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(54) Title: METHOD FOR DETECTING *NEISSERIA MENINGITIDIS* USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY

(57) Abstract: The present invention provides a method of rapidly, simply and accurately detecting *N. meningitidis*, a primer set for detecting the same, and a kit for detecting the same. The method of detecting *N. meningitidis* of the present invention comprises: amplifying a target *N. meningitidis* nucleic acid using a LAMP primer set comprising one or more types of primers each having a nucleotide sequence that is identical to or complementary to at least a part of *N. meningitidis* nucleic acid; and detecting the amplified product.



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DESCRIPTION

Method for detecting *Neisseria meningitidis* using Loop-Mediated Isothermal Amplification (LAMP) Assay

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 61/727,437 filed on 16 November, 2012.

FIELD OF THE INVENTION

The present invention relates to the application of Loop-mediated isothermal amplification (LAMP) method and primer design for the detection/diagnosis of *Neisseria meningitidis*, and discrimination/identification of six major serogroups (A, B, C, W-135, X, and Y) of *Neisseria meningitidis* that have been identified as causes of meningitis.

BACKGROUND OF THE INVENTION

Neisseria meningitidis (*N. meningitidis*) is a pathogen with global distribution. Cases of meningococcal meningitis due to *N. meningitidis* have been reported from all continents and epidemics of meningococcal meningitis have occurred with devastating consequences including high mortality for decades. Globally, *N. meningitidis* causes an estimated 1.2 million cases and 135,000 deaths each year (Stephens, Greenwood et al. 2007). *N. meningitidis* is a Gram-negative bacterium and a leading cause of severe and fatal bacterial meningitis (Virji 2009). *N. meningitidis* can colonize the human upper respiratory tract without causing the symptoms, or can cause serious blood and brain infection resulting in meningitis and septicemia. *N. meningitidis* belongs to the genus *Neisseria* together with other well-known human pathogen *N. gonorrhoeae*, which causes gonorrhea. While other 17 species of *Neisseria* genus usually don't trigger human diseases, these two species are considered as causative agents of severe human infection. Clinical isolates of *N. meningitidis* strains are usually encapsulated and the classification of *N. meningitidis* is based on the antigenic structure of the polysaccharide capsule. Twelve serogroups have been identified (A, B, C, H, I, K, L, X, Y, Z, 29E, and W-135), while six serogroups (A, B, C, W-135, X and Y) are known to be able to cause epidemics. The most important serogroups associated with disease in humans are A, B, C, Y, and W135 since

over 99% of invasive *N. meningitidis* infections are by these 5 serogroups. A number of *N. meningitidis* strains are analyzed by whole genome sequencing since the first genome sequencing result of serogroup B strains in 2000 (Tettelin, Saunders et al. 2000). A genome database of *N. meningitidis* analyzing and providing genome analysis service of
5 17 *N. meningitidis* strains is also available (Katz, Humphrey et al. 2011).

N. meningitidis is found worldwide. At any time, 5%–10% of the population may be carriers of *N. meningitidis*. Invasive disease is rare in nonepidemic areas, occurring at a rate of 0.5–10 cases per 100,000 population per year, but can occur at a rate of up to 1,000 cases per 100,000 population per year in epidemic regions. Geographic distribution and
10 epidemic potential differ according to serogroup. In Europe and other industrialized countries, serogroup B and C are the major serogroups causing the disease and the incidence rate of meningococcal disease has been reported 0.8 to 1.3 per 100,000 (Harrison, Trotter et al. 2009). A number of developed countries have been dramatically affected by meningococcal epidemics. Both the United Kingdom (UK) and New Zealand have
15 suffered major epidemics of serogroup C disease. For this reason, a novel meningococcal C vaccine was introduced into the routine immunization program of the UK and this vaccine led to significant reductions in the occurrence of meningitis epidemics. A similar experience occurred with serogroup B meningitis in New Zealand (Dyet and Martin 2006). A routinely administered serogroup B vaccine led to dramatically lower meningitis rates in
20 New Zealand. In Africa, *N. meningitidis* is the leading cause of severe, life-threatening meningitis and is responsible for thousands of cases and scores of deaths across sub-Saharan meningitis belt countries (Alonso, Bertherat et al. 2006). Seasonal large scale epidemics by serogroup A are frequent in Sub-Saharan Africa region in meningitis belt ranging from Senegal to Ethiopia.

25 Initial diagnosis of meningococcal meningitis can be made by clinical examination followed by a lumbar puncture showing a purulent spinal fluid. The bacteria can sometimes be seen in microscopic examinations of the spinal fluid. The diagnosis is supported or confirmed by growing the bacteria from specimens of spinal fluid or blood, by agglutination tests or by polymerase chain reaction (PCR). Traditionally, in well-
30 equipped laboratories, *N. meningitidis* is diagnosed using classic bacterial culture methods. These culture methods are utilized to detect *N. meningitidis* bacterium in body tissue and fluids including cerebrospinal fluid (CSF), blood, other normally sterile body fluids and, less often, in soft tissue specimens. Diagnosis using culture requires a well-equipped laboratory with appropriate biosafety facilities, specialized bacterial culture media and

reagents that aid in identifying the *N. meningitidis* species. In populations, with overuse of antibiotics, detection of *N. meningitidis* may be problematic due to inhibition of bacterial growth due to antibiotics present in human clinical samples. The identification of the serogroups and susceptibility testing to antibiotics are important to define control
5 measures. A rapid diagnostic test by immunochromatographic method for identification of *N. meningitidis* serogroups A, W-135, C and Y by utilizing monoclonal antibodies against the polysaccharides of these 4 serogroups has been developed and frequently used in endemic regions in Africa (Chanteau, Darteville et al. 2006).

Serogrouping of *N. meningitidis* infection is important for disease control and
10 vaccination strategies. A number of PCR-based methods of diagnosis of *N. meningitidis* infection by using target genes such as *ctrA*, *crgA*, *IS1106*, and *porA* and serogrouping of 6 major serogroups by using target genes polysialyltransferase *siaD* or other genes involved in serogroup-specific capsule synthesis have been developed (Taha, Alonso et al. 2005; Fraiser, Stor et al. 2009). A PCR-based identification method of serogroup 29E, X,
15 and Z has been also reported (Bennett, Mulhall et al. 2004). A real time-PCR method has been described to discriminate 6 major serogroups of *N. meningitidis* (Mothershed, Sacchi et al. 2004). Finally, a multiplex PCR assay of discriminating all 12 serogroups of *N. meningitidis* has been developed (Zhu, Wang et al. 2012). However, the lower detection limit of conventional PCR and real time PCR assay mostly ranges 10^2 – 10^3 genome copies
20 per reaction.

LAMP (Loop-mediated Isothermal Amplification) method was first developed in 2000 (Notomi, Okayama et al. 2000). By using a heat-stable DNA polymerase with strand displacement activity and a primer set specific for six distinct region of target DNA, the LAMP method has been proved to be sensitive as low as 10 genome copies or less per
25 reaction for detection of various pathogens including *Haemophilus influenzae* type b and *Mycobacterium tuberculosis* (George, Mony et al. 2011; Kim, Kilgore et al. 2011).

A LAMP method-based *N. meningitidis* detection technique has been reported showing the detection limit of 6 copies of the template per reaction and patent filed under US 2010/0304385 A1 (McKenna, Fairley et al. 2011), however, serogrouping or
30 serogroup discrimination of *N. meningitidis* using a LAMP technique has not been published or reported yet. In this invention, a new primer set for LAMP assay of *N. meningitidis* detection or diagnosis is described and six different primer sets for specific identification of six major serogroups (A, B, C, W-135, X, and Y) of *N. meningitidis* by LAMP assay are described. Detection limit and reaction efficiency, specificity, and

sensitivity of our new primer set of *N. meningitidis* detection assay will be compared to the previous publications.

Due to the continued circulation of *N. meningitidis* globally in human populations, the introduction of meningococcal vaccines has been a major public health priority. Over the past 10 years, vaccine research has led to development of a meningococcal A serogroup conjugate vaccine. This vaccine is now being introduced into routine immunization programs of sub-Saharan African countries. In addition, quadrivalent meningococcal conjugate vaccines are now available in Europe, North American countries and other regions. The introduction of meningococcal vaccines in large-scale immunization programs has the potential to dramatically reduce targeted serogroups (e.g., serogroup A in Africa).

Routine use of meningococcal vaccines has the potential for changing circulating patterns of *N. meningitidis* across large populations such as those where the serogroup A meningococcal conjugate vaccine are used widely. In such settings, a rapid, affordable, sensitive and specific assay that can differentiate between major meningococcal serogroups would provide important information to public health programs where meningococcal disease is monitored by surveillance programs. In addition, a rapid and accurate assay would provide vital clinical information for medical providers who are treating patients with suspected meningococcal meningitis.

SUMMARY OF THE INVENTION

It is an objective of the present invention to provide: a method of detecting *N. meningitidis* gene including *ctrA* (*N. meningitidis*), *crgA* (*N. meningitidis*), *IS1106* (*N. meningitidis*), *sacB* (*N. meningitidis* serogroup A), *siaD* (*N. meningitidis* serogroup B), *siaD* (*N. meningitidis* serogroup C), *synG* (*N. meningitidis* serogroup W-135), *ctrA* (*N. meningitidis* serogroup X), and *synF* (*N. meningitidis* serogroup Y); a primer set for detecting such *N. meningitidis*; and a kit for detecting such *N. meningitidis*.

The present inventors have conducted intensive studies directed towards achieving the aforementioned objective. The inventors have focused on a LAMP method, which is more excellent than an amplification reaction by a PCR method in terms of specificity and which is also excellent in terms of promptness and simplicity. As a result, they have found that the aforementioned object can be achieved by designing and using a LAMP primer set capable of specifically *N. meningitidis* nucleic acid, thereby completing the present invention.

An objective of this invention is a highly sensitive and specific nucleic acid based assay that is capable of detecting *N. meningitidis* in sample. A further objective of this invention is a sensitive nucleic acid based assay for the detection of *N. meningitidis* using Loop-Mediated Isothermal Amplification (LAMP). A further objective of this invention is a fully deployable, easy to use, thermally stable and cost effective assay.

The present invention is as follows:

According to a first aspect, a method of detecting at least a part of *N. meningitidis* nucleic acids in a sample using loop-mediated isothermal amplification (LAMP), comprising: amplifying a target *N. meningitidis* nucleic acid using a LAMP primer set comprising one or more types of primers each having a nucleotide sequence that is identical to or complementary to at least a part of *N. meningitidis* nucleic acid; and detecting the amplified product, is provided.

In the above method, the target *N. meningitidis* nucleic acid may encode an *N. meningitidis* gene selected from the group consisting of: *ctrA* (*N. meningitidis*), *crgA* (*N. meningitidis*), IS1106 (*N. meningitidis*), *sacB* (*N. meningitidis* serogroup A), *siaD* (*N. meningitidis* serogroup B), *siaD* (*N. meningitidis* serogroup C), *synG* (*N. meningitidis* serogroup W-135), *ctrA* (*N. meningitidis* serogroup X), and *synF* (*N. meningitidis* serogroup Y).

In the above method, the LAMP primer set may be selected from the group consisting of: SEQ ID Nos. 1-4; 1-5; 1-4 and 6; 1-4 and 8; 1-5 and 8; 1-6; SEQ ID Nos. 1, 2, 4 and 7; 1, 2, 4, 5 and 7; 1, 2, 4, 6 and 7; 1, 2, 4, 7 and 8; 1, 2, 4, 5, 7 and 8; SEQ ID Nos. 9-12; 9-13; 9-12 and 14; 9-14; SEQ ID Nos. 15-18; 15-19; SEQ ID Nos. 20-23; 20-24; 20-23 and 25; 20-25; SEQ ID Nos. 26-29; 26-30; SEQ ID Nos. 31-34; 31-35; 31-34 and 36; 31-36; SEQ ID Nos. 37-40; 37-41; SEQ ID Nos. 42-45; 42-46; 42-45 and 47; 42-47; SEQ ID Nos. 48-51; 48-52; 48-51 and 53; 48-53; SEQ ID Nos. 48, 51, 75 and 76; 48, 51, and 75-77; 48, 51, 53 and 75 and 76; 48, 51, 53 and 75-77; SEQ ID Nos. 54-57; 54-58; 54-57 and 59; 54-59; SEQ ID Nos. 60-63; 60-64; 60-63 and 65; 60-65; SEQ ID Nos. 60, 61, 63 and 78; 60, 61, 63, 64 and 78; 60, 61, 63, 65 and 78; 60, 61, 63-65 and 78; and a combination of any two or more of these primer sets.

