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(54) Title: METHOD FOR DETECTING PATHOGENS

(57) Abstract: The present invention relates generally to methods for detecting and identifying microorganisms and, more particularly, to methods for detecting microorganisms in a sample by incubating the sample at two temperatures to facilitate increased detection of the organism.



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METHOD FOR DETECTING PATHOGENS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application No. 60/837,469 filed August 14, 2006, where this provisional application is incorporated herein by reference in its entirety.

BACKGROUND

Technical Field

The present invention relates generally to methods for detecting and identifying microorganisms and, more particularly, to methods for detecting microorganisms by enriching the microorganism in a sample in an incubator at one temperature which prevents production of a bacterial protein of interest (or other bacterial product) but which temperature allows for optimal growth of the microorganism. After a period of time, the whole sample or a portion of the sample is transferred and incubated at a different optimal temperature which allows expression of the protein previously inhibited. This dual temperature incubated sample is then tested by assaying the sample, or a portion thereof, with an assay suitable to detect the temperature regulated protein (or other bacterial product).

20 Description of the Related Art

Microbial diseases have long been a major health concern worldwide. Significant increase in the frequency and severity of outbreaks have occurred throughout the world. New pathogenic bacteria, such as *E. coli* 0157:H7, have been identified. Furthermore, previously recognized pathogenic genera have mutated to form drug resistant highly infectious strains such as *Salmonella typhimurium* DT 104. A key feature in the prevention of such diseases is early detection and early diagnosis. Epidemiologists must look for

microbial contamination in the environment as well as in food products to find the effective disease prevention strategies.

One example is the outbreak in 1992 of Enterohemorrhagic *E. coli* (EHEC) in the Pacific Northwest of the United States due to contaminated ground beef. EHEC is a relatively “newly discovered” pathogen. EHEC was first isolated in 1975, and it was not until 1982 that *E. coli* 0157:H7 was associated with two food related outbreaks of hemorrhagic colitis in the United States. The reported incidence of *E. coli* 0157:H7 cases is increasing. Typically, *E. coli* strains are harmless commensals, but a few strains are pathogenic. EHEC is particularly virulent and can trigger deadly complications, including severe abdominal cramps and acute renal failure in children as well as cardiovascular and central nervous system problems.

As another example, *Salmonella* is the leading cause (more than 50%) of total bacterial foodborne disease outbreaks, according to the United States Centers for Disease Control (CDC) surveillance of foodborne diseases. More than 40,000 cases per year were reported to the CDC during the period 1988—1992. *Salmonella* can infect a broad variety of warm- and cold blooded animals, and can survive for long periods of time outside a host.

In a further example, *Salmonella typhimurium* DT 104 was first identified in the United Kingdom in the early 1990s. It is a highly adapted drug resistant strain of *Salmonella* known for its virulence. Resultingly, significant clinical interest has surrounded this serotype. *S. typhimurium* DT 104 contains core cell wall antigen epitopes that are highly conserved among the genus *Salmonella*.

Listeria, a genus of gram positive bacteria, is widely distributed in nature, having been isolated from soil, water, vegetation and many animal species. The detection frequency for *Listeria* in the agricultural environment appears to be increasing. For specific outbreaks of listeriosis, estimates place mortality at 30% to 40% of affected patients, however, little is known of the minimum infective dose. One particularly troublesome aspect of *Listeria* control in foods is that *Listeria* can grow at temperatures as low as -0.4°C and as high

as 44°C. These factors all contribute to the increasing significance of *Listeria* as a food pathogen.

Campylobacter jejuni and *coli* have recently been identified as the lead causes of enteritis, especially from poultry sources. This has led to an increased need to discriminate these two species from several other *Campylobacter* species which are not human pathogens. This requires the differential selection of more specific cell wall membrane antigen epitopes.

The ability to monitor potential environmental and food sources of microbial contamination quickly and easily, but with very high specificity, would reduce the risk of human infection. Therefore, an analytical method which affords high specificity to detect microorganisms, including bacteria, yeasts, molds, fungi, parasites and viruses, that requires no special or technical equipment, can be performed in the field and does not require special skills would be useful. In the case of foodborne bacterial contamination, four of the major disease-related organisms are *Salmonella*, *Listeria*, EHEC and *Campylobacter*.

