIST

PATENT LITERATURE

[0009]

15 [Patent literature 1] Japanese Unexamined Patent Publication (Kokai) No. 63-298
[Patent literature 2] WO2001/057199
[Patent literature 3] Japanese Unexamined Patent Publication (Kokai) No. 5-304990

20 SUMMARY OF INVENTION

TECHNICAL PROBLEM

25 **[0010]** According to conventional methods, microorganisms belonging to *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* could not be quickly and specifically detected. Therefore, since it is not possible to quickly diagnose the infections from *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium*, therapy is delayed, and its delay may cause prolongation and worsening of symptoms, as well as the adverse effect of infection expansion due to secondary infection. This is the current situation. If infections with these *Mycoplasmas* can be quickly detected/diagnosed, it becomes possible to administer a macrolide antibiotic effective for the *Mycoplasmas* and start the correct treatment in the early infection stage.

30 Further, *Mycoplasma pneumoniae* and *Mycoplasma genitalium* are known to be serologically very close to each other, but since the infection site (such as tissues and organs) of *Mycoplasma pneumoniae* is different from that of *Mycoplasma genitalium* as mentioned above, if it is possible to identify a molecule capable of specifically detecting the two microorganisms, it was considered that diagnosis of both infectious diseases becomes possible using the molecule as an indicator.

35 The present invention has been made in view of the problems. The object of the present invention is to specify a molecule to rapidly and specifically diagnose the *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* infections, and to provide a detection method and a detection kit using the molecule as an indicator.

40 SOLUTION TO PROBLEM

[0011] Under these circumstances, the present inventors have conducted intensive studies, and have found that DnaK of microorganisms belonging to *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* can be used as an indicator to rapidly and specifically detect *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* infections. The DnaK protein, which is also called Heat Shock Protein 70 (Hsp70), was found as a group of proteins the expression of which was increased when cells were exposed to stress conditions such as heat to protect the cells, and is currently known to participate in the intracellular transport or refolding (molecular chaperone function) of proteins translated. The advantages in using the DnaK protein as an index in immunological analysis methods reside in the facts that:

- 50 (1) the DnaK protein is always expressed, because it participates in the transport or refolding of proteins,
(2) DnaK accounts for approximately 1% of the total proteins, and
(3) DnaK is present, not as a monomer, but as a trimer, a hexamer, or further multimers.

The present invention has been achieved based on these findings.

[0012] The present invention provides as follows:

- 55 [1] A method for detecting *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, characterized by using DnaK of *Mycoplasma pneumoniae* or *Mycoplasma genitalium* as an indicator.
[2] The method of [1], wherein a DnaK protein is immunologically analyzed.

[3] An anti-DnaK antibody specific to *Mycoplasma pneumoniae* or *Mycoplasma genitalium*.

[4] A kit for detecting *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, comprising the anti-DnaK antibody of [3].

[5] The method of [1], using a DnaK gene as an indicator.

[6] A primer or probe specific to *Mycoplasma pneumoniae* or *Mycoplasma genitalium*.

5 [7] A kit for detecting *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, comprising the primer or probe of [6].

[0013] The term "the microorganisms" as used herein means *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, in particular, microorganisms which have pathogenicity and are significant to be diagnosed as microorganisms causative of the diseases described below.

10 The term "antibody which specifically reacts with the microorganisms" as used herein means an antibody which specifically reacts with the species or the genus of the microorganisms. An antibody which specifically reacts with the species of the microorganisms is particularly useful in the diagnosis of microorganism infections.

ADVANTAGEOUS EFFECTS OF INVENTION

15 [0014] According to the method for detecting microorganisms belonging to *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* using the specific molecule in the present invention as an indicator, *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* infections caused by the microorganisms can be rapidly and specifically diagnosed.

20 BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

25 FIG. 1 illustrates a comparison of nucleotide sequences (1-720) of DnaK genes (SEQ ID NO: 6) in *Mycoplasma pneumoniae* strains M129 (P1 genotype: type I) and FH (P1 genotype: type II).

FIG. 2 illustrates, following FIG. 1, a comparison of nucleotide sequences (721-1440) of the DnaK genes in both *Mycoplasma pneumoniae* strains.

FIG. 3 illustrates, following FIG. 1 and FIG. 2, a comparison of nucleotide sequences (1441-1788) of the DnaK genes in both *Mycoplasma pneumoniae* strains.

30 FIG. 4 illustrates a comparison of nucleotide sequences (1-717 for M129 strain) of P1 genes (M129 strain: SEQ ID NO: 7, and FH strain: SEQ ID NO: 8) in *Mycoplasma pneumoniae* strains M129 (P1 genotype: type I) and FH (P1 genotype: type II).

FIG. 5 illustrates, following FIG. 4, a comparison of nucleotide sequences (718-1416 for M129 strain) of the P1 genes in both *Mycoplasma pneumoniae* strains.

35 FIG. 6 illustrates, following FIG. 4 and FIG. 5, a comparison of nucleotide sequences (1417-2136 for M129 strain) of the P1 genes in both *Mycoplasma pneumoniae* strains.

FIG. 7 illustrates, following FIG. 4 to FIG. 6, a comparison of nucleotide sequences (2137-2856 for M129 strain) of the P1 genes in both *Mycoplasma pneumoniae* strains.

40 FIG. 8 illustrates, following FIG. 4 to FIG. 7, a comparison of nucleotide sequences (2857-3564 for M129 strain) of the P1 genes in both *Mycoplasma pneumoniae* strains.

FIG. 9 illustrates, following FIG. 4 to FIG. 8, a comparison of nucleotide sequences (3565-4284 for M129 strain) of the P1 genes in both *Mycoplasma pneumoniae* strains.

45 FIG. 10 illustrates, following FIG. 4 to FIG. 9, a comparison of nucleotide sequences (4285-4884 for M129 strain) of the P1 genes in both *Mycoplasma pneumoniae* strains.

DESCRIPTION OF EMBODIMENTS

[0016] The present invention now will be further illustrated in detail by, but is by no means limited to, the following embodiments of the present invention as typical examples.

50 [0017] The method of the present invention for detecting *Mycoplasma pneumoniae* or *Mycoplasma genitalium* is characterized in that DnaK of the microorganisms is used as an indicator to detect *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* (i.e., either of *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, or both *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, most preferably, both *Mycoplasma pneumoniae* and *Mycoplasma genitalium*). *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* infections can be diagnosed by detecting these microorganisms.

55 [0018] The *Mycoplasma pneumoniae* infection which may be diagnosed by the present invention is mycoplasmal pneumonia. The *Mycoplasma genitalium* infection is non-gonococcal non-chlamydial urethritis or cervicitis.

[0019] In the diagnosis of the *Mycoplasma pneumoniae* infection, a sample in which *Mycoplasma pneumoniae* may exist

may be used. Examples of the sample include pharyngeal swab, nasopharyngeal swab, nasal aspirate, nasal mucus, sputum, and bronchoalveolar lavage fluid. When the *Mycoplasma genitalium* infection is to be diagnosed, a sample in which *Mycoplasma genitalium* may exist may be used. Example of the sample include urine, urethral swab specimens, and cervical swab specimens. The identification of the two infections can be decided by the collection site of the sample

5 as a target for measurement.
[0020] DnaK which is used as an indicator in the present invention is a DnaK protein (NCBI number: NP_110122) or a DnaK gene (NCBI number: NC_000912 REGION: 521837..523624) derived from *Mycoplasma pneumoniae*, or a DnaK protein (NCBI number: AAC71527) or a DnaK gene (NCBI number: L43967 REGION: 374919..376706) derived from *Mycoplasma genitalium*. The above-mentioned proteins and genes are examples of a strain belonging to the microorganisms, and the DnaK sequences of the microorganisms within the scope of the present invention include sequences corresponding to the DnaK proteins and genes described above.

10 As shown in Example 8 described below, the DnaK genes derived from different *Mycoplasma pneumoniae* strains absolutely (100%) accorded with each other, even among strains in which the types of the P1 gene of *Mycoplasma pneumoniae* were different, and no variations were detected among strains collected from various places and over the past 50 years. From this, it is considered that the sequences of the DnaK gene and the DnaK protein of *Mycoplasma pneumoniae* are stable. Therefore, it is preferable to refer to the nucleotide sequences or the amino acid sequences published by NCBI as described above.

1. Method and kit for detecting microorganisms using antibody

20 [0021] The first embodiment of the method for detecting *Mycoplasma pneumoniae* or *Mycoplasma genitalium* of the present invention is characterized by using an anti-DnaK antibody specific to the microorganisms. When the specific antibody is selected, the specificity to the microorganisms is at least 10^5 CFU/mL or higher, preferably 10^4 CFU/mL or higher, and more preferably 10^3 CFU/mL, and the specificity to the other microorganism is at least 10^7 CFU/mL or lower, and preferably 10^8 CFU/mL or lower.

The antibody which may be used in the present invention may be a polyclonal antibody or a monoclonal antibody. These antibodies may be obtained by the following methods or other similar methods, but the method is not limited to the same.

25 [0022] As the first embodiment of the method for preparing the antibody, the complete length of the DnaK protein or its partial peptide may be used to prepare the antibody. With respect to microorganisms of which the nucleotide sequence and the amino acid sequence of the DnaK protein are known, a peptide fragment may be synthesized based on a region which shows less similarity to the amino acid sequences of DnaK proteins of other microorganisms. The length of the peptide for preparing the antibody is not limited, but in the case of the antibody against the DnaK protein, a peptide having a length capable of characterizing the protein, preferably 5 amino acids or more, and most preferably 8 amino acids or more, may be used. This peptide or the complete length of the protein alone, or a conjugate thereof crosslinked with a carrier protein such as KLH (keyhole-limpet hemocyanin) or BSA (bovine serum albumin), may be inoculated into an animal, optionally along with an adjuvant, and a serum is collected from the animal to obtain an antiserum containing an antibody (polyclonal antibody) which recognizes the DnaK protein. An antibody which is purified from the antiserum may be used. Examples of the animal which may be inoculated include a sheep, a horse, a goat, a rabbit, a mouse, and a rat, and a rabbit or a goat is preferable for preparing a polyclonal antibody. A monoclonal antibody may be obtained in accordance with a known method for preparing hybridoma cells, and a mouse is preferable in this case.

30 [0023] A fusion protein of the complete length or an amino acid sequence consisting of 5 residues or more (preferably 8 residues or more) of the protein with glutathione S-transferase or the like may be used as an antigen, after purification of the fusion protein, or without purification. The antibody may be also prepared by a genetically recombinant antibody expressed in culture cells using an immunoglobulin gene isolated by a gene cloning method and various methods described in the publication: Antibodies; A laboratory manual, E. Harlow et al., Cold Spring Harbor Laboratory Press.

35 [0024] From the antibodies prepared as described above, an antibody having a high specificity may be prepared by selecting an antibody which specifically reacts with *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* (i.e., either of *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, or both *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, most preferably, both *Mycoplasma pneumoniae* and *Mycoplasma genitalium*), and does not react with other pathogenic microorganisms, in accordance with a known method.

40 [0025] The antibody against the DnaK which may be used as the marker antigen of the present invention may be obtained by the following methods or other similar methods, but the method is not limited to the same.

45 [0026] a) With respect to microorganisms of which the nucleotide sequence and the amino acid sequence of the DnaK protein are known, a peptide fragment may be synthesized based on a region which shows less similarity to the amino acid sequences of DnaK proteins of other microorganisms, and a polyclonal or monoclonal antibody may be prepared using the peptide fragment as an antigen to obtain the antibody of interest.

50 The complete length of the nucleotide sequence of the gene may be obtained using common genetic engineering techniques, such as gene amplification by a PCR method using DNA sequences at both the termini of the known gene

as primers, or hybridization using a homologous sequence as a template probe.

Next, a protein antigen of interest may be obtained by constructing a fusion gene with other protein genes, introducing the fusion gene into a host such as *E. coli* by a known gene introduction method, overexpressing the fusion protein, and purifying the expressed protein by an affinity column chromatography method using an antibody against the protein used for preparing the fusion protein. In this case, since the complete length of the DnaK protein becomes antigens, if an antibody against an amino acid region which is conserved between microorganisms outside the scope is obtained, such an antibody cannot be used in the present invention. Therefore, with respect to an antigen obtained by this method, the antibody of interest may be obtained by obtaining hybridomas producing monoclonal antibodies and selecting a clone producing an antibody which specifically reacts with the microorganisms.

