Title: DIAGNOSTIC FORMULATION FOR TSUTSUGAMUSHI DISEASE

Abstract: The present invention relates to a diagnostic kit for tsutsugamushi disease using a mixed antigen of tsutsugamushi. More specifically, the diagnostic kit for tsutsugamushi disease comprises a test strip comprising a sample pad absorbing the sample, a gold conjugation pad binding with human antibodies in the sample, a test membrane comprising a test line containing a mixed antigen of tsutsugamushi and a control line containing a control protein, an absorption pad absorbing the residual sample. In the diagnostic kit of the present invention, the antibody against tsutsugamushi can be simply detected without using any equipment, whereby the infection with Orientia tsutsugamushi can be confirmed with the naked eye.
Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FT, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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Description

DIAGNOSTIC FORMULATION FOR TSUTSUGAMUSHI DISEASE

Technical Field

[1] The present invention relates to a diagnostic kit for tsutsugamushi disease, in which antibodies against Orientia tsutsugamushi in a biological sample are detected using a mixed antigen of Orientia tsutsugamushi.

Background Art

[2] Tsutsugamushi disease is an acute febrile illness caused by Orientia tsutsugamushi, which belongs to scrub typhus group of the family Rickettsiaceae, and transmitted by the bite of larval-stage trombiculid mites. Symptoms, such as chills, fever, and headache occur in about 10 days (1 to 3 weeks) after being bitten by a larval-stage trombiculid mite, and a rash begins to appear on the trunk of a human body around one week after the onset of symptoms, and then spreads centrifugally to the extremities. A Blister occurs at the bite site of larval-stage trombiculid mites, followed by ulcer formation with a size of 0.5 to 0.8 cm. The ulcer is covered by a black crust, which is referred to as eschar. Some patients develop respiratory symptoms, in which chest x-rays showed pulmonary infiltration is present in approximately half of the patients. In some severe cases, the central nervous system may be infected, which leads to loss of consciousness or death.

[3] Tsutsugamushi disease occurs worldwide, but is particularly frequent in Asia including Japan, China, Malaysia, Thailand, Vietnam, or the like (Tsutsugamushi disease in Korea, edited by WooHyun Jang, published by Seoheung Co., p. 21-27, 1994). The disease occurs most frequently in October, usually during the fall, September to early December (Kim, et al., Infection 20; 105-116, 1988). People working in the field or camping, may be at risk for acquiring tsutsugamushi disease.

[4] Pathogenicity of Orientia tsutsugamushi is caused by systemic vasculitis due to attacking vascular endothelial cells. After invading to the blood, Orientia tsutsugamushi replicates in the vascular endothelial cells, which causes blood vessel damage, or microvascular thrombosis and inflammation around the blood vessels. Therefore, the disease is histopathologically characterized by that blood vessels of multiple organs are damaged, which may cause dermal necrosis, rash, meningitis, hearing loss, myocarditis, and heart failure. Further, a case of hypofibrinogenemia due to intravascular coagulation has been reported. In some cases, symptoms or epidemiologic characteristics of the disease are different according to each serovar of Orientia tsutsugamushi. Tsutsugamushi bacteria belonging to the same serotype are
serologically associated with each other, and there is none or weak serologic cross-reactivity between the bacteria belonging to different serotypes. *Orientia tsutsugamushi* is classified into one species. However, there are many serotypes with different antigenicity, and they are classified into serotypes such as Gilliam, Karp, and Kato strains according to antigenicity. In Korean epidemic strains, Kangwon and Boryong strain, which have different serologic reactivity from the known epidemic prototype strains, Gilliam and Karp, have been isolated.

[5] Such serotype diversity depends on membrane protein antigens of *Orientia tsutsugamushi* such as species-specific antigen and serotype-specific antigen. Antigens were purified from Orientia tsutsugamushi, and then analyzed using polyacrylamide gel electrophoresis and immunoblotting. Consequently, it was found that major antigens with the size of 70 kDa, 60 kDa, 54 to 56 kDa, and 46 to 47 kDa are present (Takahashi K, et al., Microbiol. Immunol. 29: 475, 1985), each antigen with the size of 46 to 47 kDa, 54 to 56 kDa, and 70 kDa has strong antigenicity, and each antigen with the size of 70 kDa, and 46 to 47 kDa is a strain-specific antigen (Tamura A, et al., Microbiol. Immunol. 26: 321, 1982).

[6] It has been generally considered that the tsutsugamushi disease did not show severe clinical symptoms, and the clinical diagnosis was easy to identify from the typical symptom and sign. However, atypical symptom and sign have been reported in many cases. Therefore, in the case where specific symptoms of tsutsugamushi disease such as systemic lymphoid hyperplasia and eschar formation are not observed, it is difficult to discriminate the tsutsugamushi disease from other acute febrile illnesses such as leptospirosis, murine typhus, and haemorrhagic fever with renal syndrome.

There are some precise methods for diagnosing tsutsugamushi disease by isolation of pathogen using culture or identification of antibody using immunofluorescence test. However, in the diagnostic method using immunofluorescence test, a cell culture is required to obtain the rickettsial antigen, and it is difficult to store the antigen for a long period of time. A fluorescent microscope also requires for diagnosis, which can only be performed in the laboratories having fluorescent microscopes. Accordingly, it is difficult for a hospital in a rural area to perform an immunofluorescence test, where patients may frequently develop.

Disclosure of Invention

Technical Solution

[8] In order to overcome the above difficulties, the present inventors have made extensive studies on a diagnostic kit for tsutsugamushi disease, in which a cell culture is not needed, a chimeric recombinant protein from the serially connected each gene of three serotypes is basic antigen to improve its diagnostic sensitivity, as compared to a
recombinant antigen of single serotype gene, and the recombinant proteins from each single gene of another two serotypes additionally prepared are added as antigens to greatly improve the sensitivity.

Therefore, it is an object of the present invention is to provide a diagnostic kit for tsutsugamushi disease including a test strip comprising a mixed antigen of tsutsugamushi.

It is another object of the present invention to provide a diagnostic kit for tsutsugamushi disease, in which two test strips are connected in a plastic device for simultaneous detection of both IgM and IgG antibody.

**Brief Description of the Drawings**

Fig. 1 is a photograph showing the results of 10\% SDS-PAGE to confirm a process for purifying an expressed, recombinant antigen protein.

Fig. 2 is a photograph showing the reactivity between a protein (mixture of cr56, r21 and kr56) purified from transformed E. coli and sera from patients using a dot-blot immunoassay in order to confirm its suitability as an antigen for diagnosis.