While there are a number of *Salmonella*, *Listeria*, and EHEC detection methods presently available, trained laboratory technicians and a minimum of 2-5 days are required to obtain test results by the standard cultural methods of analysis. New, more rapid methods are based on such techniques as enzyme linked immunoassay (EIA), DNA hybridization, immunodiffusion, or growth/metabolism measurements. While taking much less time than the cultural methods, these rapid tests still require skilled technical training, a functional laboratory, and specialized equipment. These tests generally take a total of two or more days, including considerable hands-on time. *Campylobacter* detection methodology to date is technically intensive requiring fastidious media and environmental conditions, in addition to well-trained analysts.

Another recent technology in the diagnostic field involves lateral flow immunoassays. Such tests have been developed for the detection of human chorionic gonadotropin (hCG), and applied to pregnancy testing.

Typically, a monoclonal or polyclonal antibody is immobilized in a discrete band near the distal end of a solid carrier strip, called the detection zone. Another amount of antibody is labeled with a detection reagent such as an inorganic sol or dyed polystyrene particle. This labeled antibody is reversibly fixed near the proximal end of the carrier strip. Upon hydration of the proximal end with a sample fluid potentially containing the antigen, the antigen reacts with the labeled antibody and the complex passes through the zone of immobilized antibody, forming a sandwich upon reacting with the immobilized antibody. The capture of the chromogenic reagent-antigen complex causes the formation of a visible signal in the detection zone.

Two major challenges must be addressed to distinguish pathogenic bacteria, as opposed to distinguishing hormones or other soluble molecular targets. These challenges are the need to detect all of the strains of a pathogenic microorganism in the presence of numerous antigenically related organisms, with a low tolerance for false positive results and a very low, preferably zero, tolerance for false negatives. The second challenge is the physical size and heterogeneity of the microorganism itself. A typical clinical diagnostic test, such as a test for hCG in urine, is focused on detecting a single, small, unique entity (*i.e.*, a hormone) in a well characterized matrix (*e.g.*, urine). Furthermore, the structure of the analyte (hCG) is defined and uniform in size and composition.

Pathogen detection, for example, a test for *Salmonella*, must distinguish a particular pathogenic strain from nonpathogenic strains of similar microorganisms, such as *Citrobacter spp.* and *Enterobacter spp.* In contrast to the well-defined small size and structure of most hormones or marker proteins, microorganisms are very large, their surfaces are heterogeneous containing many distinct antigen epitopes that can undergo changes, such as the phase-switching of *Salmonella* flagella.

There is a need in the art for methodologies that will allow the simultaneous exposure of easily detected antigens while still allowing the microorganisms to multiply. Further, there is a need in the art to incorporate

improved selectivity for highly conserved target antigen epitopes of specific species in a population of heterogeneous microorganisms in a variety of matrices. The present invention provides these and other, related advantages.

BRIEF SUMMARY

5 One aspect of the present invention provides a method for detecting a pathogen in a sample comprising, culturing the pathogen in the sample at a first temperature wherein the first temperature prevents production of at least one endogenous protein and wherein the first temperature allows for optimal growth of the pathogen; culturing the pathogen in the sample at a
10 second temperature wherein the second temperature allows production of the at least one endogenous protein; and contacting the sample with a detection reagent that specifically binds to the at least one protein; thereby detecting the pathogen in the sample. In one embodiment, the pathogen comprises *Listeria*. In a further embodiment, the first temperature is about 37°C. In another
15 embodiment, the second temperature is about 30°C. In one embodiment, the at least one endogenous protein comprises a flagellin protein.

DETAILED DESCRIPTION

 Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used
20 hereinafter.

