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(72) Inventeurs/Inventors:
FOVET, YANNICK, DE;
DUKAN, SAM, FR;
DUCRET, ADRIEN, FR

(73) Propriétaires/Owners:
BASF SE, DE;
CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS), FR

(74) Agent: ROBIC

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(57) Abrégé/Abstract:
A method for detecting and enumerating viable microorganisms in a sample suspected of containing said microorganisms comprising: (1) contacting said sample with a cell nutritive resource and a cellular proliferation inhibitor, (2) contacting said sample with at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms, (3) detecting and quantifying the fluorescent signal, in which the microorganisms are of the species Legionella pneumophila and in which the cellular proliferation inhibitor is selected from the group consisting of ciprofloxacin and cephalexin. The invention also includes a kit comprising: (1) a cell nutritive resource, (2) a cellular proliferation inhibitor, (3) at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms, (4) a means for detecting and quantifying the fluorescent signal, in which the cellular proliferation inhibitor is selected from the group consisting of ciprofloxacin and cephalexin.

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(71) Applicants *(for all designated States except US)*: **BASF SE** [DE/DE]; 67056 Ludwigshafen (DE). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3 rue Michel-Ange, Cedex 16, F-75794 Paris (FR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **FOVET, Yannick** [FR/FR]; 55 rue du commerce, F-927000 Colombes (FR). **DUKAN, Sam** [FR/FR]; 6 rue Esperandieu, F-13004 Marseille (FR). **DUCRET, Adrien** [FR/FR]; 5 ter rue borde, F-13008 Marseille (FR).

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(54) **Title**: DETECTION AND ENUMERATION OF MICROORGANISMS

(57) **Abstract**: A method for detecting and enumerating viable microorganisms in a sample suspected of containing said microorganisms comprising: (1) contacting said sample with a cell nutritive resource and a cellular proliferation inhibitor, (2) contacting said sample with at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms, (3) detecting and quantifying the fluorescent signal, in which the microorganisms are of the species Legionella pneumophila and in which the cellular proliferation inhibitor is selected from the group consisting of ciprofloxacin and cephalexin. The invention also includes a kit comprising: (1) a cell nutritive resource, (2) a cellular proliferation inhibitor, (3) at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms, (4) a means for detecting and quantifying the fluorescent signal, in which the cellular proliferation inhibitor is selected from the group consisting of ciprofloxacin and cephalexin.

Detection and Enumeration of Microorganisms

The present invention concerns a method for detecting and enumerating viable microorganisms of the species *Legionella pneumophila* in a sample. The invention also includes a kit suitable for use in such a method. This method and kit enable viable microorganisms to be quantified more rapidly.

Legionella bacteria are ubiquitous in wet or moist environments such as soil and non-marine aquatic habitats. They can also be found in warm and cold water installations, cooling towers of air conditioning systems and water humidifiers.

Legionella, especially *Legionella pneumophila*, are pathogens that can cause an acute bacterial pneumonia, generally known as "legionnaires disease", which is often lethal for infected individuals.

Traditionally detection and enumeration of *Legionella pneumophila* are achieved by cell culturing. This method may be achieved by measuring culturable bacteria using plate count or measuring micro-colonies employing a filter membrane method. These techniques evaluate viable bacteria by their ability to form a colony or micro-colony. Unfortunately, such methods usually require between 3 and 10 days in order to allow the colonies or micro-colonies to form. Where water installations are still in operation there is an unacceptable risk of human infection during this time.

Other methods for detecting total *Legionella* microorganisms include PCR (Polymerase Chain Reaction) techniques. PCR employs DNA polymerase to amplify a piece of DNA by *in vitro* enzymatic replication. During the progression of the technique the DNA generated is used as a template for replication which brings about a chain reaction in which the DNA template is exponentially amplified. PCR enables a single or few copies of a piece of DNA to be amplified by generating millions or more copies of the DNA piece. Typically

such a method is described by Diederen et al., J Med Microbiol. 2007 Jan; 56 (Pt 1):94-101.

However a drawback of PCR is that the samples tend to contain polymerisation reaction inhibitors and therefore do not consistently provide quantitative results. Furthermore, the technique relies upon a prior DNA purification step which can result in loss of DNA with the consequential underestimation of the *Legionella* present. To some extent these disadvantages are overcome by real-time PCR which is quantitative. However, the technique cannot distinguish between viable cells and non-viable cells.

Another technique is fluorescent *in situ* hybridisation (FISH) in which an oligonucleotidic probe labelled by a fluorescent substance penetrates into the bacteria cells. Where the ribosomal nucleic acids (rRNA) have the correct sequence to the probe known as the target, the probe will attach itself to its target and will not be removed by any subsequent washing step. The bacteria in which the probe is fixed will then emit a fluorescent signal. This fluorescent signal may then be quantified by techniques such as flow cytometry, solid phase cytometry, or epifluorescent microscopy. A typical FISH technique is described by Dutil S et al J Appl Microbiol. 2006 May;100(5):955-63. However, using the FISH technique alone the total number of viable *Legionella pneumophila* could be detected but unfortunately the method could not exclusively identify only those *Legionella pneumophila* bacteria able to divide and by consequence make a colony.

