Description

The present invention relates to systems for diagnosing infectious diseases and methods for detecting and identifying microorganisms that can be used in such systems.

Infection is invasion and establishment of a foothold for growth in an organism by a pathogenic microorganism (hereinafter called bacterium). The outbreak depends on the interrelationship between the resistance of the host and the virulence of the bacterium.

Of infectious diseases, improvement in the methods of treatment of bacteremia is an important issue as shown below:

Bacteremia is not a disease caused by a specific bacterium. It is caused by the emergence and habitancy of various bacteria in blood. Its onset is clinically suspected when fever of about 40°C persists for two or more days. Bacteremia is a serious and urgent disease, whose symptom is a high fever, and a patient may die within several days, and in particular, if a patient were an infant or were suffering from terminal cancer with weakened resistance, the patient may die in one or two days.

In case of bacteremia, phagocytes including neutrophil, monocyte and macrophage primarily work for defence of the body.

Bacteria may appear in the blood of a patient suffering from bacteremia owing to the invasion of bacteria which have prevailed in the battle against phagocytes, from the tissue into the blood.

Bacteremia is a state after the above-mentioned condition has reached. To treat bacteremia, a large amount of antibiotic to which the causative bacterium or bacteria is sensitive is administered.

Generally speaking, however, antibiotics lower the functions of the body organs such as liver. One must, therefore, carefully avoid administering an ineffective antibiotic to a patient who is seriously ill.

When bacteremia is defined as a case when the phagocytosis of cells is not able to overcome the virulence of bacteria and the bacteria has spread to the blood throughout the body, sepsis is a bacteremia with serious symptoms due to toxins produced by the bacteria. A proof of sepsis (or establishment of the diagnosis) requires four important factors: 1) clinical symptoms; 2) culture; 3) gram-staining; and 4) shock state. The line of treatment is determined on the basis of these factors.

It, therefore, is essential to quickly and reliably identify the bacterium (bacteria).

According to the current method for identifying bacteria, in detection and identification of bacteria in a bacteremia-suspected specimen in a laboratory, it is a common procedure to make identification in selective media only after the specimen has been found positive in a routine process of culture bottle (hereinafter referred to as "C.B."). The rate of successful culture of bacteria from these blood specimens, however, is extremely low. Moreover, if a large dose of antibiotics were administered when bacteremia was suspected, bacteria, if any, in the blood will not be cultured and grow in many cases. The rate of C.B. positive cases, therefore, is extremely low.

Available sub-routine methods include instrumental analysis of constituents and metabolic products of bacteria (Yoshimi Benno, "Quicker identification of bacteria with gas chromatography", Rinsho Kensa, Vol.29, No.12, November 1985, Igaku Shoin), a method utilizing specific antibody (Japanese Patent Provisional Publication No. 60-224068), and a method based on hybridization based on specificity of DNA (Japanese Patent National Publication of the translated version No. 61-502376) which has a high accuracy due to the specificity. Separation and cultivation of a bacterium or bacteria, however, are essential pretreatment for all of these methods.

On the other hand, there is a method established based on the function of phagocytes in infectious diseases. According to the method, a stained smear of buffy coat in which leukocyte of the blood sample is concentrated is examined under an optical microscope.

Generally speaking, in an adult bacteremia patient the rate of detection of bacteria in buffy coat specimens is 30 % at most which is identical to that in earlobe blood specimens. For newborns, however, it was reported that bacteria had been detected in 7 cases out of 10 cases (70 %). The information concerning the presence of a bacterium or bacteria in peripheral blood, which is available through microscopic examination of smear, is an important guidance for treatment. Thus, although the diagnosis require quickness and reliability, the conventional methods are not satisfactory.

In the conventional methods which necessitate pretreatment, it takes at least one or two days to selectively isolate a bacterium or bacteria from a specimen, the cultivation takes a day, and fixation one or more days. The pretreatment, therefore, requires three to four days in total. In practice, this culture is continued till the bacterium or bacteria grow, and it frequently takes more than a week even for C.B.-positive cases. This is a factor for the high mortality of C.B.-positive patients being treated by the conventional methods. For instance, according to a report published in "The Journal of the Japanese Association for Infectious Diseases", Vol.58, No.2 p. 122, 1984, even though the blood culture positive rate was 28.6 % (163 cases/569 cases), the mortality was as high as 84.6 % (138 cases/163 cases).

Moreover, it may not be possible to distinguish contamination by indigenous bacteria.

For example, *Staphylococcus epidermidis*, one of *Staphylococci* which are frequently found to be causative bacteria of bacteremia, is present in the skin of a normal person. There is a risk of contamination of a specimen with this

bacterium when a needle is inserted into the skin.

Furthermore, one important point is that, under the circumstances as mentioned above, many bacteria in a specimen to be cultured have been taken into said phagocyte and are dead or stationary immobilized consequently, the number of bacteria that can grow is small even under appropriate conditions for cultivation. As a result, the actual rate of detection of bacteria through culture of a clinical specimen is as low as about 10 %.

In short, the present state is such that 90 % of the examination of blood cultured for one or more days after sampling from a patient who is clinically suspected to be suffering with bacteremia do not reveal the presence of bacteria. As a result, it is the present practice, as mentioned above, to start treatment when bacteremia is clinically suspected rather than to wait for the detection result. It is a trial and error method wherein an antibiotic having broad spectrum is administered first, and if the antibiotic is not effective after one or two days, another antibiotic will be tried.