[0027] b) With respect to microorganisms of which the amino acid sequence of the DnaK protein is unknown, since the amino acid sequences of the DnaK protein have a homology of 80-100%, preferably 90-100%, between different species, the protein gene of interest may be easily obtained using common genetic engineering techniques, such as gene amplification of a specific sequence region by a PCR method based on a sequence homologous to the amino acid sequence, or hybridization using a homologous sequence as a template probe.

The protein antigen of interest may be obtained by constructing a fusion gene of the protein gene with other protein genes, introducing the fusion gene into a host such as *E. coli* by a known gene introduction method, overexpressing the fusion protein, and purifying the expressed protein by an affinity column method using an antibody against the protein used for preparing the fusion protein. In this case, since the complete length of the DnaK protein becomes antigens, if an antibody against an amino acid region which is conserved between microorganisms, outside the scope is obtained, such an antibody cannot be used in the present invention. Therefore, with respect to an antigen obtained by this method, the antibody of interest may be obtained by obtaining hybridomas producing monoclonal antibodies and selecting a clone producing an antibody which specifically reacts with the microorganisms.

[0028] c) As another method in the case that the amino acid sequence of the DnaK protein is unknown, a synthetic peptide consisting of 5-30 amino acids corresponding to a common sequence region which are conserved between microorganisms in known amino acid sequences of the DnaK protein is prepared, and a polyclonal or monoclonal antibody is prepared using the peptide sequence in accordance with a known method. A highly purified DnaK protein may be obtained by purifying a cell homogenate of a microorganism of interest by affinity column chromatography using the antibody. When the purity of the protein is not sufficient, the purity may be improved by a known purification method, such as ion-exchange chromatography, hydrophobic chromatography, or gel filtration. The antibody of interest may be obtained by obtaining hybridomas using the obtained purified DnaK protein antibody, and selecting a hybridoma producing an antibody which specifically reacts with the microorganisms.

[0029] As the second embodiment of the method for preparing the antibody, *Mycoplasma pneumoniae* may be used as an antigen to prepare an antibody which reacts with the DnaK protein and is specific to *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium*, as shown in Example 1.

Similarly, *Mycoplasma genitalium* may be used as an antigen to prepare the antibody. In the case that the microorganism is used as an antigen, the antigen may be prepared in accordance with a known method. Examples of the method include sonication, heat treatment, surfactant treatment, formalin treatment, freezing and thawing treatment, and treatment with hydrochloric acid.

[0030] The antibody of the present invention which is obtained by the methods described above and is specific to the microorganisms may be used in various immunological assays, and various detection reagents and kits specific to the microorganisms of interest may be provided.

[0031] The antibody may be used in any of the known immunological assays, for example, an agglutination method using polystyrene latex particles onto which the antibody is bound, an ELISA method carried out in a microtiter plate, immunochromatography, or a sandwich method using the antibody labeled with colored particles, particles capable of developing a color, magnetic particles, an enzyme, or a fluorescent substance, alone or as a combination.

[0032] In the detection method using DnaK as an indicator of the present invention, *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* may be specifically detected without intentionally disrupting the cells, or a known method for treating microorganisms may be used to carry out detection with high sensitivity. More particularly, a treatment method using an extraction reagent comprising various surfactants such as Triton X-100, Tween-20, or SDS, an enzyme treatment method using an appropriate enzyme such as a protease, or a known method for disrupting a cell structure, such as a disruption of microorganism cells by a physical method, may be used. It is preferable that optimum conditions for extraction are selected for each microorganism by examining the combination of reagents such as surfactants.

[0033] The reagent kit for detecting the microorganism using the antibody of the present invention corresponds to the reagent kit for detection using the detection method.

The kit is not limited, so long as it contains at least one antibody of the present invention. The number, type, and combination of the antibodies used may be appropriately changed in accordance with the immunological assay to be used. The kit may contain a liquid for pretreatment in the extraction method described above, as a pretreatment of a sample.

2. Method and kit for detecting microorganisms using gene

[0034] As a method for extracting DNA, a known method may be used. Examples of the method include a solubilization of a sample with a surfactant, or deproteinization using a deproteinization agent, to obtain DNA. Preferably, so long as the DnaK gene as described below may be analyzed, for example, when the gene extracted is next amplified by a PCR method, the DNA preferably contains no inhibitors of PCR reaction.

As a method for pretreating a sample, a similar approach as described in the method for detecting the microorganisms using an antibody may be used.

[0035] The amount of DNA extracted is not limited so long as an amount capable of analyzing the DnaK gene is extracted. When the DNA is subjected to a PCR method, the amount is, for example, 5 to 50 fg or more per reaction.

[0036] The DNA extracted is used to analyse the DnaK gene. The analysis of the DnaK gene may be carried out in accordance with a known method. Examples of the method include a method for detecting the amplification of the DnaK gene by a PCR method, and a method for specifying the DnaK gene by a probe method. For example, any method for amplifying the DnaK gene by a PCR method may be used, so long as the nucleotide sequence of interest may be amplified. Any method for specifying the DnaK gene by a probe method may be used, so long as the nucleotide sequence of interest may be specified.

[0037] To amplify or specify the desired nucleotide sequence of the DnaK gene, a sequence having an 80-100% homology with respect to *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* and having a homology of preferably 60% or less with respect to other pathogenic microorganisms may be appropriately selected. The primer or probe may contain one or more variations, deletions, or additions in its nucleotide sequence, so long as the DNA fragment of interest may be amplified.

[0038] For example, when the DnaK gene of *Mycoplasma pneumoniae* is to be amplified, PCR amplification primers may be designed on the basis of the DnaK gene sequence of *Mycoplasma pneumoniae* (NCBI number: NC_000912 REGION: 521837..523624) published in NCBI, as described in the Examples below. More particularly, sense primer MpDnaK_S and antisense primer MpDnaK_A may be used to amplify the complete length of the DnaK gene.

When the DnaK gene of *Mycoplasma genitalium* is to be amplified, PCR amplification primers may be designed on the basis of the DnaK gene sequence of *Mycoplasma genitalium* (NCBI number: L43967 REGION: 374919..376706) published in NCBI.

[0039] As shown in Example 8, the DnaK genes derived from different *Mycoplasma pneumoniae* strains absolutely (100%) accorded with each other, even among strains in which the types of the P1 gene of *Mycoplasma pneumoniae* were different, and no variations were detected among strains collected from various places and over the past 50 years. From this, it is not necessary to take into consideration the difference between *Mycoplasma pneumoniae* strains in order to specifically detect the DnaK gene of *Mycoplasma pneumoniae*, and the primers or probe may be designed by taking into consideration the differences among the strains other than *Mycoplasma pneumoniae*.

Further, because it is considered that the sequence of the DnaK protein of *Mycoplasma pneumoniae* is also conservative, antibodies prepared using the DnaK protein are considered to show no difference in reactivity with respect to the genotype, the place for collection, and the time of collection, and thus, may be used over a wide area and time.

[0040] The reagent kit for detecting the microorganisms using the gene of the present invention corresponds to the reagent kit for detection using the detection method. This is a kit which is used for the method for specifically detecting *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* and which is characterized by comprising at least two types of primers for amplifying a nucleotide sequence specific to the DnaK gene of interest.

As another embodiment, the kit is characterized by comprising at least one type of probe for specifying a nucleotide sequence specific to the DnaK gene of interest.

These kits may further contain a liquid for pretreatment in the extraction method described above, as a pretreatment of a sample.

EXAMPLES

[0041] The present invention now will be further illustrated by, but is by no means limited to, the following Examples.

<<EXAMPLE 1: Preparation of antibodies specific to *Mycoplasma pneumoniae* and *Mycoplasma genitalium* and identification of a specific antigen>>

(1) Preparation of monoclonal antibodies specific to *Mycoplasma pneumoniae* and *Mycoplasma genitalium*

(1-1) Cultivation of strains for immunization and preparation of immunogens

[0042] PPLO glucose broths (containing horse serum, fresh yeast extract, and thallium acetate) were each separately

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inoculated with one of 6 strains of *Mycoplasma pneumoniae* (FH, Bru, Mac, M52, P11428, and M129-B7 strains: purchased from ATCC), and cultivation was carried out at 37°C for 7 days under aerobic conditions. Each strain collected by centrifugation was washed and suspended in PBS. These suspensions were frozen and thawed to prepare immunogens.

5 (1-2) Immunization

[0043] Six-week-old female Balb/c mice (CREA Japan, Inc.) were used for immunization. Each immunogen solution derived from a strain was emulsified with Freund's complete adjuvant (SIGMA). Each emulsion (100 µg of antigen) was subcutaneously injected into a mouse. Until an increase in antibody titer against immunogen was observed in each mouse, 50 µg of each antigen emulsified with Freund's incomplete adjuvant (SIGMA) was subcutaneously injected into the mouse every two weeks. Further, 25 µg of each antigen diluted with PBS was intraperitoneally injected into the mouse three days before cell fusion.

15 (1-3) Preparation of hybridomas

[0044] The following procedures were carried out in accordance with a conventional method. Spleen cells aseptically collected from immunized mice were fused with myeloma cells (P3U1) using polyethylene glycol 1500 (Roche), and inoculated into wells of 96-well plates. Hybridoma cells were selectively cultivated using a HAT medium, and their culture supernatants were screened under the following ELISA conditions. Immobilization for ELISA was carried out using *Mycoplasma pneumoniae* antigen (1 µg/mL) derived from each of the 6 strains used as immunogens. After a blocking treatment for wells, each culture supernatant was added to the wells and incubated at 4°C overnight. The wells were washed with a washing liquid three times, and a 2000-fold diluted HRP-labeled rabbit anti-mouse Ig antibody (Dako) was added to the wells and incubated at room temperature for 1 hour. The wells were washed with a washing liquid three times, and a substrate (TMBZ) solution was added to the wells and incubated at room temperature for 10 minutes. After the reaction was stopped, an absorbance at 450 nm was measured. The selected hybridomas were further screened by a limiting dilution method to establish clone strains. With respect to monoclonal antibodies produced from 16 strains in the established clone strains, the following experiments were carried out. The monoclonal antibodies produced from the 16 clone strains reacted with all the immunogens derived from the 6 strains.

30 (1-4) Determination of molecular weight of proteins recognized by monoclonal antibodies

[0045] The molecular weight of each protein recognized by the 16 monoclonal antibodies was determined by Western blotting. First, 10 µg of *Mycoplasma pneumoniae* antigen (FH strain) was electrophoresed by SDS-PAGE and blotted onto nitrocellulose membranes. Each culture supernatant of the 16 clones was added to the membranes and incubated at room temperature for 1 hour. The membranes were washed with a washing liquid three times, and a 1000-fold diluted HRP-labeled rabbit anti-mouse Ig antibody was added to the membranes and incubated at room temperature for 1 hour. The membranes were washed with a washing liquid three times, and a substrate (4-chloro-1-naphthol) solution was added to the membranes and incubated at room temperature for 10 minutes. After the development, the membranes were washed with a distilled water to stop the reaction.

40 As a result, it was found that 10 monoclonal antibodies recognized a molecule having a molecular weight of 62-69 kDa and 6 monoclonal antibodies recognized a molecule having a molecular weight of 40-45 kDa. From this result, we attempted to identify the antigen with respect to the molecule of 62-69 kDa which was considered to have a high immunogenicity because many clones were obtained.

45 (1-5) Identification of subclass of obtained antibodies

[0046] Iso Strip (Roche) was used to determine the subclass of 10 monoclonal antibodies which recognized the molecule of 62-69 kDa. It was found that 6 antibodies were H chain G1/L chain κ, 1 antibody was H chain G1/L chain λ, 1 antibody was H chain 2b/L chain κ, 1 antibody was H chain 2b/L chain λ, and 1 antibody was H chain 2a/L chain λ.

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(2) Identification of antigen specific to *Mycoplasma pneumoniae* and *Mycoplasma genitalium*

(2-1) Purification of antigen recognized by monoclonal antibodies

55 (2-1-1) Cultivation of strain

[0047] A *Mycoplasma pneumoniae* M129-B7 strain, of which the entire gene sequence had been already determined, was used to purify an antigen. *Mycoplasma pneumoniae* (M129-B7 strain) was inoculated into a PPLO glucose broth

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(containing horse serum, fresh yeast extract, and thallium acetate), and cultivated at 37°C for 7 days under aerobic conditions. The strain collected by centrifugation was washed and suspended in PBS. The suspension was frozen.