Fig. 3 illustrates the structure of a test strip in the diagnostic kit according to the present invention manufactured by introducing an immunochromatography technique.

Fig. 4 illustrates the structure of a final sample of diagnostic kit capped with a plastic and a method for analyzing its results in the diagnostic kit of the present invention.

Fig. 5 is a graph showing the sensitivity and specificity of the diagnostic kit of the present invention, in which (A) illustrates the result to evaluate sensitivity of a rapid diagnostic kit using sera from patients who were diagnosed by IFA(n=30), and (B) illustrates the result to evaluate specificity using the sera from healthy patients (n=10), the sera from patients with murine typhus (n=1), the sera from patients with leptospirosis (n=7), and the sera from patients with haemorrhagic fever with renal syndrome (n=8).

**Best Mode for Carrying Out the Invention**

In one embodiment, the present invention relates to a diagnostic kit for tsutsugamushi disease including a test strip comprising a mixed antigen of tsutsugamushi.

Specifically, the present invention relates to a diagnostic kit for tsutsugamushi disease including a test strip to detect antibodies against *Orientia tsutsugamushi* in a biological sample, in which a test strip comprises:

(a) a sample pad absorbing the sample;

(b) a gold conjugation pad binding with human antibodies in the sample;

(c) a test membrane comprising a test line containing a mixed antigen of tsutsugamushi and a control line containing a control protein; and
(d) an absorption pad absorbing the residual sample.

As shown in Fig. 3, a diagnostic kit of the invention for tsutsugamushi disease preferably includes a test strip comprising a sample pad, a gold conjugation pad, a test membrane, and an absorption pad. The sample pad is superposed on the gold conjugation pad to form a first overlap portion, and the gold conjugation pad is superposed on the membrane to form a second overlap portion. Further, the membrane and the absorption pad form a third overlap portion. When the biological sample is added dropwise on the sample pad, the sample migrates through the gold conjugation pad, for example, a gold conjugation pad containing a gold-labeled protein A by capillary action. The gold-labeled gold particle has preferably a diameter of 20 to 55 nm, more preferably 20 to 40 nm, and works as an indicator dye. While the biological sample migrates through the gold conjugation pad, antibodies in the sample bind with the gold-labeled protein A or the like to form a complex, and the complex migrates along the membrane.

The term "biological sample" as used herein means a serum or blood plasma from mammals including human suspected of having tsutsugamushi disease. The biological sample can be diluted or undiluted prior to adding dropwise to the sample pad of the diagnostic kit of the invention. In the case where tsutsugamushi antibodies are present in the biological sample, while the sample migrates through the test line comprising a mixed antigen of tsutsugamushi in the diagnostic kit, the antibodies binds with the mixed antigens of tsutsugamushi to make color change on the test line, which can be observed with the naked eye. However, in the case where tsutsugamushi antibodies are not present in the biological sample, the mixed antigen of tsutsugamushi cannot bind with the antibodies not to make color change on the test line.

Specifically, in a preferred embodiment of the invention, the tsutsugamushi antibodies bind to the mixed antigen of tsutsugamushi immobilized in the test line on the membrane. This antigen-antibody complex is formed as an indicator dye by the gold particle. As a result, the red purple band is observed, which indicates a positive response, that is, the presence of tsutsugamushi antibody in the biological sample.

The term "mixed antigen of tsutsugamushi" as used herein means a mixture of a recombinant antigen protein derived from Orientia tsutsugamushi Gilliam, Karp, and Kato strains, an antigen protein derived from Kangwon strains, and an antigen protein derived from Boryong strain, in which each strain causes tsutsugamushi disease. That is, the mixed antigen of tsutsugamushi is a mixture of a chimeric recombinant protein derived from recombinant fusion gene, in which genes coding for epitopes of antigen of Orientia tsutsugamushi Gilliam, Karp, and Kato strains are connected in series, and each recombinant protein derived from gene coding for antigen of Orientia tsutsugamushi Kangwon and Boryong strains.
Specifically, in the present invention, a fusion protein antigen can be produced, isolated, and purified by a one-step process, in which genes coding for 56 kDa protein fragments of standard serotype Gilliam, Karp, and Kato strains are amplified by polymerase chain reaction (PCR), the amplified DNA fragments are connected in series, the recombinant DNA is cloned into a protein expressing vector to express in E. coli, and the expressed protein is isolated and purified to be used as an antigen for diagnosing tsutsugamushi disease, on the basis of Korean Patent Application No. 2002-0020281, which discloses that 56 kDa protein of Orientia tsutsugamushi has antigenicity, thereby being usefully used as a diagnostic protein.

Further, in the invention, in addition to Orientia tsutsugamushi Gilliam, Karp, and Kato strains, a gene coding for a 56 kDa antigenic protein Of Orientia tsutsugamushi Kangwon strain and a gene coding for 21 kDa antigenic protein Of Orientia tsutsugamushi Boryong strain are amplified by PCR, the amplified DNA fragments are cloned into a protein expressing vector to express in E. coli, and the expressed proteins are isolated and purified to use as a diagnostic protein with the 56 kDa chimeric recombinant protein derived from Orientia tsutsugamushi Gilliam, Karp, and Kato strains, in order to improve diagnostic sensitivity for tsutsugamushi disease.

In one preferred embodiment, in the diagnostic kit of the invention, a 56 kDa recombinant chimeric protein of Orientia tsutsugamushi Gilliam, Karp, and Kato strains, a 56 kDa recombinant protein of Orientia tsutsugamushi Kangwon strain, and a 21 kDa recombinant protein of Orientia tsutsugamushi Boryong strain are prepared at a concentration of 1 mg/ml, respectively, and they are then mixed at a volume ratio of 5:2:1. 1 mg/ml of the mixed antigen of tsutsugamushi is added dropwise on the test line.

The positive result for tsutsugamushi disease is a case that a color is developed on the test line, in addition to the control line. The control line is to confirm whether the diagnostic kit works or not. In the negative response for tsutsugamushi disease, a color is developed only on the control line. The control line contains a control protein binding with a gold-labeled antibody. Examples thereof include a rabbit anti-goat IgG polyclonal antibody, a goat anti-human IgG polyclonal antibody or a goat anti-human IgM polyclonal antibody. The control line may contain 0.1 mg/ml to 1 mg/ml of the control proteins.

