

DESCRIPTION

RNA VIRUS DETECTION METHOD

TECHNICAL FIELD

[0001]

The present invention relates to the method for detecting RNA virus by reverse transcription-polymerase chain reaction (RT-PCR) and the kit for implementing such a method. More specifically, the present invention relates to the method for detecting the RNA virus by mixing a specimen with a surfactant under the presence of hydroxide and further adding RT-PCR reaction solution and the kit for implementing such a method.

BACKGROUND ART

[0002]

RNA virus is virus having RNA as genome. The RNA virus is categorized into coronavirus, human immunodeficiency virus, hepatitis C virus, Japanese encephalitis virus, dengue virus, and the like having envelopes as lipid bilayer membranes; and norovirus, rotavirus, rhinovirus, and the like having no envelopes. The RNA virus includes many pathogenic viruses.

[0003]

The norovirus is virus belonging to human caliciviruses, and as genome, has single-strand RNA with about 7000 bases. This virus is called small round structured virus (SRSV)

according to morphological classification observed with an electronic microscope, and as a generic term, is also called Norwalk-like virus. The virus belonging to the norovirus is categorized into two types of gene cluster including Genogroup I (GI) and Genogroup II (GII), and is further categorized into genotypes of 14, 17, or greater.

[0004]

When a human is infected with the norovirus, the symptoms of acute gastroenteritis such as vomiting and diarrhea are caused. About the half of annual food-poisoning patients in Japan results from the norovirus. About 70 percent of these patients is concentrated on November to February, and the norovirus is known as virus causing winter gastroenteritis and food poisoning. Food poisoning due to the norovirus is mainly caused due to food contamination through a chef. The norovirus is highly infectious, and easily causes, e.g., large-scale outbreak of food poisoning. The route of infection to a human is mainly oral infection. Representative examples of an infection source include feces and vomit of an infected patient and goods directly or indirectly contaminated therewith; and food contaminated with the norovirus, such as oysters and other clams. Thus, it is important for prevention of virus infection spread to specify a patient infected with the norovirus or an object contaminated with the norovirus.

[0005]

As a virus test for detecting virus infection or contamination, an immunoassay method for detecting a virus antigen or a virus gene amplification method is used (Patent Documents 1 to 3, Non-Patent Document 1). The technique of measuring the norovirus with a high degree of sensitivity includes a method in which RNA in the norovirus is amplified by RT-PCR and an amplified product amount is measured. For example, in accordance with the notification (Non-Patent Documents 2 and 3) of the Inspection and Safety Division of the Food Safety Department of the Pharmaceutical and Food Safety Bureau in the Ministry of Health, Labor and Welfare, detection of the norovirus by an RT-PCR method and quantitative detection of the norovirus by a real-time PCR method have been broadly performed.

[0006]

An RNA virus particle has such a basic structure that a core made of RNA genome and protein is enclosed in a protein shell called capsid. Thus, for detecting virus RNA by the gene amplification method, RNA needs to be extracted from the virus particle. For detecting the norovirus in feces as a specimen, e.g., the fecal specimen is suspended in distilled water or saline at a concentration of 5% to 10% (w/v). From the centrifugal supernatant thereof, RNA is extracted/purified using a commercially-available virus RNA extraction kit (e.g., QIAamp (the registered trademark) Viral RNA Mini, QIAGEN)

(Non-Patent Document 2). However, the detection process of performing the RT-PCR after the multi-stage RNA extraction/purification process is complicated. For this reason, a simple detection method has been proposed, in which fecal suspension is mixed with specimen treatment solution, shell protein is removed by thermal treatment performed for a short period of time, and RNA inside is released and is directly provided to the RT-PCR (Non-Patent Document 4). For thermally treating the mixture of the fecal suspension and the specimen treatment solution, it takes time and effort to seal a reaction container with a lid to prevent bumping or transpiration of the mixture and to detach the lid after the thermal treatment to add RT-PCR reaction solution. For improving this point, a method has been proposed, in which a specimen is mixed with a chaotropic agent such as guanidine salt and virus is detected by the RT-PCR without the thermal treatment (Patent Document 4).