(2-1-2) Purification of recognized antigen by affinity chromatography

[0048] The monoclonal antibody MCM12 obtained in (1) was bound to CNBr-activated Sepharose 4B (GE healthcare) as a column carrier to prepare an affinity column for antigen purification. The binding to the column carrier was carried out by reacting IgG 5mg/mL gel in 0.1 mol/L NaHCO₃-NaOH and 0.5 mol/L NaCl (pH 8.3) at 4 ° C overnight. Unreacted groups were blocked using a 0.2 mol/L glycine buffer (pH 8).

Proteins extracted from the *Mycoplasma pneumoniae* strain were applied to the column. After a non-adsorbed fraction was eluted, a column-adsorbed fraction was eluted using 3 mol/L sodium thiocyanate and collected. This fraction was dialyzed against 50 mmol/L PBS (pH 7) to obtain a purified product.

(2-2) Identification of protein recognized by obtained monoclonal antibodies

(2-2-1) Determination of molecular weight of recognized protein by SDS-PAGE

[0049] The purified antigen was analyzed by SDS-PAGE and Western blotting. The purified antigen (0.1 µg) was electrophoresed by SDS-PAGE and blotted onto nitrocellulose membranes. Monoclonal antibody MCM12 or monoclonal antibody MCM19 (10 µg/mL IgG solution) was separately added to the membranes and incubated at room temperature for 1 hour. The membranes were washed with a washing liquid three times, and a 1000-fold diluted HRP-labeled rabbit anti-mouse Ig antibody was added to the membranes and incubated at room temperature for 1 hour. The membranes were washed with a washing liquid three times, and a substrate (4-chloro-1-naphthol) solution was added to the membranes and incubated at room temperature. After the development, the membranes were washed with a distilled water to stop the reaction.

It was confirmed that both antibodies recognized the purified antigen.

(2-2-2) Analysis of N-terminal amino acid sequence of purified antigen

[0050] The N-terminal 10 amino acid residues of the purified antigen protein were analyzed in accordance with a conventional method. The purified antigen was electrophoresed by SDS-PAGE. A PVDF membrane on which the sample was blotted was washed with 50% methanol/0.1% trifluoroacetic acid and methanol and dried, and 10 cycles of amino acid sequencing was carried out from the N-terminus. A protein sequencer PPSQ-23A (Shimadzu) and a PTH analyzer SPD-10A (Shimadzu) were used as analyzers.

As a result, the following sequence was obtained:

S T D N G L I I G I (SEQ ID NO: 1)

A search was carried out using the database Swiss-Prot in accordance with a conventional method, and the obtained sequence completely accorded with the sequence consisting of the 2nd to 11th amino acid residues of chaperone protein DnaK of *Mycoplasma pneumoniae*. The molecular weight of the DnaK deduced from its amino acid sequence was 65 kDa, which nearly accorded with the molecular weight of the antibody-recognized antigen determined by Western blotting. As described above, it was confirmed that the antibodies obtained above were anti-DnaK antibodies specific to *Mycoplasma pneumoniae* and *Mycoplasma genitalium*.

<<EXAMPLE 2: Examination of sensitivity and cross-reactivity of obtained antibodies by ELISA method>>

[0051] In the monoclonal antibodies obtained in Example 1, monoclonal antibody MCM12 and monoclonal antibody MCM19 were used to examine the sensitivity and cross-reactivity of the antibodies.

(1) Cultivation and preparation of strains to be examined (1-1) Strains for sensitivity test

[0052] PPLO glucose broths (containing horse serum, fresh yeast extract, and thallium acetate) were each separately inoculated with one of the 8 strains of *Mycoplasma pneumoniae* shown in Table 1, and cultivation was carried out at 37°C for 4 days under aerobic conditions. Strains in which the broth reached pH 6.8 were used as test strains. To determine the number of each strain in the broth, 10-step dilution series were prepared with sterilized PBS, and 10 µL of each dilution was inoculated onto PPLO (containing horse serum, fresh yeast extract, and thallium acetate) agar media and incubated at 37°C for 10 days. Growth colonies on the agar media were counted under an optical microscope

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having a magnification of 40 to calculate the colony forming unit of each strain.

[0053]

Table 1

Strain	ATCC No.
<i>Mycoplasma pneumoniae</i> FH	15531
<i>Mycoplasma pneumoniae</i> Bru	15377
<i>Mycoplasma pneumoniae</i> Mutant 22	39505
<i>Mycoplasma pneumoniae</i> Mac	15492
<i>Mycoplasma pneumoniae</i> M52	15293
<i>Mycoplasma pneumoniae</i> PI1428	29085
<i>Mycoplasma pneumoniae</i> M129-B7	29342
<i>Mycoplasma pneumoniae</i> UTMB-10P	49894

(1-2) Strains for cross-reactivity test-1

[0054] Strains belonging to the genus *Mycoplasma* other than *Mycoplasma pneumoniae* shown in (1-1), the genus *Ureaplasma*, and the genus *Acholeplasma* were cultivated in accordance with the broths and the culture conditions shown in Table 2. The cultivation was carried out at 37°C. The terms "aerobic" and "anaerobic" in Table 2 mean aerobic cultivation and anaerobic cultivation, respectively. To determine the number of each strain in the broth, 10-step dilution series were prepared with sterilized PBS, and 10 µL of each dilution was inoculated onto PPL0 (containing horse serum, fresh yeast extract, and thallium acetate) agar and incubated at 37°C for 10 days. Growth colonies on the agar were counted under an optical microscope having a magnification of 40 to calculate the colony forming unit of each strain. The test was carried out at a number of 10⁶ to 10⁷ cfu/mL.

[0055]

Table 2

Strain	ATCC No.	Medium	Conditions
<i>Mycoplasma genitalium</i>	33530	PPL0 glucose broth (thallium ⁻)	4 days, aerobic
<i>Mycoplasma fermentans</i>	19989	PPL0 glucose broth	4 days, aerobic
<i>Acholeplasma laidlawii</i>	23206	PPL0 glucose broth	4 days, aerobic
<i>Acholeplasma oculi</i>	51735	PPL0 glucose broth	4 days, aerobic
<i>Mycoplasma penetrans</i>	55252	PPL0 glucose broth	4 days, aerobic
<i>Mycoplasma pirum</i>	25960	PPL0 glucose broth	4 days, aerobic
<i>Mycoplasma hominis</i>	23114	PPL0 arginine broth (thallium ⁻)	3 days, aerobic
<i>Mycoplasma orale</i>	23714	PPL0 arginine broth	3 days, aerobic
<i>Mycoplasma salivarium</i>	23064	PPL0 arginine broth	3 days, aerobic
<i>Mycoplasma arthritidis</i>	19611	PPL0 arginine broth	3 days, aerobic
<i>Mycoplasma buccale</i>	23636	PPL0 arginine broth	3 days, aerobic
<i>Mycoplasma faucium</i>	25293	PPL0 arginine broth	3 days, anaerobic
<i>Mycoplasma lipophilum</i>	27104	PPL0 arginine broth	3 days, aerobic
<i>Mycoplasma primatum</i>	25948	PPL0 arginine broth	3 days, aerobic
<i>Mycoplasma spermatophilum</i>	49695	PPL0 arginine broth 3	3 days, anaerobic
<i>Ureaplasma parvum</i>	700970	T-broth	2 days, aerobic
<i>Ureaplasma urealyticum</i>	27618	T-broth	2 days, aerobic

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(1-3) Strains for cross-reactivity test-2

[0056] Table 3 to Table 6 show microorganisms which were used in a cross-reactivity test of bacteria and fungi other than the genus *Mycoplasma*, the genus *Ureaplasma*, and the genus *Acholeplasma* used in (1-1) and (1-2), and the culture conditions. Heart infusion agar (Difco), trypticase soy agarII with 5% sheep blood (Becton, Dickinson and Company), chocolate agar (NISSUI), modified GAM agar (NISSUI), skirrows medium (Becton, Dickinson and Company), and Sabouraud-dextrose agar (Difco) were used as media.

These strains were cultivated on agar, and suspended in sterilized PBS at a concentration of 10⁷ to 10⁸ cfu/mL to prepare test strains. To determine the number of each strain, each test suspension in which each strain was suspended in sterilized PBS was stepwisely (10-step) diluted with the same PBS, and 50 µL of each dilution was inoculated onto agar media. Growth colonies on the media were counted by the naked eye.

The blank spaces in the "Strain No." column of the tables mean strains which were isolated and identified from clinical specimens.

[0057]

Table 3

Strain	Strain No.	Medium	Conditions
<i>Branhamella catarrhalis</i>		Heart infusion agar	37°C, 18 hours, aerobic
<i>Citrobacter freundii</i>	ATCC 8090	Heart infusion agar	37°C, 18 hours, aerobic
<i>Enterobacter cloacae</i>	ATCC 13047	Heart infusion agar	37°C, 18 hours, aerobic
<i>Escherichia coli</i>	ATCC 25932	Heart infusion agar	37°C, 18 hours, aerobic
<i>Escherichia hermannii</i>	ATCC 33650	Heart infusion agar	37°C, 18 hours, aerobic
<i>Klebsiella pneumonia</i>	ATCC 27736	Heart infusion agar	37°C, 18 hours, aerobic
<i>Leclercia adecarboxylata</i>		Heart infusion agar	37°C, 18 hours, aerobic
<i>Proteus mirabilis</i>	ATCC29906	Heart infusion agar	37°C, 18 hours, aerobic
<i>Proteus vulgaris</i>	ATCC 6380	Heart infusion agar	37°C, 18 hours, aerobic
<i>Pseudomonas aeruginosa</i>	ATCC 27853	Heart infusion agar	37°C, 18 hours, aerobic
<i>Pseudomonas maltophilia</i>	IF0 12690	Heart infusion agar	37°C, 18 hours, aerobic
<i>Salmonella choleraesuis</i> subsp.choleraesuis serovar enteritidis	JCM 1652	Heart infusion agar	37°C, 18 hours, aerobic
<i>Salmonella choleraesuis</i> subsp.choleraesuis serovar thyphimutium	JCM 6977	Heart infusion agar	37°C, 18 hours, aerobic
<i>Serratia marcescens</i>	ATCC 13880	Heart infusion agar	37°C, 18 hours, aerobic
<i>Staphylococcus aureus</i>	JCM 2151	Heart infusion agar	37°C, 18 hours, aerobic
<i>Staphylococcus aureus</i>	JCM 2179	Heart infusion agar	37°C, 18 hours, aerobic
<i>Staphylococcus epidermidis</i>	JCM 2414	Heart infusion agar	37°C, 18 hours, aerobic
<i>Staphylococcus haemolyticus</i>	ATCC29970	Heart infusion agar	37°C, 18 hours, aerobic
<i>Staphylooccus hominis</i>	ATCC27844	Heart infusion agar	37°C, 18 hours, aerobic
<i>Staphylococcus hyicus</i>	ATCC11249	Heart infusion agar	37°C, 18 hours, aerobic
<i>Staphylococcus wernerii</i>	ATCC27836	Heart infusion agar	37°C, 18 hours, aerobic

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Table 4

Strain	Strain No.	Medium	Conditions
<i>Enterococcus avium</i>	JCM8722	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Enterococcus casseliflavus</i>	JCM 5675	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Enterococcus casseliflavus</i>	JCM 5675	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Enterococcus casseliflavus</i>	JCM 5675	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Enterococcus durans</i>	JCM8725	Trypt i case soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Enterococcus faecalis</i>	ATCC51299	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Enterococcus faecalis</i>	JCM 5803	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Enterococcus faecium</i>	JCM 5804	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Enterococcus gallinarum</i>	JCM8728	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Enterococcus mundtii</i>	JCM8731	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus agalactiae</i>	ATCC13813	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus anginosus</i>		Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus bovis</i>	JCM5802 ^T	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus constellatus</i>		Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus dysgalactiae</i>	JCM5673	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus equinus</i>	JCM7879 ^T	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus milleri</i>		Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus mitis</i>		Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus mutans</i>	JCM5705 ^T	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus oralis</i>		Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus pneumoniae</i>		Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus pyogenes</i>	ATCC 10389	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic

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(continued)

Strain	Strain No.	Medium	Conditions
<i>Streptococcus salivaris subsp. salivarius</i>	JCM570 ^T	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus sanguis</i>	JCM5708 ^T	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic
<i>Streptococcus uberis</i>	JCM5709 ^T	Trypticase soy agar II with 5% sheep blood	37°C, 18 hours, aerobic

[0059]