A further method for enumerating viable *Legionella pneumophila* involves ChemChrome V6 and is described by Delgado-Viscogliosi et al Appl Environ Microbiol. 2005 Jul;71(7):4086-96. This method allows the quantification of *Legionella pneumophila* as well as discrimination between viable and non-viable bacteria. It combines specific detection of *Legionella* cells using antibodies and a bacterial viability marker (ChemChrome V6) and employing epifluorescent

microscopy for the enumeration. However, although this technique distinguishes between viable and non-viable cells it is not able to separately identify those colony-forming bacteria.

To date the only methods which permit detection of *Legionella pneumophila* bacteria able to divide has been based on the micro colony method (Scan-VIT). However this method requires around 72 hours.

Arana et al. " Detection and enumeration of viable but non-culturable transconjugants of Escherichia coli during the survival of recipient cells in river water" pp 340-346, J. App. Microbiol. Vol 83, 1997 describes the use of direct viability count (DVC) using specific markers.

Piqueres et al. "A combination of direct viable count and fluorescent in situ hybridisation for estimating Helicobacter pylori cell viability" pp 345-349 Res. Macrobiol. Vol 157, 2006 Jjemba et al. "In situ enumeration and probing of pyrene degrading soil bacteria" pp 287-298 FEMS Microbiol. Ecol. Vol. 55, 2006 both refer to DVC-FISH but for microorganisms unrelated to *Legionella pneumophila*.

EP-A-1852512 describes a method for identification and enumeration of several pathogenic microorganisms including *Legionella pneumophila*. The method incorporates the direct viability count (DVC) and the fluorescence in situ hybridisation (FISH) but also employs a helper probe in order to amplify the signal. Helper probes are unlablled oligonucleotides that bind to regions adjacent to that targeted by the specific labelled probe. This enhances in situ accessibility and hence the probe conferred signal.

The method described in EP-A-1852512 is said to employ a DNA gyrase inhibitor such as nalidixic acid, which stops cell division, increases the intracellular rRNA content and the cellular length of sensitive cells. However, in

practice nalidixic acid is not a reliably effective DNA gyrase inhibitor for *Legionella pneumophila*. Furthermore, this document does not mention problems of inaccurate enumeration that can occur due to the presence of naturally fluorescent microorganisms.

It would be desirable to provide a method for reliably quantifying viable *Legionella pneumophila* capable of forming colonies in a sample more rapidly than known techniques. In addition, it would be preferable to achieve this more accurately.

According to the present invention we provide a method for detecting and enumerating viable microorganisms in a sample suspected of containing said microorganisms comprising:

(1) contacting said sample with a cell nutritive resource and a cellular proliferation inhibitor,

(2) contacting said sample with at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms,

(3) detecting and quantifying the fluorescent signal,

in which the microorganisms are of the species *Legionella pneumophila* and in which the cellular proliferation inhibitor is selected from the group consisting of ciprofloxacin and cephalexin.

The present invention provides a method for detecting and enumerating viable microorganisms in a sample suspected of containing said microorganisms comprising:

(1) contacting said sample with a cell nutritive resource and a cellular proliferation inhibitor in which the cell nutritive resource contains a growth supplement which comprises an antioxidant,

(2) contacting said sample with at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion

of ribosomal nucleic acids of said microorganisms in which said sample is contacted with at least a first probe and a second probe in which the first probe is able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms and the second probe is able to specifically hybridise at least one different portion of ribosomal nucleic acids of the microorganisms, and

(3) detecting and quantifying the fluorescent signal,

in which the microorganisms are of the species *Legionella pneumophila* and in which the cellular proliferation inhibitor is ciprofloxacin or cephalexin.

The present invention provides a kit for more rapidly detecting and enumerating viable microorganisms of the species *Legionella pneumophila* in a sample suspected of containing said microorganisms comprising:

(1) a cell nutritive resource in which the cell nutritive resource contains a growth supplement which comprises an antioxidant,

(2) a cellular proliferation inhibitor,

(3) at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms in which said sample is contacted with at least a first probe and a second probe in which the first probe is able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms and the second probe is able to specifically hybridise at least one different portion of ribosomal nucleic acids of the microorganisms, and

(4) a means for detecting and quantifying the fluorescent signal,

in which the cellular proliferation inhibitor is ciprofloxacin or cephalexin.

Generally each of the steps are carried out sequentially in which step (2) is performed on the sample so treated in step (1) and then step (3) is conducted following step (2).

Step 1 of the inventive method is known as Direct Viable Count (DVC) which is based on incubating a bacteria in the presence of an antibiotic or DNA gyrase inhibitor which blocks the cellular division without deteriorating the cellular metabolism. Therefore the living bacteria will tend to elongate but not divide

and so can be distinguished from the dead bacteria which do not change in size. It is therefore possible to identify living bacteria by microscopy. Prior to carrying out the DVC step it may be desirable to concentrate the sample and to pre-treat it for instance by acid and/or heat treatment according to the standard method T 90-431 (ISSN 0335-3931), edited and distributed by the French Association of Normalization (AFNOR) 11, Avenue Francis de Pressensé-93571 St Denis La Plaine Cedex, France.

We have found unexpectedly that ciprofloxacin and cephalexin are very effective DNA gyrase inhibitors. Both of ciprofloxacin and cephalexin allow the *Legionella pneumophila* cells to elongate in preparation for cell division but actually block these elongated cells from dividing. By contrast nalidixic acid does not provide sufficient elongation of the *Legionella pneumophila* cells to be effective for the present method. Advantageously we find that the method of the present invention enables *Legionella pneumophila* to be identified and quantified reliably and within a timescale normally of less than 24 hours. This provides significant improvements in water sanitary control.