Prior Art

Patent Document

[0007]

Patent Document 1: WO 2002/029119

Patent Document 2: WO 2002/029120

Patent Document 3: JP-A-2004-301684

Patent Document 4: JP-A-2017-209036

Non-Patent Document

[0008]

Non-Patent Document 1: Kageyama T, et al., Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR, J Clin Microbiol, 2003 Apr; 41(4): 1548-57

Non-Patent Document 2: Inspection and Safety Division of Food Safety Department of Pharmaceutical and Food Safety Bureau in Ministry of Health, Labor and Welfare, MHLW Notice No. 1105001 (November 5, 2003) Appendix "Norovirus Detection Method," Final Revision: MHLW Notice No. 0514004 (May 14, 2007)

Non-Patent Document 3: Inspection and Safety Division of Food Safety Department of Pharmaceutical and Food Safety Bureau in Ministry of Health, Labor and Welfare, MHLW Notice No. 1105001 (November 5, 2003) Appendix "Norovirus Detection Method," Final Revision: MHLW Notice No. 1022 Article 1 (October 22, 2013)

Non-Patent Document 4: Nishimura N, et al., Detection of noroviruses in fecal specimens by direct RT-PCR without RNA purification, J Virol Methods., 2010 Feb; 163(2): 282-286

SUMMARY OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0009]

An object of the present invention is to provide a simple RNA virus detection method. Specifically, a method is provided,

in which RNA is, without thermal treatment, extracted from an RNA virus particle such as norovirus by means of one or more types of surfactant and a virus detection process is subsequently easily performed by RT-PCR reaction of released RNA. Moreover, a simpler RNA virus detection method is provided in such a manner that RNA extraction from the RNA virus particle and the RT-PCR are performed in the same container without opening/closing a lid of the container.

SOLUTIONS TO THE PROBLEMS

[0010]

The object of the present invention is achieved by the following aspects of the invention.

[1]

The method for detecting RNA virus in a specimen, including

the step (1) of suspending the specimen in distilled water, saline, or buffer solution,

the step (2) of extracting centrifugal supernatant from suspension generated in the step (1),

the step (3) of mixing the centrifugal supernatant extracted in the step (2) with specimen treatment solution containing one or more types of surfactant,

the step (4) of mixing a mixture obtained in the step (3) with one-step RT-PCR reaction solution containing reverse

transcriptase and DNA polymerase to induce RT-PCR, and
the step (5) of detecting a RT-PCR product.

[2]

The method according to [1], in which the RNA virus is selected from a group consisting of norovirus, rotavirus, rhinovirus, coronavirus, human immunodeficiency virus, hepatitis C virus, Japanese encephalitis virus, and dengue virus.

[3]

The method according to [1], in which the RNA virus is norovirus.

[4]

The method according to [3], in which a norovirus genotype is Genogroup I (GI) or Genogroup II (GII).

[5]

The method according to any one of [1] to [4], in which the specimen is derived from a sample selected from a group consisting of a biological sample, a biologically-derived sample, an environmental sample, and an environmentally-derived sample.

[6]

The method according to any one of [1] to [4], in which the specimen is derived from a sample selected from a group consisting of an excrement sample, an excrement-derived sample, a vomit sample, and a vomit-derived sample.

[7]

The method according to any one of [1] to [6], in which the surfactant is an anion surfactant.

[8]

The method according to [7], in which the anion surfactant is one or more types of anion surfactant selected from a group consisting of alkyl sulfate, alkyl ether sulfate, docusate, a sulfonate fluoro surfactant, alkyl benzen sulphonate, alkyl aryl ether phosphate, alkyl ether phosphate, alkyl carboxylate, sodium lauroyl sarcosinate, a carboxylate fluoro surfactant, sodium cholate, and sodium deoxycholate.

[9]

The method according to [7], in which the anion surfactant is alkyl sulfate.

[10]

The method according to [9], in which the alkyl sulfate is sodium dodecyl sulfate or ammonium dodecyl sulfate.

[11]

The method according to any one of [1] to [10], in which the concentration of the surfactant is 0.02% to 0.5% (w/v).

[12]

The method according to any one of [1] to [11], in which the specimen treatment solution contains hydroxide.

[13]

The method according to [12], in which the hydroxide is

sodium hydroxide or potassium hydroxide.

[14]

The method according to [12] or [13], in which the concentration of the hydroxide is 10 to 100 mM.

[15]

The method according to any one of [1] to [14], in which a mixture ratio between the centrifugal supernatant and the specimen treatment solution in the step (3) is 1:3 to 6 in terms of a volume ratio.

[16]

The method according to any one of [1] to [15], in which the reverse transcriptase is selected from a group consisting of AMV reverse transcriptase, MMLV reverse transcriptase, HIV reverse transcriptase, and variants thereof.

[17]

The method according to any one of [1] to [16], in which the DNA polymerase is selected from a group consisting of Taq DNA polymerase, Tth DNA polymerase, KOD DNA polymerase, Pfu DNA polymerase, and variants thereof.

[18]

The method according to any one of [1] to [17], in which the step (5) is performed by real-time measurement.

[19]

The method according to any one of [1] to [18], in which the step (3) is performed under a temperature of 1°C to 60°C.