Table 5

Strain	Strain No	Medium	Conditions
<i>Haemophilus aphrophilus</i>	cultiloops	Chocolate agar	37°C, 18 hours, 5%CO ₂
<i>Haemophilus haemolyticus</i>	T-30	Chocolate agar	37°C, 18 hours, 5%CO ₂
<i>Haemophilus influenzae</i>	ATCC33391	Chocolate agar	37°C, 18 hours, 5%CO ₂
<i>Haemophilus parahaemolyticus</i>	T-13	Chocolate agar	37°C, 18 hours, 5%CO ₂
<i>Haemophilus parainfluenzae</i>	T-10	Chocolate agar	37°C, 18 hours, 5%CO ₂
<i>Neisseria gonorrhoeae</i>	ATCC49981	Chocolate agar	37°C, 18 hours, 5%CO ₂
<i>Neisseria meningitidis</i> Serogroup B		Chocolate agar	37°C, 18 hours, 5%CO ₂
<i>Lactococcus garvieae</i>	JCM10343	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Lactococcus lactis subsp. Lactis</i>	JCM5805	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Lactococcus raffinolactis</i>	JCM5706	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Leuconostoc mesenteroides subsp. dextranicum</i>	JCM9700	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Leuconostoc mesenteroides subsp. mesenteroides</i>	JCM6124	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Listeria monocytogenes</i>	4b	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Pediococcus acidilactici</i>	JCM8797	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Pediococcus damnosus</i>	JCM5886	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Pediococcus pentosaceus</i>	JCM5890	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Peptostreptococcus micros</i>	ATCC33270	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Porphyromonas gingivalis</i>		Modified GAM agar	37°C, 24 hours, anaerobic
<i>Prevotelle intermedia</i>	NCTC9336	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Prevotella oris</i>	ATCC33573	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Flavobacterium meningosepticum</i>	KM 506	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Fusobacterium nucleatum sbsp. Nucleatum</i>		Modified GAM agar	37°C, 24 hours, anaerobic
<i>Acinetobacter baumannii</i>	ATCC23055	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Actinomyces maeslundii</i>	ATCC19039	Modified GAM agar	37°C, 24 hours, anaerobic
<i>Corynebacterium matruchotii</i>	ATCC14266	Modified GAM agar	37°C, 24 hours, anaerobic

[0060]

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Table 6

Strain	Strain No.	Medium	Conditions
<i>Campylobacter jejuni</i>		Skirrows medium	37°C, 48 hours, microaerobic
<i>Campylobacter coli</i>		Skirrows medium	37°C, 48 hours, microaerobic
<i>Candida albicans serotype A</i>	A207	Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Candida albicans serotype B</i>	B792	Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Candida dubliniensis</i>		Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Candida glabrata</i>		Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Candida stellatoidea</i>		Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Candida parapsilosis</i>		Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Candida guilliermondii</i>		Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Candida kefyr</i>		Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Candida tropicalis</i>		Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Candidakrusei</i>		Sabouraud-dextrose agar	25°C, 48 hours, aerobic
<i>Cryptococcus neoformans</i>	ATCC24064	Sabouraud-dextrose agar	25°C, 48 hours, aerobic

(2) Examination of sensitivity and cross-reactivity by ELISA method

(2-1) Construction of ELISA method

(2-1-1) Method for preparation of immobilized antibody and method for immobilization

[0061] Ascites fluid containing monoclonal antibody MCM19 was applied to ammonium sulfate fractionation, IgG was purified using rProteinA Sepharose FF (GE healthcare), and a quantitative analysis of protein was carried out by a BCA method. The purified IgG antibody (10 µg/mL) was immobilized on a 96-well microplate.

(2-1-2) Method for preparation of antibody for labeling with alkaline phosphatase and method for preparation of labeled antibody

[0062] Ascites fluid containing monoclonal antibody MCM12 was applied to ammonium sulfate fractionation, and IgG was purified using MEP Hypercel (Pall Corporation). The IgG was digested with pepsin to prepare F(ab')₂, and F(ab')₂ was crosslinked with alkaline phosphatase to prepare an alkaline-phosphatase-labeled antibody.

(2-1-3) Method for carrying out ELISA method

[0063] The immobilized 96-well microplate was washed, and blocked with 0.1 mmol/L TBS (pH 7.5) containing 1% BSA at room temperature for 1 hour. Each strain suspension to be tested (100 µL) was added to the microplate, and incubated at room temperature for 1 hour. The microplate was washed, and the alkaline-phosphatase-labeled antibody (10 µg/mL) was added and incubated at room temperature for 1 hour. The microplate was washed, and development was carried out using a substrate (pNPP) solution for 30 minutes. The reaction was stopped, and an absorbance at 405nm was measured.

(3) Sensitivity test

[0064] Test strains (1-1) were applied to the ELISA described above, and a test dilution which showed an absorbance of 0.05 or higher and a maximum dilution magnification was used to calculate the number of each strain. The results are shown in Table 7.

It was found from the results shown in Table 7 that the sensitivity against *Mycoplasma pneumoniae* was 10³ to 10⁴ cfu/mL by the ELISA using the monoclonal antibodies.

[0065]

Table 7

Strain	ATCC No.	Number of strain showing absorbance of 0.05 or higher (OD) by ELISA
<i>Mycoplasma pneumoniae</i> FH	15531	3.1×10^4
<i>Mycoplasma pneumoniae</i> Bru	15377	9.8×10^4
<i>Mycoplasma pneumoniae</i> Mutant 22	39505	8.0×10^4
<i>Mycoplasma pneumoniae</i> Mac	15492	2.5×10^4
<i>Mycoplasma pneumoniae</i> M52	15293	2.5×10^3
<i>Mycoplasma pneumoniae</i> PI1428	29035	3.5×10^3
<i>Mycoplasma pneumoniae</i> M129-B7	29342	3.8×10^4
<i>Mycoplasma pneumoniae</i> UTMB-10P	49894	2.3×10^3

(4) Cross-reactivity test

[0066] Test strains (1-2)[the genus *Mycoplasma* other than *Mycoplasma pneumoniae*, the genus *Ureaplasma*, and the genus *Acholeplasma* shown in Table 2] and test strains (1-3) [other bacteria and fungi shown in Table 3 to Table 6] were applied to the ELISA described above.

All the microorganisms other than *Mycoplasma genitalium* showed an absorbance of less than 0.010. With respect to *Mycoplasma genitalium*, the number thereof calculated from a test dilution which showed an absorbance of 0.05 or higher and a maximum dilution magnification was 6.9×10^4 cfu/mL.

[0067] As shown in these results, it was found that the ELISA using the monoclonal antibodies showed a cross-reactivity to *Mycoplasma genitalium*; but did not show a cross-reactivity to other microorganisms.

As described above, it was confirmed that the ELISA did not show a cross-reactivity to many bacteria and fungi which might disturb the diagnosis of a *Mycoplasma pneumoniae* or *Mycoplasma genitalium* infection.

<<EXAMPLE 3: Examination of sensitivity and cross-reactivity of obtained antibodies by immunochromatography>>

(1) Construction of immunochromatography

(1-1) Preparation of anti-*Mycoplasma-pneumoniae* antibody-conjugated colloidal gold

[0068] To a colloidal gold solution, of which pH was previously adjusted by adding 2 mL of a 50 mmol/L phosphate buffer (pH 11) to 18 mL of a colloidal gold solution (Tanaka Kikinzoku) having a diameter of 40 nm, 2.5 mL of 100 μ g/mL monoclonal antibody MCM12 solution was added and stirred. After the mixture was stirred for 1 hour, 1 mL of 1 mass% polyethylene glycol (Mw. 20000, Wako Pure Chemical Industries) aqueous solution was added and stirred, and 2 mL of 10 mass% BSA aqueous solution (SIGMA) was added and stirred. This solution was centrifuged at 4°C and 8000G for 15 minutes, and almost all the supernatant was removed so that approximately 1 mL of the supernatant was left. Colloidal gold was re-dispersed using an ultrasonic generator. The dispersed colloidal gold was dispersed into 20 mL of a phosphate buffer containing BSA, and centrifuged at 4°C and 8000G for 15 minutes. Almost all the supernatant was removed so that approximately 1 mL of the supernatant was left, and colloidal gold was re-dispersed using an ultrasonic generator to prepare an antibody-conjugated colloidal gold solution.

(1-2) Preparation of pad carrying colloidal gold

[0069] The antibody-conjugated colloidal gold solution prepared in (1-1) was diluted with the phosphate buffer containing BSA, and impregnated into a glass fiber pad (Millipore) which was previously cut to a size of 20 mm \times 300 mm. The pad was dried at room temperature overnight to prepare a pad carrying the colloidal gold antibody.

(1-3) Preparation of antibody-immobilized membrane (carrier for chromatography)

[0070] Onto a nitrocellulose membrane (Millipore) which was cut to a size of 30 mm \times 300 mm, an antibody was immobilized in accordance with the following method to prepare an antibody-immobilized membrane. A solution of

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monoclonal antibody MCM19 for immobilization (5 mg/mL) was applied in a line with a width of approximately 1 mm, using a coater (BioDot), at a position 16 mm from one of the long sides of the membrane as the bottom, and dried to prepare the antibody-immobilized membrane.

5 (1-4) Construction of kit for immunochromatography

10 [0071] The antibody-immobilized membrane, the pad carrying colloidal gold, and an absorbent pad (Pall corporation) were attached to an adhesive back sheet so that adjacent pieces overlapped with each other. The resulting overlapped structure was cut along the long side with a width of 6 mm, using a cutter, to prepare test strips for immunochromatography. Each test strip was put into a housing case to prepare test kits for immunochromatography.

(1-5) Test method

15 [0072] Cultivated strains, PBS-washed strains, culture supernatants, and pellets of cultivated strains were dissolved with a phosphate buffer containing Triton X-100 to prepare *Mycoplasma pneumoniae* antigen (or strain) solutions for test at predetermined concentrations. To each immunochromatographic kit for test, 100 μ L of *Mycoplasma pneumoniae* antigen (or strain) solution for test was added dropwise. After 15 minutes from the addition, cases where a development was detected by the naked eye at the position on which the anti-*Mycoplasma-pneumoniae* antibody was coated of each antibody-immobilized membrane were judged as "positive", and cases where no development was detected were judged as "negative".

(2) Sensitivity test

25 [0073] Test strains (1-1) in Example 2 were applied to the immunochromatography described above, and a test dilution which showed a development generated on the test line and a maximum dilution magnification was used to calculate the number of each strain. The results are shown in Table 8.

[0074]

Table 8

Strain	ATCC No.	Number of strain showing development by immunochromatography
<i>Mycoplasma pneumoniae</i> FH	15531	3.1×10^4
<i>Mycoplasma pneumoniae</i> Bru	15377	9.8×10^4
<i>Mycoplasma pneumoniae</i> Mutant 22	39505	8.0×10^4
<i>Mycoplasma pneumoniae</i> Mac	15492	2.5×10^4
<i>Mycoplasma pneumoniae</i> M52	15293	2.5×10^3
<i>Mycoplasma pneumoniae</i> PI1428	29085	3.5×10^3
<i>Mycoplasma pneumoniae</i> M129-B7	29342	3.8×10^4
<i>Mycoplasma pneumoniae</i> UTMB-10P	49894	2.3×10^3

45 [0075] It was found from the results shown in Table 8 that the sensitivity against *Mycoplasma pneumoniae* was 10^3 to 10^4 cfu/mL by immunochromatography using the monoclonal antibodies.

(3) Cross-reactivity test

50 [0076] Test strains (1-2) [the genus *Mycoplasma* other than *Mycoplasma pneumoniae*, the genus *Ureaplasma*, and the genus *Acholeplasma* shown in Table 2] and test strains (1-3) [other bacteria and fungi shown in Table 3 to Table 6] of Example 2 were applied to the immunochromatography described above.

55 All the microorganisms other than *Mycoplasma genitalium* were negative, i.e., did not show any developments. By contrast, a development was detected in *Mycoplasma genitalium*, and the number thereof calculated from a test dilution showing a maximum dilution magnification was 6.9×10^4 cfu/mL.

[0077] As shown in these results, it was found that the immunochromatography using the monoclonal antibodies showed cross-reactivity to *Mycoplasma genitalium*, but did not show a cross-reactivity to other microorganisms.

As described above, it was confirmed that the immunochromatography did not show cross-reactivity to many bacteria and fungi which might disturb the diagnosis for a *Mycoplasma pneumoniae* or *Mycoplasma genitalium* infection.

