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(54) Title: PRIMER PAIRS FOR DETECTING ORIENTIA TSUTSUGAMUSHI AND DETECTION METHOD USING THE SAME

(57) Abstract: The present invention relates to a diagnosis method of scrub typhus, more precisely, to a diagnosis method of scrub typhus by detecting *Orientia tsutsugamushi*, the cause of scrub typhus, comprising the following steps: preparing primer pairs based on the 16s ribosomal gene sequence of *Orientia tsutsugamushi*; and detecting *Orientia tsutsugamushi* using the prepared primer pairs. The diagnosis method of the present invention not only demonstrates higher diagnostic accuracy than other conventional methods amplifying and analyzing 47kDa, 57kDa, and groEL, but also is very simple and easy, suggesting that it is excellent in the aspect of cost-effect.

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Description

Title of Invention: PRIMER PAIRS FOR DETECTING ORIENTIA TSUTSUGAMUSHI AND DETECTION METHOD USING THE SAME

Technical Field

- [1] The present invention relates to primer pairs for diagnosing *Orientia tsutsugamushi* and a diagnosis method of *Orientia tsutsugamushi* using the same, more specifically, to primer pairs that are capable of amplifying 16s ribosomal RNA gene base sequence of *Orientia tsutsugamushi*, the cause of scrub typhus, and a detection method of scrub typhus by detecting *Orientia tsutsugamushi* using the same.

Background Art

- [2] Scrub typhus is an acute febrile disease, which is transmitted via chigger infected with *Orientia tsutsugamush*, and shows characteristic clinical symptoms due to systemic vasculitis. A rodent is a major host and a mite(chigger) is a host and vector.
- [3] Since scrub typhus was first reported in Korea in 1951, this major autumn febrile disease has been spread all over the country particularly in between September and November. Particularly, so many cases have been occurring as 30% of all patients in fall are diagnosed as scrub typhus.
- [4] Symptoms such as fever, chill, headache, muscle ache, and erythematous papular rash would be developed in 1-2 weeks after the chigger bite. A detection of the developed characteristic eschar would be helpful for early diagnosis of the disease. Generally, the progress of this disease is not serious and an antibiotic works well for the disease. However, in case of a late diagnosis, complications in the form of pneumonia, acute renal failure, encephal meningitis, encephalitis, upper gastrointestinal bleeding, multiple organ dysfunction, or even worse myocardial infarction or stroke can be developed. These complications might be lethal, so that rapid and accurate diagnosis is essential for the improvement of prognosis of a patient.
- [5] Up to date, the methods for diagnosing scrub typhus are methods such as by confirming *Orientiatsutsugamushi* by culturing, by detecting the antibody against *Orientiatsutsugamushi* in the serum of a patient and by examining DNA of *Orientia tsutsugamushi* separated from blood of a patient by PCR (polymerase Chain Reaction) amplification.
- [6] Among them, in the diagnosis method by culturing *Orientia tsutsugamushi*, it takes at least several weeks to culture *Orientia tsutsugamushi*, suggesting that this method is practically improper for the purpose of diagnosis of a patient. So, the most common diagnosis methods are indirect fluorescent antibody technique and immuno enzyme

technique, which are serological test methods.

- [7] However, the above methods still have disadvantages of false positive resulting from the cross-reaction with other pathogenic microorganisms, low sensitivity in case of test in an early stage of the disease due to requiring several days until an antibody is formed after symptoms of the disease begins, and additional tracking tests required for confirming diagnosis.
- [8] Another diagnosis method of scrub typhus, which is a method of detecting *Orientia tsutsugamushi* specific DNA from blood of a patient by PCR in order to make the rapid diagnosis of scrub typhus in its early stage, has been introduced. Examples of PCR comprise the conventional PCR, or nested PCR obtained by the modification of the conventional PCR and showing 100 times greater sensitivity than the conventional PCR, or real time PCR during which the results can be confirmed rapidly within two hours.
- [9] In the conventional PCR diagnosis of scrub typhus, PCR using various target protein genes such as 46kDa, 57kDa, and groEL, etc, has been tried, and nested PCR or real time PCR has been more preferred over the conventional PCR. Because when the conventional PCR was performed by using 46kDa or 57kDa gene as a target, sensitivity was as low as at best 10%. However, nested PCR has a drawback that it does take longer time to test than other PCR, with PCR performed twice to increase sensitivity. Also, real time PCR has a drawback that with very expensive testing equipments required, there was a limitation in use universally in most medical institutes.
- [10] Therefore, it is urgently requested to develop a novel diagnosis method which is mostly simple and low-cost as well as presenting high sensitivity and specificity.

Disclosure of Invention

Technical Problem

- [11] The present inventors tried to develop a novel method to detect *Orientia tsutsugamushi* with ease and simplicity from the blood sample of a patient. As a result, the present inventors completed this invention by confirming that even with the existing conventional PCR, highly accurate diagnosis could be achieved by amplifying specifically a part of 16s ribosomal RNA gene sequence of *Orientia tsutsugamushi*.
- [12] Therefore, the present invention provides a detection method of *Orientia tsutsugamushi* by amplifying 16s ribosomal RNA gene of *Orientia tsutsugamushi* using the conventional PCR.
- [13] The novel method of the present invention overcome a problem that the sensitivity in the conventional PCR is within 10%, and also overcomes the problems that in nested PCR, performing PCR twice or more leads to more labors and time than needed and higher potential possibility of contamination, and the problem that in real-time PCR,

expensive equipments are required. Therefore, the method of the present invention is an excellent method in cost-labor efficient aspects, which also provides diagnostic accuracy comparable to that of the PCRs.

Solution to Problem

- [14] To achieve the above objects, the present inventors designed a detection method of *Orientia tsutsugamushi* to amplify and identify 16s ribosomal RNA gene sequence of *Orientia tsutsugamushi*. This method can be achieved by preparing primers that can specifically amplify 16s ribosomal RNA of *Orientia tsutsugamushi*.
- [15] The preparation of the primers were designed based on the 16s ribosomal gene base sequence amplified from the blood sample of a patient infected with *Orientia tsutsugamushi* Boryoung strain, which was very common in Korea.
- [16] Thus, it is an object of the present invention to provide a method for diagnosis of scrub typhus and a method for detecting *Orientia tsutsugamushi* characterized by comprising the step of amplifying a target protein gene using the primers for 16s ribosomal RNA in blood sample of a patient suspicious with scrub typhus.
- [17] It is another object of the present invention to provide a PCR kit for the detection of *Orientiatsutsugamushi* containing the primers.
- [18]
- [19] The above and other objects, features and advantages of the present invention will become apparent from the following description of preferred embodiments and claims.

Advantageous Effects of Invention

- [20] The present invention relates to a method for diagnosis of scrub typhus, more specifically, to primers that are capable of amplifying the 16s ribosomal RNA gene base sequence of *Orientia tsutsugamushi*, the cause of scrub typhus, and a method for diagnosis and detecting *Orientia tsutsugamushi* using the same.
- [21] The diagnosis method of the present invention presents excellent and high diagnostic accuracy, compared with the conventional methods analyzing by amplifying 46kDa, 57kDa and groEL genes. Also, when the primers of the present invention are applied to the conventional PCR, comparatively improved diagnostic accuracy is obtained. Moreover, the method of the invention is not only superior in diagnostic accuracy to nested PCR or real time PCR but also simple and inexpensive.

Brief Description of Drawings

- [22] Fig. 1 is graphs illustrating the comparison of diagnostic efficiency of the detection method of *Orientia tsutsugamushi* using the primer pair composed of the sequences represented by SEQ. ID. NO. 1 and NO. 2 originated from 16s ribosomal RNA of the present invention to that of the conventional detection methods of comparative examples 1 - 6, with receiver operator characteristic (ROC) curve.

Best Mode for Carrying out the Invention

- [23] The present invention relates to a diagnosis method of scrub typhus. More precisely, the invention relates to a detection method of *Orientia tsutsugamushi* using primers for PCR to amplify the 16s ribosomal RNA gene base sequence of *Orientia tsutsugamushi*, the cause of scrub typhus.
- [24] The present invention also provides primers for PCR to amplify the 16s ribosomal RNA gene base sequence of *Orientia tsutsugamushi*, the cause of scrub typhus.
- [25] The present invention also provides a PCR kit for the detection of *Orientia tsutsugamushi* containing the primers.
- [26]
- [27] Hereinafter, the present invention is described in detail.
- [28] The present invention is characterized by preparing the primer pairs for PCR to amplify 16s ribosomal RNA gene of *Orientia tsutsugamushi* and detecting the amplified 16s ribosomal RNA gene using the primers.
- [29] The primers of the present invention were designed based on the 16s ribosomal gene base sequence amplified from the blood sample of a patient infected with *Orientia tsutsugamushi* Boryoung strain, which was very common in Korea. The primers also could detect other prototype strains such as Gilliam, Karp, Kato, and various other strains.
- [30] The present invention is also characterized by amplifying the target protein gene, which is 16s ribosomal RNA gene in the blood sample from a suspected case of scrub typhus by using the primers.
- [31] The PCR is an abbreviation for polymerase chain reaction. The PCR comprises all the PCR of the most common conventional PCR, duplex PCR and nested PCR performing PCR twice consecutively, and high-cost real time PCR during which the results can be confirmed in real time, and most preferably it comprises the conventional PCR.
- [32] The PCR kit for detection herein may include materials for PCR such as the enzyme involved in polymerization, dNTP, buffer, mineral oil, standard marker, the staining reagent bromophenol blue or xylene FF, as well as the primer pairs for PCR for the amplification of 16s ribosomal RNA gene of *Orientia Tsutsugamushi*.
- [33]
- [34] Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples. However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.
- [35]
- [36] Example 1: Primer preparation

- [37] Primer pairs were designed based on the 16s ribosomal gene base sequence amplified in the blood sample from a patient infected with tsutsugamushi Boryoung strain, which was very common in Korea, but could detect other prototype strains such as Gilliam, Karp, Kato, and various other strains. More specifically, the primer pair was prepared as a forward primer (position 403-428) and a reverse primer (position 577-601) for GenBank Accession No. bankit1356164 HM352765: *O. tsutsugamushi* Boryoung Genotype, which were represented by SEQ. ID. NO. 1 and NO. 2, respectively. Forward and reverse primers were also prepared for other regions, which were represented by SEQ. ID. NO 3 - NO. 36 (see Table 1), respectively.
- [38] Table 1