[20]

The method according to any one of [1] to [19], in which the steps (3) to (5) are performed in the same container.

[21]

The method according to any one of [1] to [20], in which in the step (5), the amplification curve of the RT-PCR product is measured using a fluorescence filter, and it is determined whether the presence of the RNA virus in the specimen is positive or negative.

[22]

An RNA virus detection kit including specimen treatment solution containing one or more types of surfactant, and one-step RT-PCR reaction solution containing reverse transcriptase and DNA polymerase.

[23]

The kit according to [22], in which the RNA virus is selected from a group consisting of norovirus, rotavirus, rhinovirus, coronavirus, human immunodeficiency virus, hepatitis C virus, Japanese encephalitis virus, and dengue virus.

[24]

The kit according to [22], in which the RNA virus is norovirus.

[25]

The kit according to [24], in which a norovirus genotype

is Genogroup I (GI) or Genogroup II (GII).

[26]

The kit according to any one of [22] to [25], in which the surfactant is an anion surfactant.

[27]

The kit according to [26], in which the anion surfactant is one or more types of anion surfactant selected from a group consisting of alkyl sulfate, alkyl ether sulfate, docusate, a sulfonate fluoro surfactant, alkyl benzen sulphonate, alkyl aryl ether phosphate, alkyl ether phosphate, alkyl carboxylate, sodium lauroyl sarcosinate, a carboxylate fluoro surfactant, sodium cholate, and sodium deoxycholate.

[28]

The kit according to [26], in which the anion surfactant is alkyl sulfate.

[29]

The kit according to [28], in which the alkyl sulfate is sodium dodecyl sulfate or ammonium dodecyl sulfate.

[30]

The kit according to any one of [22] to [29], further including a kit instruction manual.

EFFECTS OF THE INVENTION

[0011]

According to the present invention, the centrifugal

supernatant of the specimen suspension containing RNA virus particles such as the norovirus and the specimen treatment solution containing one or more types of surfactant are mixed so that RNA can be efficiently released from the virus particles without thermal treatment. Thus, a series of process of releasing RNA and subsequently adding the RT-PCR reaction solution for detecting the presence of the virus can be continuously performed in the same container, and the RNA virus can be easily detected. Further, in the present invention, the efficiency of releasing RNA from the virus particles is high, and therefore, a virus detection sensitivity is high and a virus shedding period can be detected with favorable accuracy. Consequently, the present invention is useful for detecting inapparent infection, and is specifically useful for specifying an infected patient at a recovery phase after infection.

DESCRIPTION OF EMBODIMENTS

[0012]

The present invention provides the method for detecting RNA virus in a specimen. This method includes the step (1) of suspending the specimen in distilled water, saline, or buffer solution, the step (2) of extracting centrifugal supernatant from the suspension generated in the step (1), the step (3) of mixing the centrifugal supernatant extracted in the step (2) with specimen treatment solution containing one or more types

of surfactant, the step (4) of mixing the mixture obtained in the step (3) with one-step RT-PCR reaction solution containing reverse transcriptase and DNA polymerase to induce RT-PCR, and the step (5) of detecting a RT-PCR product.

[0013]

In the present invention, the RNA virus targeted for detection in the specimen is virus having RNA as genome. The RNA virus includes, but not limited to, coronavirus, human immunodeficiency virus, hepatitis C virus, Japanese encephalitis virus, dengue virus, and the like having envelopes as lipid bilayer membranes; and norovirus, rotavirus, rhinovirus, and the like having no envelopes. Since the envelope contains lipid as a main component, the envelope is easily destroyed by an organic solvent or a surfactant such as alcohol. However, the RNA virus with no envelope, such as the norovirus, generally exhibits resistance to the organic solvent and the surfactant.

[0014]

The specimen in the present invention includes a biological sample, a biologically-derived sample, an environmental sample, an environmentally-derived sample, and the like. The biological sample includes animal and plant tissues including shellfish midgut gland; and body fluid such as blood, saliva, and tissue secretion. Specifically, the most importance is given to shellfishes as food causing food

poisoning due to the norovirus. The biologically-derived sample includes one obtained in such a manner that treatment such as sonication is performed for the biological sample. The environmental sample includes any samples such as atmospheric air, soil, dust, and water. The environmentally-derived sample includes one obtained in such a manner that treatment such as sonication is performed for the environmental sample.