The ciprofloxacin or cephalexin may be used in any effective amount. Typically this would be in concentrations of up to 20 mg/L or higher within the medium contained in the cell nutritive resource. Preferably the concentrations are between 1 and 10 mg/L. Preferably the cellular proliferation inhibitor is ciprofloxacin. We have found the greatest cellular elongation can be achieved when the concentration of ciprofloxacin is between 2 and 6 mg/L after a duration of 12 hours, especially between 3 and 5 mg/L.

The cell nutritive resource should contain any suitable growth medium composition applicable to the DVC method and suitable for *Legionella pneumophila*. A suitable medium composition may comprise a medium,

selective supplement, cellular proliferation inhibitor (ciprofloxacin or cephalexin), and growth supplement.

The medium provides the minimum nutrition required to allow growth of *Legionella pneumophila*. It may be any suitable medium as described in the literature, for instance according to the standard method prescription T 90-431 (as given above) without agar and charcoal.

The selective supplement is often required in order to limit the development of interfering microorganisms. The choice of antibiotic may be any known supplement suitable for the DVC method, for instance has described in standard method T 90-431 (as given above). Nevertheless the concentration of each antibiotic in general should be adapted for the particular liquid medium. However, it would not necessarily be detrimental in cases where these other microorganisms are not completely eliminated since step 2 will usually be sufficiently specific to overcome the effect of interfering bacteria.

Although the growth supplement is not essential to allow growth of the *Legionella pneumophila* bacteria, it can optimise its growth. *Legionella pneumophila* is characterised by doubling in 120 min (under optimum conditions). However, we have found that in some cases the viable *Legionella pneumophila* bacteria which are able to form colonies may not necessarily form colonies immediately and instead undergo a delay or lag phase before growth commences. This lag phase can be between 8 to more than 20 hours in the function of initial physiological state.

In a preferred form of the present invention the lag phase for *Legionella pneumophila* can be reduced by including as the growth supplement and antioxidant reagent into the cell nutritive resource. The antioxidant reagent may act directly upon reactive oxygen species (ROS) or by other means such as causing an effect on the metabolism of the microorganism, directly or indirectly,

which brings about a reduction in ROS. Suitable antioxidant reagents include catalase, ascorbic acid, sodium metabisulphite, dimethyl sulfoxide, TDPA (3,3'-thiodipropionic acid) and pyruvate etc. Preferably antioxidant reagent is pyruvate. Preferred doses of antioxidant reagent, especially for pyruvate, are between 0.5 and 1.5 g/L, especially around 1 g/L.

The cell nutritive resource may include at least one compound that indirectly inhibits the formation of and/or degrades the Reactive Oxygen Species (ROS), said compound may bring about reduced levels of ROS by interfering with the metabolism of the microorganism. Typically such compounds will include amino acids or their salts. A particularly preferred compound is glutamic acid or glutamate salt.

In a still further preferred form of the invention the cell nutritive resource would include glutamic acid or glutamate salt, especially the sodium salt. In general the amount of glutamic acid or glutamate will be between 0.01 and 5% by weight calculated as the sodium salt.

It is particularly preferred that the cell nutritive resource includes both pyruvic acid or pyruvate (especially the sodium salt) together with glutamic acid or glutamate (especially as the sodium salt). This combination of pyruvic acid or pyruvate with glutamic acid or glutamate seems to induce a synergistic effect in that it allows a higher estimation (and therefore more accurate estimation) of culturable *Legionella* than either compound respectively used alone. Furthermore we have found that this combination brings about a further reduction of lag phase during development of the *Legionella pneumophila,* in particular in a liquid medium. Such a reduction of lag phase in liquid medium results in a reduction of the time required to obtain a visible colony on agar plate.

Desirably the amount of pyruvate and glutamate will be as stated previously. It is particularly preferred that the ratio of glutamate to pyruvate will be in the range between 1:1 and 50:1, especially between 5:1 and 20:1 and more especially between 7:1 and 15:1.

Glutamate is not known to be an antioxidant. However, it s appear that indirectly glutamate could reduce the endogenous production of ROS naturally formed during growth or their consequences on macromolecules (oxidation).

Without being limited to theory, it thought that the glutamic acid changes the metabolism of *Legionella* to increase the effect of pyruvate and that this interference with the metabolism of *Legionella* indirectly inhibits the formation of and/or degrades intracellular ROS.

The method of the present invention can enable a shorter incubation period to be employed, desirably no more than about 24 hours. Preferably this can be reduced to 12 hours, especially when there is a low occurrence of bacterial interference and/or high occurrence of *Legionella pneumophila* bacteria.

The sample may be collected from any suitable location. This may for instance be a sample of water from recirculating water in a cooling system. However, desirably the sample may be obtained from water in the form of an aerosol. Typically the aerosol may be located in a cooling tower or air conditionner. Desirably the water condensed from the aerosol before testing according to the method of the present invention.