According ot the staining method aforementioned, the constituents of the living body are also stained together with bacteria. It, therefore, requires experience to quickly identify bacteria according to their images; there may be cases that can be hardly diagnosted as bacteremia.

Generally speaking, cells may be visualized by staining them. Cells try to digest proteins covering the exterior of invading bacteria. The exterior proteins will remain intact on the bacteria that have just entered. The exterior proteins, however, will be digested gradually, and then DNA and RNA will be destroyed. However, it is not certain to what extent these proteins, DNA and RNA have been digested.

In any case, DNA or RNA is expected to remain for a longer period than proteins. When the objective is the identification of a bacterium or bacteria in cells, it would be more efficient and accurate to pay attention to DNA or RNA of which degree of denaturation is low and which is well preserved rather than proteins which have been digested to a greater degree in cells. It is needless to say that this applies to a case when a bacterium itself has a resistance and remains in cells. Such bacteria include, for example, Mycobacterium, Tuberculosis, Listeria, Salmonella, Brucella, and Legionella Pneumophila, although these bacteria are not the primary causative bacteria of bacteremia. The diagnosing methods according to the present invention are applicable to these bacteria.

It has been experimentally demonstrated that the specificity of DNA or RNA of a bacterium is maintained after the bacterium has been taken into a cell.

Scandinavian Journal of Infectious Diseases, vol. 9, no. 6, 1987, p. 641-6 and Israel Journal Vet. Med., vol. 44, no. 1, 1988, p. 19-24, both describe a method for detecting the target DNA of a bacterium with a streptavidin-labelled enzyme and in situ hybridization using a probe prepared by extracting the DNA from the subject bacteria and labelling the biotin thereon, wherein the DNA is held in the intact bacteria existing in blood, faeces or sputum. Thus, their methods make use of in situ hybridization instead of the conventional staining method.

In diagnosing an infectious disease for treatment, the ultimate objective of the examination is to confirm the bacterium or bacteria in a specimen.

According to the invention in one aspect there is provided a method for detecting bacteria or yeast in a patient suspected of being infected, comprising:

preparing a specimen of living body constituents or a fraction or constituent containing a large amount of phagocyte obtained from the patient,

fixing the prepared specimen,

exposing the fixed phagocyte to an avidin-like protein, and

detecting any product formed by exposing the product formed to biotin bound to a labelling molecule to form a labelled product.

According to the invention in another aspect there is provided a method for identifying the bacteria in a patient suspected of being infected, comprising:

preparing a specimen of living body constituents or a fraction or constituent containing a large amount of phagocyte,

fixing the prepared specimen,

hybridizing the fixed phagocyte with a probe containing DNA fragment derived from the causative bacteria, which could be Staphylococcus aureus, Staphylococcus epidermidis, or Pseudomonas aeruginosa, and

detecting any hybridized probe.

It has been found that constituents of bacterium including yeast can be quantitatively detected, irrespective of the kinds of bacteria, by means of streptavidin or avidin bound to labelling molecules. These molecules have no affinity for phagocyte.

This is due to the avidin's affinity for biotin, the binding forces of which is as high as several hundred times compared with other substances. The outer membranes of bacterial constituents are considered to be uniformly taking in biotin. Thus through the action of avidin, the presence of a bacterium or bacteria in a cell can be visualized by means of a labelling molecule with a very high sensitivity.

Furthermore, the binding speed between avidinbiotin and living body constituents is substantially quicker than those of antibodies which have been used as detecting agents, and there is a merit that detection can be made within two to three hours.

For example, an enzyme used for a colour reaction may be coupled, via biotin, to streptavidin being able to bind to bacteria so that a bacterium or bacteria in a specimen can be detected by the emergence of a specific colour.

The fact merits attention, that phagocytes take in and collect bacteria as mentioned above. It, therefore, is quite possible to detect DNA which are still present in bacteria which have been taken in and being destroyed by directly treating phagocytes without cultivation and by performing *in situ* hybridization. As no cultivation is required, this detection method is quick and reliable.

The above-mentioned discussion may be summarized as follows:

(1) The characteristics of DNA or RNA of bacteria is substantially different from that of DNA or RNA of viruses, but DNA or RNA of bacteria has the highest concentration in phagocytes; and

(2) The DNA (RNA) of bacteria has smaller damages than bacterial proteins, and may be considerably well preserved in some cases.

As a result of attention given to the above-mentioned two points, the present invention has established a method for detecting bacterium or bacteria through hybridization for the first time.

If a non-radioactive probe such as a biotinylated one were used for hybridization, the detection process will be quick and simple since such a probe can be detected with an optical microscope in a conventional laboratory without radio isotope handling facilities.

The methods for diagnosing infectious diseases according to the present invention are capable of clearly and quickly detecting the presence of a bacterium or bacteria within just two to three hours at the shortest, and one to two days at the longest, by using the two methods having the above-mentioned effects individually or in combination. In contrast, the conventional methods, such as detection by culture, require 24 or more hours to just detect the presence of a bacterium or bacteria even for positive cases, and then require additional 24 to 48 hours or more to identify the bacterium or bacteria (determination of genus and species), thus a total of 3 to 4 days. The methods according to the present invention also identify the bacterium or bacteria of the detected specimen in a quick and reliable manner by using the *in situ* hybridization method having the above-mentioned effects. These methods, therefore, are significant because of their effects that realize significantly more accurate detection as a whole within one to two days at the longest. Even in cases when bacterial constituents have been mostly digested and are not detectable, the methods according to the present invention are capable of detecting their native DNA (RNA) by hybridization. The invention will now be illustrated by the following experiments and drawings which are photographs showing the forms of organisms that indicate the results of the investigation according to the present invention, and what are shown by the leader lines of the respective photographs are as follows:

Fig. 1: *Conococcus* (particulate signal) in human cells, on Plate 1;
Fig. 2: A bacterium (not identified) taken into human leukocyte, on Plate 2 (clinical examination);
Fig. 3: *Staphylococcus* taken into human leukocyte, on Plate 3 (in vitro);
Fig. 4: *Staphylococcus* taken into murine leukocyte, on Plate 4 (in vivo);
Fig. 5: Yeast from dialysate of a dialysis patient, on Plate 5;
Fig. 6: Hybrid with *Staphylococcus-derived* DNA taken in murine ascites, on Plate 6 (hybrid signal with *Staphylococcus*-derived probe);
Fig. 7: An example of the use of the method of Plate 6 with another probe, on Plate 7. No hybrid signal is present;
Fig. 8: Hybrid with DNA derived from *Staphylococcus* in human subphrenic abscess, on Plate 8 (hybrid signal with a probe derived from *Staphylococcus);*
Fig. 9: An example of the use of the method of Plate 8 with another probe, on Plate 9. No hybrid signal is present;
Fig. 10: Dot hybridization with Probe 24, on Plate 10;
Fig. 11: Dot hybridization with Probe 77, on Plate 11;
Fig. 12: Dot hybridization with Probe 7, on Plate 12;

Fig. 13: Dot hybridization with Probe 36, on Plate 13;
Fig. 14: Restriction enzyme maps of a *Staphylococcus*-derived probe used in an example according to the present invention;
Fig. 15: Hybrid of human blood sample and *Pseudomonas aeruginosa* derived DNA probe, on Plate 15;
Fig. 16 through Fig. 20: Hybrids between human blood samples and *Staphylococcus* derived DNA probe, on Plates 16 through 19, respectively.

Fig. 15, Fig. 16, Fig. 17 and Fig. 19 are optical microscope photographs with magnification of 1000 times. Fig. 18 is an optical microscope photograph with magnification of 400 times. Fig. 20 is an optical microscope photograph of the specimen of Fig. 17 with magnification of 400 times.

Exemplar Experiment

A. Method for Preparing Specimens

Specimens prepared by the following method of (1) are fixed on slides by the method of (2) and used as samples in the respective embodiments according to the present invention:

(1) Methods for Preparing Various Clinical Specimens by Smearing

①Preparation of Samples from Blood (Heparinized Whole Bloods of Human and Animals)

Preparation of samples from blood is made by either one of the following two methods:

①-1 Mono-poly solvent (M-PRM: Flow Laboratories, Inc.) method

3 mℓ of M-PRM was put into a test tube of 13 × 100 mm, and 3.5 mℓ of heparinized whole blood (within two hours after sampling) being the specimen is carefully stratified over the M-PRM layer. The content of the test tube is centrifuged by a swing rotor at 300 g at room temperature for 30 minutes. Just the band of polymorphonuclear leukocyte is collected, and the band is washed with PBS (isotonic phosphate bufferred saline) and centrifuged. The resulting cell precipitate is suspended in an appropriate amount of PBS (about 1 mℓ) and used as specimen.

①-2 6 % dextran method

One volume (1 mℓ) of heparinized whole blood being a specimen is mixed with 1/3-1/4 volume of 6 % dextran, and the mixture is left to stand at 37°C for one hour. The upper layer is collected and diluted with PBS, and subjected to centrifugation. The resulting cell precipitates are suspended in an appropriate amount of PBS and used as specimen.

②Preparation of Samples from Urine

Urine specimen is directly smeared.

③Preparation of Samples from Ascites

Ascites is transferred to a test tube, and centrifuged at 800 r.p.m. or 3,000 r.p.m. at room temperature for 15 to 30 minutes. The resulting cell precipitates are suspended in an appropriate amount of PBS and used as specimen.

④Preparation of Samples from Pus

Pus specimen is directly smeared.

(2) Method for Smearing and Fixing Sample on Microscope Slide

An appropriate amount of a specimen obtained by the operation of (1) is placed on a 2 %-gelatin-coated slide (preferably "H.T. Coating Slide", Boxy Brown) and extended by the side of a pipette, and completely air-dried. Next, the slide is immersed in Carnoy's B fixative (ethanol : chloroform : acetic acid = 6 : 3 : 1) for 10 minutes to fix the cells. Then the slide is lightly washed in ethanol and air-dried.

Experimental Part 1 Method for Detecting Bacterium or Bacteria by Means of Avidin-like Protein (hereinafter referred to as the streptavidin method)

Streptavidin is used as an avidin-like protein in the present experiment.

Each sample fixed on slide prepared by the above-mentioned method A is rehydrated in PBS at room temperature for 10 minutes or over, and an appropriate amount of PBS containing 5 μg/mℓ of streptavidin (Amersham), 1 % bovine serum albumin (Sigma Fraction V) and 0.1 % Tween 20 (trade name)(Sigma) is placed on the fixed sample and allowed to react at 37°C in a moisture chamber for 60 minutes. The fixed sample is then lightly shaken in an appropriate amount of PBS containing 0.1 % Tween 20 to be washed at room temperature for 10 minutes for three times.