[0015]

As another aspect of the present invention, the specimen includes an excrement sample, an excrement-derived sample, a vomit sample, a vomit-derived sample, and the like. The excrement sample and the vomit sample may be directly used, but in the step (1), e.g., 10% (w/v) of the excrement sample or the vomit sample may be suspended in the distilled water, the saline, or the buffer solution to turn into emulsion. The buffer solution includes, but not limited to, phosphate buffer solution, tris buffer solution, borate buffer solution, and Good buffer solution such as HEPES. For removing a contaminant such as gut bacteria, the emulsion of the sample may be, in the step (2), centrifugally separated at, e.g., 10000 to 12000 rpm for 2 to 20 minutes, and the centrifugal supernatant may be used as the specimen. The excrement-derived sample and the vomit-derived sample include a wiped sample. The wiped sample is, for the purpose of checking virus contamination, obtained in such a manner that an object wiped off from a finger, a dish,

a chopping board, a kitchen knife, preparation equipment, lavatory equipment, household equipment, and the like with a cotton swab, a cotton wool pad, and the like is eluted into the phosphate buffer solution or the like. The resultant eluate is subjected to ultracentrifugal separation, and the centrifugal sediment is suspended or dissolved. The resultant can be used as the specimen (Yoshiko Somura et. al., Food Hygiene and Safety Science, 2017, Vol. 58, No. 4, p. 201-204).

[0016]

The step (3) of the present invention is the step of mixing the specimen with the specimen treatment solution containing one or more types of surfactant. In the present specification, the "surfactant" is a collective term of substances acting on a substance interface and changing substance properties. The surfactant has a structure with both of a hydrophilic portion and a hydrophobic portion in a molecule. The surfactant is categorized into an anion surfactant, a cation surfactant, an ampholytic surfactant, and a non-ionic surfactant. The anion surfactant includes, but not limited to, alkyl sulfate, alkyl ether sulfate, docusate, a sulfonate fluoro surfactant, alkyl benzen sulphonate, alkyl aryl ether phosphate, alkyl ether phosphate, alkyl carboxylate, sodium lauroyl sarcosinate, a carboxylate fluoro surfactant, sodium cholate, and sodium deoxycholate. As alkyl sulfate, sodium dodecyl sulfate (SDS) and ammonium dodecyl sulfate are preferred, and sodium dodecyl

sulfate is more preferred. Sodium dodecyl sulfate is also called sodium lauryl sulfate (SLS). The cation surfactant includes, but not limited to, ethyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, and tetradecyltrimethylammonium bromide. Examples of the ampholytic surfactant include, but not limited to, betaine and alkylamino fatty acid salt. The non-ionic surfactant includes, but not limited to, nonyl phenoxy poly ethoxy ethanol (NP-40), polyoxyethylene sorbitan monooleate (Tween (the registered trademark) 80), and polyoxyethylene-p-t-octyl phenol (Triton X-100 (the registered trademark)).

[0017]

When the surfactant is added to water solution at a certain concentration or higher, surfactant monomers are aggregated into a micelle. A concentration at which the surfactant forms the micelle is called a critical micelle concentration. In the water solution, a hydrophobic region of protein or lipid is taken into a hydrophobic region inside the surfactant micelle, and protein or lipid is solubilized. In an RNA virus particle, an envelope made of capsid or lipid as a protein shell is solubilized, denatured, or destroyed under the presence of the surfactant with the critical micelle concentration or higher. As a result, RNA enclosed in capsid tends to be exposed in the water solution. The critical micelle concentration of the surfactant varies according to the type

of surfactant. For efficient exposure of virus RNA, the concentration of the surfactant in the specimen treatment solution is preferably 0.02% to 0.5% (w/v), more preferably 0.05% to 0.2% (w/v), and much more preferably 0.1% (w/v).

[0018]

A mixture ratio between the specimen and the specimen treatment solution is preferably 1:3 to 6 in terms of a volume ratio and more preferably 1:4. The specimen and the specimen treatment solution containing the surfactant are mixed with each other. Accordingly, the concentration of the surfactant in the mixture decreases, but the above-described concentration of the surfactant is maintained at the critical micelle concentration.

[0019]

In one aspect of the present invention, the specimen treatment solution contains hydroxide. The "hydroxide" in the present specification indicates such a substance that a metal ion as a cation and a hydroxide ion (OH^-) as an anion are bound to each other. Metal is alkali metal or alkali earth metal. Examples of the hydroxide include lithium hydroxide, sodium hydroxide, potassium hydroxide, magnesium hydroxide, calcium hydroxide, and barium hydroxide, and sodium hydroxide and potassium hydroxide are preferred. The hydroxide exhibits strong basic properties, and is also called alkali because the hydroxide generates hydroxide ions when dissolved in water.

The hydroxide changes the charge state of dissociative amino acid, such as aspartic acid or glutamic acid, in a protein molecule in the water solution, and denatures protein. Due to such action, capsid is destroyed when the RNA virus particle is treated with alkali. As a result, RNA enclosed in capsid tends to be exposed in the water solution. For efficient exposure of virus RNA, a hydroxide concentration in the specimen treatment solution is preferably 10 to 100 mM, more preferably 40 to 60 mM, and more preferably 50 mM.