[Table 1]

	Sequence
SEQ. ID. NO. 1	5'-AGGGATGATAATGACAGTACCTACAG-3'
SEQ. ID. NO. 2	5'-CCTCTACCATACTCTAGCCTAACAG-3'
SEQ. ID. NO. 3	5'-ATTAGTGGCAAACGGGTGAG-3'
SEQ. ID. NO. 4	5'-CACAGGCTTGGTAAGCCATTA-3'
SEQ. ID. NO. 5	5'-GGAATCTGCCCATCAGTACA-3'
SEQ. ID. NO. 6	5'-GCTGTTTCGTCCTCTCAGACC-3'
SEQ. ID. NO. 7	5'-TGCTAATACCGTATGCCCTCT-3'
SEQ. ID. NO. 8	5'-CGTGTCTCAGTCCCAGTGTG-3'
SEQ. ID. NO. 9	5'-GGAAAGATTTATCGCTGATGG-3'
SEQ. ID. NO. 10	5'-TGTCCAATATTCCCCACTGC-3'
SEQ. ID. NO. 11	5'-GCGGCAGATTAGGTAGTTGG-3'
SEQ. ID. NO. 12	5'-CATTGCTGGATCAGGCTTTC-3'
SEQ. ID. NO. 13	5'-AAGGTAATGGCTTAGCAAGCCTGTG-3'
SEQ. ID. NO. 14	5'-CAACCCTAAGGCCTTCATCA-3'
SEQ. ID. NO. 15	5'-GGTCTGAGAGGACGAACAGC-3'
SEQ. ID. NO. 16	5'-CGGGGCTTTTTCTGTAGGTA-3'
SEQ. ID. NO. 17	5'-ATATTGGACAATGGGCGAAA-3'
SEQ. ID. NO. 18	5'-TACGCCAGTAATTCCGAAC-3'
SEQ. ID. NO. 19	5'-TGATGAAGGCCTTAGGGTTG-3'
SEQ. ID. NO. 20	5'-TTAAGCCCTGGGATTTTCATC-3'
SEQ. ID. NO. 21	5'-AGAAAAAGCCCCGGCTAACT-3'
SEQ. ID. NO. 22	5'-GAAATTCCATCACCTCTACCA-3'
SEQ. ID. NO. 23	5'-GTTCGGAATTACTGGGCGTA-3'
SEQ. ID. NO. 24	5'-AGATGACAGCTTTCGCCACT-3'
SEQ. ID. NO. 25	5'-AGGGCTTAACCCTGGAAGT-3'
SEQ. ID. NO. 26	5'-CTCATCGTTTACAGCGTGGA-3'
SEQ. ID. NO. 27	5'-GCTGTCATCTGGGCCATTAC-3'
SEQ. ID. NO. 28	5'-TTAATCTTGCGACCGTACTCC-3'
SEQ. ID. NO. 29	5'-TGGGGAGCAAACAGGATTAG-3'
SEQ. ID. NO. 30	5'-GTAAGGTTTTTCGCGGATCA-3'
SEQ. ID. NO. 31	5'-ATGGTAGTCGCGAAAAATGG-3'
SEQ. ID. NO. 32	5'-AAGGGCCATGATGACTTGAC-3'
SEQ. ID. NO. 33	5'-CAGCTCGTGTCTGAGATGT-3'
SEQ. ID. NO. 34	5'-CACCGTATCGCATCACTCTG-3'
SEQ. ID. NO. 35	5'-CTACACGCGTGCTACAATGG-3'
SEQ. ID. NO. 36	5'-TGTACAAGGCCCGAGAACGTA-3'

[39] Example 2: Sample preparation and vital statistics

[40] Among patients at the age of 18 or above who went to a hospital because of acute febrile disease in 4 weeks from the outbreak, from 2007 to 2008, those who showed at least two symptoms selected from the group consisting of eschar, erythematous papular rash, headache, general prostration, muscle ache, cough, nausea, and stomachache

were selected for blood sampling.

[41] In the blood sample where IFA titer for *Orientia tsutsugamushi* was increased at least 4 times, the patient of that sample was diagnosed as scrub typhus (tsutsugamushi). So, such patients were grouped into tsutsugamushi patient group (scrub typhus group). In patients in the febrile control group (non-scrub typhus group), indirect fluorescent antibody against *Orientia tsutsugamushi* was not detected in their blood, and instead they were diagnosed as another febrile disease except scrub typhus by the tests such as serology, culture, and peripheral blood smear tests, etc, by Infectious diseases physicians.

[42] An experiment was performed with blood samples of randomly selected 115 patients diagnosed as scrub typhus and 52 patients confirmed as febrile control group (non-scrub typhus). Vital statistics were also calculated and the results are shown in Table 2.

[43] Table 2

[Table 2]

Vital statistics of patients

Characteristics	Tsutsugamushi patient group (scrub typhus group)	Febrile control group (non-scrub typhus group)	P-values
Age, mean value (range)	69 (17-91)	54 (16-93)	0.0002
Gender ratio			NS
Male	39 (33.9%)	20 (38.5%)	
Female	76 (66.1%)	32 (61.5%)	
Number	115	52	167

[44] As shown in Table 2, in the aspect of the average age of all patients (patient group: 66, control group: 53, mean value: 69 and 54 respectively), the average age of the tsutsugamushi patient group (scrub typhus group) was meaningfully higher than that of the febrile control group (non-scrub typhus group) ($p=0.0002$). There were no statistically meaningful differences between the gender ratio in the tsutsugamushi patient group (scrub typhus group) and that in the febrile control group (non-scrub typhus group) ($p=0.693$) (Table 2).

[45]

[46] Example 3: PCR diagnosis using the primer pairs of the present invention

[47] To confirm the efficacy of the primer pairs prepared and the comparative examination method, DNA was extracted from the blood samples of tsutsugamushi patients prepared in example 2. Particularly, whole blood was taken from a patient.

Buffy coat was separated. Then, DNA was separated by using QIA amp DNA mini kit (Qiagen, Germany). The separation was performed by following the protocol enclosed in the manual. The separated DNA was used as the template for detecting *Orientia tsutsugamushi*.

- [48] As a method for PCR diagnosis, the conventional PCR was selected herein and the extracted DNA was used as the template. For the PCR, 20 μl of reaction mixture was prepared by adding 2 μl of template DNA, 1 μl of 5 pmol/ μl forward primer and 1 μl of 5 pmol/ μl reverse primer, and 16 μl of sterilized deionized water to AccuPower™ PCR PreMix (1 U Top polymerase, 250 μM dNTP, 10 mM Tris-HCl (pH9.0); Bioneer, Daejeon, Korea). The tube containing the reaction mixture was well stirred, which was set in Biosystems Veriti™ 96-well Thermal cycler (Applied Biosystems, Foster City, CA), followed by PCR. PCR conditions are summarized in Table 3.
- [49] Upon completion of PCR, to confirm PCR final product, 1.5% agarose gel (Seakem[®] LE agarose; Cambrex Bio Science, Rockland, ME) containing 0.5 ng/mL EtBr (Ethidium bromide; Bioneer, Daejeon, Korea) was loaded in Bioneer electrophoresis machine (Bioneer, Korea), followed by electrophoresis at 100V (1X TAE; Bioneer, Korea) for 40 minutes.
- [50] The primers used in this experiment were the primer pair composed of sequences represented by SEQ. ID. NO. 1 and NO. 2, respectively. The amplified product of 16s ribosomal RNA gene by PCR of the invention is expected to have a size of 199 bp. The detection of a band of corresponding size confirmed whether *Orientia tsutsugamushi* was detected or not.

[51] Table 3

[Table 3]

Primers and reaction conditions of 16s conventional PCR

PCR reaction condition			
predenaturation	94 °C	2 minutes	36 cycle
denaturation	94 °C	1 minutes	
annealing	53 °C	30 seconds	
extension	72 °C	45 seconds	
extra-extension	72 °C	10 minutes	

[52] Comparative Examples 1~6:

[53] To compare the diagnostic accuracy of the detection method using the primer pairs of the present invention with others, the following comparative experiments were performed. At this time, the template DNA was the same as that prepared in example 3.

[54] The conventional PCR (47kDa C-PCR: comparative example 1), nested PCR (47kDa

N-PCR: comparative example 2) and real time PCR (47kDa Q-PCR: comparative example 3) targeting 47kDa generally used for detecting tsutsugamushi were performed. The conventional PCR (56kDa C-PCR: comparative example 4) and nested PCR (56kDa N-PCR: comparative example 5) targeting 56kDa and the conventional PCR targeting groEL (groEL C-PCR: comparative example 6) were also performed. The efficacies of those methods were compared.

[55] The primers for each PCR were prepared according to the references stated in Table 4 or designed by the inventors, and the method is explained hereinafter.