In the above method, the LAMP primer set may be SEQ ID Nos. 1-4; 1-5; 1-4 and 6; 1-4 and 8; 1-5 and 8; 1-6; 1, 2, 4 and 7; 1, 2, 4, 5 and 7; 1, 2, 4, 6 and 7; 1, 2, 4, 7 and 8; or 1, 2, 4, 5, 7 and 8, and the target *N. meningitidis* nucleic acid may encode *ctrA* (*N. meningitidis*).

In the above method, the LAMP primer set may be SEQ ID Nos. 9-12; 9-13; 9-12 and 14; 9-14; 15-18; or 15-19, and the target *N. meningitidis* nucleic acid may encode *crgA* (*N. meningitidis*)

5 In the above method, the LAMP primer set may be SEQ ID Nos. 20-23; 20-24; 20-23 and 25; 20-25; 26-29; or 26-30, and the target *N. meningitidis* nucleic acid may encode *IS1106* (*N. meningitidis*).

In the above method, the LAMP primer set may be SEQ ID Nos. 31-34; 31-35; 31-34 and 36; or 31-36, and the target *N. meningitidis* nucleic acid may encode *sacB* (*N. meningitidis* serogroup A).

10 In the above method, the LAMP primer set may be SEQ ID Nos. 37-40; or 37-41, and the target *N. meningitidis* nucleic acid may encode *siaD* (*N. meningitidis* serogroup B).

In the above method, the LAMP primer set may be SEQ ID Nos. 42-45; 42-46; 42-45 and 47; or 42-47, and the target *N. meningitidis* nucleic acid may encode *siaD* (*N. meningitidis* serogroup C).

15 In the above method, the LAMP primer set may be SEQ ID Nos. 48-51; 48-52; 48-51 and 53; 48-53; 48, 51, 75 and 76; 48, 51, and 75-77; 48, 51, 53 and 75 and 76; or 48, 51, 53 and 75-77, and the target *N. meningitidis* nucleic acid may encode *synG* (*N. meningitidis* serogroup W-135).

20 In the above method, the LAMP primer set may be SEQ ID Nos. 54-57; 54-58; 54-57 and 59; or 54-59, and the target *N. meningitidis* nucleic acid may encode *ctrA* (*N. meningitidis* serogroup X).

In the above method, the LAMP primer set may be SEQ ID Nos. 60-63; 60-64; 60-63 and 65; 60-65; 60, 61, 63 and 78; 60, 61, 63, 64 and 78; 60, 61, 63, 65 and 78; or 60, 61, 63-65 and 78, and the target *N. meningitidis* nucleic acid may encode *synF* (*N. meningitidis* serogroup Y).

25 According to a second aspect, a primer set for detection of *N. meningitidis* using loop-mediated isothermal amplification comprising a LAMP primer set selected from the group consisting of: SEQ ID Nos. 1-4; 1-5; 1-4 and 6; 1-4 and 8; 1-5 and 8; 1-6; SEQ ID Nos. 1, 2, 4 and 7; 1, 2, 4, 5 and 7; 1, 2, 4, 6 and 7; 1, 2, 4, 7 and 8; 1, 2, 4, 5, 7 and 8; SEQ ID Nos. 9-12; 9-13; 9-12 and 14; 9-14; SEQ ID Nos. 15-18; 15-19; SEQ ID Nos. 20-23; 20-24; 20-23 and 25; 20-25; SEQ ID Nos. 26-29; 26-30; SEQ ID Nos. 31-34; 31-35; 31-34 and 36; 31-36; SEQ ID Nos. 37-40; 37-41; SEQ ID Nos. 42-45; 42-46; 42-45 and 47; 42-47; SEQ ID Nos. 48-51; 48-52; 48-51 and 53; 48-53; SEQ ID Nos. 48, 51, 75 and 76; 48, 51, and 75-77; 48, 51, 53 and 75 and 76; 48, 51, 53 and 75-77; SEQ ID Nos. 54-57;

54-58; 54-57 and 59; 54-59; SEQ ID Nos. 60-63; 60-64; 60-63 and 65; 60-65; SEQ ID Nos. 60, 61, 63 and 78; 60, 61, 63, 64 and 78; 60, 61, 63, 65 and 78; 60, 61, 63-65 and 78; and a combination of any two or more of these primer sets, wherein each primer specifically hybridizes to a target *N. meningitidis* acid or the complement of a target *N.*

5 *meningitidis* nucleic acid, is provided.

In the above primer set, the target *N. meningitidis* nucleic acid may encode an *N. meningitidis* gene selected from the group consisting of: *ctrA* (*N. meningitidis*), *crgA* (*N. meningitidis*), *IS1106* (*N. meningitidis*), *sacB* (*N. meningitidis* serogroup A), *siaD* (*N. meningitidis* serogroup B), *siaD* (*N. meningitidis* serogroup C), *synG* (*N. meningitidis* serogroup W-135), *ctrA* (*N. meningitidis* serogroup X), and *synF* (*N. meningitidis* serogroup Y).

According to a third aspect, a kit for detecting *N. meningitidis* comprising the above LAMP primer set, is provided.

The details of one or more embodiments of the disclosure are set forth in the accompanying drawings and the description below. Other features, objects, and advantages will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Fig. 1 shows LAMP primer specificity.

Figure 2. Fig. 2 shows detection limit and real-time reaction of the *N. meningitidis* LAMP assay. Real-time turbidity change of reaction mixtures containing one to 10^5 copies of bacterial genome. PC-A: 10^6 genome copy of serogroup A was used as a positive control.

Figure 3. Fig. 3 shows detection limit and real-time reaction of the *N. meningitidis* LAMP assay. The relationship between the threshold times (Tt) of each reaction mixture and the log of the amount of initial template DNA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is described in detail below. The following descriptions are not intended to limit the scope of the present invention. Other than the following examples, the present invention may be modified and may be carried out, as appropriate, within a range that does not impair the intention of the present invention.

The present invention has focused on a nucleotide sequence region specific for *ctrA*, *crgA*, and *IS1106* in chromosomal DNA derived from *N. meningitidis*, *sacB* in chromosomal DNA derived from *N. meningitidis* serogroup A, *siaD* in chromosomal DNA derived from *N. meningitidis* serogroup B, *siaD* in chromosomal DNA derived from *N. meningitidis* serogroup C, *synG* in chromosomal DNA derived from *N. meningitidis* serogroup W-135, *ctrA* in chromosomal DNA derived from *N. meningitidis* serogroup X, *synF* in chromosomal DNA derived from *N. meningitidis* serogroup Y, so as to design a LAMP primer set, and thus the inventors have enabled specific detection of *N. meningitidis*. The term "LAMP primer set" is used herein to mean a primer set consisting of at least 4 types of (at maximum 6 types) of primers used in nucleic acid amplification according to a LAMP method.

The LAMP primer set is constituted by combining primers designed from 6 different regions (F3, F2, F1, B1c, B2c and B3c from the 5' end side) in a nucleotide sequence region specific for each target *N. meningitidis* nucleic acid and 6 regions complementary thereto (B3, B2, B1, F1c, F2c and F3c from the 5' end side). Specifically, the LAMP primer set is produced by combining: a Forward Inner Primer (hereinafter abbreviated as "FIP" at times) formed by ligating nucleotides in the F1c region to nucleotides in the F2 region from the 5' end side of a nucleotide sequence region specific for each gene; a Backward Inner Primer (hereinafter abbreviated as "BIP" at times) formed by ligating nucleotides in the B1c region to nucleotides in the B2 region from the 5' end side thereof; an F3 primer consisting of nucleotides in the F3 region; and a B3 primer consisting of nucleotides in the B3 region. If desired, loop primers may be further designed, and DNA may be amplified using such primers, so that an amplified product may be detected. If such loop primers are used, the time required until detection can be further reduced. Thus, the use of loop primers enables more efficient detection. As such loop primers, there can be used a Loop Primer Forward (hereinafter abbreviated as "LF" at times) consisting of nucleotides in a region between the F1c region and the F2c region and a Loop Primer Backward (hereinafter abbreviated as "LB" at times) consisting of nucleotides in a region between the B2 region and the B1 region.

In the LAMP method, an amplification reaction can be promoted only by incubation at a constant temperature capable of maintaining enzyme activity. Thus, the LAMP method does not need equipment for controlling each temperature, which is necessary for the PCR method. Accordingly, the LAMP method enables simple detection at low cost, as well as rapid detection without time loss caused by temperature change.

Primer design for *N. meningitidis* detection by LAMP

A number of *N. meningitidis* specific genes are used for identification purpose as shown in Table 1 (Taha, Alonso et al. 2005). The inventors have designed a set of LAMP primers for *ctrA*, *crgA*, and *IS1106* according to the primer design program (primerexplorer) available at Eiken Chemical Co., Ltd (<http://primerexplorer.jp/elamp4.0.0/index.html>). Four different sets of LAMP primers were designed for *ctrA* gene, two primer sets were designed for *crgA*, and two sets of LAMP primers were initially designed. All the possible LAMP primer sets were synthesized from Genotech (Daejeon, Korea) and tested for optimized results of *N. meningitidis* detection.

Table 1. *N. meningitidis* specific genes commonly used for identification purpose by PCR or sequence-based methods

Identification target	Gene	Gene product or Identification	Genbank accession number
<i>N.meningitidis</i>	<i>ctrA</i>	Capsular transport protein A	AE002098
<i>N.meningitidis</i>	<i>crgA</i>	Contact-regulated protein A	AF190471
<i>N.meningitidis</i>	<i>IS1106</i>	Insertion sequence IS1106	AF422172
<i>N.meningitidis</i>	16S rRNA	16S rRNA	CP000381.1
<i>N.meningitidis</i>	<i>porA</i>	Porin	AF143558
Serogroup A	<i>sacB</i>	capsular polysaccharide phosphotransferase, SacB	FR774048
Serogroup B	<i>siaD</i>	alpha-2,8-polysialyltransferase	CP002424
Serogroup C	<i>siaD</i>	alpha-2,8-polysialyltransferase	AM421808
Serogroup W-135	<i>synG</i>	SynG	AY234197
Serogroup X	<i>ctrA</i>	Capsular transport protein A	AY289931
Serogroup Y	<i>synF</i>	SynF	AY234201

The specificity of the LAMP reaction is tested to related *Neisseria* species such as *N. gonorrhoeae*, *N. flavescens*, *N. denitrificans*, *N. elongate*, *N. canis*, *N. cinerea*, *N. lactamica*, *N. mucosa*, and *N. sicca* as well as unrelated bacterial species such as *E. coli*, *Vibrio cholerae*, *Salmonella* spp. etc.

Detection of *N. meningitidis*

(1) Detection of *ctrA* of *N. meningitidis*

In a first embodiment of the present invention, a LAMP primer set is designed by focusing on the nucleotide sequence region of *ctrA* derived from *N. meningitidis*, so as to specifically detect *ctrA* of *N. meningitidis*.

An example of the nucleotide sequence region of *ctrA* derived from *N. meningitidis* is shown in SEQ ID NO: 66. Herein, the nucleotide sequence region may be either a region corresponding to the entire *ctrA* or a region corresponding to a portion thereof. Thus, it is not limited.

The location of each primer in the nucleotide sequence region of *ctrA* is shown in Table 2.

Table 2. LAMP primer location of targeting *ctrA* gene

Primer location	Outer primer	Inner primer				Outer primer	Loop primer	
		FIP		BIP			LF	LB
	F3	F2	F1	B1	B2	B3		
ctrA4	806-825	843-859	880-899	905-926	979-996	1014-1032	861-877	956-975
ctrA4-mod2	806-825	843-859	881-899	905-926	979-996	1014-1032	-	943-967

The term "ctrA4" or "ctrA4-mod2" in Table 2 means LAMP primer set for detecting *ctrA* gene of *N. meningitidis*. Either primer set may be used for detecting *ctrA*.

F3, B3 and BIP primers of the primer set of ctrA4-mod2 are same as that of ctrA4 and LB primer of the primer set of ctrA4-mod2 is designed based on that of ctrA4. FIP primer of the primer set of ctrA4-mod2 is designed based on that of ctrA4 and single base is altered (underlined in Table 4).