[0020]

For solubilizing, denaturing, or destroying the envelope made of capsid or lipid such that virus RNA is efficiently exposed, the surfactant and the hydroxide preferably coexist in the specimen treatment solution.

[0021]

The step (3) of the present invention for efficient exposure of virus RNA by means of capsid is preferably performed under a temperature of 1°C to 60°C, more preferably 1°C to 50°C, much more preferably 1°C to 40°C, and most preferably a room temperature of 1°C to 30°C. After the specimen and the specimen treatment solution have been mixed with each other, the resultant is preferably left stand for three minutes or longer. Thermal treatment is not necessary for the step (3) of the present invention, and for this reason, there is a low risk of bumping or transpiration of the mixture of the specimen and the

specimen treatment solution in a reaction container and the step (3) of the present invention can be performed without the reaction container being sealed with, e.g., a lid, i.e., with the reaction container being opened.

[0022]

The step (4) of the present invention is the step of mixing the mixture obtained in the step (3) with the one-step RT-PCR reaction solution containing the reverse transcriptase and the DNA polymerase to induce the RT-PCR. In one aspect of the present invention, the step (3) is performed in the non-sealed container using no lid, and therefore, the one-step RT-PCR reaction solution is directly added to the container containing the mixture obtained in the step (3) so that the steps (3), (4) can be performed in the same container. Of the surfactant contained in the mixture obtained in the step (3), SDS specifically exhibits a strong denaturalization effect for protein. For this reason, if a high concentration of SDS is introduced in the step (4), there is a probability that the enzyme activity of the reverse transcriptase and the DNA polymerase contained in the one-step RT-PCR reaction solution is inhibited and the RT-PCR does not progress. Similarly, if a high concentration of hydroxide contained in the mixture obtained in the step (3) is introduced in the step (4), such use leads to degradation of the above-described enzyme activity due to a high pH. For these reasons, a mixture ratio between

the mixture obtained in the step (3) and the one-step RT-PCR reaction solution is preferably 1:2 to 6 in terms of a volume ratio and more preferably 1:4.

[0023]

In the step (4) of the present invention, one-step RT-PCR is employed for analyzing many specimens within a short period of time. The one-step RT-PCR reaction solution is mixed with the reverse transcriptase and the DNA polymerase in advance, and reverse transcription reaction (single-strand cDNA synthesis) and the PCR can be induced in the same container.

[0024]

Reverse transcriptase contained in the one-step RT-PCR reaction solution is enzyme for generating single-strand complementary DNA (cDNA) by using virus RNA as a template. Reverse transcriptase is not specifically limited as long as the reverse transcriptase catalyzes the reverse transcription reaction, and RNA-dependent DNA polymerase derived from RNA viruses such as avian myeloblastosis virus (AMV), Moloney murine leukemia virus (M-MLV), and human immunodeficiency virus (HIV) and variants thereof can be used as the reverse transcriptase.

[0025]

The DNA polymerase contained in the one-step RT-PCR reaction solution is heat-stable DNA polymerase derived from thermophilic bacteria, and Taq, Tth, KOD, Pfu, or variants

thereof can be used as the DNA polymerase although the DNA polymerase is not limited thereto. For avoiding nonspecific amplification due to the DNA polymerase, hot start DNA polymerase may be used. The hot start DNA polymerase is, for example, DNA polymerase bound to an anti-DNA polymerase antibody or DNA polymerase that an enzyme active site is thermosensitively chemically modified. The hot start DNA polymerase is such enzyme that DNA polymerase becomes activated after an initial denaturalization step (90°C or higher) in the PCR.

[0026]

The one-step RT-PCR reaction solution contains all components for the reverse transcription reaction and the PCR under proper conditions. These components include at least the reverse transcriptase as described above, a reverse transcription reaction primer, the heat-stable DNA polymerase as described above, a PCR primer, a dNTP mixture (deoxyribonucleotide 5'-triphosphate; a mixture of dATP, dGTP, dCTP, and dTTP), and buffer solution. In one aspect of the present invention, the above-described reaction solution contains tris and magnesium. An RNA-degrading enzyme inhibitor may be also added to the above-described reaction solution. As the reverse transcription reaction primer, a primer specific to a target RNA sequence, an oligo (dT) primer, or a random primer can be used. As the PCR primer, a (forward

or reverse) primer pair specific to the sequence of cDNA generated by the reverse transcription reaction is used. The PCR primer may be the same as the above-described reverse transcription reaction primer specific to the target RNA sequence. Alternatively, two or more types of PCR primer may be added to the one-step RT-PCR reaction solution according to a DNA region to be amplified, i.e., the number of target sequences. As a composition containing the above-described components, RT-PCR reaction solution obtained in such a manner that reagents contained in a norovirus detection reagent kit (a probe method) (Shimadzu Corporation) are mixed according to a kit instruction manual can be used.