 The term "antibody" as used herein includes polyclonal, monoclonal, humanized, chimeric, and anti-idiotypic antibodies, as well as fragments thereof such as F(ab')₂ and Fab fragments and other recombinantly produced binding partners. Further, the antibodies may be covalently linked to
25 or recombinantly fused to an enzyme, such as alkaline phosphatase, horse radish peroxidase, α -galactosidase, and the like.

 The term "general enrichment media" refers to any media which is known to be useful for facilitating the growth of microorganisms. Briefly, a variety of general enrichment media are commercially available and/or can be

readily made, these include, but are not limited to, Tryptone based medium (e.g., Terrific Broth, SOB, SOC, and LB medium), NZCYM medium, minimal medium, lactose broth, buffered peptone water, Brain Heart Infusion medium, Haemophilus broth, Tryptic Soy broth, Nutrient broth and the like (see 5 Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, 1989; Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing, 1995; commercially available from Sigma Chemical Co, St. Louis, MO and Difco Laboratories Inc., Detroit, Michigan).

The present invention generally provides methods for detecting 10 pathogens in a sample by culturing the sample at a first temperature that allows optimal growth of the pathogen while preventing expression of a particular protein or other pathogen product of interest (e.g., flagella or other cell protein that can be specifically detected using any of a variety of detection reagents/assays). The sample is then grown at a second temperature at which 15 the previously inhibited protein is expressed. The sample is then subjected to any of a variety of detection assays for detecting the protein or product of interest. In this manner, the pathogen is detected in the sample.

Any of a variety of culture conditions known to the skilled artisan for the growth of pathogens can be used in the methods of the present 20 invention, such as those described in *Current Protocols in Microbiology*, (Eds. R. Coico, T. Kowalik, J. Quarles, B. Stevenson, and R. Taylor; John Wiley & Sons, Inc, NY, NY 2000-2006). The present invention uses any of several widely recognized general enrichment media such as tryptic soy broth, nutrient broth, buffered peptone water, lactose broth, brain heart infusion broth, or 25 similar media.

As would be recognized by the skilled artisan, the first and second temperatures of the present methods will vary depending on the organism to be detected and the temperature-regulated protein or product of interest. As an illustration, *Listeria* grow at 37°C and at 30°C. At 37°C, *Listeria* grow well but 30 do not produce flagella while at 30°C, *Listeria* grow and also produce flagella. When allowed to grow at 37°C for a period of time, this allows for enrichment of

the bacteria in the sample. Once allowed to grow at 30°C for a period of time, the enriched bacteria express flagella which can then be used to capture and detect the presence of the bacteria in the sample. Thus, the present method allows for the use of differential temperature for the growth of a pathogen, within the same container, to induce maximum possible growth levels and then transfer of the entire container to a second temperature to allow the production of the temperature-regulated protein. In this manner, the total assay time for detection of the pathogen is decreased and the sensitivity improved.

Accordingly, the first and second temperatures of the present invention may be any temperature appropriate for growth/inhibition of the protein of interest and growth/production of the protein of interest. For example, the first and second temperatures may range from 20°C-40°C. In certain embodiments, the first temperature may be 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40°C and the second temperature may be 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40°C.

The methods of the present invention can be used to detect any of a variety of pathogens including but not limited to *Listeria*, Enterohemorrhagic *E. coli* (EHEC), *Salmonella*, *Shigella*, *Campylobacter*, *Aeromonas hydrophilia*, *Aeromonas caviae*, *Aeromonas sobria*, *Streptococcus uberis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Bacillus sphaericus*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Serratia liquefaciens*, *Lactococcus lactis*, *Xanthomonas maltophilia*, *Staphylococcus simulans*, *Staphylococcus hominis*, *Streptococcus constellatus*, *Streptococcus anginosus*, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium fortuitum*, and *Klebsiella pneumonia*.