In one preferred embodiment, the diagnostic kit of the invention further comprises a pre-test line containing an extract of E. coli not expressing the antigen protein of tsutsugamushi. The pre-test line, in the diagnostic kit of the invention using the antigen protein of Orientia tsutsugamushi derived from E. coli, allows to remove any possibility of misdiagnosis by non specific result from the reaction of antibodies which are derived from E. coli in human, on the protein of E. coli contaminated in the
purified tsutsugamushi antigen protein. The pre-test line may contain the extract of E. coli at a concentration of 0.5 mg/ml to 0.6 mg/ml.

[31] The test membrane can contain the suitable materials such as nitrocellulose membrane (MiliporeTM XA3J072100) with a standard size of 10 x 500 nm. In the test membrane, the pre-test line, the test line, and the control line are preferably arranged in this order. The pre-test line, the test line, and the control line are arranged with an interval of 2.0 to 4.5 mm, preferably 2.5 to 3.0 mm.

[32] The gold conjugation pad can contain the suitable detecting markers. Examples of the marker include a gold-labeled protein A, a gold-labeled anti-human IgG antibody, and a gold-labeled anti-human IgM antibody. The gold conjugation pad is preferably placed adjacent to the sample pad. In the case where the gold conjugation pad contains an excessive amount of the gold-labeled marker, the excessive amplified signals, which are generated in a lower limit concentration of the tsutsugamushi antibody, increase to cause a false-positive result. Accordingly, in the invention, the gold-colloid solution is diluted to be optical density (OD) of 1 to 6, preferably 2 to 4. The absorption pad is placed at the opposite end point of the membrane and absorbs the biological sample, such as serum or blood plasma, migrating along the membrane by capillary action.

[33] In one specific embodiment, the diagnostic kit of the invention can comprise a test strip. Alternatively, as shown in Fig. 4, the diagnostic kit of the invention can comprise two strips of a first strip and a second strip branched from a sample pad. At this time, the first strip can be used for detecting human IgG antibody, and the second strip can be used for detecting a human IgM antibody against the antigen protein of tsutsugamushi. The first strip may comprise the gold conjugation pad containing a goat anti-human IgG antibody or protein A and the control line containing a rabbit anti-goat IgG polyclonal antibody. The second strip may comprise the gold conjugation pad containing a goat anti-human IgM antibody and the control line containing a goat anti-human IgM polyclonal antibody. As shown in Fig. 4, the first strip and the second strip can be preferably arranged in parallel, but are not limited thereto. The first strip and the second strip can be connected in series to arrange in opposite direction or in various directions.

[34] The test strip can be packed with a surrounding case in order to assure the safety of each diagnostic kit of the invention. The diagnostic kit can be stored at room temperature for a long period of time (at least 12 months or longer) without any loss of diagnostic activity.

[35] In one specific embodiment, diagnosis takes about 5 to 10, not exceeding 15 minutes. The diagnostic result indicates whether a patient is infected with tsutsugamushi. For example, if the biological sample is collected from the patient infected
with tsutsugamushi, the diagnostic kit of the invention gives a positive response, whereby the patient can be diagnosed as having tsutsugamushi disease. If the biological sample is collected from the patient not infected with tsutsugamushi, the diagnostic kit of the invention gives a negative response, whereby the patient can be diagnosed as not having tsutsugamushi disease.

The sensitivity and specificity of the mixed antigen of tsutsugamushi for tsutsugamushi disease was evaluated using an indirect fluorescent antibody technique, a Dot-blot immunoassay, and ELISA. As a result, it was found that the 56 kDa recombinant fusion protein derived from Orientia tsutsugamushi Gilliam, Karp, and Kato strains and the 21 kDa protein derived from Orientia tsutsugamushi Boryong strain have a sensitivity and specificity of 95% or more. Further, in the case where the serum displaying very weak positive signal was tested using the kit comprising the above proteins and the 56 kDa protein derived from Orientia tsutsugamushi Kangwon strain, the positive signal became strong, that is, its sensitivity was improved (100%). Accordingly, the mixed antigen of tsutsugamushi used in the invention was found to have excellent sensitivity and specificity.

In one embodiment, the present invention provides a diagnostic composition for tsutsugamushi disease comprising a 56 kDa fusion protein derived from Orientia tsutsugamushi Gilliam, Karp, and Kato strains, the 56 kDa protein derived from Orientia tsutsugamushi Kangwon strain, and the 21 kDa protein derived from Orientia tsutsugamushi Boryong strain as a mixed antigen of tsutsugamushi.

In one preferred embodiment, the 56 kDa protein derived from Orientia tsutsugamushi Kangwon strain in the mixed antigen of tsutsugamushi has an amino acid sequence of SEQ ID NO: 6, and the 21 kDa protein derived from Orientia tsutsugamushi Boryong strain has an amino acid sequence of SEQ ID NO: 3.

Generally, the presence of Orientia tsutsugamushi in human body has to be confirmed in order to diagnose whether human suspected of having tsutsugamushi disease is infected or not. The current diagnosis for tsutsugamushi disease is performed using immunofluorescent antibody technique. There is an advantage in that the technique has high sensitivity and specificity and discriminates between primary infection and reinfection by measuring IgG and IgM, separately. However, there are disadvantages in that equipments for cell culture are required, only highly skilled experts must perform it due to the complicated process, and high cost and time are also required. On the contrary, since the diagnostic method in the invention is performed using immunochromatography by antigen-antibody reaction, tsutsugamushi disease can be accurately and simply diagnosed in short time, around 10 minutes. Accordingly, the diagnostic method in the invention can be said to be better than the conventional diagnostic method.
The method for detecting antibody against tsutsugamushi disease using the above described diagnostic kit for tsutsugamushi disease is as follows. First, when a biological sample to be analyzed is added dropwise to the sample pad of the diagnostic kit, which is a portion absorbing the sample, the biological sample migrates to the gold conjugation pad by capillary action. At this time, the antibody in the biological sample binds to the marker in the gold conjugation pad, for example, gold-labeled protein A, gold-labeled anti-human IgG antibody, or gold-labeled anti-human IgM antibody, so as to form a colloid. The biological sample for analysis is not immobilized in the gold conjugation pad, but continues to migrate to the test line, in which the mixed antigen of tsutsugamushi is immobilized. In the case of the sample from a patient infected with tsutsugamushi disease, the sample reacts with the mixed antigen of tsutsugamushi to produce antigen-antibody reaction. That is, antibodies against tsutsugamushi, which are bound to the specific markers in the gold conjugation pad, are bound to the mixed antigen of tsutsugamushi immobilized in the test line to form a red-purple band. Therefore, the band can be observed with the naked eye. The residual markers in the gold conjugation pad, which are not reacted with test line, migrate to the control line to react with a control protein. Subsequently, a red-purple band is formed, thereby ensuring conformance to the test. As described above, the diagnostic kit of the invention is performed using immunochromatography by antigen-antibody reaction, whereby the result can be observed with the naked eye without any equipment. Further, the pre-test line of the diagnostic kit eliminates any possibility of misdiagnosis, which may be presented due to the presence of antibodies against E. coli in human serum instead of Orientia tsutsugamushi, thereby improving the accuracy of the diagnosis for tsutsugamushi disease.