The numbers described in Table 2 indicate the position of a target region in the nucleotide sequence as shown in SEQ ID NO: 66 for designing the FIP, BIP, F3, B3, LF and LB primers. For example, F3 primer in ctrA4 primer set is preferably designed from the region of bp 806 to 825 (which is hereinafter referred to as "806-825" at time, and the same holds for other primers) in the nucleotide sequence as shown in SEQ ID NO: 66, and the B3 primer in ctrA4 primer set is preferably designed from the region of 1014-1032 in the nucleotide sequence as shown in SEQ ID NO: 66.

In the first embodiment of the present invention, at least one type of (preferably at least two types of, more preferably at least four types of, and further preferably six types of) LAMP primer comprised in the LAMP primer set consists of a nucleotide sequence identical to or complementary to the sequence of a portion (partial sequence) in the nucleotide sequence of the aforementioned *ctrA*. Further, in the first embodiment of the

present invention, the LAMP primer set for detecting *ctrA* derived from *N. meningitidis* comprises each LAMP primer designed preferably from the nucleotide sequence region as shown in SEQ ID NO: 66 in the aforementioned *ctrA*. In the case of using such a LAMP primer set, the LAMP primer set is excellent in terms of detection sensitivity and detection promptness, as well as specificity, in detection of the *ctrA* of *N. meningitidis*. Moreover, in the case of the LAMP primer set, linearity is observed in an amplification curve, and good quantitative performance can also be obtained. In the present invention, an LF primer and/or an LB primer may be designed and used as loop primer(s).

With regard to LAMP primer sets of *ctrA4* and *ctrA4-mod2*, the nucleotide sequences of the primers are shown in Tables 3 and 4, respectively.

Table 3. LAMP primer set of *ctrA4* for amplification of *ctrA* for identification of *N. meningitidis* (5'-3')

CtrA4_F3: AAG AAA TCG GTT TTT CAG CC (SEQ ID NO: 1)

CtrA4_B3: TAG CGA ATG CGC ATC AGC C (SEQ ID NO: 2)

CtrA4_FIP: ACA CCA CGC GCA TCA GAA CGT GAA GCC ATT GGC CGT A (SEQ ID NO: 3)

CtrA4_BIP: TGT TCC GCT ATA CGC CAT TGG TCG TTG GAA TCT CTG CCT C (SEQ ID NO: 4)

CtrA4_LF: GAT CTT GCA AAC CGC CC (SEQ ID NO: 5)

CtrA4_LB: GGA TTG CTC AAG GTT ATG GC (SEQ ID NO: 6)

Table 4. LAMP primer set of *ctrA4-mod2* for amplification of *ctrA* for identification of *N. meningitidis* (5'-3')

CtrA4-mod2_F3: AAG AAA TCG GTT TTT CAG CC (SEQ ID NO: 1)

CtrA4-mod2_B3: TAG CGA ATG CGC ATC AGC C (SEQ ID NO: 2)

CtrA4-mod2_FIP: ACA CCA CGC GCA TCA GAA CTC AAG CCA TTG GCC GTA (SEQ ID NO: 7)

CtrA4-mod2_BIP: TGT TCC GCT ATA CGC CAT TGG TCG TTG GAA TCT CTG CCT C (SEQ ID NO: 4)

CtrA4-mod2_LB: CGT CAG GAT AAA TGG ATT GCT CAA G (SEQ ID NO: 8)

(2) Detection of *crpA* of *N. meningitidis*

In a second embodiment of the present invention, a LAMP primer set is designed by focusing on the nucleotide sequence region of *crgA* derived from *N. meningitidis*, so as to specifically detect *crgA* of *N. meningitidis*.

An example of the nucleotide sequence region of *crgA* derived from *N. meningitidis* is shown in SEQ ID NO: 67. Herein, the nucleotide sequence region may be either a region corresponding to the entire *crgA* or a region corresponding to a portion thereof. Thus, it is not limited.

The location of each primer in the nucleotide sequence region of *crgA* is shown in Table 5.

Table 5. LAMP primer location of targeting *crgA* gene

Primer location	Outer primer	Inner primer				Outer primer	Loop primer	
		FIP		BIP			LF	LB
	F3	F2	F1	B1	B2	B3		
crgA	523-543	544-562	595-616	638-657	692-709	723-740	568-592	569-685
crgA(816)	37-396	408-426	453-474	482-503	543-561	582-599	429-447	-

The term “*crgA*” or “*crgA*(816)” in Table 5 means LAMP primer set for detecting *crgA* gene of *N. meningitidis*. Either primer set may be used for detecting *crgA*.

The numbers described in Table 5 indicate the position of a target region in the nucleotide sequence as shown in SEQ ID NO: 67 for designing the FIP, BIP, F3, B3, LF and LB primers. For example, F3 primer in *crgA* primer set is preferably designed from the region of 523-543 in the nucleotide sequence as shown in SEQ ID NO: 67, and the B3 primer in *crgA* primer set is preferably designed from the region of 723-740 in the nucleotide sequence as shown in SEQ ID NO: 67.

In the second embodiment of the present invention, at least one type of (preferably at least two types of, more preferably at least four types of, and further preferably six types of) LAMP primer comprised in the LAMP primer set consists of a nucleotide sequence identical to or complementary to the sequence of a portion (partial sequence) in the nucleotide sequence of the aforementioned *crgA*. Further, in the second embodiment of the present invention, the LAMP primer set for detecting *crgA* derived from *N. meningitidis* comprises each LAMP primer designed preferably from the nucleotide sequence region as shown in SEQ ID NO: 67 in the aforementioned *crgA*. In the case of using such a LAMP primer set, the LAMP primer set is excellent in terms of detection sensitivity and detection promptness, as well as specificity, in detection of the *crgA* of *N. meningitidis*. Moreover,

in the case of the LAMP primer set, linearity is observed in an amplification curve, and good quantitative performance can also be obtained. In the present invention, an LF primer and/or an LB primer may be designed and used as loop primer(s).

With regard to a LAMP primer set of *crgA* and *crgA*(816), the nucleotide sequences of the primers are shown in Tables 6 and 7.

Table 6. LAMP primer set of *crgA* for amplification of *crgA* for identification of *N. meningitidis* (5'-3')

*crgA*_F3: AAC GAA CGC TAT CCG CAT AT (SEQ ID NO: 9)
 10 *crgA*_B3: GAC CGG CAA GCT CTT CTG (SEQ ID NO: 10)
*crgA*_FIP: GGC CCG TAA GGC AAT ATC GAC TCC GAC TTT CGC TCG TTT CT (SEQ ID NO: 11)
*crgA*_BIP: CTG CGT GCA CGC CAT CTG TTG CCG TGT TTT GCC AGG TA (SEQ ID NO: 12)
 15 *crgA*_LF: GCG TTC AAT CAG ATT GAT ATA GCC T (SEQ ID NO: 13)
*crgA*_LB: TCC GCG TAA TCG CCA GT (SEQ ID NO: 14)

Table 7. LAMP primer set of *crgA*(816) for amplification of *crgA* for identification of *N. meningitidis* (5'-3')

20 *crgA*(816)_F3: CCG GAG AAT TGG ACG ATT CC (SEQ ID NO: 15)
crgA(816)_B3: GCG GTA AAG TGC GGT GAA (SEQ ID NO: 16)
crgA(816)_FIP: CGT GCC GTG TTT TGC CAG GTA TAC GCC ATC TGT TTG ACA GC (SEQ ID NO: 17)
crgA(816)_BIP: CTA CAG AAG AGC TTG CCG GTC ACG CAT CTA AAA CCG
 25 CCC AT (SEQ ID NO: 18)
crgA(816)_LF: ACT GGC GAT TAC GCG GAA G (SEQ ID NO: 19)

(3) Detection of IS1106 of *N. meningitidis*

In a third embodiment of the present invention, a LAMP primer set is designed by focusing on the nucleotide sequence region of IS1106 derived from *N. meningitidis*, so as to specifically detect IS1106 of *N. meningitidis*.

An example of the nucleotide sequence region of IS1106 derived from *N. meningitidis* is shown in SEQ ID NO: 68. Herein, the nucleotide sequence region may be

either a region corresponding to the entire *IS1106* or a region corresponding to a portion thereof. Thus, it is not limited.

The location of each primer in the nucleotide sequence region of *IS1106* is shown in Table 8.

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Table 8. LAMP primer location of targeting *IS1106* gene

Primer location	Outer primer	Inner primer				Outer primer	Loop primer	
		FIP		BIP			LF	LB
	F3	F2	F1	B1	B2	B3		
IS1106	121-140	141-160	197-218	236-257	280-297	298-316	169-190	259-277
IS1106(68-1075)	54-73	74-93	130-150	169-190	216-234	241-259	-	191-210

The term “IS1106” or “IS1106(68-1075)” in Table 8 means LAMP primer set for detecting *IS1106* gene of *N. meningitidis*. Either primer set may be used for detecting

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The numbers described in Table 8 indicate the position of a target region in the nucleotide sequence as shown in SEQ ID NO: 68 for designing the FIP, BIP, F3, B3, LF and LB primers. For example, F3 primer in IS1106 primer set is preferably designed from the region of 121-140 in the nucleotide sequence as shown in SEQ ID NO: 68, and the B3

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In the third embodiment of the present invention, at least one type of (preferably at least two types of, more preferably at least four types of, and further preferably six types of) LAMP primer comprised in the LAMP primer set consists of a nucleotide sequence identical to or complementary to the sequence of a portion (partial sequence) in the nucleotide sequence of the aforementioned *IS1106*. Further, in the third embodiment of the present invention, the LAMP primer set for detecting *IS1106* derived from *N. meningitidis* comprises each LAMP primer designed preferably from the nucleotide sequence region as shown in SEQ ID NO: 68 in the aforementioned *IS1106*. In the case of using such a LAMP primer set, the LAMP primer set is excellent in terms of detection sensitivity and detection promptness, as well as specificity, in detection of the *IS1106* of *N. meningitidis*. Moreover, in the case of the LAMP primer set, linearity is observed in an amplification curve, and good quantitative performance can also be obtained. In the present invention, an LF primer and/or an LB primer may be designed and used as loop

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With regard to a LAMP primer set of IS1106 and IS1106(68-1075), the nucleotide sequences of the primers are shown in Tables 9 and 10.

Table 9. LAMP primer set of IS1106 for amplification of IS1106 for identification of *N.*

5 *meningitidis* (5'-3')

IS1106_F3: CCG TTT CCC GCT ATT GAA GT (SEQ ID NO: 20)

IS1106_B3: GGT GAT GAG GCT GTG TTC G (SEQ ID NO: 21)

IS1106_FIP: CGC GGT GGT CTC TAA GGT AAC GTG GAC CAG GTG ATT GAT
TGG (SEQ ID NO: 22)

10 IS1106_BIP: CTG CTG TCC ATG TTC AAA GCC GAG TTC GGG ATC GGA GAG G
(SEQ ID NO: 23)

IS1106_LF: TTG ACG GTT CAG GTA TTG TTC G (SEQ ID NO: 24)

IS1106_LB: CCT GCT CGG ACA ATG GCA C (SEQ ID NO: 25)

15 Table 10. LAMP primer set of IS1106(68-1075) for amplification of IS1106 for
identification of *N. meningitidis* (5'-3')

IS1106(68-1075)_F3: CCG TTT CCC GCT ATT GAA GT (SEQ ID NO: 26)

IS1106(68-1075)_B3: AAT CGA TGC GGG TGA TGA G (SEQ ID NO: 27)

IS1106(68-1075)_FIP: CGC GGT GGT CTC TAA GGT AAC GTG GAC CAG GTG
20 ATT GAT TGG (SEQ ID NO: 28)

IS1106(68-1075)_BIP: CTG CTG TCC ATG TTC AAA GCC GTT CGA GTT CGG
GAT CGG AG (SEQ ID NO: 29)

IS1106(68-1075)_LB: TCC TGC TCG GAC AAT GGC AC (SEQ ID NO: 30)

25 (4) Genogrouping (DNA sequence-based serogrouping) methods of *N. meningitidis*

Stephens and Swartley published a patent of serogroup specific DNA sequences (A, B, C, Y, and W-135) in 2002 (US Patent US 6,6403,306 B1). Comparison of efficiency of PCR-based methods of identification and genogrouping of 5 serogroups (A, B, C, Y, and W-135) of *N. meningitidis* among 11 reference laboratories in Europe was reported in
30 2005 (Taha, Alonso et al. 2005). Serogroups B, C, Y, and W-135 contain sialic acid in their polysaccharide capsule and the serogroup specific DNA sequences of *siaD* gene of each serogroup (encodes polysialyltransferase), could be used for genogrouping purpose. Serogroup A could be identified by using serogroup-specific gene *mynB/sacC*. Fraiser et al., reported a single multiplex PCR-based genogrouping method for identification of six

serogroups (A, B, C, X, W-135, and Y) by targeting orf-2 (serogroup A), and capsule structure specific genes (serogroups B, C, W-135, and Y), and serogroup X specific region of *ctrA* gene for serogroup X (Fraisier, Stor et al. 2009). In a real-time PCR based method developed by Mothershed et al., similar genes were utilized for identification of 6 serogroups except *xcbB* gene, which encodes a putative serogroup specific capsule polymerase was used for identification of serogroup X (Mothershed, Sacchi et al. 2004).