[56] Table 4

[Table 4]

Primer and probe sequencing information for comparative PCR

	Sequence name	Sequence	product size(bp)	Ref
47kD C-PCR/Q-PCR	OtsuFP630	(5' -AACTGATTTTATTCAACTAATGCTGCT- 3')	118	1
	OtsuRP747	(5' -TATGCCTGAGTAAGATACRTGAATRGAATT- 3')		
	OtsuPR665	[6FAM]TGGGTAGCTTTGGTGGACCGATGTTTAA TCT[BHQ1]	probe	1
47kDa N-PCR (External Primer)	OtsuFP555	(5' -TCCTTTCGGTTTAAGAGGAACA-3')	238	
	OtsuRP771	(5' -GCATTCAACTGCTTCAAGTACA- 3')		
47kDa N-PCR (Internal Primer)	OtsuFP630	(5' -AACTGATTTTATTCAACTAATGCTGCT- 3')	118	1
	OtsuRP747	(5' -TATGCCTGAGTAAGATACRTGAATRGAATT- 3')		
56kD C-PCR	P10	(5' -GATCAAGCTTCCTCAGCCTACTATAATGCC- 3')	483	2
	P11	(5' -CTAGGGATCCCGACAGATGCACTATTAGGC- 3')		
56kD N-PCR (External Primer)	P34	(5' -TCAAGCTTATTGCTAGATCTGC- 3')	1003	2
	P55	(5' -AGGGATCCCTGCTGCTGTGCTTGCTGCG- 3')		
56kD N-PCR (Internal Primer)	P10	(5' -GATCAAGCTTCCTCAGCCTACTATAATGCC- 3')	483	2
	P11	(5' -CTAGGGATCCCGACAGATGCACTATTAGGC- 3')		
groEL C-PCR	STG-F1(TF1)	(5' -ATATATCACAGTACTTTGCAAC- 3')	366	3
	STG-R1(TR2)	(5' -GTTCTAACTTAGATGTATCAT- 3')		

- [57] 1) Conventional PCR (C-PCR)
- [58] A total 20 μ l of reaction mixture was prepared by mixing 2 μ l of DNA, 1 μ l of forward primer, 1 μ l of reverse primer, AccuPowerTM PCR PreMix (1 U Top polymerase, 250 μ M dNTP, 10 mM Tris-HCl (pH9.0), 30 mM KCl, 1.5mM MgCl₂, stabilizer and tracking dye; Bioneer, Daejeon, Korea) and sterilized deionized water.
- [59] At this time, each PCR was performed with each primer as shown in Table 4 by using Biosystems VeritiTM 96-well Thermal cycler (Applied Biosystems, Foster City, CA). PCR conditions for each target gene are summarized in Table 5.
- [60] To confirm PCR product, 1.5% agarose gel (Seakem^R LE agarose; Cambrex Bio Science, Rockland, ME) containing 0.5 ng/mL Etbr (Ethidium bromide; Bioneer, Daejeon, Korea) was loaded in Bioneer electrophoresis machine (Bioneer, Korea), followed by electrophoresis at 100V (1X TAE; Bioneer, Korea) for 40 minutes.
- [61] Table 5

[Table 5]

			56kDa			47kDa			groEL		
			Temp (°C)	Time (min)	(cycle)	Temp (°C)	Time (min)	(cycle)	Temp (°C)	Time (min)	(cycle)
C-PCR		predenaturation	94	10m	35	94	10m	30	94	10m	35
		denaturation	94	1m		94	1m		94	30s	
		annealing	63	1m		60	1m		59	45s	
		extension	72	1m		72	1m		72	1m	
		extra-extension	72	7m		72	7m		72	7m	
N-PCR	1st	predenaturation	94	10m	35	94	10m	30			
		denaturation	94	1m		94	1m				
		annealing	61	1m		56	1m				
		extension	72	1m		72	1m				
		extra-extension	72	10m		72	7m				
	2nd	predenaturation	94	10m	30	94	10m	25			
		denaturation	94	30s		94	30s				
		annealing	63	30s		60	30s				
		extension	72	1m		72	1m				
		extra-extension	72	7m		72	7m				
Q-PCR		predenaturation				95	10m	45			
		denaturation				95	10s				
		annealing				60	30s				
		cooling				40	30s				

[62] 2) Nested PCR (N-PCR)

- [63] For the PCR targeting 56kDa gene, P10 and P11 (5 pmoles/ μ l) were used as internal primers and P34 and P55 (5 pmole/ μ l) were used as external primers. The first PCR reaction mixture was prepared by the same manner as used for the preparation of the

conventional PCR. The second round PCR was performed using the first PCR product (1 μ l/ 20 μ l total volume) as template DNA with the primers P10 and P11 (5 pmoles/ μ l). The final PCR product was electrophoresed on 1.5% agarose gel at 100 V. PCR targeting 47 kDa gene was performed by the same manner as described in 56kDa N-PCR except that different primers were used.

[64]

[65] 3) Real time PCR (Q-PCR)

[66] Real time PCR targeting 47kDa gene was performed with 20 μ l of reaction mixture (5 μ l of genomic DNA, 1 μ l of 5 pmoles/ μ l forward primer and 1 μ l of 5 pmoles/ μ l reverse primer (OtsuFP630 and OtsuRP747), 1 μ l of 2 pmoles/ μ l probe (OtsuPR665), 4 μ l of master mix (reaction buffer, FastStart Taq DNA polymerase, MgCl₂, dNTP (dUTP instead of dTTP)), and distilled water).

[67] The real-time PCR conditions were as follows: at 95°C for 10 minutes, two steps at 95°C for 10 seconds each, at 60°C for 30 seconds (45 cycles) and finally at 40°C for 30 seconds. The result was analyzed by Light Cycler software 4.0 program.

[68] 5' and 3' ends of the probe OtsuPR665 were labeled with FAM (6-carboxyFluo-rescein) and BHQ-1 (Black Hole Quencher-1).

[69]

[70] Example 4: Comparative analysis of diagnostic accuracy

[71] Diagnostic accuracy of the detection method of *Orientia tsutsugamushi* using the primer pair composed of sequences represented by SEQ. ID. NO. 1 and NO. 2 originated from 16s ribosomal RNA gene of the present invention and other detection methods were evaluated.

[72] For the evaluation, sensitivity and specificity were presented as receiver operator characteristic (ROC) curve concept. The diagnostic accuracy was compared with those of other detection methods by using MEDCALC software program (MedCalc Software, Mariakerke, Belgium, version 11.2.1), and the results are shown in Table 6, Fig. 1 and the below <result> (Table 6, Fig. 1).

[73]

[74] <Result>

[75] **Diagnostic accuracy of the PCR method for tsutsugamushi of the present invention**

[76] PCR was performed with the blood samples of 115 scrub typhus patients and 52 patients diagnosed as other diseases (non-scrub typhus group). From the result of 47kDa C-PCR, it was confirmed that 3 of the 115 patients (3%) were positive. From the result of 47dKa N-PCR, it was confirmed that 93 of the 115 patients were positive, indicating the sensitivity was 81%.

[77] In 47kDa Q-PCR, 38 cp or above was set as negative, and under that condition, 87 of

the 115 scrub typhus patients were positive (76% of positive rate).

[78] In C-PCR targeting 56kDa protein gene, 9 of the 115 scrub typhus patients were positive, and 92 of the 115 patients were positive in N-PCR (80% of sensitivity).

[79] In C-PCR targeting groEL gene, 76 of the 115 scrub typhus patients were positive, suggesting 66% of sensitivity.

[80] In the PCR method of the present invention targeting 16s ribosomal RNA gene (C-PCR of example 3), 100 of the 115 scrub typhus patients were positive, suggesting 86% of sensitivity.

[81] In the non-scrub typhus group, no patients were positive in any PCR test except that 2 blood samples were confirmed as positive in 47kDa Q-PCR with the non-scrub typhus group.

[82]

[83] **Comparison of diagnostic accuracy of the detection method of the present invention to that of the detection methods of comparative examples**

[84] 1) Comparison of diagnostic accuracy according to each target gene in the conventional PCR

[85] AUC of the conventional PCR was as follows. AUC of 16s C-PCR was 0.94 (95% CI, 0.89-0.97), and AUC of 47kDa C-PCR was 0.51 (95% CI, 0.44-0.59). AUC of 56kDa C-PCR was 0.54 (95% CI, 0.46-0.62), and AUC of groEL C-PCR was 0.8 (95% CI, 0.77-0.88). AUC of 16s C-PCR has statistically meaningful differences over those of other C-PCRs (16s C-PCR vs 47kDa C-PCR, $p<0.001$; 16s C-PCR vs 56kDa C-PCR, $p<0.001$). AUC of 47kDa C-PCR had no statistically meaningful differences over that of 56kDa C-PCR ($p=0.081$) (Fig. 1).

[86]

[87] 2) Comparison of diagnostic accuracy according to PCR in each target protein gene

[88] C-PCR targeting 47kDa gene demonstrated 3% (95% CI, 0.5-7.4) of sensitivity and 100% (95% CI, 93.2-100) of specificity. In the case of N-PCR targeting the gene, sensitivity was 81% (95% CI, 72.5-87.6) and specificity was 100% (95% CI, 93.2-100). Diagnostic accuracy of 47kDa Q-PCR was 76% (95% CI, 66.8-83.2) of sensitivity and 96% (95% CI, 86.8-99.5) of specificity when 38 cp was determined as cut-line for negative result. AUC of 47kDa N-PCR was 0.9 (95% CI, 0.85-0.94), and AUC of Q-PCR was 0.86 (95% CI, 0.8-0.91). AUC of C-PCR was 0.51 (95% CI, 0.44-0.59). There were statistically meaningful differences in each comparison (N-PCR vs C-PCR, $p<0.001$; N-PCR vs Q-PCR, $p=0.012$, C-PCR vs Q-PCR, $p<0.001$).

[89] In C-PCR targeting 56kDa gene, sensitivity was 8% (95% CI, 3.6-14.3) and specificity was 100% (95% CI, 93.2-100). In N-PCR targeting 56kDa gene, sensitivity was 80% (95% CI, 71.5-86.9) and specificity was 100% (95% CI, 93.2-100). In the meantime, AUC (area under the curve) of N-PCR was 0.9 (95% CI, 0.46-0.61), and

AUC of C-PCR was 0.54 (95% CI, 0.46-0.62), suggesting that there was a statistically meaningful difference (N-PCR vs C-PCR, $p < 0.001$).

- [90] Diagnostic accuracy was compared according to AUC among 47kDa N-PCR and Q-PCR, 56kDa N-PCR, and the detection method of tsutsugamushi using the primers for 16s ribosomal RNA gene. As a result, C-PCR of the present invention using the primers for 16s ribosomal RNA gene demonstrated superior AUC values to any other methods and the statistically meaningful difference (16s C-PCR vs 47kDa N-PCR, $p = 0.017$; 16s C-PCR vs 56 kDa N-PCR, $p = 0.03$; 16s C-PCR vs 47kDa Q-PCR, $p < 0.001$) (Fig. 2).