Next, a PBS containing 5 μg/mℓ of biotinylated alkaline phosphatase (E. Y LABS. Inc.), 1 % bovine serum albumin and 0.1 % Tween 20 is placed on the fixed sample on slide, and is allowed to react at 37°C in a moisture chamber for 60 minutes. Then the fixed sample is lightly shaken in a PBS containing 0.1 % Tween 20 and washed at room temperature for 10 minutes once. Furthermore, the fixed sample is lightly shaken in AP7.5 (100 mM Tris·HCl pH 7.5, 100 mM NaCl) containing 0.05 % Triton (Triton X-100, Sigma) (hereinafter referred to as TX) and washed at room temperature for 10 minutes twice. Then the fixed sample is lightly shaken in AP9.5 (100 mM Tris·HCl pH9.5, 100 mM NaCl, 10 mM MgCl) and washed at room temperature for 10 minutes three times.

The fixed sample is then placed in AP9.5 containing 0.3 mg/mℓ of NBT (nitroblue tetrazolium, Bethesda Research Laboratories) and 0.17 mg/mℓ of BCIP (5-bromo-4-chloro-3-indolyl phosphate, Bethesda Research Laboratories) at 37°C in the dark to make colour reaction. The reaction is terminated by immersing the sample in 10 mM EDTA for several minutes. The sample is then air-dried.

Finally, contrast staining of the sample is made with Naphthol Blue Black (Sigma) of an appropriate concentration. The sample is washed in running water, and completely air-dried. The sample is then oil-immersed and observed under a microscope to check for the presence of a bacterium or bacteria (signal).

Bacteria are observed as purple signals and cells such as neutrophil as water-colour or blue signals.

In the following, the results of the exemplary experiments on the respective plates will be explained with reference to the leader lines drawn on the microphotographs of the plates.

An optical microscope with magnification of 1000 times was used.

Plate 1 A bacterium *(Gonococcus)* in urine (clinical specimen):
Gonococcus in human cells were observed as particulate signals stained in blue. (Fig. 1)

Plate 2 A bacterium (not identified) detected in human blood (clinical specimen);
The blood preparation method was the dextran method.
The bacterium taken in human leukocyte was observed. (Fig. 2)

Plate 3 *Staphylococcus* detected in human blood (in vitro);
The blood preparation method was the mono-poly solvent method. Blood and *Staphylococcus* were incubated for 20 minutes.
Signals indicating *Staphylococcus* taken in human leukocyte were observed. (Fig. 3)

Plate 4 *Staphylococcus* detected in human blood (in vivo);
Staphylococcus was injected into a mouse intravenously, and after 4 hours, blood was sampled according to the conventional method.
Signals indicating *Staphylococcus* taken in murine leukocyte were observed. (Fig. 4)

Plate 5 Yeast detected in dialysate after peritoneal dialysis;
The specimen was obtained from a dialysis patient.
Signals indicating yeast from the dialysate of the dialysis patient were observed. (Fig. 5)

The preparation method of Plates 2, 3 and 4 may be either the dextran method or the mono-poly solvent method.

Experimental Part 2 Detection and Identification of Bacterium or Bacteria (Infectious Bacterium or Bacteria) in or out of Cells through *In Situ* Hybridization with Biotinylated Probe (hereinafter referred to as the probe method)

Each sample fixed on a slide prepared by the above-mentioned method A is immersed in PBS at room temperature for 10 minutes or over to rehydrate it, and then immersed in a PBS containing saponin and TX by 0.25 % (w/v), respectively, and lightly shaken at room temperature to treat it for 10 minutes.

Next, about 100 mℓ of PBS-saponin (0.05 %) solution containing 5 mg/mℓ each of lysozyme (Sigma) and of Lysostaphin (trade name, Sigma) and 0.5 mg/mℓ of N-acetylmuramidase SG (trade name, Seikagaku-Kogyo) is added to each well. The sample is allowed to stand at room temperature or at 37°C in a moisture chamber for 120 minutes. Then the sample is washed with physiological saline containing 0.2 N HCl for 20 minutes. Next, the sample is immersed in 0.1M triethanolamine-HCl buffer (pH 8.0) containing 0.5 % acetic anhydride for 20 minutes.

After that, each slide is washed with 75 % ethanol, and then with 95 % ethanol, and sufficiently air-dried. Next, the slide is immersed in physiological saline solution containing 70mM NaOH for three minutes, and immediately washed with 70 % ethanol and then with 95 % ethanol, and then sufficiently air-dried and used as a sample for *in situ* hybridization.

Next, an appropriate amount of a hybridization solution of the following composition is placed on the sample fixed on the slide, and the sample is allowed to react at 37°C in a moisture chamber for one day.

(Composition)

- 45 % formamide
- 2 × SSC
- 1 × Denhardt's solution [0.02 % polyvinylpyrrolidone (Sigma), 0.02 % Ficoll® (Pharmacia Fine Chemicals), 0.02 % bovine serum albumin]
- 250 μg/mℓ of salmon sperm DNA [denatured beforehand by heating it at 100°C for five minutes and chilled in ice (Pharmacia Fine Chemicals)]
- 10 % Dextran Sulphate (Pharmacia Fine Chemicals)
- An appropriate amount of biotinylated probe DNA (denatured beforehand). Biotinylation of the probe DNA is prepared by nick translation. For example, a nick translation kit (Bethesda Research Laboratories) is used.

Next, the sample is washed in 2 × SSC containing 50 % formamide at 37°C for 30 minutes, then in 1 × SSC containing 50 % formamide at 37°C or over for 30 minutes. Furthermore, the sample is lightly shaken in 1 × SSC and washed at room temperature for 20 minutes three times. Then the sample is immersed in PBS at room temperature for 10 minutes.