(5) Detection of *sacB* of *N. meningitidis* serogroup A

In a forth embodiment of the present invention, a LAMP primer set is designed by focusing on the nucleotide sequence region of *sacB* of *N. meningitidis* serogroup A, so as to specifically detect *N. meningitidis* serogroup A.

An example of the nucleotide sequence region of *sacB* of *N. meningitidis* serogroup A is shown in SEQ ID NO: 69. Herein, the nucleotide sequence region may be either a region corresponding to the entire *sacB* of *N. meningitidis* serogroup A or a region corresponding to a portion thereof. Thus, it is not limited.

The location of each primer in the nucleotide sequence region of *sacB* of *N. meningitidis* serogroup A is shown in Table 11.

Table 11. LAMP primer location in *sacB* gene of *N. meningitidis* serogroup A

Primer location	Outer primer	Inner primer				Outer primer	Loop primer	
		FIP		BIP			LF	LB
	F3	F2	F1	B1	B2	B3		
MenA3	435-454	475-496	520-604	577-604	643-662	677-698	499-518	608-631

The term "MenA3" in Table 11 means LAMP primer set for detecting *sacB* gene of *N. meningitidis* serogroup A.

The numbers described in Table 11 indicate the position of a target region in the nucleotide sequence as shown in SEQ ID NO: 69 for designing the FIP, BIP, F3, B3, LF and LB primers. For example, F3 primer in MenA3 primer set is preferably designed from the region of 435-454 in the nucleotide sequence as shown in SEQ ID NO: 69, and the B3 primer in MenA3 primer set is preferably designed from the region of 677-698 in the nucleotide sequence as shown in SEQ ID NO: 69.

In the forth embodiment of the present invention, at least one type of (preferably at least two types of, more preferably at least four types of, and further preferably six types of) LAMP primer comprised in the LAMP primer set consists of a nucleotide sequence

identical to or complementary to the sequence of a portion (partial sequence) in the nucleotide sequence of the aforementioned *sacB* gene of *N. meningitidis* serogroup A. Further, in the forth embodiment of the present invention, the LAMP primer set for detecting *sacB* derived from *N. meningitidis* serogroup A comprises each LAMP primer
 5 designed preferably from the nucleotide sequence region as shown in SEQ ID NO: 69 in the aforementioned *sacB* of *N. meningitidis* serogroup A. In the case of using such a LAMP primer set, the LAMP primer set is excellent in terms of detection sensitivity and detection promptness, as well as specificity, in detection of *sacB* derived from *N. meningitidis* serogroup A. Moreover, in the case of the LAMP primer set, linearity is
 10 observed in an amplification curve, and good quantitative performance can also be obtained. In the present invention, an LF primer and/or an LB primer may be designed and used as loop primer(s).

With regard to a LAMP primer set of MenA3, the nucleotide sequences of the primers are shown in Table 12.

Table 12. LAMP primer set of MenA3 for amplification of *sacB* for identification of *N. meningitidis* serogroup A(5'-3')

MenA3_F3: CGT AAA TGA AAT TTG GAC AG (SEQ ID NO: 31)

MenA3_B3: TTA TGA TCT TCT TCA TAG GGT A (SEQ ID NO: 32)

MenA3_FIP: GAA CTC TAA TCT GAA CCA AAA TTG AGA GTT GAC ATG AAA
 CTC AGC ACA G (SEQ ID NO: 33)

MenA3_BIP: CCT ACA GCT AAC AGA TAT TCT AGA AAA CGA ATA GTT TCG
 TAT GCC TTC (SEQ ID NO: 34)

MenA3_LF: ATA GAT GAA CTT AAA GTT CT (SEQ ID NO: 35)

MenA3_LB: GGA AGC ACT CTA TTA AAA ATA ATC (SEQ ID NO: 36)

(6) Detection of *siaD* of *N. meningitidis* serogroup B

In a fifth embodiment of the present invention, a LAMP primer set is designed by focusing on the nucleotide sequence region of *siaD* of *N. meningitidis* serogroup B, so as
 30 to specifically detect *N. meningitidis* serogroup B.

An example of the nucleotide sequence region of *siaD* of *N. meningitidis* serogroup B is shown in SEQ ID NO: 70. Herein, the nucleotide sequence region may be either a region corresponding to the entire *siaD* of *N. meningitidis* serogroup B or a region corresponding to a portion thereof. Thus, it is not limited.

The location of each primer in the nucleotide sequence region of *siaD* of *N. meningitidis* serogroup B is shown in Table 13.

Table 13. LAMP primer location in *siaD* gene of *N. meningitidis* serogroup B

Primer location	Outer primer	Inner primer				Outer primer	Loop primer	
		FIP		BIP			LF	LB
	F3	F2	F1	B1	B2	B3		
MenB3.11	891-907	922-941	962-981	1013-1035	1077-1095	1099-1122	-	1039-1065

The term “MenB3.11” in Table 13 means LAMP primer set for detecting *siaD* gene of *N. meningitidis* serogroup B.

The numbers described in Table 13 indicate the position of a target region in the nucleotide sequence as shown in SEQ ID NO: 70 for designing the FIP, BIP, F3, B3, and LB primers. For example, F3 primer in MenB3.11 primer set is preferably designed from the region of 891-907 in the nucleotide sequence as shown in SEQ ID NO: 70, and the B3 primer in MenB3.11 primer set is preferably designed from the region of 1099-1122 in the nucleotide sequence as shown in SEQ ID NO: 70.

In the fifth embodiment of the present invention, at least one type of (preferably at least two types of, more preferably at least four types of, and further preferably six types of) LAMP primer comprised in the LAMP primer set consists of a nucleotide sequence identical to or complementary to the sequence of a portion (partial sequence) in the nucleotide sequence of the aforementioned *siaD* gene of *N. meningitidis* serogroup B. Further, in the fifth embodiment of the present invention, the LAMP primer set for detecting *siaD* derived from *N. meningitidis* serogroup B comprises each LAMP primer designed preferably from the nucleotide sequence region as shown in SEQ ID NO: 70 in the aforementioned *siaD* of *N. meningitidis* serogroup B. In the case of using such a LAMP primer set, the LAMP primer set is excellent in terms of detection sensitivity and detection promptness, as well as specificity, in detection of *siaD* derived from *N. meningitidis* serogroup B. Moreover, in the case of the LAMP primer set, linearity is observed in an amplification curve, and good quantitative performance can also be obtained. In the present invention, an LF primer and/or an LB primer may be designed and used as loop primer(s).

With regard to a LAMP primer set of MenB3.11, the nucleotide sequences of the primers are shown in Table 14.

Table 14. LAMP primer set of MenB3.11 for amplification of *siaD* for identification of *N. meningitidis* serogroup B (5'-3')

MenB3.11_F3: AAA CCC TCG GCT GGT AG (SEQ ID NO: 37)

MenB3.11_B3: CTT AAT AAT CTC TAA GTG TTC TTG (SEQ ID NO: 38)

5 MenB3.11_FIP: GGC CAG GCC TAT AAT TCC TTC CTT TTC TAA TTG AGC CCC T (SEQ ID NO: 39)

MenB3.11_BIP: CAC CCT CAA CCC AAT GTC TTT CTC ATT TCA GTG TTT TCC ACC (SEQ ID NO: 40)

MenB3.11_LB: GGA GAG TTA ATT ATT AAC TTA ATT CAA (SEQ ID NO: 41)

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(7) Detection of *siaD* of *N. meningitidis* serogroup C

In a sixth embodiment of the present invention, a LAMP primer set is designed by focusing on the nucleotide sequence region of *siaD* of *N. meningitidis* serogroup C, so as to specifically detect *N. meningitidis* serogroup C.

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An example of the nucleotide sequence region of *siaD* of *N. meningitidis* serogroup C is shown in SEQ ID NO: 71. Herein, the nucleotide sequence region may be either a region corresponding to the entire *siaD* of *N. meningitidis* serogroup C or a region corresponding to a portion thereof. Thus, it is not limited.

The location of each primer in the nucleotide sequence region of *siaD* of *N.*

20 *meningitidis* serogroup C is shown in Table 15.

Table 15. LAMP primer location in *siaD* gene of *N. meningitidis* serogroup C

Primer location	Outer primer	Inner primer				Outer primer	Loop primer	
		FIP		BIP			LF	LB
	F3	F2	F1	B1	B2	B3		
MenC	374-392	400-417	442-465	493-517	556-576	578-598	426-441	522-543

The term "MenC" in Table 15 means LAMP primer set for detecting *siaD* gene of *N. meningitidis* serogroup C.

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The numbers described in Table 15 indicate the position of a target region in the nucleotide sequence as shown in SEQ ID NO: 71 for designing the FIP, BIP, F3, B3, LF and LB primers. For example, F3 primer in MenC primer set is preferably designed from the region of 374-392 in the nucleotide sequence as shown in SEQ ID NO: 71, and the B3 primer in MenC primer set is preferably designed from the region of 578-598 in the nucleotide sequence as shown in SEQ ID NO: 71.

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In the sixth embodiment of the present invention, at least one type of (preferably at least two types of, more preferably at least four types of, and further preferably six types of) LAMP primer comprised in the LAMP primer set consists of a nucleotide sequence identical to or complementary to the sequence of a portion (partial sequence) in the nucleotide sequence of the aforementioned *siaD* gene of *N. meningitidis* serogroup C. Further, in the sixth embodiment of the present invention, the LAMP primer set for detecting *siaD* derived from *N. meningitidis* serogroup C comprises each LAMP primer designed preferably from the nucleotide sequence region as shown in SEQ ID NO: 71 in the aforementioned *siaD* of *N. meningitidis* serogroup C. In the case of using such a LAMP primer set, the LAMP primer set is excellent in terms of detection sensitivity and detection promptness, as well as specificity, in detection of *siaD* derived from *N. meningitidis* serogroup C. Moreover, in the case of the LAMP primer set, linearity is observed in an amplification curve, and good quantitative performance can also be obtained. In the present invention, an LF primer and/or an LB primer may be designed and used as loop primer(s).

With regard to a LAMP primer set of MenC, the nucleotide sequences of the primers are shown in Table 16.

Table 16. LAMP primer set of MenC for amplification of *siaD* for identification of *N. meningitidis* serogroup C (5'-3')

MenC_F3: TGC TCT TCA ATT AAA GCG G (SEQ ID NO: 42)

MenC_B3: GGT AAC AAT TAA TCC CCG TCT (SEQ ID NO: 43)

MenC_FIP: CCT ACT ACC CAA TGT CTG TCA ATT TTG TTG GGC TGT ATG GTG (SEQ ID NO: 44)

MenC_BIP: AGT CGA TGT CAG TCC AAT AAT TCC TGT AGT GAT TAA TGA ACC CCC T (SEQ ID NO: 45)

MenC_LF: GGG CAA ATC GTG ATT G (SEQ ID NO: 46)

MenC_LB: GGG TTG TTA AAT AAA TTA GTG G (SEQ ID NO: 47)

(8) Detection of *synG* of *N. meningitidis* serogroup W-135

In a seventh embodiment of the present invention, a LAMP primer set is designed by focusing on the nucleotide sequence region of *synG* of *N. meningitidis* serogroup W-135, so as to specifically detect *N. meningitidis* serogroup W-135.

An example of the nucleotide sequence region of *synG* of *N. meningitidis* serogroup W-135 is shown in SEQ ID NO: 72. Herein, the nucleotide sequence region may be either a region corresponding to the entire *synG* of *N. meningitidis* serogroup W-135 or a region corresponding to a portion thereof. Thus, it is not limited.

5 The location of each primer in the nucleotide sequence region of *synG* of *N. meningitidis* serogroup W-135 is shown in Table 17.