- [91] Table 6

[Table 6]

Analysis of diagnostic accuracy by ROC curve according to target gene and PCR method

	Sensitivity/specificity	AUC	p value (compared with example 3)
Example 3 (This invention)	86%/100%	0.94(95% CI, 0.89-0.97)	-
Comparative Example 1 (47kDa C-PCR)	3%/100%	0.51(95% CI, 0.44-0.59)	<0.001
Comparative Example 2 (47kDa N-PCR)	81%/100%	0.9(95% CI, 0.85-0.94)	0.017
Comparative Example 3 (47kDa Q-PCR)	76%/96%	0.86(95% CI, 0.8-0.91)	<0.001
Comparative Example 4 (56kDa C-PCR)	8%/100%	0.54(95% CI, 0.46-0.62)	<0.001
Comparative Example 5 (56kDa N-PCR)	80%/100%	0.9(95% CI, 0.46-0.61)	0.03
Comparative Example 6 (groEL)	61%/100%	0.8(95% CI, 0.77-0.88)	<0.001

- [92] That is to say, the comparison of diagnostic accuracy with AUC for tsutsugamushi shows that the diagnostic accuracy of the detection method of tsutsugamushi of the present invention (example 3) using the primers for 16s ribosomal RNA was superior to that of other conventional detection methods (comparative examples 1-6), and the difference in diagnostic accuracy was statistically meaningful. Moreover, it shows that the conventional PCR with the primers for 16s ribosomal RNA gene invented by the present inventors demonstrated much higher diagnostic accuracy than nested PCR and real-time PCR amplifying 47kDa gene which had been known to be very accurate and

appropriate for disease diagnosis.

[93]

[94] Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

[95]

[96] <Reference>

[97] 1. Jiang J, Chan TC, Temenak JJ, Dasch GA, Ching WM, Richards AL. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. Am J Trop Med Hyg. 2004 Apr;70(4):351-6.

[98]

[99] 2. Kim DM, Yun NR, Yang TY, Lee JH, Yang JT, Shim SK, et al. Usefulness of nested PCR for the diagnosis of scrub typhus in clinical practice: A prospective study. Am J Trop Med Hyg. 2006 Sep;75(3):542-5.

[100]

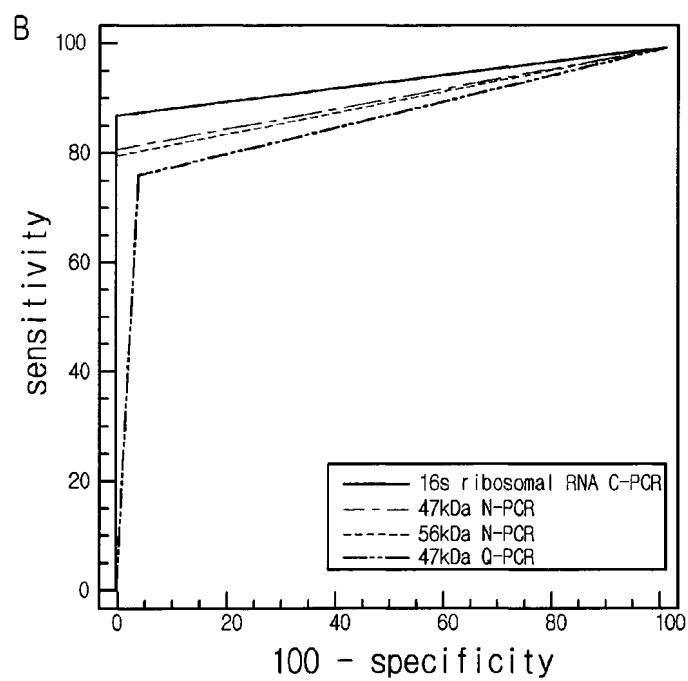
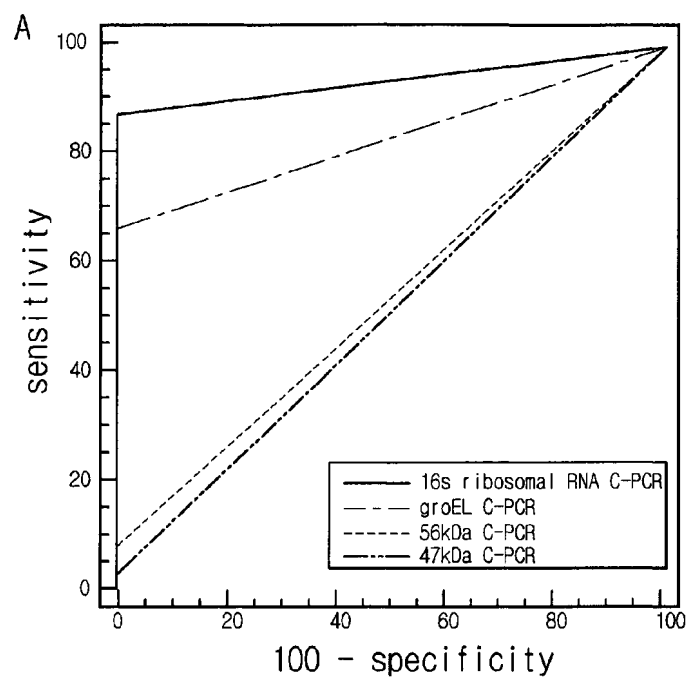
[101] 3. Park HS, Lee JH, Jeong EJ, Kim JE, Hong SJ, Park TK, Kim TY, Jang WJ, Park KH, Kim BJ, Kook YH, Lee SH. Rapid and simple identification of *Orientia tsutsugamushi* from other group rickettsiae by duplex PCR assay using groEL gene. Microbiol Immunol. 2005;49(6):545-9.

[102]

Claims

- [Claim 1] A primer pair for the diagnosis of scrub typhus composed of two sequences selected from the group consisting of:
oligonucleotide sequences represented by SEQ. ID. NO. 1 ~ NO. 36;
and
sequences of at least 18 mer having at least 90% homology with said sequences.
- [Claim 2] The primer pair for the diagnosis of scrub typhus according to claim 1, composed of the sequences represented by SEQ. ID. NO. 1 and NO. 2.
- [Claim 3] A diagnosis method of scrub typhus using the primer pair of claim 1.
- [Claim 4] The diagnosis method of scrub typhus according to claim 3, wherein the primer pair composed of the sequences represented by SEQ. ID. NO. 1 and NO. 2.
- [Claim 5] The diagnosis method of scrub typhus according to claim 3 or claim 4, comprising detecting *Orientia tsutsugamushi* with polymerase chain reaction (PCR).
- [Claim 6] The diagnosis method of scrub typhus according to claim 3 or claim 4, wherein the detecting *Orientia tsutsugamushi* comprises the following steps of;
amplifying 16s rRNA gene sequence of *Orientia tsutsugamushi* or a part of the sequence; and
identifying thereof.
- [Claim 7] The diagnosis method according to claim 5, wherein the polymerase chain reaction (PCR) is the conventional PCR.
- [Claim 8] A PCR kit for detecting *Orientia tsutsugamushi* by the amplification of 16s rRNA gene sequence or a part of the sequence, comprising the primer pairs of claim 1 or 2.
- [Claim 9] A PCR kit for detecting *Orientia tsutsugamushi* by the amplification of 16s rRNA gene sequence or a part of the sequence.
- [Claim 10] A PCR kit for detecting *Orientia tsutsugamushi* by amplification of 16s rRNA gene sequence or a part of the sequence, comprising primer pair composed of the sequences represented by SEQ. ID. NO. 1 and NO. 2.

[Fig. 1]



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2010/005765**A. CLASSIFICATION OF SUBJECT MATTER***C12N 15/11(2006.01)i, C12Q 1/68(2006.01)i*

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/11

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

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal), Google, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Odorico, D.M. et al., New Orientia tsutsugamushi strain from scrub typhus in Australia, Emerg. Infect. Dis., Oct.-Dec. 1998, Vol. 4, No. 4, pp. 641-644 See abstract; p. 643	1,2,8-10
X	Ohashi, N. et al., Phylogenetic position of Rickettsia tsutsugamushi and the relationship among its antigenic variants by analyses of 16S rRNA gene sequences, FEMS Microbiol. Lett., 15 Jan. 1995, Vol. 125, No. 2-3, pp. 299-304 See abstract	1,2,8-10
Y	Nakayama, K. et al., The Whole-genome sequencing of the obligate intracellular bacterium Orientia tsutsugamushi revealed massive gene amplification during reductive genome evolution, DNA Res., Aug. 2008, Vol. 15, No. 4, pp. 185-199 See the whole document	1,2,8-10
Y	Edwards, U. et al., Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA, Nucleic Acids Res., 11 Oct. 1989, Vol. 17, No. 19, pp. 7843-7853 See the whole document	1,2,8-10

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 AUGUST 2011 (25.08.2011)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2010/005765

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

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

1. ☒ Claims Nos.: 3-7
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 3-7 pertain to methods for treatment of the human body by therapy, as well as diagnostic methods, and thus relate to a subject matter which this International Searching Authority is not required to search under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2010/005765

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
None			



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权利要求书1页 说明书11页

序列表7页 附图2页

(54) 发明名称

用于检测恙虫病东方体的引物对和使用所述引物对的检测方法

(57) 摘要

本发明涉及恙虫病的诊断方法,更具体而言,本发明涉及通过对恙虫病东方体(引起恙虫病的原因)进行检测的诊断方法,所述方法包括如下步骤:基于恙虫病东方体的 16s 核糖体 RNA 基因序列制备引物对;然后使用所制备的引物对对恙虫病东方体进行检测。本发明的诊断方法不仅显示出高于其它传统方法(对 47kDa、56kDa 和 groEL 进行扩增和分析的方法)的诊断准确度,而且还非常简单方便,这表明所述方法在成本效益方面很出色。

1. 一种用于对恙虫病进行诊断的引物对,所述引物对由选自于由如下序列所组成的组中的两种序列组成:

由 SEQ. ID. NO. 1-SEQ. ID. NO. 36 表示的寡核苷酸序列;以及
与上述序列具有至少 90% 同源性的、至少为 18mer 的序列。

2. 根据权利要求 1 所述的用于对恙虫病进行诊断的引物对,所述引物对由 SEQ. ID. NO. 1 和 SEQ. ID. NO. 2 所示的序列组成。

3. 一种使用权利要求 1 所述的引物对的恙虫病诊断方法。

4. 根据权利要求 3 所述的恙虫病诊断方法,其中,所述引物对由 SEQ. ID. NO. 1 和 SEQ. ID. NO. 2 所示的序列组成。

5. 根据权利要求 3 或 4 所述的恙虫病诊断方法,所述方法包括用聚合酶链式反应(PCR)对恙虫病东方体进行检测。

6. 根据权利要求 3 或 4 所述的恙虫病诊断方法,其中,所述对恙虫病东方体进行检测包括下列步骤:

对恙虫病东方体的 16s rRNA 基因的序列或所述序列的一部分进行扩增;以及
对其进行鉴定。

7. 根据权利要求 5 所述的诊断方法,其中,所述聚合酶链式反应(PCR)为常规 PCR。

8. 一种用于对恙虫病东方体进行检测的 PCR 试剂盒,所述试剂盒通过扩增 16s rRNA 基因的序列或所述序列的一部分对恙虫病东方体进行检测,所述试剂盒包含权利要求 1 或 2 所述的引物对。

9. 一种用于对恙虫病东方体进行检测的 PCR 试剂盒,所述试剂盒通过扩增 16s rRNA 基因的序列或所述序列的一部分对恙虫病东方体进行检测。

10. 一种用于对恙虫病东方体进行检测的 PCR 试剂盒,所述试剂盒通过扩增 16s rRNA 基因的序列或所述序列的一部分对恙虫病东方体进行检测,所述试剂盒包含由 SEQ. ID. NO. 1 和 SEQ. ID. NO. 2 所示的序列组成的引物对。

用于检测恙虫病东方体的引物对和使用所述引物对的检测方法

技术领域

[0001] 本发明涉及用于诊断恙虫病东方体(*Orientia tsutsugamushi*)的引物对和使用所述引物对的诊断恙虫病东方体的方法,更具体而言,本发明涉及能够对恙虫病东方体(引发恙虫病(*scrub typhus*)的原因)的 16s 核糖体 RNA 基因的碱基序列进行扩增的引物对和使用所述引物对通过对恙虫病东方体进行检测的恙虫病检测方法。

背景技术

[0002] 恙虫病为一种急性热病(*febrile disease*),该病通过感染了恙虫病东方体的恙螨进行传播,并由于系统性血管炎而显示出特征性的临床症状。啮齿动物为主要宿主,幼螨(恙螨)为宿主和媒介。

[0003] 自恙虫病 1951 年首次在韩国报道以来,这种主要的秋季热病已蔓延至全国各地,特别是在九月至十一月期间。尤其是,已发生了如此之多的病例,以至于秋季中 30% 的患者都被诊断为恙虫病。

[0004] 恙螨叮咬后的 1-2 周内将产生如下症状:例如发热、发冷、头疼、肌肉疼痛和红斑性丘疹。对产生的特征焦痂的检测将有助于该疾病的早期诊断。通常,这一疾病的发展并不严重,并且抗生素对该疾病很有效。然而,如果发生延误诊断,则可产生如下形式的并发症:肺炎、急性肾衰竭、脑膜炎、脑炎、上胃肠道出血、多器官功能障碍、或者甚至更糟的心肌梗死或中风。这些并发症可能是致命的,因此快速而准确的诊断是改善患者预后所必不可少的。

[0005] 到目前为止,用于诊断恙虫病的方法包括如下方法:例如,通过培养以确认恙虫病东方体的方法、通过对患者血清中的抗恙虫病东方体的抗体进行检测的方法、以及通过 PCR(聚合酶链式反应)扩增对从患者血液中分离的恙虫病东方体的 DNA 进行检查的方法。

[0006] 在这些方法中,通过对恙虫病东方体进行培养的诊断方法需要至少几周来培养恙虫病东方体,这表明这一方法实际上不适合用于诊断患者的目的。所以,最常见的诊断方法为间接荧光抗体技术和免疫酶技术,其均为血清学检测方法。

[0007] 然而,上述方法仍具有如下缺点:由与其它病原微生物的交叉反应所造成的假阳性;从疾病症状开始后直至形成抗体需要几天,导致该疾病早期测试的低灵敏度;以及需要额外的跟踪测试来对诊断进行确认。

[0008] 介绍了恙虫病的另一诊断方法,该方法是通过 PCR 对来自患者血液的恙虫病东方体特异性 DNA 进行检测、以在恙虫病的早期阶段对其进行快速诊断的方法。PCR 的实例包括:常规 PCR;或巢式 PCR,巢式 PCR 通过对常规 PCR 进行改进而得到,并显示出高于常规 PCR 100 倍的灵敏度;或实时 PCR,在实时 PCR 的过程中,可在 2 小时内快速确认结果。

[0009] 在恙虫病的常规 PCR 诊断中,已尝试了使用各种靶蛋白基因(如 47kDa、56kDa 和 groEL 等)的 PCR,相比常规 PCR,更优选巢式 PCR 或实时 PCR。因为当使用 47kDa 或 56kDa 基因作为靶标来进行常规 PCR 时,灵敏度低至至多 10%。然而,巢式 PCR 具有如下缺点:由于将

PCR 进行了两次来增加灵敏度,因而确实需要比其它 PCR 更长的测试时间。同时,实时 PCR 具有如下缺点:由于需要非常昂贵的测试设备,在大多数医疗机构普遍使用方面存在局限。

[0010] 因此,迫切需要开发一种新的诊断方法,所述诊断方法相当简单而且低成本,并呈现高灵敏度和特异性。

发明内容

[0011] 技术问题

[0012] 本发明人试图开发新的方法来对来自患者血液样品的恙虫病东方体进行方便易行的检测。结果,本发明人通过确认了如下内容而完成了本发明:通过对恙虫病东方体的 16s 核糖体 RNA 基因序列的一部分进行特异性扩增,甚至用现有的常规 PCR,也能够实现高度准确的诊断。

[0013] 因此,本发明提供了恙虫病东方体的检测方法,该方法通过使用常规 PCR 对恙虫病东方体的 16s 核糖体 RNA 基因进行扩增。

[0014] 本发明的新方法克服了在常规 PCR 中灵敏度在 10% 以内的问题,同时还克服了在巢式 PCR 中进行两次或更多次 PCR 而导致需要更多工作量和时间以及高的污染潜在可能性的问题,并克服了在实时 PCR 中需要昂贵设备的问题。因此,本发明的方法在成本-工作量的效益方面是出色的方法,所述方法还提供了可与上述 PCR 相比拟的诊断准确度。

[0015] 技术方案

[0016] 为了实现上述目的,本发明人设计了恙虫病东方体的检测方法,以对恙虫病东方体的 16s 核糖体 RNA 基因序列进行扩增和鉴定。这一方法可通过制备能特异性扩增恙虫病东方体的 16s 核糖体 RNA 的引物而实现。

[0017] 以从感染有恙虫病东方体 Boryoung 菌株(该菌株在韩国非常常见)的患者的血液样品中扩增的 16s 核糖体 RNA 基因的碱基序列为基础来设计制备所述引物。

[0018] 因此,本发明的目的是提供用于诊断恙虫病的方法和用于检测恙虫病东方体的方法,其特征在于,所述方法包括在疑似患有恙虫病的患者的血液样品中使用 16s 核糖体 RNA 的引物扩增靶蛋白基因的步骤。

[0019] 本发明的另一目的是提供用于对恙虫病东方体进行检测的 PCR 试剂盒,所述 PCR 试剂盒包含所述引物。

[0020] 本发明的上述以及其它目的、特征和优点将在以下对优选实施方式和权利要求的描述中变得显而易见。

[0021] 有益效果

[0022] 本发明涉及用于诊断恙虫病的方法,更具体而言,本发明涉及能够扩增恙虫病东方体(引起恙虫病的原因)的 16s 核糖体 RNA 基因的碱基序列的引物和使用所述引物对恙虫病东方体进行诊断和检测的方法。

[0023] 与通过扩增 47kDa、56kDa 和 groEL 基因进行分析的传统方法相比,本发明的诊断方法具有出色且高的诊断准确度。同时,当将本发明的引物用于常规 PCR 时,获得了相对提高的诊断准确度。另外,本发明的方法不仅在诊断准确度方面优于巢式 PCR 或实时 PCR,而且还简单便宜。

附图说明

[0024] 图 1 为利用受试者工作特征(receiver operating characteristic, ROC)曲线来阐释对如下检测方法的诊断效率进行比较的图:使用由 SEQ. ID. NO. 1 和 SEQ. ID. NO. 2 表示的序列(来源于本发明的 16s 核糖体 RNA)组成的引物对的恙虫病东方体检测方法;以及比较例 1-6 中的传统检测方法。

具体实施方式

[0025] 本发明涉及恙虫病的诊断方法。更准确而言,本发明涉及使用 PCR 引物对恙虫病东方体(引起恙虫病的原因)的 16s 核糖体 RNA 基因的碱基序列进行扩增的恙虫病东方体检测方法。

[0026] 本发明还提供了用于扩增恙虫病东方体(引起恙虫病的原因)的 16s 核糖体 RNA 基因的碱基序列的 PCR 引物。

[0027] 本发明还提供了用于对恙虫病东方体进行检测的 PCR 试剂盒,所述 PCR 试剂盒包含所述引物。

[0028] 下文对本发明进行详细描述。

[0029] 本发明的特征在于制备 PCR 引物对来对恙虫病东方体的 16s 核糖体 RNA 基因进行扩增,以及对使用所述引物扩增的 16s 核糖体 RNA 基因进行检测。

[0030] 基于从感染有恙虫病东方体 Boryoung 菌株(该菌株在韩国非常常见)的患者的血液样品中扩增的 16s 核糖体 RNA 基因的碱基序列来设计本发明的引物。所述引物还可对其它模式菌株(如 Gilliam、Karp、Kato)和各种其它菌株进行检测。