Hereinafter, the present invention will be described in more detail with reference to Examples. However, these Examples are for the illustrative purpose only, and the invention is not intended to be limited by these Examples.

Mode for the Invention

Example 1: Antigen protein expression and isolation

1.1. Expression of 56 kDa chimeric recombinant protein (cr56)

The chimeric recombinant protein (cr56) comprising the major epitopes of Orientia tsutsugamushi Gilliam, Karp, and Kato strains was expressed, isolated, and purified in the same manners as Examples disclosed in Korean Patent No. 2002-0020281.

Specifically, Orientia tsutsugamushi Gilliam, Karp, and Kato strains were cultured in mouse L-929 cells, collected and purified. The purified cells were digested with enzymes, and then their DNA was extracted with phenol and ethanol. On the basis of a basic sequence of 56 kDa protein of Orientia tsutsugamushi, each DNA portion having
30% or more amino acid sequence homology among Boryong and Gilliam, Karp, and Kato serotypes was selected, and a pair of oligonucleotide primer for each Gilliam, Karp, and Kato strain was prepared. PCR was carried out with each primer and the purified DNA of *Orientia tsutsugamushi* as a template to amplify DNA fragments of Gilliam, Karp, and Kato strains. The PCR products were purified and cloned to be ligated at restriction enzyme digestion sites of a pTYB12 vector. First, the DNA fragment of Gilliam strain was introduced into a pTYB12 vector to prepare a vector, pTG3, and then the DNA fragment of Karp strain was introduced into the pTG3 vector to prepare a vector, pTGPl. The DNA fragment of Kato strain was introduced into the pTGPl vector to prepare a vector, pTGPT2. Further, the DNA fragment connected with the DNA sequences of Gilliam, Karp, and Kato strains in series was cut from the pTGPT2 vector. The DNA fragment was introduced into a pET22b(+) vector to prepare a vector, pETb7. E. coli was transformed by the prepared expression vector, and then the transformed E. coli was cultured to induce expression of the fusion protein. The fusion protein was confirmed using electrophoresis and western blotting, isolated and purified.

1-2. Expression of 21 kDa recombinant protein (r21) of *Orientia tsutsugamushi* Boryong strain

A gene having species-specific antigenicity in *Orientia tsutsugamushi* Boryong strains was expressed in E. coli to isolate and purify the recombinant protein (r21) as the following method.

(1) Purification of *O. tsutsugamushi* DNA

Mouse L-cells infected with *O. tsutsugamushi* Boryong strain were centrifuged to collect a cell pellet. The cell pellet was washed with a phosphate buffer three times, and the whole DNA was purified to use. A Tris-HCl buffer (10 mM pH 8.0, 10 mM EDTA, 150 mM NaCl) containing 1% SDS and proteinase K (100 ug/ml) was added to the cell pellet. The cell pellet was subjected to reaction at 56°C for 1 hour to dissolve, and then reacted with the same amount of a phenol-chloroform-isoamylalcohol mixture three times to purify.

(2) PCR amplification

The modified primers including Nde I and Xho I restriction sites (Table 1) were added to 100 ng of the purified and stored DNA to be 20 pmole. PCR was carried out with a 10 x Tag polymerase buffer (100 mM Tris-HCl, 15 mM MgCl2, 500 mM KCl, 1 mg/ml gelatin, pH 8.3), dNTPs, and Tag polymerase. The amplification of DNA was confirmed by electrophoresis with 1.2% agarose gel.
Primer sequence for cloning 21 kDa recombinant protein of Boryong strain

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<th>SEQ ID NO: NO 1</th>
<th>Primer sequence</th>
<th>Enzyme site</th>
<th>Cloning vector</th>
<th>Polypeptide (kDa)</th>
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</thead>
<tbody>
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<td></td>
<td>5′-GGACATATGACTAAGCTCCATAAA-3′</td>
<td>Nde I</td>
<td>pET22b</td>
<td>21 kDa</td>
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<tr>
<td></td>
<td>5′-AAGCTTCGGGTATTGATTGATCTT-3′</td>
<td>Xho I</td>
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</tr>
</tbody>
</table>

* Plasmid pET22b expresses a His-Tagged fusion protein

(3) Gene cloning

The DNA amplified by polymerase chain reaction was separated by electrophoresis with 1.2% agarose gel, and then recovered using a QIAGEN gel elution kit (QIAGEN Inc., USA). The amplified DNA band with a right size was cut on a UV transiluminator. The band was transferred to a microtube and completely dissolved with a NaI solution (three volume of the agarose gel) at 60°C for 5 minutes. Glass milk was added thereto, and the microtube was vortexed for 10 seconds. The solution was centrifuged at 17,000 g (Hanil, AL5S-24, 12,000rpm) at room temperature for 1 minute to remove a supernatant. An elution buffer was added to the residual solution, and then the microtube was vortexed to centrifuge at 17,000 g (12,000 rpm). A new supernatant was transferred to a new microtube. 40ng of the purified DNA was cloned into the pET22b(+) vector.

(4) Competent cell preparation and transformation

E.coli BL21 cells cultured in LB medium for 18 hours were diluted to 100-fold with LB medium. E.coli BL21 cells were recultured to be an absorbance of 0.3 at 600 nm. After placed in ice for 30 minutes, the supernatant was discarded. 0.1M CaCl$_2$ was added to be half volume of the medium, and then the bacteria were suspended. The suspended bacteria were placed in ice for 1 hour, and centrifuged at 2,000 g (4,100 rpm) for 10 minutes. The pellet was suspended in 0.1M CaCl$_2$ with one tenth volume of the culture medium to be used for the transformation.