Table 17. LAMP primer location in *synG* gene of *N. meningitidis* serogroup W-135

Primer location	Outer primer	Inner primer				Outer primer	Loop primer	
		FIP		BIP			LF	LB
	F3	F2	F1	B1	B2	B3		
MenW3	745-768	773-791	821-844	864-887	913-933	946-967	792-814	890-905

10 The term "MenW3" in Table 17 means LAMP primer set for detecting *synG* gene of *N. meningitidis* serogroup W-135. The present inventors designed "MenW3.22" primer set based on MenW3 primer set. Either primer set may be used for detecting *synG* gene of *N. meningitidis* serogroup W-135. F3 and BIP primers of the primer set of MenW3.22 are same as that of MenW3 and B3, FIP and LF primers of the primer set of MenW3.22 is
 15 designed based on that of MenW3 (the modified bases are underlined in Table 18-2).

The numbers described in Table 17 indicate the position of a target region in the nucleotide sequence as shown in SEQ ID NO: 72 for designing the FIP, BIP, F3, B3, LF and LB primers. For example, F3 primer in Men W3 primer set is preferably designed from the region of 745-768 in the nucleotide sequence as shown in SEQ ID NO: 72, and
 20 the B3 primer in MenW3 primer set is preferably designed from the region of 946-967 in the nucleotide sequence as shown in SEQ ID NO: 72.

In the seventh embodiment of the present invention, at least one type of (preferably at least two types of, more preferably at least four types of, and further preferably six types of) LAMP primer comprised in the LAMP primer set consists of a nucleotide sequence
 25 identical to or complementary to the sequence of a portion (partial sequence) in the nucleotide sequence of the aforementioned *synG* gene of *N. meningitidis* serogroup W-135. Further, in the seventh embodiment of the present invention, the LAMP primer set for detecting *synG* derived from *N. meningitidis* serogroup W-135 comprises each LAMP primer designed preferably from the nucleotide sequence region as shown in SEQ ID NO: 72 in the aforementioned *synG* of *N. meningitidis* serogroup W-135. In the case of using
 30 such a LAMP primer set, the LAMP primer set is excellent in terms of detection

sensitivity and detection promptness, as well as specificity, in detection of *synG* derived from *N. meningitidis* serogroup W-135. Moreover, in the case of the LAMP primer set, linearity is observed in an amplification curve, and good quantitative performance can also be obtained. In the present invention, an LF primer and/or an LB primer may be designed and used as loop primer(s).

With regard to LAMP primer sets of MenW3 and MenW3.22, the nucleotide sequences of the primers are shown in Tables 18-1 and 18-2, respectively.

Table 18-1. LAMP primer set of MenW3 for amplification of *synG* for identification of *N. meningitidis* serogroup W-135 (5'-3')

MenW3_F3: GAC AAT AAG TTA CAA AAC CGT ATC (SEQ ID NO: 48)

MenW3_B3: CAC CAG TTT TAA AAA CAC AAC C (SEQ ID NO: 49)

MenW3_FIP: CCT CAC TTT CTG ATG TCA TGA TCA TCA AAG GTG AAT CTT CCG A (SEQ ID NO: 50)

MenW3_BIP: GGA AGG CAT GGT GTA TGA TAT TCC GTT ACT GTA ATC ATT CGC TCC (SEQ ID NO: 51)

MenW3_LF: CTG TAT TTT CAT AAA TTT CCT GC (SEQ ID NO: 52)

MenW3_LB: TCG TTG TAT ATG ATT T (SEQ ID NO: 53)

Table 18-2. LAMP primer set of MenW3.22 for amplification of *synG* for identification of *N. meningitidis* serogroup W-135 (5'-3')

MenW3.22_F3: GAC AAT AAG TTA CAA AAC CGT ATC (SEQ ID NO: 48)

MenW3.22_B3: TCA CCA GTT TTA AAA ACA CAA CC (SEQ ID NO: 75)

MenW3.22_FIP: CTC ACT TTC TGA TGT CAT GAT CAG GTT ATT CAA AGG TGA ATC TTC CGA (SEQ ID NO: 76)

MenW3.22_BIP: GGA AGG CAT GGT GTA TGA TAT TCC GTT ACT GTA ATC ATT CGC TCC (SEQ ID NO: 51)

MenW3.22_LF: TCT GTA TTT TCA TAA ATT TCC TGC (SEQ ID NO: 77)

(9) Detection of *ctrA* of *N. meningitidis* serogroup X

In a eighth embodiment of the present invention, a LAMP primer set is designed by focusing on the nucleotide sequence region of *ctrA* of *N. meningitidis* serogroup X, so as to specifically detect *N. meningitidis* serogroup X.

An example of the nucleotide sequence region of *ctrA* of *N. meningitidis* serogroup X is shown in SEQ ID NO: 73. Herein, the nucleotide sequence region may be either a region corresponding to the entire *ctrA* of *N. meningitidis* serogroup X or a region corresponding to a portion thereof. Thus, it is not limited.

- 5 The location of each primer in the nucleotide sequence region of *ctrA* of *N. meningitidis* serogroup X is shown in Table 19.

Table 19. LAMP primer location in *ctrA* gene of *N. meningitidis* serogroup X

Primer location	Outer primer	Inner primer				Outer primer	Loop primer	
		FIP		BIP			LF	LB
	F3	F2	F1	B1	B2	B3		
MenX	68-86	105-125	154-178	179-201	231-252	273-296	126-149	209-229

- 10 The term “MenX” in Table 19 means LAMP primer set for detecting *ctrA* gene of *N. meningitidis* serogroup X.

The numbers described in Table 19 indicate the position of a target region in the nucleotide sequence as shown in SEQ ID NO: 73 for designing the FIP, BIP, F3, B3, LF and LB primers. For example, F3 primer in MenX primer set is preferably designed from the region of 68-86 in the nucleotide sequence as shown in SEQ ID NO: 73, and the B3 primer in MenX primer set is preferably designed from the region of 273-296 in the nucleotide sequence as shown in SEQ ID NO: 73.

- In the eighth embodiment of the present invention, at least one type of (preferably at least two types of, more preferably at least four types of, and further preferably six types of) LAMP primer comprised in the LAMP primer set consists of a nucleotide sequence identical to or complementary to the sequence of a portion (partial sequence) in the nucleotide sequence of the aforementioned *ctrA* gene of *N. meningitidis* serogroup X. Further, in the eighth embodiment of the present invention, the LAMP primer set for detecting *ctrA* derived from *N. meningitidis* serogroup X comprises each LAMP primer designed preferably from the nucleotide sequence region as shown in SEQ ID NO: 73 in the aforementioned *ctrA* of *N. meningitidis* serogroup X. In the case of using such a LAMP primer set, the LAMP primer set is excellent in terms of detection sensitivity and detection promptness, as well as specificity, in detection of *ctrA* derived from *N. meningitidis* serogroup X. Moreover, in the case of the LAMP primer set, linearity is observed in an amplification curve, and good quantitative performance can also be

obtained. In the present invention, an LF primer and/or an LB primer may be designed and used as loop primer(s).

With regard to a LAMP primer set of MenX, the nucleotide sequences of the primers are shown in Table 20.

5

Table 20. LAMP primer set of MenX for amplification of *ctrA* for identification of *N. meningitidis* serogroup X (5'-3')

MenX_F3: GCC TTA TAC AAA GAC TGC G (SEQ ID NO: 54)

MenX_B3: AAT AGG GGA TAG ATA ATT AGA GGT (SEQ ID NO: 55)

10 MenX_FIP: GCC GAG TGC TAA GAA AGT AGA ATC TCA ATC AAT TCC ACT
TCA GGG A (SEQ ID NO: 56)

MenX_BIP: CCT GTT GTT GGC AAA GAA CTA CAA AAT GCA AAT TCA ATT
GGT TGG (SEQ ID NO: 57)

MenX_LF: GTC AGG TAT CTT CTG AAA CTC AAA (SEQ ID NO: 58)

15 MenX_LB: ACC ATT GTA GCG GTC ATA AGT (SEQ ID NO: 59)

(10) Detection of *synF* of *N. meningitidis* serogroup Y

In a ninth embodiment of the present invention, a LAMP primer set is designed by focusing on the nucleotide sequence region of *synF* of *N. meningitidis* serogroup Y, so as
20 to specifically detect *N. meningitidis* serogroup Y.

An example of the nucleotide sequence region of *synF* of *N. meningitidis* serogroup Y is shown in SEQ ID NO: 74. Herein, the nucleotide sequence region may be either a region corresponding to the entire *synF* of *N. meningitidis* serogroup Y or a region corresponding to a portion thereof. Thus, it is not limited.

25 The location of each primer in the nucleotide sequence region of *synF* of *N. meningitidis* serogroup Y is shown in Table 21.

Table 21. LAMP primer location in *synF* gene of *N. meningitidis* serogroup Y

Primer location	Outer primer	Inner primer				Outer primer	Loop primer	
		FIP		BIP			LF	LB
	F3	F2	F1	B1	B2	B3		
MenY5.2	96-111	129-149	177-199	204-229	272-289	290-309	152-173	241-264

30 The term "MenY5.2" in Table 21 means LAMP primer set for detecting *synF* gene of *N. meningitidis* serogroup Y. The present inventors designed "MenY5.2-mod" primer

set based on MenY5.2 primer set. Either primer set may be used for detecting *synF* gene of *N. meningitidis* serogroup Y. F3, B3, BIP, LF and LB primers of the primer set of MenY5.2-mod are same as that of MenY5.2 and FIP primer of the primer set of MenY5.2-mod is designed based on that of MenY5.2 (the modified base is underlined in Table 22-2).

5 MenY5.2-mod primer set shows improved sensitivity compared to MenY5.2 primer set.

The numbers described in Table 21 indicate the position of a target region in the nucleotide sequence as shown in SEQ ID NO: 74 for designing the FIP, BIP, F3, B3, LF and LB primers. For example, F3 primer in MenY5.2 primer set is preferably designed from the region of 96-111 in the nucleotide sequence as shown in SEQ ID NO: 74, and the
10 B3 primer in MenY5.2 primer set is preferably designed from the region of 290-309 in the nucleotide sequence as shown in SEQ ID NO: 74.

In the ninth embodiment of the present invention, at least one type of (preferably at least two types of, more preferably at least four types of, and further preferably six types of) LAMP primer comprised in the LAMP primer set consists of a nucleotide sequence
15 identical to or complementary to the sequence of a portion (partial sequence) in the nucleotide sequence of the aforementioned *synF* gene of *N. meningitidis* serogroup Y. Further, in the ninth embodiment of the present invention, the LAMP primer set for detecting *synF* derived from *N. meningitidis* serogroup Y comprises each LAMP primer designed preferably from the nucleotide sequence region as shown in SEQ ID NO: 74 in
20 the aforementioned *synF* of *N. meningitidis* serogroup Y. In the case of using such a LAMP primer set, the LAMP primer set is excellent in terms of detection sensitivity and detection promptness, as well as specificity, in detection of *synF* derived from *N. meningitidis* serogroup Y. Moreover, in the case of the LAMP primer set, linearity is observed in an amplification curve, and good quantitative performance can also be
25 obtained. In the present invention, an LF primer and/or an LB primer may be designed and used as loop primer(s).

With regard to LAMP primer sets of MenY5.2 and MenY5.2-mod, the nucleotide sequences of the primers are shown in Tables 22-1 and 22-2, respectively.

30 Table 22-1. LAMP primer set of MenY5.2 for amplification of *synF* for identification of *N. meningitidis* serogroup Y (5'-3')

MenY5.2_F3: TGT CAA AAC CTC CAG C (SEQ ID NO: 60)

MenY5.2_B3: CGC TAA ACG ATA CAT TTC CA (SEQ ID NO: 61)

MenY5.2_FIP: CGG GTT TGA AGA ATT GTT GAT GGT GAC ATT CCA GAA AAT
GTT AG (SEQ ID NO: 62)

MenY5.2_BIP: CAC TGC CCA CTA TAA GCA TGT TTT GAG TTG AAG AGGATG
AGT GA (SEQ ID NO: 63)

5 MenY5.2_LF: GAA TAA AAA GGA ATA TTT CGG C (SEQ ID NO: 64)

MenY5.2_LB: TCT TTA TTA TCT GAA GAA GAT AGC (SEQ ID NO: 65)

Table 22-2. LAMP primer set of MenY5.2-mod for amplification of *synF* for
identification of *N. meningitidis* serogroup Y (5'-3')

10 MenY5.2_F3: TGT CAA AAC CTC CAG C (SEQ ID NO: 60)

MenY5.2_B3: CGC TAA ACG ATA CAT TTC CA (SEQ ID NO: 61)

MenY5.2_FIP: CGG GTT TGA AGA ATT GTT GAT GGT GAC TTT CCA GAA AAT
GTT AG (SEQ ID NO: 78)

MenY5.2_BIP: CAC TGC CCA CTA TAA GCA TGT TTT GAG TTG AAG AGGATG
15 AGT GA (SEQ ID NO: 63)

MenY5.2_LF: GAA TAA AAA GGA ATA TTT CGG C (SEQ ID NO: 64)

MenY5.2_LB: TCT TTA TTA TCT GAA GAA GAT AGC (SEQ ID NO: 65)

(11) Preparation of LAMP primers, detection operations, etc.