[0031] 本发明的特征还在于通过使用所述引物扩增靶蛋白基因,所述靶蛋白基因为来自恙虫病疑似病例的血液样品中的 16s 核糖体 RNA 基因。

[0032] PCR 为聚合酶链式反应的简称。PCR 包括所有 PCR:最常见的常规 PCR、双重 PCR 和巢式 PCR(连续运行两次 PCR)、以及高成本的实时 PCR(在实时 PCR 过程中,可实时确认结果),最优选包括常规 PCR。

[0033] 本文中用于检测的 PCR 试剂盒可包括用于 PCR 的材料,例如参与聚合的酶、dNTP、缓冲液、矿物油、标准标记物、染色剂溴酚蓝或二甲苯 FF 以及用于扩增恙虫病东方体的 16s 核糖体 RNA 基因的 PCR 引物对。

[0034] 实施例

[0035] 如下实施例中所示的本发明实践的以及目前优选的实施方式仅用作说明。然而,可以理解的是,本领域技术人员在基于这一公开的考虑上可进行修改和改进,所述修改和改进在本发明的精神和保护范围之内。

[0036] 实施例 1:引物制备

[0037] 基于从感染有恙虫 Boryoung 菌株(该菌株在韩国非常常见)的患者的血液样品中扩增的 16s 核糖体 RNA 基因的碱基序列来设计引物对,然而,还可对其它模式菌株(如 Gilliam、Karp、Kato)和各种其它菌株进行检测。更具体而言,将所述引物对制备为用于 GenBank 登录号 bankit1356164HM352765:恙虫病东方体 Boryoung 基因型的正向引物(403-428 位)和反向引物(577-601 位),其分别由 SEQ. ID. NO. 1 和 SEQ. ID. NO. 2 表示。还制备了用于其它区域的正向引物和反向引物,所述引物分别由 SEQ. ID. NO. 3-SEQ. ID. NO. 36

表示(参见表 1)。

[0038] [表 1]

[0039]

	序列
SEQ. ID. NO. 1	5'-AGGGATGATAATGACAGTACCTACAG-3'
SEQ. ID. NO. 2	5'-CCTCTACCATACTCTAGCCTAACAG-3'
SEQ. ID. NO. 3	5'-ATTAGTGGCAAACGGGTGAG-3'
SEQ. ID. NO. 4	5'-CACAGGCTTGGTAAGCCATTA-3'
SEQ. ID. NO. 5	5'-GGAATCTGCCCATCAGTACA-3'
SEQ. ID. NO. 6	5'-GCTGTTCTGTCCTCTCAGACC-3'
SEQ. ID. NO. 7	5'-TGCTAATACCGTATGCCCTCT-3'
SEQ. ID. NO. 8	5'-CGTGTCTCAGTCCCAGTGTG-3'
SEQ. ID. NO. 9	5'-GGAAAGATTATCGCTGATGG-3'
SEQ. ID. NO. 10	5'-TGTCCAATATTCCCCACTGC-3'
SEQ. ID. NO. 11	5'-GCGGCAGATTAGGTAGTTGG-3'
SEQ. ID. NO. 12	5'-CATTGCTGGATCAGGCTTTC-3'
SEQ. ID. NO. 13	5'-AAGGTAATGGCTTAGCAAGCCTGTG-3'
SEQ. ID. NO. 14	5'-CAACCCTAAGGCCTTCATCA-3'
SEQ. ID. NO. 15	5'-GGTCTGAGAGGACGAACAGC-3'
SEQ. ID. NO. 16	5'-CGGGGCTTTTTCTGTAGGTA-3'
SEQ. ID. NO. 17	5'-ATATTGGACAATGGGCGAAA-3'
SEQ. ID. NO. 18	5'-TACGCCCAGTAATTCCGAAC-3'
SEQ. ID. NO. 19	5'-TGATGAAGGCCTTAGGGTTG-3'
SEQ. ID. NO. 20	5'-TTAAGCCCTGGGATTTCATC-3'
SEQ. ID. NO. 21	5'-AGAAAAAGCCCCGGCTAACT-3'
SEQ. ID. NO. 22	5'-GAAATTCCATCACCCCTCTACCA-3'
SEQ. ID. NO. 23	5'-GTTCTGGAATTACTGGGCGTA-3'
SEQ. ID. NO. 24	5'-AGATGACAGCTTTCGCCACT-3'
SEQ. ID. NO. 25	5'-AGGGCTTAACCCTGGAAGTG-3'
SEQ. ID. NO. 26	5'-CTCATCGTTTACAGCGTGGA-3'
SEQ. ID. NO. 27	5'-GCTGTCATCTGGGCCATTAC-3'
SEQ. ID. NO. 28	5'-TTAATCTTGCGACCGTACTCC-3'
SEQ. ID. NO. 29	5'-TGGGGAGCAAACAGGATTAG-3'
SEQ. ID. NO. 30	5'-GTAAGGTTTTTCGCGGATCA-3'
SEQ. ID. NO. 31	5'-ATGGTAGTCGCGAAAAATGG-3'
SEQ. ID. NO. 32	5'-AAGGGCCATGATGACTTGAC-3'
SEQ. ID. NO. 33	5'-CAGCTCGTGTCTGAGATGT-3'
SEQ. ID. NO. 34	5'-CACCGTATCGCATCACTCTG-3'
SEQ. ID. NO. 35	5'-CTACACGCGTGCTACAATGG-3'
SEQ. ID. NO. 36	5'-TGTACAAGGCCCGAGAACGTA-3'

[0040] 实施例 2:样品制备和人口统计(vital statistics)

[0041] 从 2007-2008 年,在由于发作开始 4 周内的急性热病而来医院的 18 岁或以上的患者中,选择表现出选自于由如下症状所组成的组中的至少两种症状的患者来取血样:焦痂、红斑性丘疹、头疼、全身虚脱、肌肉疼痛、咳嗽、恶心和胃疼。

[0042] 对于恙虫病东方体的 IFA 效价增加至少四倍的血液样品,将这些样品的患者诊断为恙虫病。因此,该类患者被划分入恙虫病患者组(恙虫病组)。在发热对照组(非恙虫病组)的患者中,在他们的血液中未检测到抗恙虫病东方体的间接荧光抗体,而通过测试(例如血清学测试、培养测试和外周血涂片测试等),他们被传染病医师诊断为除恙虫病外的另一热病。

[0043] 用随机选择的 115 名被诊断为恙虫病的患者的血液样品和 52 名确认为发热对照组(非恙虫病)的患者的血液样品进行实验。还对人口统计进行了计算,结果显示在表 2 中。

[0044] [表 2]

[0045] 患者的人口统计

[0046]	特征	恙虫病患者组 (恙虫病组)	发热对照组 (非恙虫病组)	P 值
	年龄, 平均值 (范围)	69 (17-91)	54 (16-93)	0.0002
	性别比			NS
	男性	39 (33.9%)	20 (38.5%)	
	女性	76 (66.1%)	32 (61.5%)	
	数量	115	52	167

[0047] 如表 2 中所示,在所有患者的平均年龄方面(患者组:66,对照组:53,平均值:分别为 69 和 54),恙虫病患者组(恙虫病组)的平均年龄有意义地高于发热对照组(非恙虫病组)的平均年龄($p=0.0002$)。恙虫病患者组(恙虫病组)与发热对照组(非恙虫病组)的性别比之间不存在统计学上有意义的差异($p=0.693$) (表 2)。

[0048] 实施例 3:使用本发明的引物对进行的 PCR 诊断

[0049] 为了确认所制备的引物对和相应的检查方法的效力,从实施例 2 中制备的恙虫病患者血液样品中提取 DNA。特别是,从患者中取全血。将血沉棕黄层(buffy coat)分离。然后,通过使用 QIA amp DNA mini 试剂盒(Qiagen, Germany)分离 DNA。通过遵循在手册中所附的方案进行分离。将分离的 DNA 用作检测恙虫病东方体的模板。

[0050] 作为用于 PCR 诊断的方法,在本文中选择常规 PCR,并将所提取的 DNA 用作模板。对于所述 PCR,通过向 AccuPower™ PCR PreMix(1U Top 聚合酶, 250 μ M dNTP, 10mM Tris-HCl (pH9.0); Bioneer, Daejeon, Korea) 中加入如下成分制备 20 μ l 反应混合物: 2 μ l 模板 DNA、1 μ l 15pmol/ μ l 的正向引物和 1 μ l 15pmol/ μ l 的反向引物、和 16 μ l 灭菌去离子水。充分振荡含有所述反应混合物的管(所述管被设置在 Biosystems Veriti™96 孔热循环仪 (Applied Biosystems, Foster City, CA) 中),接着进行 PCR。表 3 中总结了 PCR 条件。

[0051] PCR 完成后,为确认 PCR 终产物,将含 0.5ng/mL EtBr (溴化乙锭; Bioneer, Daejeon, Korea) 的 1.5% 琼脂糖凝胶 (Seakem[®] LE 琼脂糖; Cambrex Bio Science, Rockland, ME) 装载在 Bioneer 电泳仪 (Bioneer, Korea) 中,然后在 100V 下电泳 (1×TAE; Bioneer, Korea) 40 分钟。

[0052] 在这一实验中使用的引物为分别由 SEQ. ID. NO. 1 和 SEQ. ID. NO. 2 表示的序列所组成的引物对。预期由本发明的 PCR 而得到的 16s 核糖体 RNA 基因的扩增产物的大小为 199bp。对相应大小的条带进行检测来确认是否检测到恙虫病东方体。

[0053] [表 3]

[0054] 16s 常规 PCR 的反应条件

[0055]	PCR 反应条件			36 个循环
	预变性	94℃	2 分钟	
	变性	94℃	1 分钟	
	退火	53℃	30 秒	
	延伸	72℃	45 秒	
	额外延伸	72℃	10 分钟	

[0056] 比较例 1-6：

[0057] 为了对使用本发明的引物对进行的检测方法与其它检测方法的诊断准确度进行比较,进行了如下比较试验。此时,模板 DNA 与实施例 3 中制备的相同。

[0058] 进行了以 47kDa 为靶标的常规 PCR (47kDa C-PCR :比较例 1)、巢式 PCR (47kDa N-PCR :比较例 2)和实时 PCR (47kDa Q-PCR :比较例 3),所述 47kDa 通常用于对恙虫病进行检测。还进行了以 56kDa 为靶标的常规 PCR (56kDa C-PCR :比较例 4)和巢式 PCR (56kDa N-PCR :比较例 5)以及以 groEL 为靶标的常规 PCR (groEL C-PCR :比较例 6)。比较了这些方法的效力。