<<EXAMPLE 4: Evaluation of clinical specimens>>

[0078] Pharyngeal swabs were collected from 3 patients suspected of suffering with a mycoplasma infection and 33 healthy persons, and a detection of *Mycoplasma pneumoniae* was carried out in accordance with the immunochromatography of Example 3. As a result, a positive reaction was observed in the 3 patients suspected of suffering with a mycoplasma infection, and the 33 healthy persons were negative, as shown in Table 9.

DNAs were extracted from the same samples in accordance with a conventional method, and a gene detection of *Mycoplasma pneumoniae* was carried out using a modified method derived from the qualitative PCR method of Jensen et al. (APMIS. 1989;97(11): 1046-8.), in which part of a *Mycoplasma pneumoniae* P1 gene (*M. pneumoniae* M129-B7 NCBI number: NC_000912) was amplified, and both were compared to each other. Both positive and negative results accorded with each other, as shown in Table 9.

[0079]

Table 9

		Immunochromatography		Total
		Positive	Negative	
PCR	Positive	3	0	3
	Negative	0	33	33
Total		3	33	36
Positive agreement rate :100% (3/3)				
Negative agreement rate : 100% (33/33)				
Overall agreement rate : 100% (36/36)				

[0080] Next, DNAs derived from the samples which showed positive by both the immunochromatography and the qualitative PCR method was used, and a gene detection of *Mycoplasma genitalium*, was carried out using a modified method derived from the qualitative PCR method of Yoshida et al. (J Clin Microbiol. 2002; 40(4): 1451-5.) for *Mycoplasma genitalium*, in which part of a *Mycoplasma genitalium* 16s rRNA region (*M. genitalium* G7 NCBI number: L43967) was amplified. As a result, the gene derived from *Mycoplasma genitalium* was not detected in any of the samples, as shown in Table 10.

In this manner, it was confirmed that the gene derived from *Mycoplasma genitalium* could be amplified by this method.

[0081]

Table 10

Clinical specimen	<i>M. genitalium</i> PCR
Sample A	—
Sample B	—
Sample C	—

[0082] As described above, it was shown that the antibody of the present invention was used to specifically detect *Mycoplasma pneumoniae*, and a mycoplasma infection can be diagnosed.

<<EXAMPLE 5: Amplification of DnaK gene of *Mycopiasma pneumoniae* culture strains>>

[0083] As samples to be measured, 8 strains of *Mycoplasma pneumoniae* purchased from ATCC (*M. pneumoniae* FH: ATCC No. 15531, *M. pneumoniae* Bru: ATCC No. 15377, *M. pneumoniae* Mac: ATCC No. 15492, *M. pneumoniae* Mutant 22: ATCC No.39505, *M. pneumoniae* M52: ATCC No.15293, *M. pneumoniae* P11428: ATCC No. 29085, *M. pneumoniae* M129-B7: ATCC No. 29342, and *M. pneumoniae* UTMB-10P: ATCC No. 49894) were used. These 8 strains of *Mycoplasma pneumoniae* were cultivated in a PPLO medium, and DNAs were extracted.

The DNA extraction was carried out using a Sumitest EX-R&D kit (Medical & Biological Laboratories), and each DNA

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was suspended in 10 mmol/L Tris-HCl, 1mmol/L EDTA Buffer pH 8.0 (Nippon Gene)(hereinafter referred to as TE Buffer) and cryopreserved at -40°C.

[0084] With respect to the extracted DNAs, the number of gene copies was determined by a mycoplasma common quantitative PCR for 16s rRNA region. Each DNA was diluted with TE buffer to prepare 10-fold diluted preparations from 2×10^6 to 2×10^0 copies/ μ L. These were used in detecting the DnaK gene.

The mycoplasma common quantitative PCR for 16s rRNA region was carried out as follows.

Primers which were common to the genus *Mycoplasma* for amplifying the 16s rRNA region were designed, and the number of gene copies in each extracted *M. pneumoniae* DNA was calculated by a real-time PCR method using a standard. The real-time PCR was carried out using LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science).

The following primer sequences were used.

M. pneumoniae M129-B7 complete genome: GenBank Accession No. NC_000912

FmY4: 5'-TGGGGAGCAAA(C/T)AGGATTAG-3' (SEQ ID NO: 2) nt 119,081-119,100 20mer

MGSO-2: 5'-CACCATCTGTCACTCTGTAAACCTC-3' (SEQ ID NO: 3) nt 119,332-119,356 25mer

With regard to the PCR conditions, a reaction at 95°C for 10 minutes was carried out and a cycle composed of reactions at 94°C for 10 seconds for denaturing, at 60°C for 2 seconds for annealing, and at 72°C for 12 seconds was repeated 50 times.

As the standard, a diluted series (10^7 , 10^5 , 10^3 , 10^2 , and 10^1 copies/test) of pT7Blue T-Vector (Takara Bio) in which part of 16s rRNA (771 bp: 302-1072 for 16s rRNA) of *M. pneumoniae* (M129 strain) was recombined was used. The number of copies in the standard was calculated on the basis of the following equations:

$$\text{DNA concentration } (\mu\text{g/mL}) = \text{ABS } (260 \text{ nm}) \times 50$$

$$1 \text{ pmol of } 1 \text{ kbp DNA} = 0.66 \mu\text{g}$$

[Math. 1]

$$\text{copy}(\text{copies / mL}) = \frac{1}{0.66} \times \left\{ \frac{1000\text{bp}}{L_p + L_r} \times (A_{260} \times 50) \times (6.02 \times 10^{23}) \right\} \times 10^{-12}$$

Lp: Length of plasmid

Lr: Length of recombinant DNA

[0085] Next, the DnaK gene of *Mycoplasma pneumoniae* was amplified by PCR as follows. With regard to a PCR reaction liquid, 25 μ L of Premix EX Taq Hot Start Version (TaKaRa), 1 μ L of 10pmol/ μ L sense primer MpDnaK_S, and 1 μ L of 10 pmol/ μ L antisense primer MpDnaK_A were added to 18 μ L of Otsuka distilled water (Otsuka Pharmaceutical) to prepare 45 μ L of a master mixture, and 5 μ L of each extracted DNA was added to the master mixture to adjust the total volume to 50 μ L. TE buffer was used as a PCR negative control. To amplify the DnaK gene having a complete length of 1,788 bp, the sense primer was designed at 81 bp 5'-upstream from the starting codon of the DnaK gene, and the antisense primer was designed at 53 bp 3'-downstream from the stop codon. More particularly, sense primer MpDnaK_S corresponded to the 521, 756-521, 782 nucleotide sequence of *M. pneumoniae* M129 (GenBank Acc No. NC_000912), and antisense primer MpDnaK_A corresponded to the 523,655-523,677 nucleotide sequence.

MpDnaK_S: 5'-CTCAAACGCTAAAAGTGCTAACG-3' 23mer (SEQ ID NO: 4)

MpDnaK_A: 5'-AAACCATTATTACAGGTCAAATAAGAC-3' 27mer (SEQ ID NO: 5)

In the PCR reaction, using a Mastercycler (Eppendorf), a cycle composed of reactions at 94°C for 30 seconds for denaturing, at 50°C for 30 seconds for annealing, and at 72°C for 2 minutes was repeated 50 times, and finally a reaction at 72°C for 5 minutes was carried out. After the PCR reaction, 5 μ L of each PCR product was subjected to 2% agarose electrophoresis, and the agarose gel was stained with ethidium bromide and irradiated with ultraviolet light to confirm an amplified band of approximately 1,900 bp.

The 8 *Mycoplasma pneumoniae* stains prepared above were examined to confirm that all the 8 strains could be amplified up to 10^2 copies/test.

<<EXAMPLE 6: Cross-reactivity to mycoplasma culture strains isolated from human>>

[0086] As samples to be measured, 17 mycoplasma strains purchased from ATCC (*M. genitalium*: ATCC No. 33530, *M. hominis*: ATCC No.23114, *Ureaplasma parvum*: ATCC No. 700970, *U. urealyticum*: ATCC No. 27618, *M. fermentans*:

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ATCC No. 19989, *Acholeplasma laidlawii*: ATCC No. 23206, *A. oculi*: ATCC No. 51735, *M. penetrans*: ATCC No. 55252, *M. pirum*: ATCC No. 25960, *M. orale*: ATCC No. 23714, *M. salivarium*: ATCC No. 23064, *M. arthritis*: ATCC No. 19611, *M. buccale*: ATCC No. 23636, *M. faucium*3 ATCC No. 25293, *M. lipophilum*: ATCC No. 27104, *M. primatum*: ATCC No. 25948, and *M. spermatophilum*: ATCC No. 49695) were used. These 17 mycoplasma strains were cultivated in a PPLO medium. Similar to Example 5, DNAs were extracted, the number of gene copies was determined by the quantitative PCR for 16s rRNA region, and each DNA was diluted to 2×10^5 copies/ μ L.

[0087] The procedures described in Example 5 were repeated, except that the 17 *Mycoplasma* strains were used as the samples to be measured, to carry out the PCR for the DnaK gene of *Mycoplasma pneumoniae*, and no amplified band was detected in any of the 17 strains. Because no cross-reactivity was detected when the concentration of the DNA sample was 10,000 times that of DNA capable of amplifying the DnaK gene of *M. pneumoniae*, it was found that the PCR for the DnaK gene of *Mycoplasma pneumoniae* had an extremely high specificity.

<<EXAMPLE 7: Amplification of DnaK gene of *Mycoplasma pneumoniae* from clinical specimens>>

[0088] As samples to be measured, extracted DNAs from 46 cases of positive clinical specimens (40 cases of pharyngeal swabs, 2 cases of nasal mucus, 1 case of nasopharyngeal aspirates, and 3 cases of nasopharyngeal swabs) and 30 cases of negative specimens (10 cases of pharyngeal swabs from healthy persons, 10 cases of pharyngeal swab from clinical specimens, 4 cases of nasal mucus, 3 cases of nasopharyngeal aspirates, and 3 cases of nasopharyngeal swabs) were tested by a nested PCR for the *Mycoplasma pneumoniae* P1 gene region, described in the "*Mycoplasma pneumoniae*" section of National Institute of Infectious Diseases, "Pathogen Detection Manual" p. 1309-1344.

[0089] The PCR for the DnaK gene of *Mycoplasma pneumoniae* was carried out to confirm that the DnaK gene was amplified in all 46 cases of P1 gene PCR positive. By contrast, the DnaK gene was not amplified in any of the 30 cases of P1 gene PCR negative.

[0090]

Table 11

		DnaK gene PCR		Total
		Positive	Negative	
P1 gene PCR	Positive	46	0	46
	Negative	0	30	30
Total		46	30	76

<<EXAMPLE 8: Analysis of DnaK gene nucleotide sequence from culture strains and clinical specimens>>

[0091] The nucleotide sequences of PCR products from the 8 ATCC strains of Example 5 and the 8 clinical specimens (7 cases of pharyngeal swabs and 1 case of nasopharyngeal swabs) of Example 7, in which the amplification was detected by the PCR for DnaK gene, were determined using a BigDye Terminator v3.1 (Applied Biosystems) and a 3130x1 Genetic Analyzer (Applied Biosystems).

As a result, with respect to the DnaK gene (1,788 bp, SEQ ID NO: 6), the PCR products from the 8 ATCC strains and the 8 clinical specimens absolutely (100%) accorded with each other, and also absolutely (100%) accordea with the M129 strain (Acc No. NC_000912) and FH strain (Acc No. CP002077) registered in GenBank. The alignment between the M129 stain and the FH strain is shown in Figures 1 to 3.

With respect to the P1 gene, differential typing was carried out by a PCR-RLFP method in accordance with the reference: JOURNAL OF CLINICAL MICROBIOLOGY, 1996, p. 447-449 Vol. 34, No. 2, and the 8 ATCC strains of Example 5 were classified into two groups. More particularly, 4 strains including M129-B7, M52, P11428, and Mutant 22 were classified into Type I, and 4 strains including FH, Bru, Mac, and UTMB-10P were classified into Type II. The alignment between the M129 strain (SEQ ID NO: 7) and the FH strain (SEQ ID NO: 8) as typical strains is shown in Figures 4 to 10.

It was considered from these results that the obtained antibodies show no difference in reactivity with respect to the genotype, the place for collection, and the time of collection, because the DnaK genes absolutely (100%) accorded with each other, even among strains in which the types of the P1 gene were different, and no variations in the nucleotide sequence were detected among strains collected from various places and over the past 50 years.