The ligation product prepared in the above and the competent cells were mixed to be placed in ice, and then heat shocked at 42°C. The LB medium was added thereto,
and cultured at 37°C for 1 hour. The culture medium was spread onto an LB agar plate to culture at 37°C.

(5) Protein expression and isolation

The transformed E.coli BL21 cells were cultured at 37°C for 18 hours, and then diluted about 100-fold to be OD600 of 0.5. IPTG was added thereto to be a concentration of 0.3 mM, and then cultured for 2 hours. The transformed E.coli BL21 cells were centrifuged at 5,800 g (7,000 rpm). The pellet was suspended in a 1 X binding buffer (5 mM imidazole, 20 mM Tris-HCl pH 7.9, 0.5 M NaCl) to sonicate with an ultrasonicator at 18 Hz for 15 seconds six times. The sonicated solution was centrifuged at 23,000 g (14,000 rpm). The supernatant was used as a soluble fraction, and the pellet was used as an insoluble fraction. SDS-PAGE was carried out with each fraction to confirm which fraction has the recombinant protein.

(6) Protein purification

A column was filled with 2 ml of Hisbind resin (Novagen), and then prepared with distilled water, a 1 X charge buffer, and a 1 X binding buffer. After the antigen protein was passed through the column, the column was washed with a 1 X washing buffer. An elution buffer (100 mM to 300 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl pH 7.9) was passed through the column to recover each 1 ml of the protein solution. A concentration of the protein in each fraction was determined by the Lowry assay. SDS-PAGE and western blotting were carried out to confirm the protein (SEQ ID NO: 3) (Fig. 1).

1-3. Expression of 56 kDa recombinant Kangwon protein (kr56)

With reference to the result of phylogenic analysis (FEMA Microbiology Letters, 1999, 180:163-169), as considering that Orientia tsutsugamushi is classified into world wide prototype strains, Karp, Gilliam, and Kato, and domestic prevalent serotypes, Boryong and Yonchon (most similar to Kangwon), these five strains were thought to be suitable as the antigen. The Karp, Gilliam, and Kato strains presented the cr56 protein, and the Boryong strain presented the r21 protein as the antigen. Further, the Yonchon strain presented the kr56 protein derived from Kangwon strain, similar to Yonchon. Therefore, the present inventers supposed to use 56 kDa recombinant Kangwon protein (kr56) with the r21 protein as additional antigens, to the chimeric recombinant protein (cr56).

(1) Overview of expression of 56 kDa recombinant protein of Kangwon strain (kr56)
A portion of amino acid sequence, 84 (250 bp) to 445 (1,335 bp), which is known as a major antigenic domain of Kangwon 87-61, was modified to include Nco I and Sal I restriction sites. Subsequently, a pair of primers was prepared (Table 2) and subjected to PCR reaction. A PCR product and an expression vector, pET30a were digested with the restriction enzymes, Nco I and Sal I, and then ligated with each other. E.coli BL21 was transformed with the plasmid, and then its protein was extracted.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Enzyme site</th>
<th>Cloning vector</th>
<th>Polypeptide (kDa)</th>
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<tr>
<td>F:5’-CCATGGCGCCAGGATTYAGACCA-3’</td>
<td>Nco I</td>
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</table>

* Plasmid pET30a expresses a His-Tagged fusion protein

(2) Gene cloning and expression

The DNA amplified by polymerase chain reaction (362 amino acids of amino acid 84 (250 bp) to 445 (1,335 bp) (total 1,086 bp)) was separated by electrophoresis with 1.2% agarose gel, and then recovered using a QIAGEN gel elution kit (QIAGEN Inc., USA). The amplified DNA band with a right size was cut on a UV transilluminator. The band was transferred to a microtube, and completely dissolved with a NaI solution (three volume of the agarose gel) at 60°C for 5 minutes. Glass milk was added thereto, and the microtube was vortexed for 10 seconds. The solution was centrifuged at 17,000 g (Hanil, AL5S-24, 12,000rpm) at room temperature for 1 minute to remove a supernatant. The microtube was left at room temperature for 5 minutes, and dried. An elution buffer was added thereto, and then the microtube was vortexed to centrifuge at 17,000 g (12,000 rpm). A new supernatant was transferred to a new microtube. The purified PCR product and pET30a vector were digested with the restriction enzymes, Nco I and Sal I, and then recovered by the same method using Geneclean kit II. 100 ng of the restricted vector and 100 ng of the restricted PCR product were mixed with each other, and then the mixture of 10 X ligase buffer (250 mM Tris-HCl pH 7.8, 100 mM MgCl₂, 20 mM DTT and 4 mM ATP) and ligase was added thereto, and then subjected
to reaction at 4°C for 18 hours.

(3) Competent cell preparation and transformation

E. coli BL21 cells cultured in LB medium for 18 hours were diluted to 100-fold with LB medium. E. coli BL21 cells were recultured to be an absorbance of 0.3 at 600 nm. After placed in ice for 30 minutes, the supernatant was discarded. 0.1M CaCl$_2$ was added to be half volume of the medium, and then the bacteria were suspended. The suspended bacteria were placed in ice for 1 hour, and centrifuged at 2,000 g (4,100 rpm) for 10 minutes. The pellet was suspended in 0.1M CaCl$_2$ with one tenths volume of the culture medium to be used for the transformation.

(4) Transformation

The ligation product prepared in the above and the competent cells were mixed to be placed in ice, and then heat shocked at 42°C for one and half minutes. The LB medium was added thereto, and cultured at 37°C for 1 hour. The culture medium was spread onto an LB agar plate to culture at 37°C.

(5) Isolation of plasmid DNA from transformed E. coli

Single colony of transformed E. coli was inoculated in LB medium containing kanamycin, and then shaking-cultured at 37°C. The extraction of plasmid DNA was carried out using AccuPrep Plasmid Extraction kit. The culture medium was centrifuged at 750 g (2,500 rpm) at room temperature for 10 minutes to recover a cell pellet. A resuspension buffer was added to the cell pellet, and then vortexed. A lysis buffer was added thereto, and then left at room temperature for 5 minutes. A neutralization buffer was added thereto, left at room temperature, and then centrifuged at 17,000 x g (12,000 rpm). The supernatant was transferred into a binding column tube, and then centrifuged at 17,000 x g (12,000 rpm) at room temperature to remove the extracted solution. 80% ethanol was added thereto, centrifuged, and then the binding column was only placed in a new tube. An elution buffer was added thereto, left at room temperature, and then centrifuged to transfer a supernatant into a new microtube.