The mode of incubation can be as defined in literature describing the DVC procedure. Such a method can include sample filtration and then filter incubation on pads soaked with the medium. Preferably according to the present invention the sample, or concentrated sample, is directly incubated with the medium and then filtrated. In this way we can reduce the risk of losing

bacteria during the incubation process and provide better conditions during incubation, for instance oxygen transfer or agitation of the sample.

Step 2 of the inventive method can employ a standard FISH protocol.

In the first part of the FISH procedure the cells may be fixed by treating the external membrane or envelope of the cell to make it permeable to the oligonucleotidic probes. Generally this will be achieved by using fixing solutions that are aqueous solutions of alcohols or aldehydes which are miscible with water at 25°C. Suitable alcohols or aldehydes include formaldehyde, paraformaldehyde, ethanol and/or methanol. Typically solutions of formaldehyde or paraformaldehyde can be up to 10% by weight and preferably between 1 and 5%. Usually the alcohols will be at least 50% by weight and generally between 60 and 90% by weight. Preferably this treatment can be achieved by sequential treatment with one or more of these solutions. Preferably the treatment may comprise a solution of between 1 and 5% formaldehyde or paraformaldehyde followed by two or three solutions of ethanol or methanol of increasing strength between 50% and 90%.

The FISH procedure then employs a hybridisation procedure by employing a hybridisation buffer containing at least one fluorescence labelled oligonucleotidic probe comprising an oligonucleotide with attached fluorescent marker in which the oligonucleotide is capable of targeting a specific sequence within the cell. The oligonucleotidic probes penetrate the external membrane of the cells and bind to the target sequence corresponding to the oligonucleotide. Binding will be understood to mean the formation of hydrogen bonds between complimentary nucleic acid pieces. In the FISH technique the oligonucleotidic probes are complimentary and capable of binding to a certain region of the ribosomal target sequence within the microorganism. Typically the probes comprise between 15 and 30 bases in length as single-stranded

deoxyribonucleic acid pieces and are directed to a specific target region specific to the microorganism.

The oligonucleotidic probe should desirably be a specific ribosomal ARN 16S probe of *Legionella pneumophila*. Any oligonucleotidic probes which specifically target microorganisms of the species *Legionella pneumophila* may be employed. Preferably the probe will be selected from the group consisting of PNE 1 probe described by Grimm et al 1998 with a 5' 3' base sequence of ATC TGA CCG TCC CAG GTT, LEGPNE 1 probe (SEQ ID No 22) described by Grimm et al 1998 and Declerck et al 2003 having a 5' 3' base sequence of ATCTG ACCGT CCCAG GTT, and LP2 probe (SEQ ID No 23) described by Yamamoto et al 1993 having a 5' 3' base sequence of AGCTT TCATC CAAAG ATA.

It may also be desirable to alternatively employ probes with sequences having at least 70%, preferably at least 80% and more preferably at least 90% identity with any of the three probes PNE 1 probe, LEGPNE 1 probe, or LP2 probe.

In order to overcome the risk of falsely identified microorganisms that are naturally fluorescent, a preferred form of the invention employs two nucleotidic probes. One probe targets all microorganisms from the *Legionella* genus and the second probe is designed to hybridise specifically microorganisms of *Legionella pneumophila* species. Each probe is labelled with different fluorescent dyes. The microorganisms that are naturally fluorescent will be fluorescent in only a specific wavelength or narrow band of wavelengths and consequently using the two probes with different dyes that fluoresce at different wavelengths allows naturally fluorescent microorganisms that are not specifically *Legionella pneumophila* to be eliminated.

In this preferred form of the invention the first probe will hybridise microorganisms belonging to the *Legionella* genus, which includes but is not

limited to *Legionella longbeachae, Legionella jordanis, Legionella anisa, Legionella pneumophila*. This first probe can comprise a nucleotide that can bind with a target ribosomal sequence from any of the bacteria within the *Legionella* genus. Typically the first probe can be any of the probes selected from the group consisting of the LEG705 probe (SEQ ID n°7) described in Manz *et al.* (Manz *et al.* 1995) with a 5' 3' base sequence of CTGGT GTTCC TTCCG ATC, the LEG226 probe (SEQ ID n°8) described in Manz *et al.* (Manz *et al.* 1995), with a 5' 3' base sequence of TCGGA CGCAG GCTAA TCT, the Legall11 probe (SEQ ID n°9) described in Leskela *et al.* (Leskela *et al.* 2005) with a 5' 3' base sequence of CCTCC TCCCC ACTGA AAGT, the Legall22 probe (SEQ ID n°10) described in Leskela *et al.* (Leskela *et al.* 2005) with a 5' 3' base sequence of CACTG TATGT CAAGG GTAGG, the Leg120v probe (SEQ ID n°11) described in Buchbinder *et al.* (Bushbinder *et al.* 2002) with a 5' 3' base sequence of AAGGC ATATT CCTAC GCG.

It may also be desirable to alternatively employ probes with sequences having at least 70%, preferably at least 80% and more preferably at least 90% identity with any of the three probes LEG705 probe, LEG226 probe, Legall11 probe, Legall22 probe, and Leg120v probe.

The second probe can hybridise only the specific species of *Legionella pneumophila* and can be any of the aforementioned nucleotidic probes with this characteristic e.g. any of the three probes PNE 1 probe, LEGPNE 1 probe, or LP2 probe.