INDUSTRIAL APPLICABILITY

[0092] According to the present invention, *Mycoplasma pneumoniae* and/or *Mycoplasma genitalium* can be specifically

detected with high sensitivity in specimens such as oral swab specimens, nasal cavity swab specimens, urine, tissue samples, or body fluids, or samples derived from culture. In particular, the present invention is important for the diagnosis of atypical pneumonia caused by *Mycoplasma pneumoniae* or the diagnosis of nongonococcal urethritis and sexually transmitted disease caused by *Mycoplasma genitalium*, and is industrially applicable to the manufacture of pharmaceuticals.

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Although the present invention has been described with reference to specific embodiments, various changes and modifications obvious to those skilled in the art are possible without departing from the scope of the appended claims.

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SEQUENCE LISTING

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20 Claims

1. A method for detecting *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, **characterized by** using DnaK of *Mycoplasma pneumoniae* or *Mycoplasma genitalium* as an indicator.
- 25 2. The method according to claim 1, wherein a DnaK protein is immunologically analyzed.
3. An anti-DnaK antibody specific to *Mycoplasma pneumoniae* or *Mycoplasma genitalium*.
- 30 4. A kit for detecting *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, comprising the anti-DnaK antibody according to claim 3.
5. The method according to claim 1, using a DnaK gene as an indicator.
- 35 6. A primer or probe specific to *Mycoplasma pneumoniae* or *Mycoplasma genitalium*.
7. A kit for detecting *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, comprising the primer or probe according to claim 6.

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Figure 1

M129_DnaK	ATGAGTACAGATAACGGCTTAATTATCGGCATTGACCTGGTACCTAACTCCTGTGTG	60
FH_DnaK	ATGAGTACAGATAACGGCTTAATTATCGGCATTGACCTGGTACCTAACTCCTGTGTG	60

M129_DnaK	TCGGTCATGGAGAATGGACGCCAGTAGTGTGGAAAACCTGAAGGTAACGCACCACC	120
FH_DnaK	TCGGTCATGGAGAATGGACGCCAGTAGTGTGGAAAACCTGAAGGTAACGCACCACC	120

M129_DnaK	CCTTCGATTGTTCTTACAAGAACAACGAAATTATTGTGGTGATGCTGCCAAACGGCAA	180
FH_DnaK	CCTTCGATTGTTCTTACAAGAACAACGAAATTATTGTGGTGATGCTGCCAAACGGCAA	180

M129_DnaK	ATGGTAACTAACCCCTAATACTATTGTTCCATTAAGCGTTTAAATGGGTACCTCCAATAAG	240
FH_DnaK	ATGGTAACTAACCCCTAATACTATTGTTCCATTAAGCGTTTAAATGGGTACCTCCAATAAG	240

M129_DnaK	GTAACCGTTAAGAATCCTGATGGTCTACCAAAGAGTTAACTCCTGAAGAGGTATCAGCG	300
FH_DnaK	GTAACCGTTAAGAATCCTGATGGTCTACCAAAGAGTTAACTCCTGAAGAGGTATCAGCG	300

M129_DnaK	CAAATCTTGAGCTACCTCAAGGACTATGCGGAAAAGAAGATTGGTAAAACGATTCCCGT	360
FH_DnaK	CAAATCTTGAGCTACCTCAAGGACTATGCGGAAAAGAAGATTGGTAAAACGATTCCCGT	360

M129_DnaK	GCTGTTATTACCGTACCTGCTTACTTTAACGATGCAGAACGGAACGCTACTAAAACCGCT	420
FH_DnaK	GCTGTTATTACCGTACCTGCTTACTTTAACGATGCAGAACGGAACGCTACTAAAACCGCT	420

M129_DnaK	GGTAAGATTGCTGGTTAAACGTTGAGCGGATTATTAACGAACCTACCGCGCTGCATTG	480
FH_DnaK	GGTAAGATTGCTGGTTAAACGTTGAGCGGATTATTAACGAACCTACCGCGCTGCATTG	480

M129_DnaK	GCTTATGGGATCGACAAGTCTAACCGAGAAATGAAAGTCTTGGTGTACGACCTTGGTGGT	540
FH_DnaK	GCTTATGGGATCGACAAGTCTAACCGAGAAATGAAAGTCTTGGTGTACGACCTTGGTGGT	540

M129_DnaK	GGTACCTTTGACGTTTCCTTACTTGACATTGCTGAAGGTACCTCGAAGTATTAGCCACT	600
FH_DnaK	GGTACCTTTGACGTTTCCTTACTTGACATTGCTGAAGGTACCTCGAAGTATTAGCCACT	600

M129_DnaK	GCTGGGACAACCGTTTGGGTGGTGATGACTGGGACAACAAGATTATTGAGTTCATCTTA	660
FH_DnaK	GCTGGGACAACCGTTTGGGTGGTGATGACTGGGACAACAAGATTATTGAGTTCATCTTA	660

M129_DnaK	GCGCACATTGCCAAGAACAACAATGGGCTTAACTTGCCAATGACAAGATGGCTATGCAA	720
FH_DnaK	GCGCACATTGCCAAGAACAACAATGGGCTTAACTTGCCAATGACAAGATGGCTATGCAA	720

Figure 2

M129_DnaK	CGCTTAAAGGAAGCGGCTGAACGTGCTAAGATTGAAC TTCCGCCCAACTAGAAGCAATT	780
FH_DnaK	CGCTTAAAGGAAGCGGCTGAACGTGCTAAGATTGAAC TTCCGCCCAACTAGAAGCAATT	780

M129_DnaK	ATCTCTTACC GTTCTTAACGGTTACCGAAAAGGGTCCGGTAAACGTTGAACTTAAGCTA	840
FH_DnaK	ATCTCTTACC GTTCTTAACGGTTACCGAAAAGGGTCCGGTAAACGTTGAACTTAAGCTA	840

M129_DnaK	ACCCGTGCTAAGTTTGAAGAAATTACCAACAATTACTAGAACG TACTCGCAACCCAATT	900
FH_DnaK	ACCCGTGCTAAGTTTGAAGAAATTACCAACAATTACTAGAACG TACTCGCAACCCAATT	900

M129_DnaK	TCGGATGTTTTACGTGAAGCCAAGATTAACCAGAAGAAATTAATGAAATCTTGTGGTG	960
FH_DnaK	TCGGATGTTTTACGTGAAGCCAAGATTAACCAGAAGAAATTAATGAAATCTTGTGGTG	960

M129_DnaK	GGTGGATCGACCCGGATGCCAGCAGTGCAAAA ACTAGTGAATCAATGGTACCAGGACAC	1020
FH_DnaK	GGTGGATCGACCCGGATGCCAGCAGTGCAAAA ACTAGTGAATCAATGGTACCAGGACAC	1020

M129_DnaK	AGTCCAAACCGCTCAATTAACCCGGATGAGGTGGTAGCCATTGGTGTGCCATCCAAGGG	1080
FH_DnaK	AGTCCAAACCGCTCAATTAACCCGGATGAGGTGGTAGCCATTGGTGTGCCATCCAAGGG	1080

M129_DnaK	GGTGTGTACGCGGTGATGTAAGGACGTGTTACTGTTGGACGTTACTCCTTTAACGCTC	1140
FH_DnaK	GGTGTGTACGCGGTGATGTAAGGACGTGTTACTGTTGGACGTTACTCCTTTAACGCTC	1140

M129_DnaK	TCGATTGAAACCCCTTGGTGGTGTAGCAACTCCGTTAATTAAGCGTAACACCACCATTCT	1200
FH_DnaK	TCGATTGAAACCCCTTGGTGGTGTAGCAACTCCGTTAATTAAGCGTAACACCACCATTCT	1200

M129_DnaK	GTAAGTAAGAGTCAAATCTTCTCTACAGCGCAAGACAACCAAGAATCAGTGGATGTGGTG	1260
FH_DnaK	GTAAGTAAGAGTCAAATCTTCTCTACAGCGCAAGACAACCAAGAATCAGTGGATGTGGTG	1260

M129_DnaK	GTTTGTCAAGGGGAACGCCAATGGCACGTGACAACAAGTCTTTGGGTCGCTTTAACTTA	1320
FH_DnaK	GTTTGTCAAGGGGAACGCCAATGGCACGTGACAACAAGTCTTTGGGTCGCTTTAACTTA	1320

M129_DnaK	GGGGCATCCAACCAGCACCCAAGGGTAAACCCCAAATTGAAATTACCTTTAGCTTGGAC	1380
FH_DnaK	GGGGCATCCAACCAGCACCCAAGGGTAAACCCCAAATTGAAATTACCTTTAGCTTGGAC	1380

M129_DnaK	GCCAACGGGATCTTAAACGTGAAGGCTAAAGATTTAACCACTCAAAGGAAAACAGTATT	1440
FH_DnaK	GCCAACGGGATCTTAAACGTGAAGGCTAAAGATTTAACCACTCAAAGGAAAACAGTATT	1440

Figure 3

M129_DnaK	ACTATTAGTGACAACGGCAACTGTCCGAAGAGGAAATCCAAAAGATGATTCGTGATGCG	1500
FH_DnaK	ACTATTAGTGACAACGGCAACTGTCCGAAGAGGAAATCCAAAAGATGATTCGTGATGCG	1500

M129_DnaK	GAAGCCAACAAGGAGCGTGACAATGTGATTCGTGAACGCATTGAGCTCCGTAACGAAGGT	1560
FH_DnaK	GAAGCCAACAAGGAGCGTGACAATGTGATTCGTGAACGCATTGAGCTCCGTAACGAAGGT	1560

M129_DnaK	GAAAGCATCGTGAGCAGGATTAAGGAGATTCTCCAAGTCCCGAAGCGAAGGACTTCCCT	1620
FH_DnaK	GAAAGCATCGTGAGCAGGATTAAGGAGATTCTCCAAGTCCCGAAGCGAAGGACTTCCCT	1620

M129_DnaK	AAAGAAGAGAAGGAAAACTCGACAAGATTACCGGTGGTATTGATGCAGCAATTAAGGCC	1680
FH_DnaK	AAAGAAGAGAAGGAAAACTCGACAAGATTACCGGTGGTATTGATGCAGCAATTAAGGCC	1680

M129_DnaK	AATGACTACACCAAGTAAAAGCCGAAATCGAAAACCTCAAGAAGTGAAGGGAAGAAATG	1740
FH_DnaK	AATGACTACACCAAGTAAAAGCCGAAATCGAAAACCTCAAGAAGTGAAGGGAAGAAATG	1740

M129_DnaK	GCCAAGAAGTACAACCCTAACGGGGATCAAGGTCAACCAGCACAATAA	1788
FH_DnaK	GCCAAGAAGTACAACCCTAACGGGGATCAAGGTCAACCAGCACAATAA	1788

Figure 5

M129_P1	AAGGTGAAGAACGCCGAGGCGGACCCGCAAG--AGCAATGAAAAACTCCA-GGGCGCT	774
FH_P1	AAGGTGAAGGATGCAACCGTGGATAGTTCGAAGCAATCAACGGAAAGCTTAAAGGGCGAA	780
	***** * ** * ** * ***** * ** * ** * *****	
M129_P1	GAGGCCACTGGTTCTTCAACCACATCTGGATCTGGCCAATCCACCCAACGTGGGGGTTCG	834
FH_P1	GAATCGAGTTCAGTTCACCACATCT---TCCACCTCCACCACCCAACGTGGGGGTTCG	837
	** * * * ** ***** * * * *****	
M129_P1	TCAGGGACACCAAAGTCAAGGCTTTAAAAATAGAGGTGAAAAAGAAATC--GACTCGG	892
FH_P1	TCAAATGAAAACAAAGTCAAGGCGTTCAGGTGGCGGTGAAAAAGAAATCCGGGAGTCAG	897
	** * * ***** * * * ***** * * *	
M129_P1	AG-----GACAATGGTCAGCTGCAGTTAGAAAAAATGATCTCGCCAAC	936
FH_P1	GGCAACTCCGGTGACCAAGGCACCGAACAGGTGGAAGTTGAATCTAATGATTTAGCCAAC	957
	* ** * * * ** ***** * *****	
M129_P1	GCTCCATTAAGCGGAGCGAGGAGTCCGGTCAGTCCGTCCTCAACTCAAGGCGGACGATTT	996
FH_P1	GCCCCGATTAACGGGGCTCCAATAACAACCAGCAAGTCCAACTCAAGGCGGACGATTT	1017
	** ** ***** * * ** *****	
M129_P1	GGTACTGCCCTTCCAGTTCGGGATCAGGCGCAACTCCAATCCCGGTTCCCCACCCCC	1056
FH_P1	GGTACTGCCCTTCCAGTTCGGGATCAGGCA-----CCCAAGATGGCACCCCCACCCCC	1071
	***** ***** * * * *****	
M129_P1	TGAAGCCGTTGGCTTGCGACTGAGCAAATTCACAAGGACCTCCCAAATGATCCGCCTCG	1116
FH_P1	TGAACGCCGTGGTTAACGACTGAGCAAATTCACAACGACCCCGCAAATTCGCGCCTCG	1131
	**** ***** * ***** * ***** *****	
M129_P1	ATCCTGATTCTGTACGATGCGCCTTATGCGCGCAACCGTACCGCATTGACCGGTTGAT	1176
FH_P1	ATCCTGATTCTGTACGATGCGCCTTATGCGCGCAACCGTACCGCATTGACCGGTTGAT	1191