[0059] 用于各 PCR 的引物根据表 4 中所示的参考文献制备或由本发明人设计,下文将对方法进行解释。

[0060] [表 4]

[0061] 用于比较 PCR 的引物和探针序列信息

[0062]

	序列名称	序列	产物大小 (bp)	Ref
47kD C- PCR/Q- PCR	OtsuFP63 0	(5' -AACTGATTTTATTCAACTAATGCTGCT-3')	118	1
	OtsuRP74 7	(5' -TATGCCTGAGTAAGATACTGAATRGAATT-3')		
	OtsuPR66 5	[6FAM]TGGGTAGCTTTGGTGGACCGATGTTTAA TCT[BHQ1]	探针	1
47kDa N-PCR (外部 引物)	OtsuFP55 5	(5' -TCCTTTCGGTTTAAGAGGAACA-3')	238	
	OtsuRP77 1	(5' -GCATTCAACTGCTTCAAGTACA-3')		
47kDa N-PCR (内部 引物)	OtsuFP63 0	(5' -AACTGATTTTATTCAACTAATGCTGCT-3')	118	1
	OtsuRP74 7	(5' -TATGCCTGAGTAAGATACTGAATRGAATT-3')		
56kD C- PCR	P10	(5' -GATCAAGCTTCCTCAGCCTACTATAATGCC-3')	483	2
	P11	(5' - CTAGGGATCCCGACAGATGCACTATTAGGC-3')		
56kD N- PCR (外部 引物)	P34	(5' -TCAAGCTTATTGCTAGATCTGC-3')	1003	2
	P55	(5' -AGGGATCCCTGCTGCTGTGCTTGTGCTGCG-3')		
56kD N- PCR (内部 引物)	P10	(5' -GATCAAGCTTCCTCAGCCTACTATAATGCC-3')	483	2
	P11	(5' - CTAGGGATCCCGACAGATGCACTATTAGGC-3')		
groEL C- PCR	STG- F1(TF1)	(5' -ATATATCACAGTACTTTGCAAC-3')	366	3
	STG- R1(TR2)	(5' -GTTCCCTAACTTAGATGTATCAT-3')		

[0063] 1) 常规 PCR (C-PCR)

[0064] 通过将 2 μ l DNA、1 μ l 正向引物、1 μ l 反向引物、AccuPowerTM PCR PreMix (1U Top 聚合酶、250 μ M dNTP、10mM Tris-HCl(pH9.0)、30mM KCl、1.5mM MgCl₂、稳定剂以及示踪染料;Bioneer, Daejeon, Korea) 和灭菌去离子水进行混合制备总共 20 μ l 的反应混合物。

[0065] 此时, 通过使用 Biosystems VeritiTM96 孔热循环仪(Applied Biosystems, Foster City, CA), 用如表 4 中所示的各引物进行各 PCR。表 5 中总结了各靶基因的 PCR 条件。

[0066] 为确认 PCR 产物, 将含 0.5ng/mL EtBr (溴化乙锭;Bioneer, Daejeon, Korea) 的 1.5% 琼脂糖凝胶(Seakem^R LE 琼脂糖;Cambrex Bio Science, Rockland, ME)装载在 Bioneer 电泳仪(Bioneer, Korea)中, 然后在 100V 下电泳(1 $\times$ TAE;Bioneer, Korea)40 分钟。

[0067] [表 5]

			56kDa			47kDa			groEL		
			温度 (°C)	时间 (min)	(循环)	温度 (°C)	时间 (min)	(循环)	温度 (°C)	时间 (min)	(循环)
C-PCR		预变性	94	10m	35	94	10m	30	94	10m	35
		变性	94	1m		94	1m		94	30s	
		退火	63	1m		60	1m		59	45s	
		延伸	72	1m		72	1m		72	1m	
		额外延伸	72	7m		72	7m		72	7m	
N-PCR	1st	预变性	94	10m	35	94	10m	30			
		变性	94	1m		94	1m				
		退火	61	1m		56	1m				
		延伸	72	1m		72	1m				
		额外延伸	72	10m		72	7m				
	2nd	预变性	94	10m	30	94	10m	25			
		变性	94	30s		94	30s				
		退火	63	30s		60	30s				
		延伸	72	1m		72	1m				
		额外延伸	72	7m		72	7m				
Q-PCR		预变性				95	10m	45			
		变性				95	10s				
		退火				60	30s				
		冷却				40	30s				

[0069] 2) 巢式 PCR (N-PCR)

[0070] 对于以 56kDa 基因为靶标的 PCR, 将 P10 和 P11 (5pmole/ μ L) 用作内部引物, 并将 P34 和 P55 (5pmole/ μ L) 用作外部引物。第一轮 PCR 反应混合物通过与用于常规 PCR 的制备方法相同的方式进行制备。第二轮 PCR 使用引物 P10 和 P11 (5pmole/ μ L) 并使用第一轮 PCR 的产物 (1 μ L/20 μ L 总体积) 作为模板 DNA 进行。将最终的 PCR 产物在 100V 下、1.5% 琼脂糖凝胶上进行电泳。除使用不同的引物外, 通过与 56kDa N-PCR 中所述相同的方式进行以 47kDa 基因为靶标的 PCR。

[0071] 3) 实时 PCR (Q-PCR)

[0072] 用 20 μ L 反应混合物 (5 μ L 基因组 DNA、1 μ L 5pmole/ μ L 的正向引物和 1 μ L 5pmole/ μ L 的反向引物 (OtsuFP630 和 OtsuRP747)、1 μ L 2pmole/ μ L 的探针 (OtsuPR665)、4 μ L master mix (反应缓冲液、FastStartTaq DNA 聚合酶、MgCl₂、dNTP (dUTP 代替 dTTP))、以及蒸馏水) 进行以 47kDa 基因为靶标的实时 PCR。

[0073] 所述实时 PCR 的条件如下: 在 95°C 下 10 分钟; 在 95°C 下 10 秒、在 60°C 下 30 秒的两个步骤 (45 个循环); 以及最后在 40°C 下 30 秒。通过 Light Cycler 软件 4.0 程序分析结果。

[0074] 探针 OtsuPR665 的 5' 末端和 3' 末端标记有 FAM (6-carboxyFluo-rescein) 和

BHQ-1 (Black Hole Quencher-1)。

[0075] 实施例 4 : 诊断准确度的比较分析

[0076] 对如下检测方法的诊断准确度进行了评价 : 使用由 SEQ. ID. NO. 1 和 SEQ. ID. NO. 2 所示的序列 (所述序列源自本发明的 16s 核糖体 RNA 基因) 所组成的引物对进行的恙虫病东方体的检测方法和其它检测方法。

[0077] 对于所述评价, 以受试者工作特征 (ROC) 曲线的方式表示灵敏度与特异性。通过使用 MEDCALC 软件程序 (MedCalc Software, Mariakerke, Belgium, version 11.2.1) 进行与其它检测方法的诊断准确度的比较, 并在表 6、图 1 和下面的 < 结果 > 中示出了结果 (表 6 和图 1)。

[0078] < 结果 >

[0079] 本发明的恙虫病的 PCR 方法的诊断准确度

[0080] 用 115 名恙虫患者的血液样品和 52 名被诊断为其它疾病的患者 (非恙虫病组) 的血液样品进行 PCR。从 47kDa C-PCR 的结果中, 确认 115 名患者中的 3 名 (3%) 为阳性。从 47kDa N-PCR 的结果中, 确认 115 名患者中的 93 名为阳性, 说明灵敏度为 81%。

[0081] 在 47kDa Q-PCR 中, 将 38cp 或以上设置为阴性, 在该条件下, 115 名恙虫病患者中的 87 名为阳性 (76% 的阳性率)。

[0082] 在以 56kDa 蛋白基因为靶标的 C-PCR 中, 115 名恙虫病患者中的 9 名为阳性 ; 在 N-PCR 中, 115 名患者中的 92 名为阳性 (灵敏度为 80%)。

[0083] 在以 groEL 基因为靶标的 C-PCR 中, 115 名恙虫病患者中的 76 名为阳性, 表明灵敏度为 66%。

[0084] 在以 16s 核糖体 RNA 基因为靶标的本发明的 PCR 方法 (实施例 3 的 C-PCR) 中, 115 名恙虫病患者中的 100 名为阳性, 表明灵敏度为 86%。

[0085] 在非恙虫病组中, 除非恙虫病组中的 2 个血液样品在 47kDa Q-PCR 中被确定为阳性外, 在任何 PCR 测试中没有患者为阳性。

[0086] 本发明检测方法与比较例检测方法的诊断准确度的比较

[0087] 1) 常规 PCR 中根据各靶基因的诊断准确度的比较

[0088] 常规 PCR 的 AUC 如下。16s C-PCR 的 AUC 为 0.94 (95%CI, 0.89-0.97), 而 47kDa C-PCR 的 AUC 为 0.51 (95%CI, 0.44-0.59)。56kDa C-PCR 的 AUC 为 0.54 (95%CI, 0.46-0.62), 而 groEL C-PCR 的 AUC 为 0.8 (95%CI, 0.77-0.88)。16s C-PCR 的 AUC 与其它 C-PCR 的 AUC 具有统计学上有意义的差异 (16s C-PCR vs 47kDa C-PCR, $p < 0.001$; 16s C-PCR vs 56kDa C-PCR, $p < 0.001$)。47kDa C-PCR 的 AUC 与 56kDa C-PCR 的 AUC 不具有统计学上有意义的差异 ($p = 0.081$) (图 1)。