Any of the fluorescent dyes known to be compatible with the appropriate emission/excitation spectra of FITC (for instance Syto9, Alexa 488 etc) or Cy3 (for instance Rhodamine, Alexa 583 etc) can be used.

Step 3 involves quantifying relevant bacteria using a microscope. This can be achieved manually or automatically, for instance using an epifluorescent

microscope. Preferably the detection and counting of bacteria labelled by *in situ* hybridization and fixed on a filter need the use of microscope equipped with an epifluorescence system.

Suitable detection devices include ChemScan RDI and ScanVIT- Legionella TM (Vernicon AG, Munich, Germany). It possible to use chemscan (solid cytometry developed by AESChemunex) to detect and count labelled bacteria. However, this system can be use only 1 set of emission/excitation mirror (488 nm) and thus limit our protocol to use only one labelled probe.

ScanVIT- Legionella TM is preferred especially according to the aforementioned preferred aspect of the invention employing at least two probes, since this technique permits the use of two different fluorescent signals in order to eliminate naturally fluorescent microorganisms that are not *Legionella pneumophila*.

The method according to present invention facilitates the accurate and rapid quantitative determination for the existence of *Legionella pneumophila*. The method is suitable for detecting *Legionella pneumophila* in samples derived from any of the group selected from industrial cooling waters, drinking waters, and natural waters.

The present invention also incorporates a kit for more rapidly detecting and enumerating viable microorganisms of the species *Legionella pneumophila* in a sample suspected of containing said microorganisms comprising:

(1) a cell nutritive resource,

(2) a cellular proliferation inhibitor,

(3) at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms,

(4) a means for detecting and quantifying the fluorescent signal,

in which the cellular proliferation inhibitor is selected from the group consisting of ciprofloxacin and cephalexin.

In a preferred form a kit may comprise the following solutions: a medium composition (as defined above) which can be dehydrated for long storage; a solution for fixing the external membrane of bacteria, for instance formaldehyde; a hybridisation solution, for instance as described above, which should be made up shortly before use; a wash solution, for instance as described above, which should be made up shortly before use; nucleic acid fluorescence dye, for instance DAPI which could be dehydrated for long storage; an anti fading mounting reagent. Preferably the kit may comprise filters. More preferably the kit may additionally comprise a physiological buffer; ethanol solutions of strength varying between 50 and 90%; and sterile water. The kit may also contain Eppendorf, for instance 2 ml.

Kit may also contain any of the embodiments described in regard to the first aspect of the invention.

The kit is suitable for use with the method of the present invention and enables rapidly and reliable enumeration of *Legionella pneumophila*.

The following examples illustrate the invention.

Example 1

A *Legionella pneumophila* suspension at final concentration of 10^6 bacteria/ml is dispatched in 2 suspensions of same volume. Only the first suspension (S1) is fixed with 3.7 % (v/vl) formaldehyde at ambient temperature (20 to 22 °C) for 30 min. The both suspension are then washed three times by centrifugation (6,000 x g, 5 min at 20°C), in PBS pH 7.4.

The both suspension are finally mixed in accord with table 1.

Table 1

% viable Cell	S1 [ml]	S2 [ml]
100	0	2
50	1	1
10	1.8	0.2
1	1.98	0.02
0	2	0

Each mix is treated according to the experimental protocol below.

Results

The results are represented in figure 1. Figure 1 shows the correlation between the standard method AFNOR (8 days) and the method of the present invention requiring less than 24 hours. The results indicate a good correlation between the two methods in terms of accuracy of identification of *Legionella pneumophila*.

Experimental protocol

Samples (V_1) were filtered through 25-mm-diameter, 0.2-µm-pore-size white polycarbonate membrane (Millipore, GTTP02500). Filters were rinsed two times with 10 ml of Solution A and were disposed in a sterile tube (Eppendorf 2ml) containing 1 ml of solution A. Tubes were shaken 1 min at 30Hz (4°C). After filters removing, each suspension was dispatched like follow:

1. 500 µl of suspension is disposed aseptically in a sterile tube containing 500 µl of solution B. Tubes were then incubated 24 hours at 37°C or 45°C with agitation. After incubation, suspensions were filtered through 25-mm-diameter, 0.2-µm-pore-size white polycarbonate membrane (Millipore, GTTP02500) and were fixed with solution C at ambient temperature (20 to 22°C) for 30 min. Filters were rinsed two times with phosphate buffered saline (pH 7.4) and air dried
2. 500 µl of suspensions were directly filtered through 25-mm-diameter, 0.2-µm-pore-size white polycarbonate membrane (Millipore, GTTP02500) and were fixed with solution C at ambient temperature (20 to 22°C) for 30 min. Filters were rinsed two times with phosphate buffered saline (pH 7.4) and air dried

Filters were dehydrated by sequential washes in Solution D, Solution E and Solution F (2 min each) and then dried by incubation 30 min at 37°C. Filters were disposed on slide and 50 µl of Solution G was applied to the filter. A cover slip was disposed on filter to avoid dehydration. Hybridization was performed for 2 hours at 46 ± 1°C in a moisture chamber. Then the filters were washed three times 5 ml of solution H preheated at 46°C and then rinsed two times with sterile water. Filters were incubated 15 min with 1 ml of Solution I and rinse two times with sterile water. After air dried, filters were finally mounted on a slide with 20 µl of solution J. Hybridized cells were visualized by epifluorescence microscopy with an x100 immersion objective lens and 2 emission/excitation filters: a 510 to 550 nm excitation filter and a 590 nm barrier filter.