[0027]

In the case of detecting norovirus RNA, a PCR primer as described in, e.g., Patent Documents 1 and 2, Non-Patent Document 3, and JP-A-2018-78806 is used so that Genogroup I (GI) and Genogroup II (GII) in a norovirus genotype can be detected, but the present invention is not limited to above. The above-described norovirus detection reagent kit (the probe method) contains the PCR primer described in Non-Patent Document 3.

[0028]

Reaction temperature conditions for the reverse transcription reaction in the RT-PCR and PCR conditions (a temperature, a time, and the number of cycles) can be easily

set by those skilled in the art.

[0029]

The step (5) of the present invention is the step of detecting the product of the RT-PCR performed in the step (4). In one aspect of the present invention, the PCR product is detected by real-time measurement. In the case of performing the real-time measurement, the RT-PCR of the step (4) and the step (5) of detecting the RT-PCR product are performed in the same container. In one aspect of the present invention, the step (3) is performed in the non-sealed container using no lid, and therefore, the one-step RT-PCR reaction solution is directly added to the container containing the mixture obtained in the step (3) so that the steps (3), (4) can be performed in the same container. Thus, in one aspect of the present invention, the steps (3) to (5) can be performed in the same container.

[0030]

The real-time measurement of the PCR product is also called real-time PCR. In the real-time PCR, a PCR-amplified product is normally detected using fluorescence. As a fluorescence detection method, there are a method using an intercalating fluorescent dye and a method using a fluorescence-labeled probe. SYBR (the registered trademark) Green I is used as the intercalating fluorescent dye, but the present invention is not limited to above. The intercalating

fluorescent dye is bound to double-strand DNA synthesized by the PCR, thereby emitting fluorescence in response to irradiation with excitation light. The intensity of such fluorescence is measured so that the amount of generation of the PCR-amplified product can be measured.

[0031]

The fluorescence-labeled probe includes, but not limited to, a TaqMan probe, Molecular Beacon, a cycling probe, and the like. The TaqMan probe is oligonucleotide that a 5' end is modified with a fluorescent dye and a 3' end is modified with a quencher substance. The TaqMan probe is hybridized specifically to template DNA at an annealing step of the PCR. Since the quencher is present on the probe, generation of fluorescence is suppressed even upon irradiation with excitation light. At a subsequent elongation reaction step, when the TaqMan probe hybridized to the template DNA is decomposed by the 5'→3' exonuclease activity of Taq DNA polymerase, the fluorescent dye is released from the probe, and suppression in generation of fluorescence by the quencher is cancelled and fluorescence is generated. The intensity of such fluorescence is measured so that the amount of generation of the amplified product can be measured. The fluorescent dye includes, but not limited to, FAM, ROX, and Cy5. The quencher includes, but not limited to, TAMRA (the registered trademark) and MGB. For distinctively detecting two or more types of DNA

target sequence, the PCR is induced using two or more types of oligonucleotide probe (e.g., the TaqMan probe) to which different fluorescent dyes are bound.

[0032]

At the step (5), the amplification curve of the RT-PCR product is measured using a fluorescence filter corresponding to the fluorescent dye used. In a case where the fluorescence intensity increases according to the number of PCR cycles, it is determined that the presence of the RNA virus targeted for analysis in the specimen is positive. On the other hand, in a case where the fluorescence intensity does not increase in the PCR, it is determined that the presence of the RNA virus is negative.

[0033]

In one aspect of the present invention, an RNA virus detection kit is provided, which includes the specimen treatment solution containing one or more types of surfactant and the one-step RT-PCR reaction solution containing the reverse transcriptase and the DNA polymerase.

[Examples]

[0034]

Next, the present invention will be described in more detail with reference to examples, but is not limited to these examples.

[Example 1]

[0035]

[Effects of Surfactant and Hydroxide on Virus RNA Exposure in Specimen]

(1) Specimen

Of Human feces (ten examples) containing norovirus, 100 mg was collected and was suspended in 1 mL of distilled water. In this manner, about 10% (w/v) of fecal emulsion was obtained. The fecal emulsion was centrifugally separated at 10000 rpm for five minutes at a room temperature, and centrifugal supernatant was taken as a specimen.

(2) Specimen Treatment Solution

Specimen treatment solution containing the following components was prepared:

50 mM of sodium hydroxide (NaOH);

0.1% (w/v) of sodium dodecyl sulfate (SDS); and

625 μ M of dNTP (dATP, dGTP, dCTP, and dTTP).