In succession, to effect blocking, PBS containing 10 % normal rabbit serum (Vector Laboratories Inc.) is placed on the sample fixed on slide, and the sample is then incubated at 37°C in a moisture chamber for 60 minutes, and immersed in PBS at room temperature for several minutes.

Next, PBS containing 2 μg/mℓ of streptavidin, 1 % bovine serum albumin, and 0.1 % Tween 20 is placed on the sample fixed on slide, and the sample was incubated at 37°C in a moisture chamber for 60 minutes, and then lightly shaken and washed in PBS containing 0.1 % Tween 20 at room temperature for 10 minutes three times.

Next, PBS containing 2 μg/mℓ of biotinylated alkaline phosphatase, 1 % BSA, 0.1 % Tween 20 is placed on the sample fixed on slide, and the sample is incubated at 37°C in a moisture chamber for 60 minutes. Then the sample is lightly shaken and washed in PBS containing 0.1 % Tween 20 at room temperature for 10 minutes. The sample is then lightly shaken and washed in AP7.5 containing 0:05 % TX at room temperature for 10 minutes twice. Then the sample is lightly shaken and washed in AP9.5 at room temperature for 10 minutes three times.

Next, the sample is immersed in AP9.5 containing 330 μg/mℓ of NBT and 170 μg/mℓ of BCIP at 37°C in the dark to make colour reaction for an appropriate time. The reaction is terminated by immersing the sample in 10 mM EDTA for several minutes, and the sample is air-dried.

Finally, contrast staining of the sample is made with Naphthol Blue Black of an appropriate concentration. The sample is washed in running water, and completely air-dried. The sample is then oil-immersed and observed under a microscope to check for the presence of a bacterium or bacteria on the basis of colour reaction and unique form.

In the following, the results of the experiments will be illustrated with reference to the leader lines drawn on the microphotographs of the plates. An optical microscope with magnification of 1000 times was used.

Illustration of Experiment 1 Preparation of *Staphylococcus*-derived DNA Probe

The properties of the *Staphylococcus-derived* DNA probe used in the following Exemplary Experiments 2 and 3 were as follows:

The preparation method used the cloning technology established in a text, for example, "Guide to Molecular Cloning Techniques", Methods in ENZYMOLOGY, vol., 152, Academic Press 1987, and *Staphylococcus* chromosomal DNA was inserted into a vector (e.g. pBR).

Clones having DNA fragments unique to *Staphylococcus* were selected from the resulting clones, and they were used as probes.

These probes do not cross hybridize with other bacteria (such as *Escherichia coli, Klebsiella. Pseudomonas, Enterobacter,* and *Staphylococcus epidermidis* of the same genus).

The restriction map of these probes is as shown in Fig. 14.

The reactivity between these probes and clinical strains *(Staphylococcus aureus* and *Staphylococcus epidermidis*; a priority was given to these species since they are the most frequently causative bacteria of infectious diseases and they are the same species) was examined as follows:

Preparation Method

The preparation method was based on the above-mentioned text, and clinical bacterial strains were cultured and their DNA were extracted.

A certain amount (e.g. 5 μℓ) of each extract was spotted on a nylon filter and subjected to alkaline denaturation. After that, they were used as samples of hybridization.

Dot Blot Hybridization

According to said text, hybridization was made with the above-mentioned ^{32}P-labelled probes in 50 % formamide 5 × SSC at 42°C all night.

After that, samples were washed with 0.1 × SSC, 0.1 % SDS, at 55°C for 20 minutes twice, then subjected to irradiation at -70°C all night. Then the samples were developed.

The reactivities between these probes and clinical strains *(Staphylococcus aureus* and *Staphylococcus epidermidis)* are shown by Plates 10, 11, 12 and 13.

In each case, signals are observable in places where *Staphylococcus aureus* was spotted (spots of the upper two rows of Figs. 10, 11, 12, and 13), and no signals are observable in places where *Staphylococcus epidermidis* was spotted (spots of the lower two rows of said figures). Hence these probes reliably detect *Staphylococcus aureus* but do not cross with *Staphylococcus epidermidis* which is the same species as *Staphylococcus aureus.*

Exemplary Experiment 2 Detection of *Staphylococcus* in Murine Ascites

Staphylococcus was injected into the abdominal cavity of a mouse, and laparotomy was made 4-6 hours after the injection to collect ascites. The ascites was treated according to the above-mentioned preparation method A (1)③ and the resulting centrifuge precipitate was suspended in 100 μℓ of PBS, and 20-50 μℓ of the suspension was placed in a well of each object glass to prepare a fixation sample. According to the method described in the above-mentioned Exemplary Embodiment 1, the following probes were used to hybridize the sample.

The results of the experiment are shown by each experimental plate.

Plate 6 Hybridization between *Staphylococcus* taken in ascites and *Staphylococcus-derived* DNA probe; Signals indicating hybrid were observed. The signals indicated dispersion of leukocyte. (Fig. 6)

Plate 7 Hybridization using *Escherichia coli*–derived and *Klebsiella*-derived probes; No signal of hybridization was detected. (Fig. 7)

Exemplary Experiment 3 Detection of *Staphylococcus* in Human Subphrenic Abscess

Specimen of human subphrenic abscess was suspended in physiological saline, and 20 μℓ of the suspension was smeared and spread in each well and then air-dried (for 40 minutes). After that, the samples were pretreated in the same manner as Plates 6 and 7, and then subjected to *in situ* hybridization by the method of Experimental Part 1.

The results of the experiment are shown below by experimental plate.