By "bacteria" is meant one or more viable bacteria existing or co-existing collectively in a test sample. The term may refer to a single bacterium (e.g., *Aeromonas hydrophilia*, *Aeromonas caviae*, *Aeromonas sobria*, *Streptococcus uberis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Bacillus sphaericus*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Serratia*

liquefaciens, *Lactococcus lactis*, *Xanthomonas maltophilia*, *Staphylococcus simulans*, *Staphylococcus hominis*, *Streptococcus constellatus*, *Streptococcus anginosus*, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium fortuitum*, and *Klebsiella pneumonia*), a genus of bacteria (e.g., streptococci, pseudomonas and enterococci), a number of related species of bacteria (e.g., coliforms), an even larger group of bacteria having a common characteristic (e.g., all gram-negative bacteria), a group of bacteria commonly found in a food product, an animal or human subject, or an environmental source, or a combination of two or more bacteria listed above. The bacteria include those described or referred to in *Bergey's Manual of Systematic Bacteriology*, 1989, Williams and Wilkins, U.S.A., incorporated by reference herein.

By "sample" or "test sample" is meant a piece, fraction, aliquot, droplet, portion, fragment, volume, or tidbit taken from a food product such as ground beef or chicken, a human or animal test subject, a soil, water, air or other environmental source, or any other source whose bacterial concentration is to be measured. A test sample may be taken from a source using techniques known to one skilled in the art, including, but not limited to, those described or referred to in *Compendium of Methods for the Microbiological Examination of Foods*, Third Edition, Edited by Carl Vanderzant and Don F. Splittstoesser, Compiled by the APHA Technical Committee on Microbiological Methods for Foods, incorporated by reference herein.

As would be recognized by the skilled artisan, any protein whose expression is temperature-dependent may be useful in the methods of the present invention. The illustrative proteins described herein are bacterial flagella proteins but other temperature-regulated proteins are also contemplated herein.

In certain embodiments, the methods of detecting are specific for *Listeria*, Enterohemorrhagic *E. coli* (EHEC), *Salmonella*, or *Campylobacter*. In one embodiment, the method includes introducing the cultured sample into a detection system, such as a visual immunoprecipitate assay, an enzyme linked immunoassay, chemiluminescence, immunoblotting, or similar detection

technology containing an antibody specific for the target protein or other product of a microorganism thereby producing a highly accurate result.

An additional aspect of the present invention is the use of a visual immunoprecipitate assay to detect the presence of a microorganism in a test sample. In the visual immunoprecipitate assay, the antibodies, including the "antibody-detection-reagent" initially located in the reagent zone, is typically either a polyclonal or monoclonal antibody. Further, when using a polyclonal antibody the antibody is preferably affinity column purified prior to its utilization the present invention. The production of such antibodies is well known in the art. (See, e.g., *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Suitable affinity purified antibodies can also be procured from commercially available sources. For example, a polyclonal antisera specific for *Salmonella* is available from Kirkegaard and Perry Laboratories, Gaithersburg, Maryland. A preferred visual immunoprecipitate assay is that which is described by U.S. Patent No. 5,658,747. Briefly, U.S. Patent No. 5,658,747 utilizes a lateral flow diagnostic device which comprises a reagent zone containing an antibody-detection reagent and a detection zone located downstream of the reagent zone and comprising an immobile binding partner capable of specifically binding said complex between the target microorganism and the antibody detection reagent.

Polyclonal antibodies can be readily generated by one of ordinary skill in the art via immunization of a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, turkeys, rabbits, mice, or rats. Briefly, the target microorganism, or an antigen specifically associated with the target microorganism, is utilized to immunize the animal. The immunogenicity of the protein or peptide of interest may be increased through the use of an adjuvant such as Freund's complete or incomplete adjuvant or by coupling to another protein such as ovalbumin or keyhole limpet hemocyanin (KLH).

Monoclonal antibodies can also be readily generated using well-known techniques. (See, e.g., *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKearn, and

Bechtol (eds.), 1980, and *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), *supra*.) Briefly, as one example, a subject animal is immunized as with the production of a polyclonal antibody. Alternatively, *in vitro* immunization techniques suitable for the production of monoclonal antibodies are also known
5 in the art. Antibody-producing cells are then fused to immortal myeloma cells to provide an immortal hybridoma cell line. Following the fusion, the cells are placed into culture plates containing a suitable medium, traditionally HAT medium, although other suitable media are known in the art. After about seven days, the resulting fused cells or hybridomas may be screened in order to
10 determine the presence of antibodies which recognize the desired antigen. Following several clonal dilutions and reassays, hybridoma producing antibodies that bind to the protein of interest can be isolated.