(3-1) Specimen Treatment

After 4 μ L of the above-described specimen treatment solution had been taken into a lidless PCR reaction tube and 1 μ L of the above-described specimen had been further added to the PCR reaction tube, the resultant was left stand for three minutes at the room temperature.

(3-2) Specimen Treatment in Combination with Thermal Treatment

By way of comparison, the specimen was treated using specimen treatment solution containing 15 mM of NaOH, but not

containing SDS. As such specimen treatment solution, specimen treatment solution (Sample Treatment Reagent) contained in a norovirus detection reagent kit (a probe method) (Shimadzu Corporation, Product Number 241-09325-91) was used. Then, 9 μL of the above-described specimen treatment solution was taken into the PCR reaction tube, and 1 μL of the above-described specimen was further added to the PCR reaction tube and was agitated and mixed. After the PCR reaction tube had been spun down by a small centrifuge, the resultant was placed in a constant-temperature apparatus at 90°C , and was heated for five minutes. After such thermal treatment, the PCR reaction tube was spun down by the small centrifuge, and was directly cooled with ice.

(4) One-Step RT-PCR Reaction

Then, 20 μL of one-step RT-PCR reaction solution was added to the PCR reaction tube containing 5 μL of the treatment solution obtained in 3-1 above, or 15 μL of one-step RT-PCR reaction solution was added to the PCR reaction tube containing 10 μL of the treatment solution obtained in 3-2 above. After the resultant had been agitated and mixed, the PCR reaction tube was spun down by the small centrifuge. Thereafter, reaction was immediately started using a real-time PCR apparatus (GVP-9600, Shimadzu Corporation).

(5) Composition of One-Step RT-PCR Reaction Solution

The one-step RT-PCR reaction solution was added to 5 μL

of the treatment solution obtained in 3-1 above such that the following reaction solution composition is made. One-step RT-PCR reaction solution prepared in such a manner that reagents (NoV Reagents A, B, and C) contained in the norovirus detection reagent kit (the probe method) (Shimadzu Corporation, Product Number 241-09325-91) are mixed was added to 10 μ L of the treatment solution obtained in 3-2 above. The composition in the reaction is as follows:

40 mM of tris;

0.025 units/ μ L of Taq polymerase;

1 unit/ μ L of reverse transcriptase;

3.75 mM of magnesium chloride;

400 nM of a PCR primer set (COG1F/COG1R and COG2F/COG2R)

(see Non-Patent Document 3 and Table 11); and

200 nM of a fluorescence-labeled probe (a TaqMan probe: G1A, G1B, and G2).

(6) Conditions for Setting RT-PCR

After reverse transcription reaction at "45°C/5 minutes," initial denaturalization at "95°C/3 minutes" was performed. Subsequently, 45 cycles of PCR at "95°C/1 second to 56°C/10 seconds" were performed. Photometry was performed in a step of 56°C/10 seconds.

(7) Results and Discussion

Photometry results are shown in Table 1. Table 1 shows comparison between the case of treatment with the specimen

treatment solution according to the present invention and the case of thermal treatment as a typical method, and shows Ct values. The Ct value indicates the number of cycles at which the amplification curve and a threshold (Threshold) cross each other in the real-time PCR. According to Table 1, the Ct value is, for all specimens, the substantially same between the case of the thermal treatment as the typical method and the case of the treatment with the specimen treatment solution according to the present invention. These results show that the substantially same initial template amount is shown in any types of treatment. That is, it shows that the specimen treatment of the present invention that the thermal treatment can be omitted by means of the surfactant and the hydroxide provides a virus RNA exposure effect similar to that of the typical method in which the thermal treatment is performed.

[0036]

[Table 1]

Specimen Number	Ct Value	
	Method of Present Invention	Typical Method in which Thermal Treatment is Performed
1	22.8	22.7
2	21.6	20.7
3	29.0	28.3
4	27.7	27.4
5	20.5	20.2
6	29.2	27.9
7	24.8	25.6
8	24.8	24.5
9	32.5	30.8
10	28.3	27.7

CLAIMS

1. A method for detecting RNA virus in a specimen,
comprising:
 - a step (1) of suspending the specimen in distilled water,
saline, or buffer solution;
 - a step (2) of extracting centrifugal supernatant from
suspension generated in the step (1);
 - a step (3) of mixing the centrifugal supernatant
extracted in the step (2) with specimen treatment solution
containing one or more types of surfactant;
 - a step (4) of mixing a mixture obtained in the step (3)
with one-step RT-PCR reaction solution containing reverse
transcriptase and DNA polymerase to induce RT-PCR; and
 - a step (5) of detecting a RT-PCR product.
2. The method according to claim 1, wherein the RNA virus
is selected from a group consisting of norovirus, rotavirus,
rhinovirus, coronavirus, human immunodeficiency virus,
hepatitis C virus, Japanese encephalitis virus, and dengue
virus.
3. The method according to claim 1, wherein the RNA virus
is norovirus.
4. The method according to claim 3, wherein a norovirus

genotype is Genogroup I (GI) or Genogroup II (GII).