(6) Protein expression and isolation

The transformed E. coli BL21 cells were cultured at 37°C for 18 hours, and then diluted about 100-fold to be OD600 of 0.5. IPTG was added thereto to be a concentration of 0.3 mM, and then cultured for 2 hours. The transformed E. coli BL21 cells were centrifuged at 5,800 g (7,000 rpm). The pellet was suspended in a 1X binding buffer (5 mM imidazole, 20 mM Tris-HCl pH 7.9, 0.5 M NaCl) to sonicate with an ultrasonicator at 18 Hz for 15 seconds six times. The sonicated solution was centrifuged
at 23,000 g (14,000 rpm). The supernatant was used as a soluble fraction, and the pellet was used as an insoluble fraction. SDS-PAGE was carried out with each fraction to confirm which fraction has the recombinant protein.

(7) Protein purification

A column was filled with 2 ml of Hisbind resin (Novagen), and then prepared with distilled water, a 1 X charge buffer, and a 1 X binding buffer. After the antigen protein was passed through the column, the column was washed with a 1 X washing buffer. An elution buffer (300 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl pH 7.9) was passed through the column to recover each 1 ml of the protein solution. A concentration of the protein in each fraction was determined by the Lowry assay. SDS-PAGE and western blotting were carried out to confirm the protein (SEQ ID NO: 6) (Fig. 1).

Example 2: Diagnostic analysis of purified antigen protein using ELISA.

The present test was performed in order to assure the possibility of diagnosing tsutsugamushi disease by antigen-antibody reaction with the antibody in the serum from the patient with tsutsugamushi disease, using the cr56 protein, which was expressed in E.coli by fusion of three prototypes of Orientia tsutsugamushi, and the kr56 protein and the r21 protein, which were respectively expressed by two serotypes, Kangwon and Boryong.

In the case of using only cr56 protein, its sensitivity and specificity was 93.2% and 94.2% in ELISA, respectively, which is not sufficient. Moreover, the measured value was frequently observed around the absorbance (OD) of diagnostic standard limit. As a result, the r21 and kr56 proteins were added to mix with the cr56 protein. The problems were solved by using the mixed protein of three proteins.

The purified protein antigens were diluted with a 0.05 M carbonate buffer (pH 9.6) to be as Table 3. A 96 well plate was coated with the diluted antigen, and then subjected to reaction for 18 hours. The plate was washed with a phosphate buffer (PBS) containing 0.05% Tween 20 twice, and then 100 µl of 3% bovine serum albumin was added to the wells to block at 37°C for 2 hours. A 2-fold serial dilution of a patient's serum was preformed. 100 µl of the diluted serum was used as a primary antibody. A peroxidase conjugated goat anti-human IgG was diluted to 1:6,000 to use as a secondary antibody. 100 µl of the prepared secondary antibody was added to the wells. After washing three times, 100 µl of citrate phosphate buffer (pH 4.9) containing 1 mg/ml OPD (o-phenylene diamine dihydrochloride) and 0.03% hydrogen peroxide was added to the wells. The plate was shielded from light at room temperature for 30 minutes, and then 50 µl of 1 M sulfuric acid was added thereto to terminate the reaction. Its absorbance was measured at 490 nm using a MicroELISA reader. The
result is shown in Table 3. The mixed antigen of cr56 and r21 was found to have sensitivity and specificity of 95% or more. Further, in the case of adding the kr56 protein, the weak positive signal in the patient’s serum became strong, that is, its sensitivity was found to be improved (100%). Therefore, it can be seen that the addition of kr56 protein gives an effect of improving diagnostic sensitivity.

**[107]**

**[108]** [Table 3]

**[109]** Antigenicity analysis of purified antigen protein using ELISA

**[HO]**

<table>
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<th>IFA [titer]</th>
<th>ELISA (OD at 490 nm)</th>
</tr>
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<tr>
<td></td>
<td>Gilliam</td>
<td>Karp</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>99-241</td>
<td>160</td>
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**[111]** (1) Each antigen protein (cr56 and r21) was diluted to be 1.25 µg/ml. The cr56 protein was mixed with the same amount of the r21 protein. 100 µl of the mixed antigen protein was added to each well. Healthy person: OD=0.085 ± 0.042

**[112]** (2) The cr56, r21, and kr56 antigen proteins were diluted to be 1.25 µg/ml, 0.25 µg/ml, and 0.5 µg/ml, respectively. The cr56 protein was mixed with the same amount of the r21 and kr56 proteins. Healthy person: OD=0.078 ± 0.038

**[113]** (3) cr56: 1.25 µg/ml, r21: 0.25 µg/ml. Healthy person: OD=0.081 ± 0.044

**[114]** (4) kr56: 0.6 µg/ml. Healthy person: OD=0.075 ± 0.035

**[115]**
Example 3: Diagnostic analysis of purified antigen protein using dot-blot

The present test was performed in order to assure the possibility of diagnosing tsutsugamushi disease by antigen-antibody reaction with the antibody in the serum from the patient with tsutsugamushi disease, using the cr56 protein, which was expressed in E.coli by fusion of three prototypes of Orientia tsutsugamushi, and the kr56 protein and the r21 protein, which were respectively expressed by two serotypes, Kangwon and Boryong. 1 µg of the purified protein antigen (1 µg/µl) was added dropwise to a nitrocelulose membrane, and then dried at 37°C for 1 hour. The dried membrane was blocked with a TBST (Tris-buffered saline containing 0.5% Tween 20) solution containing 5% skim milk for 1 hour. The patient's serum was diluted to 1:3,000 as a primary antibody, and then added to the membrane. The reaction was performed for 1 hour. A peroxidase conjugated goat anti-human IgG was used as a secondary antibody. The result is shown in Fig. 2 and Table 4. As a result, the mixed antigen of cr56, r21, and kr56 proteins was found to have the highest sensitivity and specificity.

[Table 4]

Antigenicity analysis of purified antigen protein using dot-blot
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<tr>
<th>Patient No.</th>
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<th>IgM</th>
<th>IFA (titer)</th>
<th>Dot-blotting</th>
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<td>+(weak)</td>
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<tr>
<td>Karp</td>
<td>cr56/r21 (5:1)</td>
<td>(2)</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Kangwon</td>
<td>kr56</td>
<td>(3)</td>
<td></td>
<td></td>
<td>++</td>
<td>+(weak)</td>
</tr>
<tr>
<td></td>
<td>cr56/r21/kr56</td>
<td>(4)</td>
<td></td>
<td></td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>

(1) Each antigen protein was mixed (cr56 : r21 = 1 : 1), and used with 1 µg/µl/dot.
(2) Each antigen protein was mixed (cr56 : r21 = 5 : 1), and used with 1 µg/µl/dot.
(3) Kangwon: 0.6 µg
(4) Each antigen protein was mixed (cr56 : r21 : kr56 = 5 : 1 : 2), and used with 1 µg/µl/dot.