20 The LAMP primers used in the present invention can be prepared by chemical
synthesis using a DNA automatic synthesizer, for example. In the present invention, each
LAMP primer means an oligonucleotide, which has a certain nucleotide sequence as
described above, is capable of forming a base pair with another nucleotide, and comprises
an --OH group acting as a base point of complementary strand synthesis at the 3' end
25 thereof. Thus, as far as such conditions are satisfied, the backbone thereof is not
necessarily limited to that due to a phosphodiester bond. For example, it may be a
phosphothioate form that has not P but S as a backbone or a peptide nucleic acid based on
a peptide bond.

In the present invention, the type of a template-dependent nucleic acid synthase
30 that can be used in the LAMP method is not particularly limited, as long as it has strand
displacement activity. Examples of such an enzyme include *Bst* DNA polymerase (large
fragment), *Bca*(exo-) DNA polymerase, *E. coli* DNA polymerase I Klenow fragment,
Vent(Exo-) DNA polymerase (obtained by eliminating exonuclease activity from Vent
DNA polymerase), DeepVent(Exo-) DNA polymerase (obtained by eliminating

exonuclease activity from DeepVent DNA polymerase), and KOD DNA polymerase. Of these, *Bst* DNA polymerase (large fragment) is preferable. When such *Bst* DNA polymerase is used, the reaction is preferably carried out at a reaction optimum temperature that is between approximately 60°C and 67°C.

Moreover, known techniques can be applied to detect an amplification product. For example, a labeled oligonucleotide that specifically recognizes an amplified gene sequence is used, or an amplification product can easily be detected also by directly subjecting a reaction solution obtained after termination of the reaction to agarose gel electrophoresis. Furthermore, a LAMP primer used in the present invention may also be allowed to bind to a solid phase, as with a DNA chip. When such a solid phase primer is used as a synthesis initiation point, a nucleic acid synthesis reaction product is captured by a solid phase, and thus, separation and detection can be easily carried out.

Further, since an amplification reaction is efficiency carried out at an accelerated rate by the LAMP method, amplification can be confirmed by previously adding into a reaction solution, an intercalator specifically incorporated into a molecule of double-stranded nucleic acid, such as ethidium bromide or SYBR® Green I. A fluorescent labeling agent such as calcein can also be used for detecting amplified products under UV light irradiation. Still further, in the LAMP method, a large amount of substrate is consumed by the synthesis of nucleic acid, and pyrophosphoric acid generated as a by-product reacts with co-existing magnesium, so that it is converted to magnesium pyrophosphate. As a result, the reaction solution becomes clouded to such an extent that it can be confirmed by naked eye. Such a clouded state is observed after completion of the reaction, or an increase in such turbidity is observed over time (in real time) from initiation of the reaction, so that amplification can be confirmed. When the clouded state is confirmed over time, a change in absorbance at 650 nm may be observed using an optical measurement apparatus (e.g. a spectrophotometer, etc.), for example. In addition, according to such a method of confirming the clouded state over time, it is also possible to quantify the amount of chromosomal DNA (the amount of template DNA) derived from each *N. meningitidis* gene in a test sample.

Detection Kit

Various types of reagents necessary for an amplification reaction by the LAMP method have previously been packaged, and thus they can be provided in the form of a kit for detecting various genes of *N. meningitidis*. Specifically, the kit of the present invention

includes the aforementioned LAMP primer set used in detection of various genes of *N. meningitidis*. In addition to such a LAMP primer set, the kit of the present invention may also comprise, as necessary, reagents necessary for detection of a synthetic reaction product, such as dNTP used as a substrate for the synthesis of a complementary strand,
 5 DNA polymerase used in the synthesis of a strand-displacement-type complementary strand, and a buffer solution that provides conditions preferable for an enzyme reaction. Moreover, the present kit may further comprise a reagent (e.g. betaine, etc.) for destabilizing the double strand of nucleic acid.

Hereafter, the present invention will be more specifically described in the
 10 following examples. However, these examples are not intended to limit the scope of the present invention.

15 EXAMPLES

Example 1: Reducing non-specific amplification reaction of LAMP

In LAMP assay, some primer sets can cause non-specific amplification reaction because of interactions between primers or interactions in each primer. Non-specific amplification reaction in LAMP assay using ctrA4 primer set is shown in Table 23-1.

20 Table 23-1. ctrA4 non-specific primer test (65°C for 90 minutes)

	ALL	F3(-)	B3(-)	FIP(-)	BIP(-)	LF(-)
1	58.1	0	0	0	0	0
2	87.4	0	0	0	84	0
3	0	61.5	0	0	0	75.8
4	78.4	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	76.2	0
7	0	0	66.4	0	0	0

Table 23-1 shows a comparison of non-specific amplification reaction time with removing primers. For example, "ALL" in Table 23-1 means an amplification reaction
 25 with all primers, and "F3(-)" in Table 23 means an amplification reaction without F3 primer only. There are some non-specific amplification reactions at 65°C within 90 minutes for using ctrA4 primer set. The primer set is left at room temperature for 30 minutes before the reaction in order to facilitate an observation of non-specific

amplifications under a severe condition. Therefore, the present inventors modified FIP of ctrA4 primer set and replaced LF of ctrA4 primer set with LB. The modified primer set named as ctrA4-mod2 primer set. There is no non-specific amplification reaction at 67°C within 90 minutes for using ctrA4-mod2 primer set.

- 5 Non-specific amplification reaction in LAMP assay using MenW3 primer set is also examined (See Table 23-2).

Table 23-2. MenW3 non-specific primer test (63°C for 90 minutes)

	ALL	F3(-)	B3(-)	FIP(-)	BIP(-)	LF(-)	LB(-)
1	40.5	0	0	0	0	0	0
2	0	0	0	0	82.6	0	59.1
3	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0
5	0	0	0	0	97.5	0	0

- 10 Table 23-2 shows a comparison of non-specific amplification reaction time with removing primers. There are some non-specific amplification reactions at 65°C within 90 minutes for using MenW3 primer set. The primer set is left at room temperature for 30 minutes before the reaction in order to facilitate an observation of non-specific amplifications under a severe condition. Therefore, the present inventors modified B3, FIP
 15 and LF of MenW3 primer set. The modified primer set named as MenW3.22 primer set. There is no non-specific amplification reaction at 64°C within 90 minutes for using MenW3.22 primer set.

Example 2: Specificity confirmation test

- 20 The detection method of the present invention was carried out and the specificity was confirmed using the above primer sets. The details are described below.

(1) Preparation of Chromosomal DNA

- 25 First, chromosomal DNA was purified from various types of strains that were to be subjected to a test, and DNA used as a template of an amplification reaction was prepared. Such chromosomal DNA was obtained by extracting it from various types of strains, and then purifying it. Extraction and purification operations were carried out in accordance with manuals included with the kits used.

In the present test, chromosomal DNAs were extracted from total 15 strains (6 serogroups of *N. meningitidis* and 9 strains other than *N. meningitidis*), and they were used in the specificity confirmation test. The 15 strains are shown in Fig. 1.

5 (2) LAMP Reaction

The LAMP primer sets (crtA4, crtA4-mod2, crgA, crgA(816), IS1106, IS1106(68-1075), MenA3, MenB3.11, MenC, MenW3, MenW3.22, MenX, MenY5.2 and MenY5.2-mod) were used, and a LAMP reaction was carried out using, as a template, the chromosomal DNA derived from various types of strains purified in the above.

10 The reaction mixture 25 µl containing 1.6 µM concentrations (each) of FIP and BIP primers, 0.2 µM concentrations (each) of F3 and B3 primers, 0.4 µM LF, 8 U of *Bst* DNA polymerase large fragment (New England Biolabs, Ipswich, MA), 1.4 mM deoxyribonucleoside triphosphates, 0.8 M betaine (Sigma, St. Louis, MO), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄, 0.1% Tween 20, and
15 template DNA 2 µl. The reaction mixture was incubated at optimum temperature of each primer set (i.e., 65°C or 67°C) for 60 minutes and then heated at 80°C for 5 minutes to terminate the reaction.

The LAMP reaction product (LAMP amplicon) will be directly detected with the naked eyes by adding 0.1% SYBR Green I to the reaction mixture. The solution will turn
20 green in the presence of a LAMP amplicon, while it remains orange with no amplicon. The LAMP amplicon will be further confirmed by gel electrophoresis in 2% agarose gel.

(3) Confirmation of presence or absence of amplification

The presence or absence of amplification was detected by directly looking at the
25 reaction tube by naked eye and observing the presence or absence of cloudiness of the LAMP reaction solution. That is to say, magnesium pyrophosphate is generated as a by-product of the reaction in an amount proportional to the amount of a replication sequence when such a replication sequence is present, and as a result, the LAMP reaction solution becomes clouded. On the other hand, when such a replication sequence is not present, the
30 LAMP reaction solution remains transparent. Thus, an amplification product was detected using such cloudiness as an indicator.

Moreover, the presence or absence of amplification was also confirmed by subjecting the amplification product to agarose gel electrophoresis (2% agarose gel;

ethidium bromide staining). As a result, a replication sequence appeared as a ladder-like pattern characteristic for the LAMP reaction (not shown in figures).

(4) Test results

5 The results of the aforementioned test are shown in Fig 1. With regard to the results, a case where amplification (cloudiness) was confirmed by visual observation after incubation for 60 minutes was expressed with the symbol “+,” and a case where such amplification was not confirmed after such incubation for 60 minutes was expressed with the symbol “-.”

10 As a result, in the case of using the LAMP primer sets of crtA4, crtA4-mod2, crgA, crgA(816), IS1106, and IS1106(68-1075), a large amount of amplification product was confirmed only when chromosomal DNAs of six serogroups (A, B, C, X, Y and W-135) of *N. meningitidis* were used as a template. In contrast, in the case of using other types of strains, no amplification products were confirmed.

15 In the case of using the LAMP primer set MenA3, a large amount of amplification product was confirmed only when chromosomal DNA derived *N. meningitidis* serogroup A was used as a template. In contrast, in the case of using other types of strains, no amplification products were confirmed.

20 In the case of using the LAMP primer set MenB3.11, a large amount of amplification product was confirmed only when chromosomal DNA derived *N. meningitidis* serogroup B was used as a template. In contrast, in the case of using other types of strains, no amplification products were confirmed.

25 In the case of using the LAMP primer set MenC, a large amount of amplification product was confirmed only when chromosomal DNA derived *N. meningitidis* serogroup C was used as a template. In contrast, in the case of using other types of strains, no amplification products were confirmed.

30 In the cases of using the LAMP primer set MenW3 and MenW3.22, a large amount of amplification product was confirmed only when chromosomal DNA derived *N. meningitidis* serogroup W was used as a template. In contrast, in the case of using other types of strains, no amplification products were confirmed.

 In the case of using the LAMP primer set MenX, a large amount of amplification product was confirmed only when chromosomal DNA derived *N. meningitidis* serogroup X was used as a template. In contrast, in the case of using other types of strains, no amplification products were confirmed.

In the cases of using the LAMP primer set MenY5.2 and MenY5.2-mod, a large amount of amplification product was confirmed only when chromosomal DNA derived *N. meningitidis* serogroup Y was used as a template. In contrast, in the case of using other types of strains, no amplification products were confirmed.

- 5 From the result, it was confirmed that the method of detecting *N. meningitidis* of the present invention is excellent in terms of specificity.

Example 3: Sensitivity confirmation test

Detection sensitivity obtained using the above LAMP primer sets was confirmed.

- 10 The details are described below.