M129_P1	CACTTGATCCCAAGGCCATGACCGCAACTATCCGCCAGTTGAAGAAGCCCAAGTGA	1236
FH_P1	CACTTGATCCCAAGGCCATGACCGCAACTATCCGCCAGTTGAAGAAGCCCAAGTGA	1251

M129_P1	AACCACCACGGTTGTGGGACTGAAAGGCGCGGATGTTTGCTCCAAACCACCGGGTTC	1296
FH_P1	AACCACCACGGTTGTGGGACTGAAAGGCGCGGATGTTTGCTCCAAACCACCGGGTTC	1311

M129_P1	TTCAACCCGCGCGCCACCCGAGTGGTTTGTGATGGCGGGCAGACGGTCGCGGATAACGAA	1356
FH_P1	TTCAACCCGCGCGCCACCCGAGTGGTTTGTGATGGCGGGCAGACGGTCGCGGATAACGAA	1371

M129_P1	AAGACCGGTTTGTGATGTGGATAACTCTGAAAACACCAAGCAGGGCTTCAAAGGAAGCT	1416
FH_P1	AAGACCGGTTTGTGATGTGGATAACTCTGAAAACACCAAGCAGGGCTTCAAAGGAAGCT	1431

Figure 6

M129_P1	GACTCCGACAAGTCGGCCCCGATCGCCCTCCCGTTTGAAGCGTACTTCGCCAACATTGGC	1476
FH_P1	GACTCCGACAAGTCGGCCCCGATCGCCCTCCCGTTTGAAGCGTACTTCGCCAACATTGGC	1491

M129_P1	AACCTCACCTGGTTCGGGCAAGCGCTTTTGGTGTGGTGGCAATGGCCATGTTACCAAG	1536
FH_P1	AACCTCACCTGGTTCGGGCAAGCGCTTTTGGTGTGGTGGCAATGGCCATGTTACCAAG	1551

M129_P1	TCGGCCACACCGCGCCTTTGAGTATAGGTGTCTTTAGGGTGGCTATAATGCAACTGGT	1596
FH_P1	TCGGCCACACCGCGCCTTTGAGTATAGGTGTCTTTAGGGTGGCTATAATGCAACTGGT	1611

M129_P1	ACCAGTGCTACTGTAACCTGGTTGACCATATGCCTTACTGTTCTCAGGCATGGTCAACAAA	1656
FH_P1	ACCAGTGCTACTGTAACCTGGTTGACCATATGCCTTACTGTTCTCAGGCATGGTCAACAAA	1671

M129_P1	CAAACGACGGGTTAAAGGATCTACCCTTTAACAAATAACCGCTGGTTGAATATGTACCA	1716
FH_P1	CAAACGACGGGTTAAAGGATCTACCCTTTAACAAATAACCGCTGGTTGAATATGTACCA	1731

M129_P1	CGGATGGCAGTTGCTGGCGCTAAGTTCGTTGGTAGGGAACCGTTTTAGCGGGTACCATT	1776
FH_P1	CGGATGGCAGTTGCTGGCGCTAAGTTCGTTGGTAGGGAACCGTTTTAGCGGGTACCATT	1791

M129_P1	ACCATGGGTGATACCGCTACCGTACCTCGCTTACTGTACGATGAACTTGAAAGCAACCTG	1836
FH_P1	ACCATGGGTGATACCGCTACCGTACCTCGCTTACTGTACGATGAACTTGAAAGCAACCTG	1851

M129_P1	AACTTAGTAGCGCAAGGCCAAGGTCTTTACGCGAAGACTTGCAACTCTTCACACCCTAC	1896
FH_P1	AACTTAGTAGCGCAAGGCCAAGGTCTTTACGCGAAGACTTGCAACTCTTCACACCCTAC	1911

M129_P1	GGATGAGCCAATCGTCCGATTACCAATCGGGCTTGAAGTAGTAGTAGTAGTAGT	1956
FH_P1	GGATGAGCCAATCGTCCGATTACCAATCGGGCTTGAAGTAGTAGTAGTAGTA---GT	1968
***** *		
M129_P1	CACAACGCACCCTACTACTTCCACAATAACCCCGATTGACAAGACCGTCCAATCCAAAAT	2016
FH_P1	CACAACGCACCCTACTACTTCCACAATAACCCCGATTGACAAGACCGTCCAATCCAAAAT	2028
***** *		
M129_P1	GTGGTTGATGCCTTTATTAAGCCCTGAGAGGACAAGAACGGTAAGGATGATGCCAAATAC	2076
FH_P1	GTGGTTGATGCCTTTATTAAGCCCTGAGAGGACAAGAACGGTAAGGATGATGCCAAATAC	2088

M129_P1	ATCTACCCTTACCGTTACAGTGGCATGTGAGCTTGACAGGTATACAACTGGTCCAATAAG	2136
FH_P1	ATCTACCCTTACCGTTACAGTGGCATGTGAGCTTGACAGGTATACAACTGGTCCAATAAG	2148

Figure 7

M129_P1	CTCACTGACCAACCATTAAGTGCTGACTTTGTCAATGAGAATGCTTACCAACCAAACCTCC	2196
FH_P1	CTCACTGACCAACCATTAAGTGCTGACTTTGTCAATGAGAATGCTTACCAACCAAACCTCC	2208

M129_P1	TTGTTTGCTGCTATTCTCAATCCGGAATGTTAGCAGCTCTCCCGACAAGGTTAAATAC	2256
FH_P1	TTGTTTGCTGCTATTCTCAATCCGGAATGTTAGCAGCTCTCCCGACAAGGTTAAATAC	2268

M129_P1	GGTAAGGAAAACGAGTTTGTGCTAACGAGTACGAGCGCTTTAACAGAAGTTAACGGTA	2316
FH_P1	GGTAAGGAAAACGAGTTTGTGCTAACGAGTACGAGCGCTTTAACAGAAGTTAACGGTA	2328

M129_P1	GCTCCTACCAAGGAACAAACTGATCCCACTTCTCCCCACGCTTCCCGTTTCTCCACC	2376
FH_P1	GCTCCTACCAAGGAACAAACTGATCCCACTTCTCCCCACGCTTCCCGTTTCTCCACC	2388

M129_P1	GGGTTCACCTTGTGGGGTCGGTCTCGACCAGGTGTTGGATTATGTGCCCTGGATTGGG	2436
FH_P1	GGGTTCACCTTGTGGGGTCGGTCTCGACCAGGTGTTGGATTATGTGCCCTGGATTGGG	2448

M129_P1	AATGGGTACAGGTATGGCAATAACCACCGGGCGTGGATGATATAACCGCGCTCAAACC	2496
FH_P1	AATGGGTACAGGTATGGCAATAACCACCGGGCGTGGATGATATAACCGCGCTCAAACC	2508

M129_P1	AGCGCGGGTCTGCCAGCGGAATTAGTACGAACACAAGTGGTTCGCGTTCCTTCTCCCG	2556
FH_P1	AGCGCGGGTCTGCCAGCGGAATTAGTACGAACACAAGTGGTTCGCGTTCCTTCTCCCG	2568
***** *****		
M129_P1	ACGTTTTCCAACATCGGCGTCGGCCTCAAAGCGAATGTCCAAGCCACCCTCGGGGCGAGT	2616
FH_P1	ACGTTTTCCAACATCGGCGTCGGCCTCAAAGCGAATGTCCAAGCCACCCTCGGGGCGAGT	2628

M129_P1	CAGACGATGATTACAGGCGGTTGCGCTCGAAGAACCCTCGACCAAGCCAACCTCCAGCTC	2676
FH_P1	CAGACGATGATTACAGGCGGTTGCGCTCGAAGAACCCTCGACCAAGCCAACCTCCAGCTC	2688

M129_P1	TGAACGGGGCGGGGTGAAGGAATGATAAGGCTTCAAGTGGACAAAGTGACGAAAACCAC	2736
FH_P1	TGAACGGGGCGGGGTGAAGGAATGATAAGGCTTCAAGTGGACAAAGTGACGAA---CCAC	2745
***** ****		
M129_P1	ACCAAGTTCAGAGCGCTACGGGGATGGACCAGCAGGACAATCAGGTACCTCCGCGGGG	2796
FH_P1	ACCAAGTTCAGAGCGCTACGGGGATGGGCCAGCAGGAACAATCAGGTACCTCCGCGGGG	2805
***** ***** *****		
M129_P1	AATCCCGACTCGTTAAAGCAGGATAATATTAGTAAGAGTGGGGATAGTTTAACCACGCAG	2856
FH_P1	AATCCCGACTCGTTAAAGCAGGATAAGATTAGTAAGAGTGGGGATAGTTTAACCACGCAG	2865
***** *****		

Figure 8

M129_P1	GACGGCAATGCGATCGATCAACAAGAGGCCACCAACTACACCAACCTCCCCCAACCTC	2916
FH_P1	GACGGCAATGCGATGGATCAACAAGAGGCCACCAACTACACCAACCTCCCCCAACCTC	2925

M129_P1	ACCCACCGCTGATTGACCGAACGCGCTGTATTACCAACAAGAACAACGCGCAGCGC	2976
FH_P1	ACCCACCGCTGATTGACCGAACGCGCTGTATTACCAACAAGAACAACGCGCAGCGC	2985

M129_P1	GCCCAGCTCTTCTCCGCGGCTTGTGGGCAGCATCCCGGTGTTGGTGAATCGAAGTGGG	3036
FH_P1	GCCCAGCTGTTCTGCGCGCCTGTTGGGCAGCATCCCGGTGTTGGTGAATAAGTCCGGC	3045
	***** **	
M129_P1	TCCGATTCCAACA---AATTCCAAGCCACCGACCAAAAATGGTCTACACCGACTTACAT	3093
FH_P1	CAAGATGATAACAGTAAGTTTAAAGCGGAGGACCAAAAATGGTCTACACCGACTTACAG	3105
	*** ** * * * *****	
M129_P1	TCGGACCAAACTGAACCTCCCGCTTACGGTGAGGTGAATGGGTTGTTGAATCCG	3153
FH_P1	TCGGACCAAACTGAACCTCCCGCTTACGGTGAGGTGAATGGGTTGTTGAATCCG	3165

M129_P1	GCGTTGGTGAAACCTATTTTGGGAACACGCGAGCGGGTGGTTCGGGGTCCAACACGACC	3213
FH_P1	GCGTTGGTGAAACCTATTTTGGGAACACGCGAGCGAGTGGTTCGGGGTCCAACACGACC	3225

M129_P1	AGTTCACCCGGTATCGGTTTTAAAATTCGGAACAAAATA-----ATGAT-TCCAAA	3264
FH_P1	AGTTCACCCGGTATCGGTTTTAAAATTCGGAACAAAAGTGGCACAAACACAACGTCGAAG	3285
	***** * * * * *	
M129_P1	GCCACCCTGATCACCCCGGTTGGCTTGAACGCCAGGACGTCGGTAACCTCGTTGTC	3324
FH_P1	GCTGTGCTGATCACCCCGGTTGGCTTGAACGCCAGGACGTCGGTAACCTCGTTGTC	3345
	** *****	
M129_P1	AGTGGCACCAGTTCAGCTTCCAGCTCGGCGGGTGGCTGGTACCTTACGACTTTGTC	3384
FH_P1	AGTGGCACCAGTTCAGCTTCCAGCTCGGCGGGTGGTACCTTACGACTTTATC	3405
	***** * *****	
M129_P1	AAACCCGCGCGGTTACCTCGGCTCCAGTTAACGGGCTTGGATGCAAGTATGCGACG	3444
FH_P1	AAACCCGCGCTGTTACCTCGGCTCCAGTTAACGGGCTTGGATGCAAGTATGCGACG	3465