Plate 8 Hybridization with the DNA probe used for Plates 6 and 7; Signals of hybrid were observed. (Fig. 8)

Plate 9 Hybridization with the same probe used for Plates 6 and 7; When *Escherichia coli*-derived and *Klebsiella*-derived DNA probes were used, no signal of hybrid was detected.

Experimental Part 3 Comparative Experiment of *In Situ* Hybridization Method and Blood Culture Method

From a patient for whom no bacterium was detected by the streptavidin method of the above-mentioned Experimental Part 1, human blood samples were prepared by using the 6 % dextran method of said A (1)① 2 and the method for smearing and fixing sample on slide of said A (2). According to the method described in Experimental Part 1, the samples were hybridized with the *Staphylococcus*-derived DNA probe prepared in Exemplary Experiment 1 (see Plates 16 through 19 of Table I below, and Figs 16 through 19).

Pseudomonas aeruginosa-derived DNA probe was also prepared by a method similar to the method for preparing the DNA probe of Exemplary Experiment 1. To be more specific, chromosome DNA of *Pseudomonas aeruginosa* was fragmented by restriction enzyme Hind III and inserted into an appropriate vector (e.g. pBR322) to amplify the fragments.

Those which do not cross with other bacterial DNAs (such as *Escherichia coli, Klebsiella, Enterobacter,* and *Staphylococcus)* were selected. Then those having appropriate length (for example, 10 kb, 5 kb, 4.5 kb) were selected as probes.

In accordance with the method described in said Experimental Part 1, one of the probes was hybridized with the human blood samples prepared as described above (see Plate 15 of Table I below, and Fig. 15).

Using a blood collecting unit "Roche", blood was aseptically collected from the vein of the same patient from whom the above-mentioned human blood samples were taken. Blood was collected in Liquoid BC bottle "Roche" (Nippon Roche KK), 10 mℓ each. The specimens were incubated at room temperature, and the specimens were monitored day by day. As a result, as shown in Table I below, no bacterium was detected in Bottles 15 through 19 (no microphotographs are indicated).

The above-mentioned results are summarized in Table I below.

Table I Results of Comparative Experiment of the Probe Method and the Blood Culture Method

Plate or Bottle No.	Name of bacterium detected by the probe method	Blood culture method, in 1 month
15	*Pseudomonas aeruginosa*	- *
16	*Staphylococcus aureus*	-
17	ditto	-
18	ditto	- **
19	ditto	-

Notes:

* *Pseudomonas aeruginosa* was detected from sputum.

** *Staphylococcus aureus* was detected from feces.

As clearly shown by the results of Table I above, it was demonstrated that the use of the probe method allowed detection of a bacterium from specimens from which no bacterium had been detected by the conventional blood culture method and the direct streptavidin method, and that the detection capability of the probe method was excellent.

First, the *in situ* hybridization method, which paid attention to phagocyte, was capable of quickly and reliably identifying a bacterium in a specimen which was not positive according to the blood culture. Hence the establishment of this diagnostic method will give a good guidance to the treatment of bacteremia, and a reduction in mortality is expected.

According to the conventional blood culture method, it was necessary, in the preparation of a specimen, to take about 5 to 10 mℓ of blood sample from a patient day after day till the bacterium is detected, and the patient had to suffer physically as well as mentally. In contrast with it, according to the diagnostic method of the present invention, it is sufficient to take a blood sample from a patient only once, and no exact sterile operation is needed. Furthermore, a very small sample quantity will do. For instance, the dextran method requires only about 1 mℓ. Thus physical and mental suffering of the patient can be reduced.

According to the method for diagnosing infectious diseases of the present invention, which uses the probe method alone, it is possible to detect and identify bacterium or bacteria in a specimen from which no bacterium was detected

by the streptavidin method, and the detection and identification capabilities are markedly great.

Furthermore, the detection method of the present invention, which uses the streptavidin method alone, quickly gives a very clear detection result. This invention marks a big progress in the sense that it allows confirmation of bacteremia in circumstances for which the conventional approach had to tentatively start treatment with antibiotics on the basis of clinically suspected bacteremia.

The detection and identification methods of the present invention, which use the probe method alone, have the following effects with regard to the identification of causative bacterium or bacteria of bacteremia. According to the present hybridization method, one specimen allows identification of the kind of bacterium, and the time required can be significantly reduced to one to two days at the longest, and the detection rate is markedly high, whereas the conventional method took three or four days (and the detection rate was low).

When non-radioactive labels, in particular, biotinylated probes are used, the method can be used easily irrespective of the place.

However, with regard to this DNA hybridization method, further research is needed to find probes of which specificity is as high as possible.

For example, if a DNA probe which is specific to *Staphylococcus aureus* only was developed and used, the detection result will be negative for a specimen in which no *Staphylococcus aureus is* present. If the specimen was checked by changing the kinds of probe one after another and found to be negative for all DNA, the presence of a bacterium will be uncertain.

Of the methods for daiagnosing infectious diseases according to the present invention, the diagnostic method using the streptavidin method and the probe method in combination quickly detects a specimen in which a bacterium is present and then quickly and reliably identifies the bacterium of the specimen. The whole process of the method, therefore, is quick and reliable.

In other words, the confirmation of the presence or absence of bacterium by means of the streptavidin method is quite useful in preventing costly repetition of hybridization of specimens having no bacterium.

Furthermore, in contrast with the conventional staining method, the present method does not stain the living body constituents. It specifically stains bacterium or the like in the stained specimen. It, therefore, allows quick and reliable judgement of the infection state, and no special skill is required.