(1) Preparation of chromosomal DNA

- 15 In the present test, chromosomal DNA was purified from six serogroups (A, B, C, X, Y and W-135) of *N. meningitidis* by the same method as that described in Example 1 above. The purified chromosomal DNA was used as a template. The concentration of template DNA in the reaction solution (copy number) was quantified at a molecular size of 2.2 Mbp.

(2) LAMP method and PCR method

- 20 The template DNA solution quantified in (1) above was repeatedly diluted by a factor of 10, so as to prepare solutions diluted by a factor of 1 to 1,000,000. The prepared solutions were used as template DNA solutions in the LAMP reaction, so that a detection limit was confirmed. On the other hand, as a negative control, the detection limit of a solution with a template DNA concentration of 0 was also examined. In terms of the
- 25 additive amount of a template DNA solution and the additive amounts of other additives, the same LAMP reaction solution as that used in the specificity confirmation test of Example 2 was used, with the exception that the concentration of the template DNA solution was different. Moreover, the LAMP reaction was promoted by incubating the reaction solution at optimum temperature of each primer set (i.e. 63°C or 67°C) for 60
- 30 minutes, and finally, the solution was heated at 80°C for 5 minutes so as to terminate the reaction.

(3) Confirmation of presence or absence of amplification

With regard to the presence or absence of amplification by the LAMP reaction, turbidity was measured over time and when the turbidity became 0.1 or greater, it was determined that an amplification product was confirmed.

Furthermore, as in the case of the specificity confirmation test of Example 2, the presence or absence of amplification was also confirmed by visual observation and 2% agarose gel electrophoresis (not shown in figures).

(4) Test results

With regard to test results, a case where an amplification product was confirmed was expressed with the symbol "+," and a case where such an amplification product was not confirmed was expressed with the symbol "-." The test results are shown in the following Tables 24-27.

Table 24. Comparison of sensitivity of PCR and LAMP test of *ctrA* of *N. meningitidis*

Nm serogroup (ctrA)	Assay	Detection with <i>N. meningitidis</i> genome copy number per reaction									
		10 ⁸	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	0
A	PCR	+	+	+	+	-	-	-	-	-	-
	LAMP	+	+	+	+	+	+	+	-	-	-
B	PCR	+	+	+	+	+	-	-	-	-	-
	LAMP	+	+	+	+	+	+	+	+	+	-
C	PCR	+	+	+	+	-	-	-	-	-	-
	LAMP	+	+	+	+	+	+	+	+	-	-
X	PCR	+	+	+	+	+	-	-	-	-	-
	LAMP	+	+	+	+	+	+	+	+	-	-
Y	PCR	+	+	+	+	-	-	-	-	-	-
	LAMP	+	+	+	+	+	+	+	+	+	-
W	PCR	+	+	+	+	+	-	-	-	-	-
	LAMP	+	+	+	+	+	+	+	+	-	-

As shown in Table 24, in all cases of using LAMP primer sets ctrA4-mod2, more than 10² copies of template DNA could be detected by performing the LAMP reaction for 60 minutes. Among others, serogroup B and serogroup Y were able to detect 10⁰ copies of template DNA. Thus, their sensitivity was particularly high.

PCR amplification of *ctrA* gene has detection limit of up to 10⁴ genome copies per reaction in all serogroups, while the LAMP reaction showed positive results when the 10⁰ copies of genome were used.

Table 25. Comparison of sensitivity of PCR and LAMP test of *crgA* of *N. meningitidis*

Gene	sensitivity for Nm PCR					
	A	B	C	X	Y	W-135
<i>crgA</i>	10 ³	10 ⁴	10 ⁴	10 ⁴	10 ⁴	10 ³

<i>crgA</i>	LAMP sensitivity									
67°C	10 ⁸	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	0
serogroup										
A	+	+	+	+	+	+	-	-	-	-
B	+	+	+	+	+	+	+	+	-	-
C	+	+	+	+	+	+	+	+	-	-
X	+	+	+	+	+	+	+	+	-	-
Y	+	+	+	+	+	+	+	+	-	-
W-135	+	+	+	+	+	+	+	-	-	-

<i>crgA</i> (816)	LAMP sensitivity									
63°C	10 ⁸	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	0
Serogroup										
A	+	+	+	+	+	+	±	-	-	-
B	+	+	+	+	+	+	-	-	-	-
C	+	+	+	+	+	+	+	+	-	-
X	+	+	+	+	+	+	-	-	-	-
Y	+	+	+	+	+	+	+	+	-	-
W-135	+	+	+	+	+	+	+	-	-	-

As shown in Table 25, in all cases of using LAMP primer sets of *crgA* and *crgA*(816), more than 10³ copies of template DNA could be detected by performing the LAMP reaction for 60 minutes. Among others, serogroups B, C, X and Y were able to detect 10¹ copies of template DNA when *crgA* primer set is used and serogroups C and Y were able to detect 10¹ copies of template DNA when *crgA*(816) primer set is used. Thus, their sensitivity was particularly high.

PCR amplification of *crgA* gene has detection limit of up to 10³ genome copies per reaction in all serogroups, while the LAMP reaction showed positive results.

Table 26. Comparison of sensitivity of PCR and LAMP test of IS1106 of *N. meningitidis*

Gene	sensitivity for Nm PCR					
	A	B	C	X	Y	W-135
IS1106	10 ⁴	10 ⁴	10 ⁴	10 ³	10 ⁴	10 ⁴

IS1106	LAMP sensitivity									
65°C	10 ⁸	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	0
serogroup										
A	+	+	+	+	+	+	+	+	-	-
B	+	+	+	+	+	+	+	+	-	-
C	+	+	+	+	+	+	+	+	-	-
X	+	+	+	+	+	+	+	+	±	-
Y	+	+	+	+	+	+	+	+	-	-
W-135	+	+	+	+	+	+	+	+	±	-

IS1106 (68-1075)	LAMP sensitivity									
65°C	10 ⁸	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	0
serogroup										
A	+	+	+	+	+	+	+	+	-	-
B	+	+	+	+	+	+	+	±	-	-
C	+	+	+	+	+	+	+	+	+	-
X	+	+	+	+	+	+	+	+	-	-
Y	+	+	+	+	+	+	+	+	-	-
W-135	+	+	+	+	+	+	+	+	-	-

- 5 As shown in Table 26, in all cases of using LAMP primer sets of IS1106 and IS1106(68-1075), more than 10¹ copies of template DNA could be detected by performing the LAMP reaction for 60 minutes. Among others, serogroups X and W-135 were able to detect 10⁰ copies of template DNA when IS1106 primer set is used and serogroup C was able to detect 10⁰ copies of template DNA when IS1106(68-1075) primer set is used. Thus,
- 10 their sensitivity was particularly high.

PCR amplification of IS1106 gene has detection limit of up to 10³ genome copies per reaction in all serogroups, while the LAMP reaction showed positive results.

Table 27. Sensitivity of LAMP reactions of serogroup discrimination

	LAMP reaction					
	A	B	C	X	W-135	Y
Serogroup A	10 ² copies	(-)	(-)	(-)	(-)	(-)
Serogroup B	(-)	10 ² copies	(-)	(-)	(-)	(-)
Serogroup C	(-)	(-)	10 ² copies	(-)	(-)	(-)
Serogroup X	(-)	(-)	(-)	10 ² copies	(-)	(-)
Serogroup W-135	(-)	(-)	(-)	(-)	10 ² copies	(-)
Serogroup Y	(-)	(-)	(-)	(-)	(-)	10 ² copies

The serogroup specific LAMP reactions reliably showed sensitivity of 100 copies per reaction. Smaller amounts (down to a single or 10 genomes equivalent) were detected in some experiments.

Example 4: Real-time turbidity measurement test

With regard to the LAMP reaction using ctrA4-mod2 LAMP primer set, a real-time turbidity measurement was carried out, and the quantitative performance of template DNA was analyzed.

In the present test, the template DNA concentration per reaction tube was adjusted to 0 to 10⁵ copies, and the LAMP reaction was then carried out using the ctrA4-mod2 primer sets. During the reaction, absorbance at 650 nm was measured.

The results of a real-time turbidity measurement in the case of using the LAMP primer set ctrA4-mod2 are shown in Fig. 2. As shown in Fig. 2, it was confirmed that the turbidity became 0.1 or greater within 60 minutes if the template DNA concentration was 10² copies or more. This result corresponded to the result of the confirmation of the presence or absence of amplification by visual observation and electrophoresis in the sensitivity test of Example 3. Furthermore, it was confirmed that the threshold time (the time required until the turbidity exceeded 0.1) became shorter, as the concentration of the initially used template DNA was increased.

The graph as shown in Fig. 3 shows the relationship between the threshold time (Tt) obtained in the case of using ctrA4-mod2 and the common logarithm of the initial template DNA concentration. A linearity was observed between these factors, and a high

correlation (correlation coefficient $R^2=0.983$) was shown. This means that not only the presence of template DNA but also the concentration thereof can be quantified when the initial concentration of the template DNA is unknown. Even in the case of a sample whose concentration is unknown, for example, if diluted solutions of different dilution magnifications are prepared, a LAMP reaction is then carried out using each diluted solution, and a threshold time is then measured in each LAMP reaction so as to produce a regression line based on such threshold times, the unknown initial concentration of the template DNA can be determined.

10 Example 5: Application of LAMP to the clinical specimens

1,574 CSF specimens from suspected meningitis patients were collected for the presence of *N. meningitidis* (568 CSF specimens collected in Vietnam, 536 CSF samples collected in China, and 470 CSF specimens collected in Korea during 2001-2002). The CSF specimens were collected during a 2-year prospective study of bacterial meningitis in three countries (Anh, Kilgore et al. 2006). A LAMP reaction using a target gene *ctrA* was applied to the specimens. 20 specimens were shown to be *N. meningitidis* positive among selected specimens collected in Vietnam, 5 out of Chinese specimens were positive in *N. meningitidis* LAMP reaction, and 6 were *N. meningitidis* positive among Korean samples (Table 28). Some of the LAMP-positive specimens were positive in *ctrA* PCR also and some of them were negative. All of these specimens will be applied to compare the sensitivity of LAMP and PCR. Three serogroup discrimination LAMP reactions were applied to the *ctrA* LAMP positive specimens. Two different serogroup discrimination methods based on PCR (Taha, Alonso et al. 2005; Fraissier, Stor et al. 2009) were applied to a number of CSF specimens, however, the sensitivity of the PCR methods was not sufficient enough to discriminate the serogroups of the *N. meningitidis* DNA in the CSF specimens. Therefore, no serogroups could be identified by PCR-based methods.

Subsequently, 31 positive specimens from Nm LAMP were examined regarding serogroup using serogroup specific LAMP primer sets. All of the 31 positive specimens did not show positive when a LAMP reaction test targeting *Haemophilus influenzae* type b or *Streptococcus pneumoniae* is conducted. As a result, all of the 31 positive specimens were classified into one serogroup of 6 serogroups (Table 29).

Table 28. Comparison of LAMP and PCR result in 1574 CSF specimens for *N. meningitidis*

Country	LAMP	PCR		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		Positive	Negative				
Vietnam	Positive	15	5	100.0	99.1	75.0	100.0
	Negative	0	548				
China	Positive	5	0	100.0	100.0	100.0	100.0
	Negative	0	531				
Korea	Positive	5	1	100.0	99.8	83.3	100.0
	Negative	0	464				
Total	Positive	25	6	100.0	99.6	80.6	100.0
	Negative	0	1543				

5

Table 29. Serogrouping LAMP Result of Nm LAMP (+) 31 CSF specimens

Total	Hib LAM P	Sp LAM P	Nm LAM P	Serogrouping LAMP Result						
				A	B	C	X	Y	W-135	Non typable
Vietnam (n=568)	0	0	20	4 (20%)*	4 (20%)	2 (10%)	2 (10%)	3 (15%)	4 (20%)	1 (5%)
China (n=536)	0	0	5	0	1 (20%)	1 (20%)	1 (20%)	2 (40%)	0	0
Korea (n=470)	0	0	6	1 (16.7%)	0	0	2 (33.3%)	0	2 (33.3%)	1 (16.7%)
Total	0	0	31	5	5	3	5	5	6	2

*, number (%)

The foregoing description of the embodiments of this invention has been presented for the purposes of illustration and description. It is not intended to be exhaustive or to limit the embodiments of the invention to the form disclosed; modifications and variations are possible. Such modifications and variations that may be apparent to a person skilled in the art are intended to be included within the scope of this invention as defined by the accompanying claims.