Solutions

Table 2

Solution	Details	General Description
A	Posphate Buffer Saline pH 7.4 (PBS)	Physiological buffer
B	Medium	See Table 3
C	Formaldehyde [3.7%] (Sigma, F-1635)	
D	Ethanol 50%	
E	Ethanol 80%	
F	Ethanol 90%	
G	Hybridization Solution	See Table 4
H	Wash Solution	See Table 5
I	DAPI [0,5µg/ml] (Sigma, D-95421)	Nucleic Acid Fluorescent Dye
J	Citifluor (Ted Pella, INC., 19476-A)	Anti-fading Mounting Reagent

Medium 2X

Table 3

	Compounds	Concentration	CAS
Medium	Yeast Extract	20 g/L	
	Ferric Pyrophosphate	0.5 g/L	
	L-Cysteine (chlorydrate)	0.8 g/L	
	α-ketoglutarate	2 g/L	
	Buffer ACES/Potassium Hydroxide	2 g/L	

Selective Supplement	Glycine	6 mg/L	
	Vancomycine	2 µg/L	
	Polymyxine	160 UI/L	
	Cycloheximide	160 µg/L	
Cell Division Blocking Antibiotic	Ciprofloxacin	8 mg/L	85721-33-1
Growth Supplement	Pyruvate	2 g/L	

All reactive were sterilized by filtration trough 0.2-µm-pore-size nitrocellulose membrane (Millipore, SLGS025 0S). For details of each set of reagent see below.

Hybridization solution

Table 4

Compounds	Concentration
formamide (Sigma, F-9037)	20%
NaCl (Sigma, S-9625)	0.9 M
SDS (Sigma, L-4522)	0.1 %
Tris-HCl pH 7.2 (Sigma, T-2538)	20 mM

All reactive were sterilized by filtration trough 0.2-µm-pore-size nitrocellulose membrane (Millipore, SLGS025 0S).

Probe used for FISH detection are: LEG705 (Eurogentec: 5'-CTGGTGTTCCTTCCGATC-3'), specific of *Legionellaceae* and labelled with

FITC (Fluorescéine isothiocyanate) and PNE1 (Eurogentec: 5'-CTGGTGTTCCTTCCGATC-3'), specific of *Legionella pneumophila* genus and labelled with Cy3.

Probes were added to hybridization solution at final concentration of 1 ng/µl.

Wash solution

Table 5

Compounds	Concentration
NaCl (Sigma, S-9625)	215 mM
SDS (Sigma, L-4522)	0.1 %
Tris-HCl pH 7.2 (Sigma, T-2538)	20 mM

All reactive were sterilized by filtration trough 0.2-µm-pore-size nitrocellulose membrane (Millipore, SLGS025 0S).

Example 2

The experimental protocol of Example 1 is applied to a sample employing leg705 first probe labelled with a green fluorescent dye and PNE1 second probe labelled with a red fluorescent dye.

Figure 2 shows four cases which allow *Legionella pneumophila* to be distinguished from naturally fluorescent bacteria. Referring to Figure 2:
Case 1-Neither Green, neither red fluorescent bacterium - bacterium doesn't belong to *Legionella* genus and to *Legionella pneumophila* species;

Case 2-Only Green fluorescent bacterium - the bacterium belongs to *Legionella* genus OR is naturally fluorescent;

Case 3-Only Red bacterium - Despite red fluorescence, bacterium does not

belong to *Legionella pneumophila* species because it is not green fluorescent and thus doesn't belong to *Legionella* genus. This bacterium is naturally red fluorescent; and

Case 4-Green AND Red fluorescent bacterium - the bacterium belongs to *Legionella* genus AND *Legionella pneumophila* species.

Using this method we can accurately detect *Legionella pneumophila* by limiting the detection of naturally fluorescent bacteria which would otherwise falsely indicate this bacteria.

The PNE1 probe is first described in Grimm *et al.* (Grimm *et al.*, 1998). Specific sequence is : 5'-ATC TGA CCG TCC CAG GTT-3'

The Leg705 probe is first described in Manz *et al.* (Manz *et al.*, 1995). Specific sequence is : 5'-CTGGTGTTCCTTCCGATC-3'

Three dyes were used in this test are used as follows:

The first dye is used to stain nucleic acid of all micro organisms. DAPI is used to stain bacteria in blue under UV excitation. This dye is not coupled with an oligonucleotidic probe.

The second two dyes are coupled with oligonucleotidic probe to allow specific detection. These two dyes must be characterized by two different spectra of excitation/emission. In the test FITC and Cy3 are used, which are the most commonly dyes used to FISH detection, but many dyes with respectively the same spectra are available.

Table 6

Dyes used	λ Excitation [nm]	λ Emission [nm]	Dyes Available
DAPI	350	461	
FITC	494	521	Alexa 488, Hylite 488, Dylight 488,
Cy3	550	570	Rhodamine, Alexa 555, Hylite 555, …

Claims

1. A method for detecting and enumerating viable microorganisms in a sample suspected of containing said microorganisms comprising:

(1) contacting said sample with a cell nutritive resource and a cellular proliferation inhibitor in which the cell nutritive resource contains a growth supplement which comprises an antioxidant,

(2) contacting said sample with at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms in which said sample is contacted with at least a first probe and a second probe in which the first probe is able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms and the second probe is able to specifically hybridise at least one different portion of ribosomal nucleic acids of the microorganisms, and

(3) detecting and quantifying the fluorescent signal,

in which the microorganisms are of the species *Legionella pneumophila* and in which the cellular proliferation inhibitor is ciprofloxacin or cephalexin.