**Example 4:** Study for removal of reaction between protein derived from E.coli in purified antigen and antibody in human serum

The antigen was purified using a His-bind affinity chromatography. However, the protein derived from E.coli could not be completely removed. That is, it was difficult to remove completely the protein derived from E.coli by purification. Therefore, E.coli host cell, which does not express the antigen protein of tsutsugamushi, was cultured to extract. The protein of extracted host cell was sprayed for the pre-test line before the test line, thereby human antibody reacting with the protein derived from E.coli. was removed.

**Example 5:** Production of stick type sample of diagnostic kit
In order to confirm the efficacy of the antigen protein of tsutsugamushi (cr56, kr56, r21) as a diagnostic agent for tsutsugamushi disease, a test strip sample for diagnosis was manufactured using a dispenser purchased from NANO ENG Co.. The test strip, as shown in Fig. 3, consisted of a nitrocellulose membrane, a sample pad, an absorption pad, and a gold conjugation pad (glass fiber). The nitrocellulose membrane was purchased from Millipore to use. In order to avoid the membrane damage, a part of the membrane was attached with a transparent plastic. Three proteins were sprayed on the nitrocellulose membrane. One of three proteins is the mixed protein, in which cr56, kr56, and r21 antigen proteins expressed in E.coli were mixed with the suitable amount, to detect the antibody against tsutsugamushi in patient's serum, and used as a test line. Another one of three proteins is used as a control line to confirm whether the developing system works well or not. The other one of three proteins is the E.coli extract not expressing the antigen protein of tsutsugamushi, and used as a pre-test line to remove human antibody reacts with the protein derived from E.coli. Each protein was sprayed on the pre-test line, the test line, and the control line with the suitable amount using the dispenser, and then dried at 37°C for 2 hours. The glass fiber was absorbed with the colloidal gold conjugate in a 5% trehalose solution, and then dried at a vacuum dry oven for 2 hours. The sample pad and the absorption pad were cut with the predetermined size. The sample pad was immersed in a 250 mM Tris solution containing 1% tween 20 in order to absorb the serum or blood plasma well, and then completely dried at 37°C for 4 hours. Subsequently, the gold conjugation pad (glass fiber) sprayed with the colloidal gold conjugates was placed below the nitrocellulose membrane, and the sample pad was placed therebelow. The absorption pad was placed above the nitrocellulose membrane. The final diagnostic stick was cut with a width of 4 mm and a height of 60 mm. Two kinds of diagnostic kit samples were prepared according to their use. The proteins used herein are shown in Table 5. The samples 1 and 2 are able to detect only IgG against the antigen protein of tsutsugamushi in the human serum, and the samples 3 and 4 are able to detect only IgM. Therefore, with respect to its use, the samples 1 and 2 can be diagnostic tool to detect middle-late stage patients and the samples 3 and 4 can be a useful diagnostic tool to detect early stage patients. In the present invention, two strips were placed in one plastic, so as to diagnose simultaneously. The efficacy test was performed for the sample of the diagnostic kit using sera from patient and healthy person. As a result, the sample of the diagnostic kit was found to be fast, accurate, and simple diagnostic kit for tsutsugamushi disease. Further, the cross-reactivity was not found to occur from the test result using sera from patients with other acute febrile illness (haemorrhagic fever with renal syndrome, murine typhus, leptospirosis). Accordingly, the diagnostic kit in the invention was found to have more improved sensitivity than the conventional kit.
Protein used for manufacturing sample of diagnostic kit

The test strips manufactured herein were placed in one plastic device to produce the final diagnostic kit as shown in Fig. 4. The manual is as follows. The plasma blood or serum from the patient was diluted with a suitable solution. 400 µl of the diluted plasma blood or serum was added dropwise to a small well on the bottom to left for 10 minutes. It is observed whether red purple color is developed in the test line (position marked as T) and the control line (position marked as C) or not. In the case where red purple color is developed in both of two lines, the result is positive. In the case where red purple color is only developed in the control line, the result is negative. In the case where red purple color is not developed in any of two lines, there is something wrong with the development system, whereby another test should be performed with a new diagnostic kit. The test was performed for the optimum concentration and amount of the protein used in the sample of the diagnostic kit. As the result shown in Table 6, the optimum amount of the protein was determined to manufacture the sample of diagnostic kit.
Optimum composition of protein for manufacturing sample of diagnostic kit (stick with a width of 4 mm)

<table>
<thead>
<tr>
<th>Pre-test line</th>
<th>Test line</th>
<th>Control line</th>
<th>Gold conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 µg</td>
<td>0.1 µg to 0.4 µg</td>
<td>0.04 µg to 0.4 µg</td>
<td>Absorbance=10 20 µl</td>
</tr>
</tbody>
</table>

Example 6: Efficacy test for strip sample of diagnostic kit

In order to confirm the efficacy of the manufactured strip sample of the diagnostic kit, the test was performed using sera from 65 persons containing patients with tsutsugamushi, healthy persons, and patients with other acute febrile illness. The manual is as follows. The sera from the patients or healthy persons were diluted with a suitable solution. 400 µl of the diluted sera were added dropwise to a small well on the bottom and it is left for 10 minutes. It is observed whether red purple color is developed in the test line (position marked as T) and the control line (position marked as C) or not. In the case where red purple color is developed in both of two lines, the result is positive. In the case where red purple color is only developed in the control line, the result is negative. The result is shown in Fig. 5. The test results of the 30 cases of patients with tsutsugamushi diagnosed by IFA were found to be all positive. Therefore, its sensitivity was 100%. Further, strong positive responses were observed in sera from 11 patients of 30 patients (37%) to IgM, and weak positive responses were observed in sera from 4 patients of 30 patients (13%) to IgM. Accordingly, it can be seen that the sample for the diagnostic kit has significant efficacy to detect the early stage patients.