[0089] 2) 各靶蛋白基因中根据 PCR 的诊断准确度的比较

[0090] 以 47kDa 基因为靶标的 C-PCR 显示出 3% (95%CI, 0.5-7.4) 的灵敏度和 100% (95%CI, 93.2-100) 的特异性。在以该基因为靶标的 N-PCR 的情况下, 灵敏度为 81% (95%CI, 72.5-87.6), 特异性为 100% (95%CI, 93.2-100)。当将 38cp 确定为阴性结果的分割线时, 47kDa Q-PCR 的诊断准确度为 76% (95%CI, 66.8-83.2) 的灵敏度和 96% (95%CI, 86.8-99.5) 的特异性。47kDa N-PCR 的 AUC 为 0.9 (95%CI, 0.85-0.94), Q-PCR 的 AUC 为 0.86 (95%CI, 0.8-0.91)。C-PCR 的 AUC 为 0.51 (95%CI, 0.44-0.59)。在各比较中存在统计学上有意义

的差异(N-PCR vs C-PCR, $p < 0.001$; N-PCR vs Q-PCR, $p = 0.012$; C-PCR vs Q-PCR, $p < 0.001$)。

[0091] 在以 56kDa 基因为靶标的 C-PCR 中, 灵敏度为 8% (95%CI, 3.6-14.3), 特异性为 100% (95%CI, 93.2-100)。在以 56kDa 基因为靶标的 N-PCR 中, 灵敏度为 80% (95%CI, 71.5-86.9), 特异性为 100% (95%CI, 93.2-100)。与此同时, N-PCR 的 AUC (曲线下面积) 为 0.9 (95%CI, 0.46-0.61), 而 C-PCR 的 AUC 为 0.54 (95%CI, 0.46-0.62), 表明存在统计学上有意义的差异(N-PCR vs C-PCR, $p < 0.001$)。

[0092] 根据 47kDa N-PCR 和 Q-PCR、56kDa N-PCR、以及使用 16s 核糖体 RNA 基因的引物进行的恙虫病检测方法间的 AUC 对诊断准确度进行比较。结果, 使用 16s 核糖体 RNA 基因的引物进行的本发明的 C-PCR 显示出优于任何其它方法的 AUC 值, 并具有统计学上有意义的差异(16s C-PCR vs 47kDa N-PCR, $p = 0.017$; 16s C-PCR vs 56kDa N-PCR, $p = 0.03$; 16s C-PCR vs 47kDa Q-PCR, $p < 0.001$) (图 1)。

[0093] [表 6]

[0094] 根据靶基因和 PCR 方法通过 ROC 曲线进行的诊断准确度分析

[0095]

	灵敏度/特异性	AUC	P 值 (与实施例 3 相比)
实施例 3 (本发明)	86%/100%	0.94(95% CI, 0.89-0.97)	-
比较例 1 (47kDa C-PCR)	3%/100%	0.51(95% CI, 0.44-0.59)	<0.001
比较例 2 (47kDa N-PCR)	81%/100%	0.9(95% CI, 0.85-0.94)	0.017
比较例 3 (47kDa Q-PCR)	76%/96%	0.86(95% CI, 0.8-0.91)	<0.001
比较例 4 (56kDa C-PCR)	8%/100%	0.54(95% CI, 0.46-0.62)	<0.001
比较例 5 (56kDa N-PCR)	80%/100%	0.9(95% CI, 0.46-0.61)	0.03
比较例 6 (groEL)	66%/100%	0.8(95% CI, 0.77-0.88)	<0.001

[0096] 也就是说, 用 AUC 进行的恙虫病诊断准确度的比较显示出本发明的恙虫病诊断方法(实施例 3) (所述方法使用 16s 核糖体 RNA 的引物) 的诊断准确度优于传统检测方法(比较例 1-6), 并且在诊断准确度方面的差异具有统计学意义。另外, 显示出由本发明人发明的使用 16s 核糖体 RNA 基因的引物的常规 PCR 表现出远远高于扩增 47kDa 基因的巢式 PCR 和实时 PCR 的诊断准确度, 已知所述扩增 47kDa 基因的巢式 PCR 和实时 PCR 非常准确且适于疾病诊断。

[0097] 本领域技术人员将理解的是, 在上述的描述中所公开的概念和具体实施方式可以容易地被用作修改或设计用于实施本发明的相同目的其它实施方式的基础。本领域技术

人员也将理解,此类等同实施方式并未脱离如所附权利要求中所提出的本发明的精神和保护范围。

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[0001]

<110> Industry-Academic Cooperation Foundation, Chosun University

<120> 用于检测恙虫病东方体的引物对和使用所述引物对的检测方法

<130> PP-2013-0001/CN

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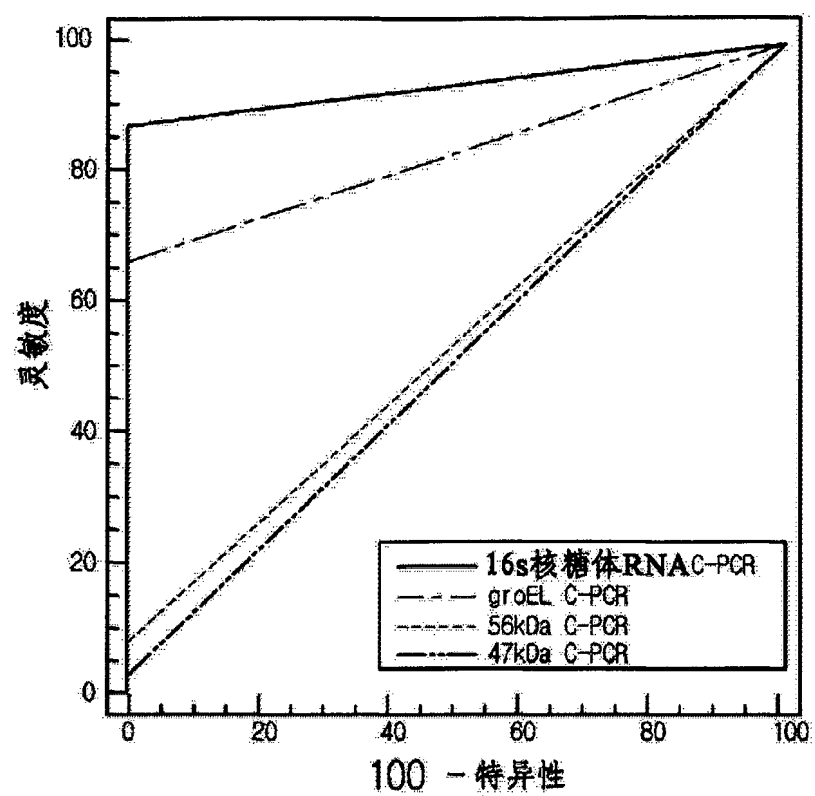


图 1A

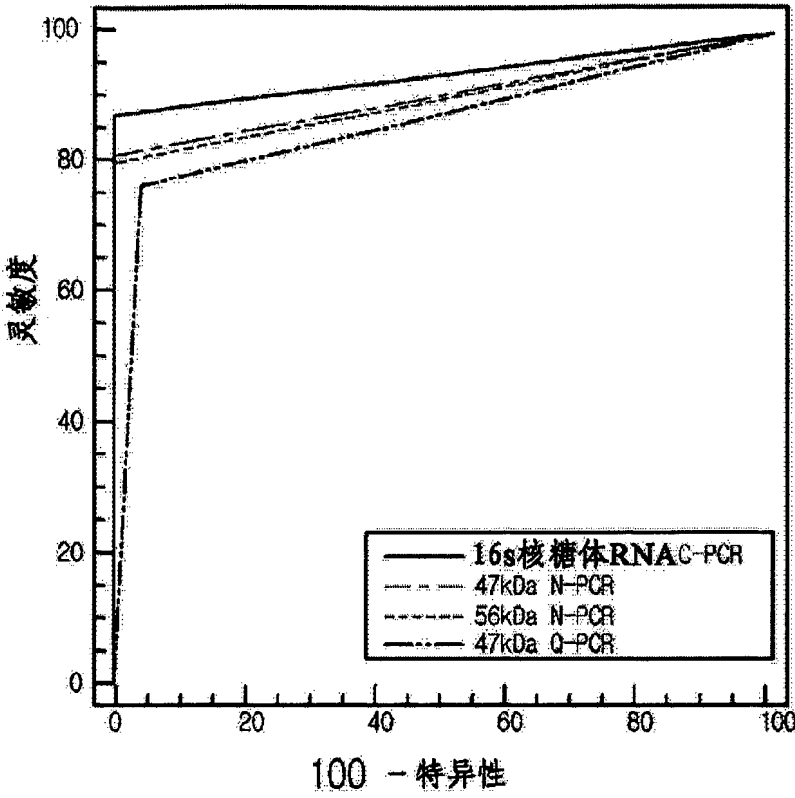


图 1B

Our Ref: 13HKI032-101

說明書摘要

發明名稱：用於檢測恙蟲病東方體的引物對和使用所述引物對的檢測方法

本發明涉及恙蟲病的診斷方法，更具體而言，本發明涉及通過對恙蟲病東方體（引起恙蟲病的原因）進行檢測的診斷方法，所述方法包括如下步驟：基於恙蟲病東方體的 16s 核糖體 RNA 基因序列製備引物對；然後使用所製備的引物對對恙蟲病東方體進行檢測。本發明的診斷方法不僅顯示出高於其它傳統方法（對 47kDa、56kDa 和 groEL 進行擴增和分析的方法）的診斷準確度，而且還非常簡單方便，這表明所述方法在成本效益方面很出色。

ABSTRACT

Title of Invention : PRIMER PAIRS FOR DETECTING ORIENTIA TSUTSUGAMUSHI
AND DETECTION METHOD USING THE SAME

The present invention relates to a diagnosis method of scrub typhus, more precisely, to a diagnosis method of scrub typhus by detecting *Orientia tsutsugamushi*, the cause of scrub typhus, comprising the following steps: preparing primer pairs based on the 16s ribosomal gene sequence of *Orientia tsutsugamushi*; and detecting *Orientia tsutsugamushi* using the prepared primer pairs. The diagnosis method of the present invention not only demonstrates higher diagnostic accuracy than other conventional methods amplifying and analyzing 47kDa, 57kDa, and groEL, but also is very simple and easy, suggesting that it is excellent in the aspect of cost-effect.