2. The method of claim 1 in which the antioxidant is pyruvic acid or salt thereof.

3. The method according to claim 1 or 2 in which the cell nutritive resource of step (1) contains glutamic acid or salt thereof.

4. The method according to any one of claims 1 to 3 in which the cell nutritive resource of step (1) contains glutamic acid or salt thereof and pyruvic acid or salt thereof.

5. The method according to any one of claims 1 to 4 in which the oligonucleotidic probe comprises a nucleic acid molecule as set forth in SEQ ID NO: 22, 23 or as set forth by the following sequence: ATCTGACCGTCCCAGGTT.

6. The method according to any one of claims 1 to 5 in which in step (2) said sample is contacted with the at least first probe and second probe in which the first probe is able to specifically hybridise the at least one portion of ribosomal nucleic acids all belonging to *Legionella* genus and in which the second probe is able to specifically hybridise the at least one portion of the ribosomal nucleic acids all belonging to *Legionella pneumophila* species.

7. The method according to claim 6 in which the first probe comprises a nucleic acid molecule as set forth in SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 11.

8. The method according to any one of claims 1 to 7 in which step (3) is performed automatically using an epifluorescent microscope.

9. The method according to any one of claims 1 to 8 in which the sample is derived from industrial cooling waters, drinking waters or natural waters.

10. Kit for more rapidly detecting and enumerating viable microorganisms of the species *Legionella pneumophila* in a sample suspected of containing said microorganisms comprising:

(1) a cell nutritive resource in which the cell nutritive resource contains a growth supplement which comprises an antioxidant,
(2) a cellular proliferation inhibitor,
(3) at least one fluorescence labelled oligonucleotidic probe able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms in which said sample is contacted with at least a first probe and a second probe in which the first probe is able to specifically hybridise at least one portion of ribosomal nucleic acids of said microorganisms and the second probe is able to specifically hybridise at least one different portion of ribosomal nucleic acids of the microorganisms, and
(4) a means for detecting and quantifying the fluorescent signal,

in which the cellular proliferation inhibitor is ciprofloxacin or cephalexin.