Claims

1. A method for detecting bacteria or yeast in a patient suspected of being infected, comprising:

   preparing a specimen of living body constituents or a fraction or constituent containing a large amount of phagocyte obtained from the patient,

   fixing the prepared specimen,

   exposing the fixed phagocyte to an avidin-like protein, and

   detecting any product formed by exposing the product formed to biotin bound to a labelling molecule to form a labelled product.

2. A method as claimed in Claim 1 in which the avidin-like protein is avidin or streptavidin.

3. A method as claimed in Claim 1 or Claim 2 in which the labelling molecule is an enzyme, an antibody, a dye, or gold.

4. A method as claimed in any preceding claim in which the specimen is blood, ascites fluid or pus.

5. A method as claimed in any preceding claim in which the fixing step comprises application of a fixative comprising ethanol, chloroform and acetic acid in a ratio of 6:3:1 to the specimen.

6. A method as claimed in any preceding claim further comprising identification of the specific bacteria detected by reaction with biotin by hybridizating the fixed phagocyte with a probe containing DNA fragment derived from the causative bacteria.

7. A method as claimed in Claim 6 in which the causative bacteria is Staphylococcus aureus, Staphylococcus epidermidis or Pseudomonas aeruginosa.

by the streptavidin method, and the detection and identification capabilities are markedly great.

Furthermore, the detection method of the present invention, which uses the streptavidin method alone, quickly gives a very clear detection result. This invention marks a big progress in the sense that it allows confirmation of bacteremia in circumstances for which the conventional approach had to tentatively start treatment with antibiotics on the basis of clinically suspected bacteremia.

The detection and identification methods of the present invention, which use the probe method alone, have the following effects with regard to the identification of causative bacterium or bacteria of bacteremia. According to the present hybridization method, one specimen allows identification of the kind of bacterium, and the time required can be significantly reduced to one to two days at the longest, and the detection rate is markedly high, whereas the conventional method took three or four days (and the detection rate was low).

When non-radioactive labels, in particular, biotinylated probes are used, the method can be used easily irrespective of the place.

However, with regard to this DNA hybridization method, further research is needed to find probes of which specificity is as high as possible.

For example, if a DNA probe which is specific to *Staphylococcus aureus* only was developed and used, the detection result will be negative for a specimen in which no *Staphylococcus aureus is* present. If the specimen was checked by changing the kinds of probe one after another and found to be negative for all DNA, the presence of a bacterium will be uncertain.

Of the methods for daiagnosing infectious diseases according to the present invention, the diagnostic method using the streptavidin method and the probe method in combination quickly detects a specimen in which a bacterium is present and then quickly and reliably identifies the bacterium of the specimen. The whole process of the method, therefore, is quick and reliable.

In other words, the confirmation of the presence or absence of bacterium by means of the streptavidin method is quite useful in preventing costly repetition of hybridization of specimens having no bacterium.

Furthermore, in contrast with the conventional staining method, the present method does not stain the living body constituents. It specifically stains bacterium or the like in the stained specimen. It, therefore, allows quick and reliable judgement of the infection state, and no special skill is required.

Claims

1. A method for detecting bacteria or yeast in a patient suspected of being infected, comprising:

   preparing a specimen of living body constituents or a fraction or constituent containing a large amount of phagocyte obtained from the patient,

   fixing the prepared specimen,

   exposing the fixed phagocyte to an avidin-like protein, and

   detecting any product formed by exposing the product formed to biotin bound to a labelling molecule to form a labelled product.

2. A method as claimed in Claim 1 in which the avidin-like protein is avidin or streptavidin.

3. A method as claimed in Claim 1 or Claim 2 in which the labelling molecule is an enzyme, an antibody, a dye, or gold.

4. A method as claimed in any preceding claim in which the specimen is blood, ascites fluid or pus.

5. A method as claimed in any preceding claim in which the fixing step comprises application of a fixative comprising ethanol, chloroform and acetic acid in a ratio of 6:3:1 to the specimen.

6. A method as claimed in any preceding claim further comprising identification of the specific bacteria detected by reaction with biotin by hybridizating the fixed phagocyte with a probe containing DNA fragment derived from the causative bacteria.

7. A method as claimed in Claim 6 in which the causative bacteria is Staphylococcus aureus, Staphylococcus epidermidis or Pseudomonas aeruginosa.

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8. A method for identifying the bacteria in a patient suspected of being infected, comprising:

preparing a specimen of living body constituents or a fraction or constituent containing a large amount of phagocyte,

fixing the prepared specimen,

hybridizing the fixed phagocyte with a probe containing DNA fragment derived from the causative bacteria, and

detecting any hybridized probe.

9. A method as claimed in Claim 8 in which the causative bacteria is Staphylococcus aureus, Staphylococcus epidermidis, or Pseudomonas aeruginosa.

10. A method as claimed in Claim 8 or Claim 9 in which the specimen is blood, ascites fluid or pus.

11. A method as claimed in any of claims 8 to 10 in which the fixing step comprises application to a fixative comprising ethanol, chloroform and acetic acid in a ratio of 6:3:1 to the specimen.

Patentansprüche

1. Eine Methode zum Nachweis von Bakterien oder Hefepilzen bei Patienten mit Infektionsverdacht, die folgendes umfaßt:

Herstellung einer Probe aus Bestandteilen des lebenden Organismus oder einer Fraktion bzw. einem Bestandteil, die (der) eine große Anzahl von Phagozyten des Patienten enthält;

Fixierung der hergestellten Probe;

Einwirkung eines avidinähnlichen Proteins auf die fixierten Phagozyten;

Nachweis eines Produkts, das durch Einwirkung von Biotin, das zum Zwecke der Bildung eines markierten Produkts an ein Markierungsmolekül gebunden ist, auf das gebildete Produkt entsteht.