Specificity test was carried out with sera from healthy persons (n=10), the sera from patients with murine typhus (n=1), the sera from patients with leptospirosis (n=7), and the sera from patients with hemorrhagic fever with renal syndrome (n=8). As a result, the positive response was observed in one sample of sera from patients with leptospirosis to IgG, and the response was not observed in the rest samples of the sera to IgG. The number of sample for specificity test was not sufficient, so it cannot be said that the specificity test was accurate. The specificity was 97.2% to IgG. Further, the specificity was 100% to IgM from the result of cross-reactivity test with IgM (µ-specific).

Industrial Applicability

In the diagnostic kit for tsutsugamushi disease in the present invention, the diagnosis is performed using proteins, which have antigenicity of Gilliam, Karp, and
Kato, Kangwon and Boryong strains of *Orientia tsutsugamushi* by immunochromatography method. Therefore, the diagnostic result can be observed simply, accurately and fast with the naked eye, as compared to the conventional immunofluorescent antibody technique or the like.
Claims

[1] A diagnostic kit for tsutsugamushi disease including a test strip to detect antibodies against tsutsugamushi antigen in a biological sample, in which the test strip comprises:
(a) a sample pad absorbing the sample;
(b) a gold conjugation pad binding with human antibodies in the sample;
(c) a test membrane comprising a test line containing a mixed antigen of tsutsugamushi and a control line containing a control protein; and
(d) an absorption pad absorbing the residual sample.

[2] The diagnostic kit according to claim 1, further comprising a pre-test line, placed before the test line on the test membrane, in which an E.coli extract does not contain the antigen protein of tsutsugamushi.

[3] The diagnostic kit according to claim 2, wherein the pre-test line, the test line, and the control line are placed in this order.

[4] The diagnostic kit according to claim 2, wherein 0.5 mg/ml to 0.6 mg/ml of the E.coli extract is absorbed in the pre-test line.

[5] The diagnostic kit according to claim 1 or 2, wherein 1 mg/ml of the mixed antigen of tsutsugamushi and 0.1 mg/ml to 1 mg/ml of the control protein are present.

[6] The diagnostic kit according to claim 1 or 2, wherein the mixed antigen of tsutsugamushi comprises a 56 kDa fusion protein of Orientia tsutsugamushi Gilliam, Karp, and Kato strains, a 56 kDa protein of Orientia tsutsugamushi Kangwon strain, and a 2IkDa protein of Orientia tsutsugamushi Boryong strain.

[7] The diagnostic kit according to claim 6, wherein the 56 kDa fusion protein of Orientia tsutsugamushi Gilliam, Karp, and Kato strains, the 56 kDa protein of Orientia tsutsugamushi Kangwon strain, and the 2IkDa protein of Orientia tsutsugamushi Boryong strain are prepared at a concentration of 1 mg/ml, respectively, and mixed at a volume ratio of 5 : 2 : 1 to prepared 1 mg/ml of the mixed antigen of tsutsugamushi.

[8] The diagnostic kit according to claim 1 or 2, wherein the control protein is a rabbit anti-goat IgG polyclonal antibody and/or a goat anti-human IgM polyclonal antibody.

[9] The diagnostic kit according to claim 1 or 2, wherein the gold conjugation pad comprise at least one marker selected from the group consisting of a gold-labeled protein A, a gold-labeled anti-human IgG antibody, and a gold-labeled anti-human IgM antibody.

[10] The diagnostic kit according to claim 1 or 2, wherein the human antibody is IgG
or IgM.

[11] The diagnostic kit according to claim 1 or 2, wherein the pre-test line, the test line, and the control line of the membrane are arranged with an interval of 2.5 to 3.0 mm.

[12] A diagnostic kit for tsutsugamushi disease comprising two test strips of claim 1 connected in one plastic device, wherein one strip of them consists of a first strip detecting a human IgG antibody against the antigen protein of tsutsugamushi, the other strip consists of a second strip detecting a human IgM antibody against the antigen protein of tsutsugamushi, the first strip comprises a gold conjugation pad containing a goat anti-human IgG antibody or a protein A and a control line containing a rabbit anti-goat IgG polyclonal antibody, and the second strip comprises a gold conjugation pad containing a goat anti-human IgM antibody and a control line containing a goat anti-human IgM polyclonal antibody.

[13] The diagnostic kit according to claim 12, wherein the first strip and the second strip are connected in series or in parallel.

[Fig. 1]

1a cr56 antigen

1: SIZE MAKER
2: Column eluent 1
3: Column eluent 2
4: Column eluent 3
5: Column eluent 4
6: Column eluent 5
7: Column eluent 6
8: Column eluent 7
9: Column eluent 8
10: Column eluent 9
11: Column eluent 10

1b kr56 antigen

1: Column eluent 1
2: Column eluent 2
3: Column eluent 3
4: Column eluent 4
5: Column eluent 5
6: Column eluent 6
7: Column eluent 7
8: Column eluent 8
9: SIZE MAKER

[Fig. 2]

Sera from patients

Sera from healthy persons
[Fig. 3]

Absorption pad

Material: nitrocellulose membrane

Control line

Test line

Pre-test line

Colloidal gold conjugate

Sample pad
Box No. 1  Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1  With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of

   a  type of material
      [ ] a sequence listing
      [ ] table(s) related to the sequence listing

   b  format of material
      [X] on paper
      [ ] in electronic form

   c  time of filing/furnishing
      [ ] contained in the international application as filed
      [X] filed together with the international application in electronic form
      [ ] furnished subsequently to this Authority for the purposes of search

2  In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished

3  Additional comments
A. **CLASSIFICATION OF SUBJECT MATTER**

**GOIN 33/53(2006.01)i**

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

B. **FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

- IPC8 GOIN

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

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

- eKIPASS (KIPO Internal), USPTO, Google Scholar, Delphion ("tsutsgamushi, test strip, mixed antigen etc")

C. **DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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<td>KRI 020020020281 (PACIFIC PHAMACEUTICAL CO., LTD ) 15 03 2002</td>
<td>1-5, 8-13</td>
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<td>A</td>
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<td>6,7,14</td>
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<td>A</td>
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<td>US 6,001,658 A (Robert A Fredrickson) 14 12 1999</td>
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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**

10 JULY 2007 (10 07 2007)

**Date of mailing of the international search report**

11 JULY 2007 (11.07.2007)

**Name and mailing address of the ISA/KR**

- **Korean Intellectual Property Office**
  - 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea
- **Facsimile No** 82-42-472-7140

**Authorized officer**

- CHO, Myung Sun
- **Telephone No** 82-42-481-5594

Form PCT/ISA/210 (second sheet) (April 2007)
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