5. The method according to any one of claims 1 to 4, wherein the specimen is derived from a sample selected from a group consisting of a biological sample, a biologically-derived sample, an environmental sample, and an environmentally-derived sample.

6. The method according to any one of claims 1 to 4, wherein the specimen is derived from a sample selected from a group consisting of an excrement sample, an excrement-derived sample, a vomit sample, and a vomit-derived sample.

7. The method according to any one of claims 1 to 6, wherein the surfactant is an anion surfactant.

8. The method according to claim 7, wherein the anion surfactant is one or more types of anion surfactant selected from a group consisting of alkyl sulfate, alkyl ether sulfate, docusate, a sulfonate fluoro surfactant, alkyl benzen sulphonate, alkyl aryl ether phosphate, alkyl ether phosphate, alkyl carboxylate, sodium lauroyl sarcosinate, a carboxylate fluoro surfactant, sodium cholate, and sodium deoxycholate.

9. The method according to claim 7, wherein the anion

surfactant is alkyl sulfate.

10. The method according to claim 9, wherein the alkyl sulfate is sodium dodecyl sulfate or ammonium dodecyl sulfate.

11. The method according to any one of claims 1 to 10, wherein a concentration of the surfactant is 0.02% to 0.5% (w/v).

12. The method according to any one of claims 1 to 11, wherein the specimen treatment solution contains hydroxide.

13. The method according to claim 12, wherein the hydroxide is sodium hydroxide or potassium hydroxide.

14. The method according to claim 12 or 13, wherein a concentration of the hydroxide is 10 to 100 mM.

15. The method according to any one of claims 1 to 14, wherein a mixture ratio between the specimen and the specimen treatment solution in the step (3) is 1:3 to 6 in terms of a volume ratio.

16. The method according to any one of claims 1 to 15, wherein the reverse transcriptase is selected from a group consisting of AMV reverse transcriptase, MMLV reverse transcriptase, HIV reverse transcriptase, and variants thereof.

17. The method according to any one of claims 1 to 16, wherein the DNA polymerase is selected from a group consisting of Taq DNA polymerase, Tth DNA polymerase, KOD DNA polymerase, Pfu DNA polymerase, and variants thereof.

18. The method according to any one of claims 1 to 17, wherein the step (5) is performed by real-time measurement.

19. The method according to any one of claims 1 to 18, wherein the step (3) is performed under a temperature of 1°C to 60°C.

20. The method according to any one of claims 1 to 19, wherein the steps (3) to (5) are performed in an identical container.

21. The method according to any one of claims 1 to 20, wherein in the step (5), an amplification curve of the RT-PCR product is measured using a fluorescence filter, and it is determined whether a presence of the RNA virus in the specimen is positive or negative.

22. An RNA virus detection kit comprising:

specimen treatment solution containing one or more types of surfactant; and

one-step RT-PCR reaction solution containing reverse

transcriptase and DNA polymerase.

23. The kit according to claim 22, wherein the RNA virus is selected from a group consisting of norovirus, rotavirus, rhinovirus, coronavirus, human immunodeficiency virus, hepatitis C virus, Japanese encephalitis virus, and dengue virus.

24. The kit according to claim 22, wherein the RNA virus is norovirus.

25. The kit according to claim 24, wherein a norovirus genotype is Genogroup I (GI) or Genogroup II (GII).

26. The kit according to any one of claims 22 to 25, wherein the surfactant is an anion surfactant.

27. The kit according to claim 26, wherein the anion surfactant is one or more types of anion surfactant selected from a group consisting of alkyl sulfate, alkyl ether sulfate, docusate, a sulfonate fluoro surfactant, alkyl benzen sulphonate, alkyl aryl ether phosphate, alkyl ether phosphate, alkyl carboxylate, sodium lauroyl sarcosinate, a carboxylate fluoro surfactant, sodium cholate, and sodium deoxycholate.

28. The kit according to claim 26, wherein the anion surfactant is alkyl sulfate.

29. The kit according to claim 28, wherein the alkyl sulfate is sodium dodecyl sulfate or ammonium dodecyl sulfate.

30. The kit according to any one of claims 22 to 29, further comprising:

a kit instruction manual.