Figure 1

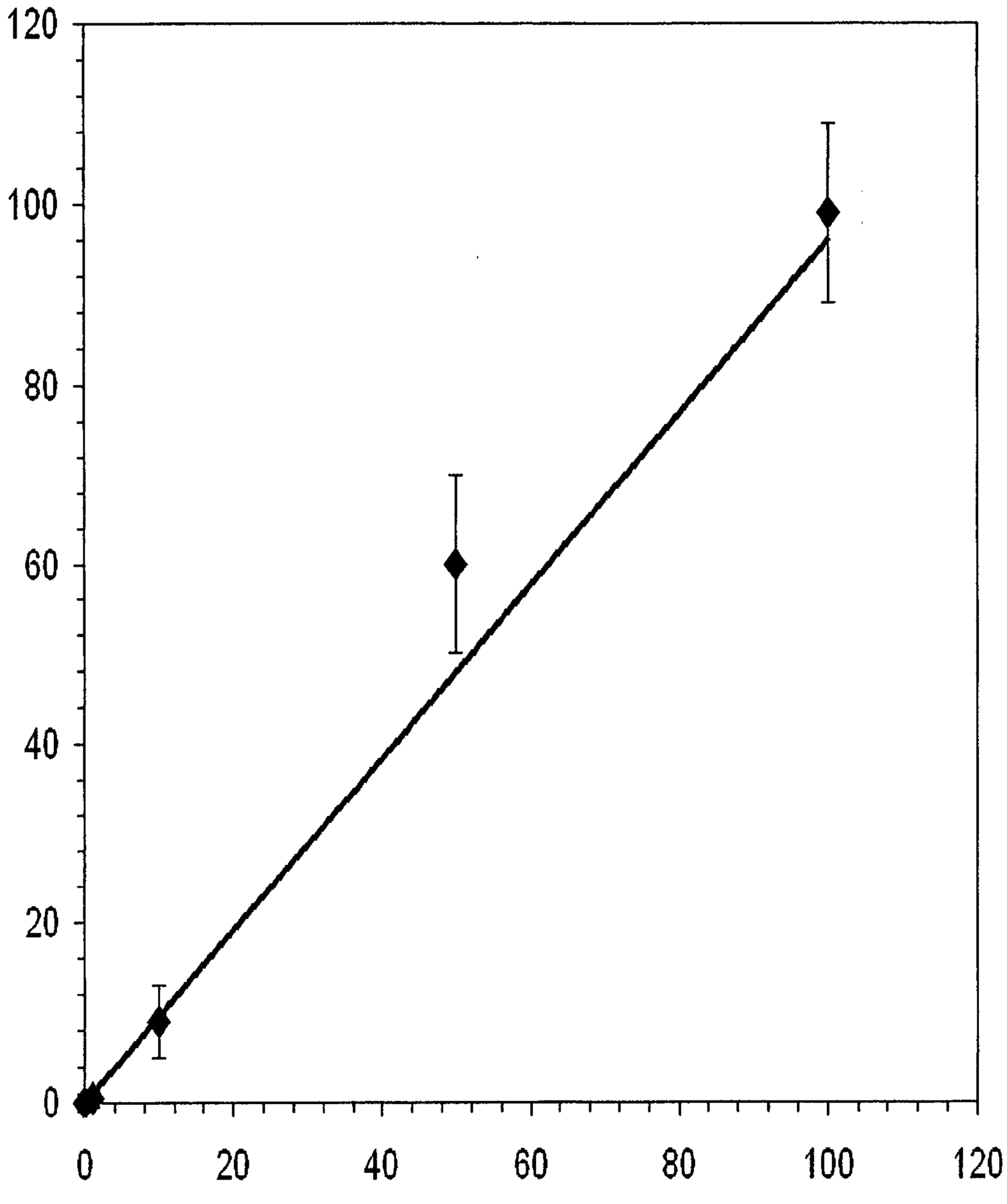
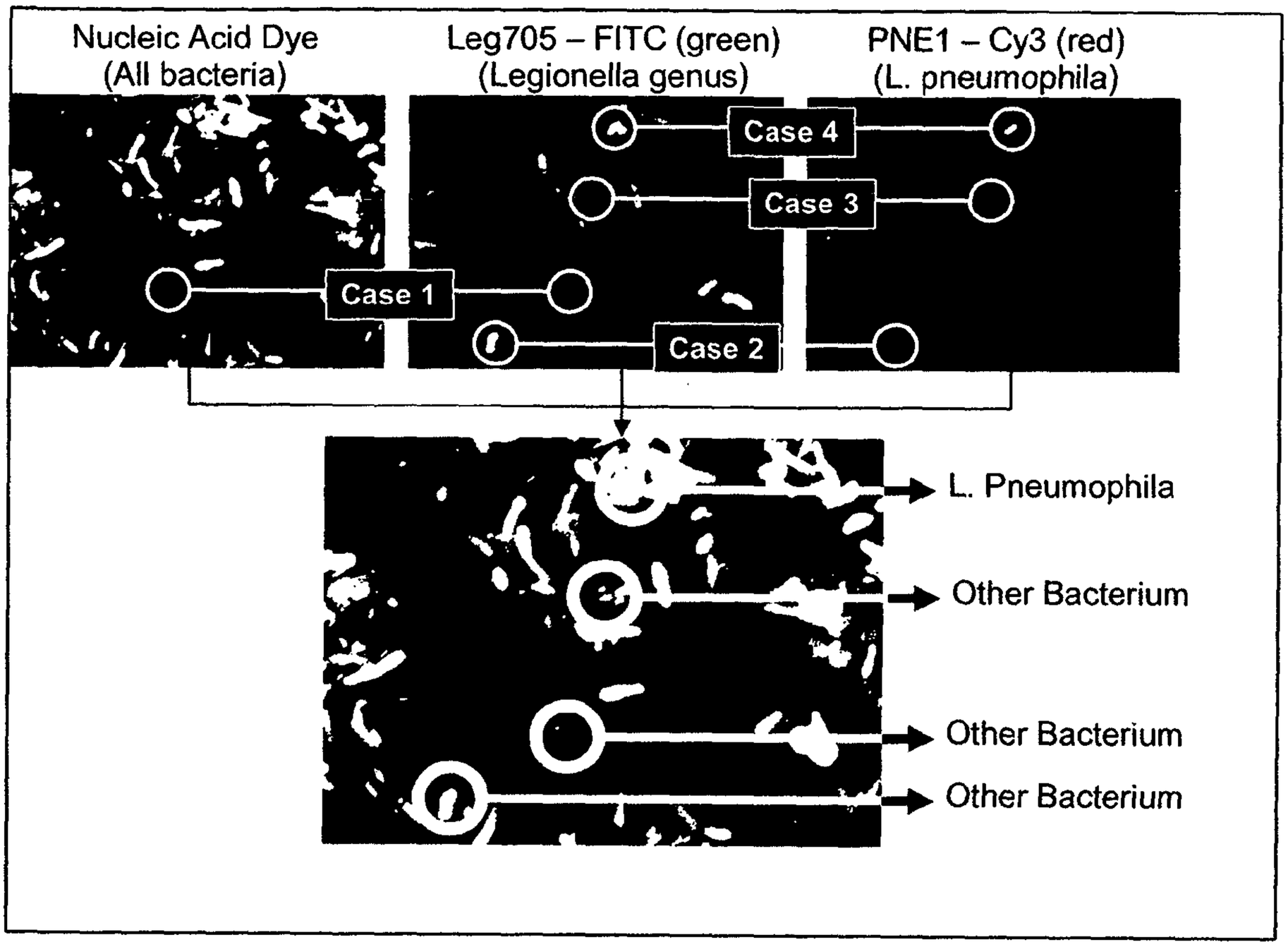


Figure 2


Nucleic Acid Dye
(All bacteria)
Leg705 – FITC (green)
(Legionella genus)
PNE1 – Cy3 (red)
(L. pneumophila)
Case 4
Case 3
Case 1
Case 2
L. Pneumophila
Other Bacterium
Other Bacterium
Other Bacterium