Other techniques can also be utilized to construct monoclonal antibodies or binding partners. (See, e.g., Huse et al., "Generation of a Large
15 Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," *Science* 246:1275-1281, 1989; Sastry et al., "Cloning of the Immunological Repertoire in *Escherichia coli* for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," *Proc. Natl. Acad. Sci. USA* 86:5728-5732, 1989; Altling-Mees et al.,
20 "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," *Strategies in Molecular Biology* 3:1-9, 1990; Larrick et al., "Polymerase Chain Reaction Using Mixed Primers: Cloning of Human Monoclonal Antibody Variable Region Genes From Single Hybridoma Cells," *BioTechnology* 7:934-938, 1989.)

25 Once a suitable antibody has been obtained, it may be isolated or purified by many techniques well known to those of ordinary skill in the art (see *Antibodies: A Laboratory Manual*, Harlow and Lane, *supra*).

Antibodies useful in the present invention are preferably capable of selectively detecting all of the strains of a target microorganism in the
30 presence of numerous antigenically related organisms. Further, the antibodies are preferably capable of such detection with a low tolerance for non-specific

binding (which leads to a false positive result) and a very low, preferably zero, failure to bind target the microorganism (which leads to a false negative result).

In certain embodiments, the sample is a solution containing, or consisting essentially of, an unpurified field sample such as a food, water, dirt, cosmetic, wastewater, industrial, pharmaceutical, botanical, environmental and other types of samples analyzed by enrichment-detection methods. Alternatively, the sample may be a biological fluid such as a body fluid. In a further embodiment, the sample may be partially or substantially purified prior to culturing the sample according to the methods of the present invention.

Following incubation of the sample under appropriate conditions including the first and second temperatures, the results are detected preferably using a rapid detection method such as, but not limited to, visual immunoprecipitate assay, enzyme linked immunoassay, chemiluminescence, immunoblotting, or similar detection technology. Such methodologies are described in greater detail in U.S. Patent No. 5,658,747 and PCT WO 95/30903.

Another aspect of the present invention provides a method for detecting a microorganism in a test sample wherein the test sample is incubated in an appropriate medium and a first temperature for sufficient time to enrich levels of microorganisms followed by incubation in an appropriate medium and a second temperature suitable for production of a temperature-regulated detectable protein by the microorganism. Subsequently, the presence of pathogenic microorganisms is detected by utilizing immuno-based detection methodologies, which include but are not limited to, immuno-affinity, visual immunoprecipitation, enzyme linked immunoassay, chemiluminescence, immunoblotting, and the like. Alternatively, the exposure of antigen in a sample may be enhanced by treatment with detergent prior to or contemporaneously with detection. In a further alternative embodiment, the exposure of antigen in a sample may be enhanced by heating the sample in the presence of the detergent, prior to or contemporaneously with detection

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or
5 listed in the Application Data Sheet, U.S. Provisional Patent Application No. 60/837,469 filed August 14, 2006 are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

10 These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of
15 equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

What is claimed is:

1. A method for detecting a pathogen in a sample comprising:
culturing the pathogen in the sample at a first temperature wherein the first temperature prevents production of at least one endogenous protein and wherein the first temperature allows for optimal growth of the pathogen;
culturing the pathogen in the sample at a second temperature wherein the second temperature allows production of the at least one endogenous protein; and
contacting the sample with a detection reagent that specifically binds to the at least one protein;
thereby detecting the pathogen in the sample.
2. The method of claim 1 wherein the pathogen comprises *Listeria*.
3. The method of claim 2 wherein the first temperature is about 37°C.
4. The method of claim 2 wherein the second temperature is about 30°C.
5. The method of claim 2 wherein the at least one endogenous protein comprises a flagellin protein.