15

CLAIMS

1. A method for detecting at least a part of *N. meningitidis* nucleic acids in a sample using loop-mediated isothermal amplification (LAMP), comprising:

5 amplifying a target *N. meningitidis* nucleic acid using a LAMP primer set comprising one or more types of primers each having a nucleotide sequence that is identical to or complementary to at least a part of *N. meningitidis* nucleic acid; and detecting the amplified product

10 2. The method of claim 1, wherein the target *N. meningitidis* nucleic acid encodes an *N. meningitidis* gene selected from the group consisting of: *ctrA* (*N. meningitidis*), *crgA* (*N. meningitidis*), IS1106 (*N. meningitidis*), *sacB* (*N. meningitidis* serogroup A), *siaD* (*N. meningitidis* serogroup B), *siaD* (*N. meningitidis* serogroup C), *synG* (*N. meningitidis* serogroup W-135), *ctrA* (*N. meningitidis* serogroup X), and *synF* (*N. meningitidis* serogroup Y).

3. The method of claim 1, wherein the LAMP primer set is selected from the group consisting of:

SEQ ID Nos. 1-4; 1-5; 1-4 and 6; 1-4 and 8; 1-5 and 8; 1-6;

20 SEQ ID Nos. 1, 2, 4 and 7; 1, 2, 4, 5 and 7; 1, 2, 4, 6 and 7; 1, 2, 4, 7 and 8; 1, 2, 4, 5, 7 and 8;

SEQ ID Nos. 9-12; 9-13; 9-12 and 14; 9-14;

SEQ ID Nos. 15-18; 15-19;

SEQ ID Nos. 20-23; 20-24; 20-23 and 25; 20-25;

25 SEQ ID Nos. 26-29; 26-30;

SEQ ID Nos. 31-34; 31-35; 31-34 and 36; 31-36;

SEQ ID Nos. 37-40; 37-41;

SEQ ID Nos. 42-45; 42-46; 42-45 and 47; 42-47;

SEQ ID Nos. 48-51; 48-52; 48-51 and 53; 48-53;

30 SEQ ID Nos. 48, 51, 75 and 76; 48, 51, and 75-77; 48, 51, 53 and 75 and 76; 48, 51, 53 and 75-77;

SEQ ID Nos. 54-57; 54-58; 54-57 and 59; 54-59;

SEQ ID Nos. 60-63; 60-64; 60-63 and 65; 60-65;

SEQ ID Nos. 60, 61, 63 and 78; 60, 61, 63, 64 and 78; 60, 61, 63, 65 and 78; 60, 61, 63-65 and 78; and

a combination of any two or more of these primer sets.

- 5 4. The method of claim 1, wherein the LAMP primer set is SEQ ID Nos. 1-4; 1-5; 1-4 and 6; 1-4 and 8; 1-5 and 8; 1-6; 1, 2, 4 and 7; 1, 2, 4, 5 and 7; 1, 2, 4, 6 and 7; 1, 2, 4, 7 and 8; or 1, 2, 4, 5, 7 and 8, and the target *N. meningitidis* nucleic acid encodes *ctrA* (*N. meningitidis*).
- 10 5. The method of claim 1, wherein the LAMP primer set is SEQ ID Nos. 9-12; 9-13; 9-12 and 14; 9-14; 15-18; or 15-19, and the target *N. meningitidis* nucleic acid encodes *crgA* (*N. meningitidis*).
- 15 6. The method of claim 1, wherein the LAMP primer set is SEQ ID Nos. 20-23; 20-24; 20-23 and 25; 20-25; 26-29; or 26-30, and the target *N. meningitidis* nucleic acid encodes IS1106 (*N. meningitidis*).
- 20 7. The method of claim 1, wherein the LAMP primer set is SEQ ID Nos. 31-34; 31-35; 31-34 and 36; or 31-36, and the target *N. meningitidis* nucleic acid encodes *sacB* (*N. meningitidis* serogroup A).
- 25 8. The method of claim 1, wherein the LAMP primer set is SEQ ID Nos. 37-40; or 37-41, and the target *N. meningitidis* nucleic acid encodes *siaD* (*N. meningitidis* serogroup B).
9. The method of claim 1, wherein the LAMP primer set is SEQ ID Nos. 42-45; 42-46; 42-45 and 47; or 42-47, and the target *N. meningitidis* nucleic acid encodes *siaD* (*N. meningitidis* serogroup C).
- 30 10. The method of claim 1, wherein the LAMP primer set is SEQ ID Nos. 48-51; 48-52; 48-51 and 53; 48-53; 48, 51, 75 and 76; 48, 51, and 75-77; 48, 51, 53 and 75 and 76; or 48, 51, 53 and 75-77, and the target *N. meningitidis* nucleic acid encodes *synG* (*N. meningitidis* serogroup W-135).

11. The method of claim 1, wherein the LAMP primer set is SEQ ID Nos. 54-57; 54-58; 54-57 and 59; or 54-59, and the target *N. meningitidis* nucleic acid encodes *ctrA* (*N. meningitidis* serogroup X).

5 12. The method of claim 1, wherein the LAMP primer set is SEQ ID Nos. 60-63; 60-64; 60-63 and 65; 60-65; 60, 61, 63 and 78; 60, 61, 63, 64 and 78; 60, 61, 63, 65 and 78; or 60, 61, 63-65 and 78, and the target *N. meningitidis* nucleic acid encodes *synF* (*N. meningitidis* serogroup Y).

10 13. A primer set for detection of *N. meningitidis* using loop-mediated isothermal amplification comprising a LAMP primer set selected from the group consisting of:

SEQ ID Nos. 1-4; 1-5; 1-4 and 6; 1-4 and 8; 1-5 and 8; 1-6;

SEQ ID Nos. 1, 2, 4 and 7; 1, 2, 4, 5 and 7; 1, 2, 4, 6 and 7; 1, 2, 4, 7 and 8; 1, 2, 4, 5, 7 and 8;

15 SEQ ID Nos. 9-12; 9-13; 9-12 and 14; 9-14;

SEQ ID Nos. 15-18; 15-19;

SEQ ID Nos. 20-23; 20-24; 20-23 and 25; 20-25;

SEQ ID Nos. 26-29; 26-30;

SEQ ID Nos. 31-34; 31-35; 31-34 and 36; 31-36;

20 SEQ ID Nos. 37-40; 37-41;

SEQ ID Nos. 42-45; 42-46; 42-45 and 47; 42-47;

SEQ ID Nos. 48-51; 48-52; 48-51 and 53; 48-53;

SEQ ID Nos. 48, 51, 75 and 76; 48, 51, and 75-77; 48, 51, 53 and 75 and 76; 48, 51, 53 and 75-77;

25 SEQ ID Nos. 54-57; 54-58; 54-57 and 59; 54-59;

SEQ ID Nos. 60-63; 60-64; 60-63 and 65; 60-65;

SEQ ID Nos. 60, 61, 63 and 78; 60, 61, 63, 64 and 78; 60, 61, 63, 65 and 78; 60, 61, 63-65 and 78; and

a combination of any two or more of these primer sets,

30 wherein each primer specifically hybridizes to a target *N. meningitidis* nucleic acid or the complement of a target *N. meningitidis* nucleic acid.

14. The primer set of claim 13, wherein the target *N. meningitidis* nucleic acid encodes *N. meningitidis* gene selected from the group consisting of: *ctrA* (*N. meningitidis*), *crgA* (*N.*

meningitidis), IS1106 (*N. meningitidis*), *sacB* (*N. meningitidis* serogroup A), *siaD* (*N. meningitidis* serogroup B), *siaD* (*N. meningitidis* serogroup C), *synG* (*N. meningitidis* serogroup W-135), *ctrA* (*N. meningitidis* serogroup X), and *synF* (*N. meningitidis* serogroup Y).

5

15. A kit for detecting *N. meningitidis* comprising the LAMP primer set according to claim 13.

10

Fig. 2

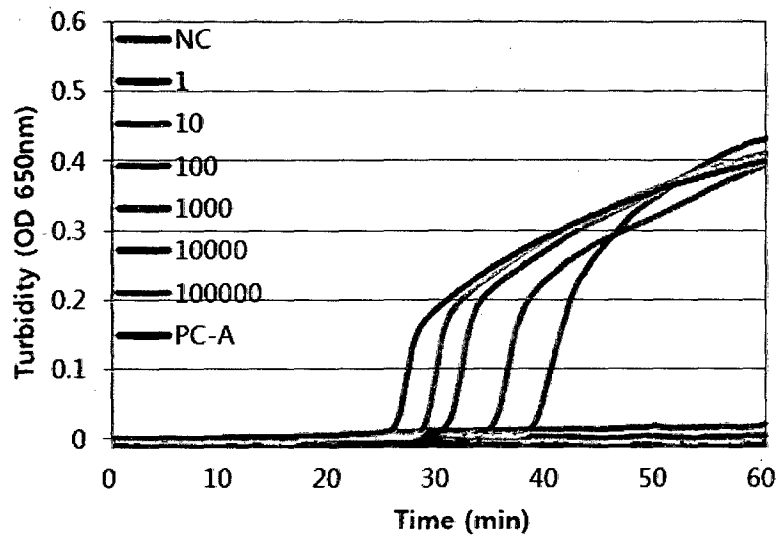
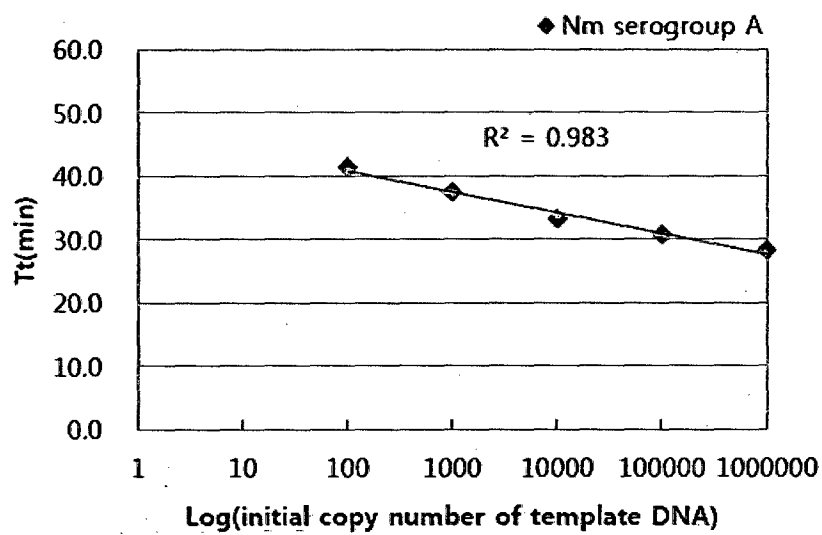


Fig. 3



INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2013/081572

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

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

B. FIELDS SEARCHED

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

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JAMES P. MCKENNA ET AL: "Development and clinical validation of a loop-mediated isothermal amplification method for the rapid detection of Neisseria meningitidis", DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE, vol. 69, no. 2, 1 February 2011 (2011-02-01), pages 137-144, XP55106665, ISSN: 0732-8893, DOI: 10.1016/j.diagmicrobio.2010.10.008 abstract and atb. 1	1-15
X	WO 2009/063243 A2 (BELFAST HEALTH AND SOCIAL CARE [GB]; COYLE PETER [GB]; FAIRLEY DEREK []) 22 May 2009 (2009-05-22) abstract, tab. 1, p. 19-20 and claims 1-9 ----- -/-	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

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24/03/2014

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INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2013/081572

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAHA MUHAMED-KHEIR ET AL: "Interlaboratory comparison of PCR-based identification and genogrouping of Neisseria meningitidis", JOURNAL OF CLINICAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 43, no. 1, 1 January 2005 (2005-01-01), pages 144-149, XP002520039, ISSN: 0095-1137, DOI: 10.1128/JCM.43.1.144-149.2005 abstract and tab. 2 and 3 -----	1-15
A	X. WANG ET AL: "Clinical Validation of Multiplex Real-Time PCR Assays for Detection of Bacterial Meningitis Pathogens", JOURNAL OF CLINICAL MICROBIOLOGY, vol. 50, no. 3, 14 December 2011 (2011-12-14), pages 702-708, XP055106871, ISSN: 0095-1137, DOI: 10.1128/JCM.06087-11 abstract -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/JP2013/081572

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		WO 2009063243 A2	22-05-2009