2. Eine Methode nach Anspruch 1, gekennzeichnet dadurch, daß das avidinartige Protein Avidin oder Streptavidin ist.

3. Eine Methode nach Anspruch 1 oder 2, gekennzeichnet dadurch, daß das Markierungsmolekül ein Enzym, ein Antikörper, ein Farbstoff oder Gold ist.

4. Eine Methode nach einem der vorausgegangenen Ansprüche, gekennzeichnet dadurch, daß die Probe Blut, Aszitesflüssigkeit oder Eiter ist.

5. Eine Methode nach einem der vorausgegangenen Ansprüche, gekennzeichnet dadurch, daß die Fixierung das Aufbringen eines Fixierungsmittels, das Ethanol, Chloroform und Essigsäure in einem Verhältnis von 6:3:1 enthält, auf die Probe umfaßt.

6. Eine Methode nach einem der vorausgegangenen Ansprüche, die außerdem die Bestimmung der spezifischen durch eine Reaktion mit Biotin nachgewiesenen Bakterien umfaßt, bei der die fixierten Phagozyten mit einer Sonde hybridisiert werden, die von den Erregerbakterien abgeleitete DNA-Fragmente enthält.

7. Eine Methode nach Anspruch 6, gekennzeichnet dadurch, daß es sich bei den Erregerbakterien um *Staphylococcusaureus, Staphylococcus epidermidis* oder *Pseudomonas aeruginosa handelt.*

8. Eine Methode zum Nachweis von Bakterien bei Patienten mit Infektionsverdacht, die folgendes umfaßt:

Herstellung einer Probe aus Bestandteilen eines lebenden Organismus oder einer Fraktion bzw. einem Bestandteil, die (der) eine große Anzahl von Phagozyten enthält;

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Fixierung der hergestellten Probe;

Hybridisierung der fixierten Phagozyten mit einer Sonde, die ein von den Erregerbakterien abgeleitetes DNA-Fragment enthält;

Nachweis von hybridisierten Sonden.

9. Eine Methode nach Anspruch 8, gekennzeichnet dadurch, daß es sich bei den Erregerbakterien um *Staphylococcus aureus*, *Staphylococcus epidermidis* oder *Pseudomonasaeruginosa* handelt.

10. Eine Methode nach Anspruch 8 oder 9, gekennzeichnet dadurch, daß die Probe Blut, Aszitesflüssigkeit oder Eiter ist.

11. Eine Methode nach einem der Ansprüche 8 bis 10, gekennzeichnet dadurch, daß die Fixierung das Aufbringen eines Fixierungsmittels, das Ethanol, Chloroform und Essigsäure in einem Verhältnis von 6:3:1 enthält, auf die Probe umfaßt.

Revendications

1. Procédé pour détecter des bactéries ou des levures chez un patient suspecté d'être infecté, comprenant :

la préparation d'un prélèvement de constituants du corps vivant ou d'une fraction ou du constituant contenant une grande quantité de phagocyte obtenue à partir du patient,

la fixation du prélèvement préparé,

l'exposition du phagocyte fixée à une protéine analogue à l'avidine et

la détection de tous produits formée par exposition du produit formé pour assurer une liaison biotine à une molécule de marquage, en vue de former un produit marqué.

2. Procédé selon la revendication 1, dans lequel la protéine analogue à l'avidine est l'avidine ou la streptavidine.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel la molécule de marquage est une enzyme, un anticorps, un colorant ou de l'or.

4. Procédé selon l'une quelconque des revendications précédentes, dans lequel le prélèvement est du sang, du fluide ascitiques ou du pus.

5. Procédé selon l'une quelconque des revendications précédentes, dans lesquelles l'étape de fixation comprend l'application d'un fixateur comprenant de l'éthanol, du chloroforme et de l'acide acétique, selon un rapport 6:3:1 par rapport au prélèvement.

6. Procédé selon l'une quelconque des revendications précédentes, comprenant en outre l'identification de la bactérie spécifique détectée par réaction avec de la biotine, par hybridation du phagocyte fixe avec une sonde contenant un fragment DNA dérivé de la bactérie en cause.

7. Procédé selon la revendication 6, dans lequel la bactérie en cause est Staphylococcus aureus, Staphylococcus epidermidis ou Pseudomonas aeruginosa;

8. Procédé pour identifier la bactérie chez un patient suspecté d'être infecté, comprenant :

la préparation d'un prélèvement de constituants de corps vivant ou d'une fraction ou du constituant contenant une grande quantité de phagocyte,

la fixation du prélèvement préparé,

l'hybridation du phagocyte fixe avec une sonde contenant un fragment DNA dérivé de la bactérie en cause, et

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la détection de toute sonde hybridisée.

9. Procédé selon la revendication 8, dans lequel la bactérie en cause est Staphylococcus aureus, Staphylococcus epidermidis ou Pseudomonas aeruginosa.

10. Procédé selon la revendication 8, ou la revendication 9, dans lequel le prélèvement est du sang, du fluide ascites ou du pus.

11. Procédé selon l'une quelconque des revendications 8 à 10, dans lequel les tables de fixation comprend l'application à un fixateur comprenant de l'éthanol, du chloroforme et de l'acide acétique selon un rapport 6:3:1 par rapport au prélèvement.

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