M129_P1	CAGCGCCCTCATTGGGCCCGCCCTGAGCGCCTTTCGTGGCAGTTGGGTCAAC	3504
FH_P1	CAGCGCTCTCATTGGGCCCGCCCTGAGCGCCTTTCGTGGCAGTTGGGTCAAC	3525

M129_P1	CGGTTGGGCCGCGTGGAGAGTGTGTGGGATTGAAGGGGTGTGGGCGATCAAGCTCAG	3564
FH_P1	CGGTTGGGCCGCGTGGAGAGTGTGTGGGATTGAAGGGGTGTGGGCGATCAAGCTCAG	3585

Figure 9

M129_P1	TCCGACTCGCAAGGATCTACCACCACCGCAACAAGGAACGCCTTACCGGAGCACCCGAAT	3624
FH_P1	TCCGACTCGCAAGGATCTACCACCACCGCAACAAGGGAGCGCCTTACCGGAGCACCCGAAT	3645

M129_P1	GCTTTGGCCTTTCAGGTGAGTGTGGTGAAGCGAGTGCTTACAAGCCAAACACGAGCTCC	3684
FH_P1	GCTTTGGCCTTTCAGGTGAGTGTGGTGAAGCGAGTGCTTACAAGCCAAACACGAGCTCC	3705

M129_P1	GGCCAAACCCAATCCACTAACAGTTCCTCCCTACCTGCACCTGGTGAAGCCTAAGAAAGTT	3744
FH_P1	GGCCAAACCCAATCCACTAACAGTTCCTCCCTACCTGCACCTGGTGAAGCCTAAGAAAGTT	3765

M129_P1	ACCCAATCCGACAAGTTAGACGACGATCTTAAAAACCTGTTGGACCCCAACAGGTTCCG	3804
FH_P1	ATCCAATCCGACAAGTTAGACGACGATCTTAAAAACCTGTTGGACCCCAACAGGTTCCG	3825
* *****		
M129_P1	ACCAAGCTGCGCAAAGCTTTGGTACAGACCATTCCACCCAGCCCAGCCCAATCGCTC	3864
FH_P1	ACCAAGCTGCGCAAAGCTTTGGTACAGACCATTCCACCCAGCCCAGCCCAATCGCTC	3885

M129_P1	AAAACAACGACACCGGTATTTGGGACGAGTAGTGGTAACCTCAGTAGTGTGCTTAGTGGT	3924
FH_P1	AAAACAACGACACCGGTATTTGGGACGAGTAGTGGTAACCTCAGTAGTGTGCTTAGTGGT	3945

M129_P1	GGGGGTGCTGGAGGGGGTTCTTCAGGCTCAGGTCAATCTGGCGTGGATCTCTCCCCGTT	3984
FH_P1	GGGGGTGCTGGAGGGGGTTCTTCAGGCTCAGGTCAATCTGGCGTGGATCTCTCCCCGTT	4005

M129_P1	GAAAAAGTGAGTGGTGGCTTGTGGGCAGTTACCAAGCAGAGTGACGAAACACCTCC	4044
FH_P1	GAAAAAGTGAGTGGTGGCTTGTGGGCAGTTACCAAGCAGAGTGACGAAACACCTCC	4065

M129_P1	TCCACCAACAACCTCGCGCTAATACTAATACGGGGAATGATGTGGTGGGGTTGGTTCGA	4104
FH_P1	TCCACCAACAACCTCGCGCTAATACTAATACGGGGAATGATGTGGTGGGGTTGGTTCGA	4125

M129_P1	CTTCTGAAAGCAACGCCGCAAAGATGAATGACGATGTTGATGGTATTGTACGCACCCCA	4164
FH_P1	CTTCTGAAAGCAACGCCGCAAAGATGAATGACGATGTTGATGGTATTGTACGCACCCCA	4185

M129_P1	CTCGTGAAGTGTAGATGGGAAGGACAAACAGCTGACACTGGTCCACAAAGCGTGAAG	4224
FH_P1	CTCGTGAAGTGTAGATGGGAAGGACAAACAGCTGACACTGGTCCACAAAGCGTGAAG	4245

M129_P1	TTCAAGTCTCCTGACCAAATTGACTTCAACCGCTTGTTACCCACCCAGTCACCGATCTG	4284
FH_P1	TTCAAGTCTCCTGACCAAATTGACTTCAACCGCTTGTTACCCACCCAGTCACCGATCTG	4305

Figure 10

M129_P1	TTTGATCCGGTAACTATGTTGGTGTATGACCAGTACATACCGCTGTTTATTGATATCCCA	4344
FH_P1	TTTGATCCGGTAACTATGTTGGTGTATGACCAGTACATACCGCTGTTTATTGATATCCCA	4365

M129_P1	GCAAGTGTGAACCCTAAAATGGTTCGTTTAAAGGTCTTGAGCTTTGACACCAACGAACAG	4404
FH_P1	GCAAGTGTGAACCCTAAAATGGTTCGTTTAAAGGTCTTGAGCTTTGACACCAACGAACAG	4425

M129_P1	AGCTTAGGTCTCCGCTTAGAGTCTTTAAACCTGATCAAGATACCCAACCAAAACAACAAC	4464
FH_P1	AGCTTAGGTCTCCGCTTAGAGTCTTTAAACCTGATCAAGATACCCAACCAAAACAACAAC	4485

M129_P1	G TTCAGGTCAATCCGAATAACGGTGACTTCTTACCCTGTTAACGGCTCCAGTCAAGGT	4524
FH_P1	G TTCAGGTCAATCCGAATAACGGTGACTTCTTACCCTGTTAACGGCTCCAGTCAAGGT	4545

M129_P1	CCCCAACCTTGTTTAGTCCGTTTAAACCAGTGACCTGATTACGTGTGCCGTTAGCGATC	4584
FH_P1	CCCCAACCTTGTTTAGTCCGTTTAAACCAGTGACCTGATTACGTGTGCCGTTAGCGATC	4605

M129_P1	ACTGTACCTATTGTTGTGATTGTGCTCAGTGTACCTTAGGACTTGCCATTGGAATCCCA	4644
FH_P1	ACTGTACCTATTGTTGTGATTGTGCTCAGTGTACCTTAGGACTTGCCATTGGAATCCCA	4665

M129_P1	ATGCACAAGAACAAACAGGCCTTGAAGGCTGGGTTTGCCTATCAAACCAAAAGGTTGAT	4704
FH_P1	ATGCACAAGAACAAACAGGCCTTGAAGGCTGGGTTTGCCTATCAAACCAAAAGGTTGAT	4725

M129_P1	GTGTTGACCAAAGCGGTTGGTAGTGTCTTTAAGGAAATCATTAAACCGCACAGGTATCAGT	4764
FH_P1	GTGTTGACCAAAGCGGTTGGTAGTGTCTTTAAGGAAATCATTAAACCGCACAGGTATCAGT	4785

M129_P1	CAAGCGCCAAAACGCTTGAACAAACCAGTGCGGCTAAACCAGGAGCACCCGCCACCA	4824
FH_P1	CAAGCGCCAAAACGCTTGAACAAACCAGTGCGGCTAAACCAGGAGCACCCGCCACCA	4845

M129_P1	GTACCACCAAAGCCAGGGGCTCCTAAGCCACCAGTGCAACCACCTAAAAAACCGCTTAG	4884
FH_P1	GTACCACCAAAGCCAGGGGCTCCTAAGCCACCAGTGCAACCACCTAAAAAACCGCTTAG	4905

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2010/071652

A. CLASSIFICATION OF SUBJECT MATTER G01N33/569(2006.01)i, C07K16/12(2006.01)i, C12N15/09(2006.01)i, C12Q1/68(2006.01)i, G01N33/53(2006.01)i According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) G01N33/569, C07K16/12, C12N15/09, C12Q1/68, G01N33/53 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2011 Kokai Jitsuyo Shinan Koho 1971-2011 Toroku Jitsuyo Shinan Koho 1994-2011 Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) JSTPlus/JMEDPlus/JST7580 (JDreamII), CPlus (STN)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/A	WO 01/57199 A1 (Asahi Kasei Corp.), 09 August 2001 (09.08.2001), page 2, lines 19 to 22 & US 2004/0014943 A1 & AU 3053201 A & CN 1406279 A & TW 282368 B	6, 7/1-5
A	JP 05-304990 A (Behringwerke AG.), 19 November 1993 (19.11.1993), & US 5641638 A & EP 537497 A2 & DE 4134297 A & AU 2712192 A & CA 2080713 A	1-6
A	JP 63-000298 A (Cetus Corp.), 05 January 1988 (05.01.1988), & EP 254384 A2 & AU 7068587 A	1-6
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search 31 January, 2011 (31.01.11)	Date of mailing of the international search report 08 February, 2011 (08.02.11)	
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer	
Facsimile No.	Telephone No.	

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2010/071652

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DASCHER C.C. et al., Heat Shock Response in Mycoplasmas, Genome-Limited Organisms, J. Bacteriol., 1990.04, Vol.172, No.4, P.1823-1827	1-6
A	US 5158870 A (BASEMAN J. B.), 27 October 1992 (27.10.1992), (Family: none)	1-6
A	JP 2002-306169 A (Pfizer Products Inc.), 22 October 2002 (22.10.2002), & EP 1245677 A1 & DE 60125507 D & CA 2340455 A & AT 349530 T & DK 1245677 T & ES 2277893 T & PT 1245677 E & CA 2340455 A1	1-6
A	JP 2004-73185 A (The Institute for Genomic Research), 11 March 2004 (11.03.2004), & US 6537773 B1 & EP 756006 A2 & CA 2178526 A	1-6

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2010/071652

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.

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Continuation of Box No.III of continuation of first sheet(2)

The technical feature common to the invention of claim 1 and the invention of claim 6 is detection of *Mycoplasma pneumoniae* or *Mycoplasma genitalium*. The common technical feature, however, cannot be considered as a special technical feature since it does not make a contribution over the prior art in view of the disclosure of document 1 (WO 2001/057199 A1). In addition, there is no other same or corresponding special technical feature among those inventions. Consequently, the claims of this international application contain the following two (groups of) inventions.

(Invention group 1) the inventions of claims 1-5

Inventions related to a method for detecting *Mycoplasma pneumoniae* or *Mycoplasma genitalium*, which is characterized by using DnaK of *Mycoplasma pneumoniae* or *Mycoplasma genitalium* as an indicator; an antibody used for the method; and a kit used for the method

(Invention group 2) the inventions of claims 6 and 7

Inventions related to a primer or probe which is specific to *Mycoplasma pneumoniae* or *Mycoplasma genitalium*

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- JP 63000298 A [0009]
- WO 2001057199 A [0009]
- JP 5304990 A [0009]

Non-patent literature cited in the description

- **E. HARLOW et al.** Antibodies; A laboratory manual. Cold Spring Harbor Laboratory Press [0023]
- **JENSEN et al.** *APMIS*, 1989, vol. 97 (11), 1046-8 [0078]
- **YOSHIDA et al.** *J Clin Microbiol.*, 2002, vol. 40 (4), 1451-5 [0080]
- Pathogen Detection Manual. National Institute of Infectious Diseases, 1309-1344 [0088]
- *JOURNAL OF CLINICAL MICROBIOLOGY*, 1996, vol. 34 (2), 447-449 [0091]

专利名称(译)	检测属于肺炎支原体和/或生殖支原体的微生物的方法		
公开(公告)号	EP2508887A1	公开(公告)日	2012-10-10
申请号	EP2010834639	申请日	2010-12-03
[标]申请(专利权)人(译)	三菱化学美迪恩斯株式会社		
申请(专利权)人(译)	三菱化学医药股份有限公司		
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IPC分类号	G01N33/569 C07K16/12 C12N15/09 C12Q1/68 G01N33/53		
CPC分类号	C07K16/1253 C12Q1/689 C12Q2521/301 G01N33/56933 G01N2469/10		
代理机构(译)	法思博事务所		
优先权	2009276115 2009-12-04 JP 2010023102 2010-02-04 JP		
其他公开文献	EP2508887A4 EP2508887B1		
外部链接	Espacenet		

摘要(译)

提供了用于快速且特异性地诊断肺炎支原体和/或生殖支原体感染的检测方法和检测试剂盒。肺炎支原体或生殖支原体的DnaK用作指示剂。

