STREPTOCOCCAL DIAGNOSTIC METHOD


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3 Claims

STREPTOCOCCAL DIAGNOSTIC METHOD

A method of detecting streptococcal organisms, especially Group A, beta-hemolytic streptococci, is provided wherein separate smears on microscope slides comprising negative control and positive control organisms are treated with antibody conjugated to peroxidase, and the slides then compared under the microscope, after dyeing with enzyme substrates to determine the presence of Group A streptococci.

DISCLOSURE OF THE INVENTION

This invention relates to a new and novel method of utilizing labeled antibodies especially to detect the presence of Group A streptococci. In, for example, a swab sample. Serological grouping of human beta-hemolytic streptococcal infections is important to the medical profession, especially the presence of Group A streptococci which necessitates therapy. As has been reported, a significant error may result from the assumption that all beta-hemolytic streptococci from the human nasopharynx belong to Group A.

Accurate and rapid identification of group A beta-hemolytic streptococci is a vital step towards adequate treatment and eventual prevention of the possible sequelae of this infection, i.e., scarlet fever, rheumatic fever, glomerulonephritis. Such an identification of group A streptococcal pharyngitis requires laboratory assistance to demonstrate the organism directly.

A number of known methods are used to identify group A streptococci. One involves resorting to a fluorescent antibody that make for a specific identification of infectious organisms. However, this laboratory procedure involves a costly fluorescent microscope. In another method used in detecting streptococci, a bacterial culture is grown. However, positive identification of Group A streptococci requires 48 hours, involving in some cases an undesirable time lag of proper diagnosis and treatment.

The method of the invention here incorporates advantages of sensitivity, rapidity, and uses only of an ordinary light microscope for identification purposes rather than resorting to an expensive fluorescent microscope.

The diagnostic method here, in brief, involves a method of detecting the presence of Group A streptococci in the presence of other streptococci and staphylococci organisms in an unknown sample. First is prepared a series of at least 3 dry slide smears, one of said smears acting as a negative control comprising a suspension of only Group G streptococci and Staphylococcus aureus. Also prepared is a second positive control smear comprising a suspension of only Group A streptococci. Lastly a third smear is prepared comprising a suspension of unknown organisms to be tested for presence of Group A streptococci. Separately prepared is an antibody conjugate. This is prepared by conjugating an antibody to streptococcus with peroxidase.

The resultant conjugate is added to each smear which are then incubated. Thereafter, hydrogen peroxide is added to each smear as well as an electron donor dye. The three thus treated slide smears are observed under a microscope with the unknown smear being compared with the positive control to determine the presence of group A streptococci. Normally, the smears prior to addition of hydrogen peroxide and electron donor are rinsed with a buffer solution and dried again.

In more detail, the first step in the invention involves development of an antibody in a captive rabbit by resort to a test animal such as a rabbit. A conjugate of the antibody to Group A streptococcus and peroxidase is then made by bridging or conjugating the two by resort to a coupling agent, such as glutaldehyde or diisocyanate. Here, peroxidase enzymes which can be used are a horse radish or lacto peroxidase.

As is known each antibody has a counterpart in some antigen such as a group A streptococcus. An antibody specific to an antigen is made from the globulin of serum or plasma from a host animal which was stimulated to produce that antibody, then the antigen and antibody specific to that antigen will become attached to each other. However, in subsequent microscopic work one cannot see the antigen-antibody organism. Thus, in the case here the peroxidase acts as a type of amplifier which will later allow the organism to be properly observed under a microscope by resort to a dye.

A preferred method of preparing the reagent here is to form a conjugate of antibody and peroxidase, and then add the conjugate to the group A streptococcus. Smears are made of a swab sample and a positive and negative control sample are prepared in the usual manner. The lower control limit organisms (negative control) are prepared from a NCDC-derived panel of group G streptococci and Staphylococcus aureus which are coagulase positive, non-viable and have been reconstituted from a lyophilized stock. The upper control limit organisms likewise are obtained from a NCDC-derived panel of group A streptococci which are non-viable and reconstituted from a lyophilized stock. The upper control limit organisms, of course, act as a positive control. The three air-dried smears are usually rinsed in a buffer such as a tris-saline buffer, pH 7.5 (buffer) for a few minutes and thereafter drained, usually with a filter paper. Of course, all three smears may be placed on a single slide, if desired, in separate inscribed areas.

One drop of the conjugated reagent prepared as described above is placed on each dry smear and they are incubated. In a typical situation, incubation may be carried out in a moist chamber for 20 minutes at room temperature. The slides are then rinsed again with buffer solution and drained. In one specific technique the slides are immersed in a buffer solution for about 5 minutes.

The slides are then added a detecting dye usually an electron donor dye. A typical dye used here is 3-aminio-9-ethyl carbazole. Other suitable dyes which may be used include 3,3'-diaminobenzidine, p-chloroaniline and a mixture of u-naphthol and p-phenylenediamine dihydrochloride. Along with the dye is added an oxidizing agent such as hydrogen peroxide. Other oxidizing agents which may be used are methyl peroxide and ethyl peroxide (CH₃OOH; C₂H₅OOH).

In one specific technique two drops of 0.1% 3-aminio-9-ethyl carbazole dye in a buffer containing 0.3% hydrogen peroxide are added to each smear. Then after a suitable waiting period, say 15 minutes the slides are then again washed with tap water and each observed under a microscope. The streptococci stains appear as a bright red stain.

In the procedure here it is believed that the peroxidase enzyme breaks down the hydrogen peroxide. The hydrogen peroxide breakdown product in turn causes the
dye to become colored, which colored material in turn becomes attached to the Streptococci. The following examples are illustrative of this invention.

**EXAMPLE I**

Preparation and fixation of organisms from throat swabs

The pharyngeal mucosal swab is swirled vigorously for approximately 30 seconds in 0.5 ml saline in a Kahn or similar tube. The saline is expressed from the swab. If desired, the same swab can be used to inoculate a blood agar plate for isolation of β-hemolytic Streptococci. Immediately thereafter, a disposable Pasteur pipette is used to transfer one drop (approximately 25 microliters) of the saline suspension of bacteria onto the inoculated area of a microscope slide. Samples (0.01 ml, 0.0001 ml) of the same saline rinse (bacterial suspension) can be taken into a calibrated loop and spread on blood agar plates to titrate the saline wash. The test area, on the microscope slide is then allowed to air dry. The smear is fixed by immersion in absolute methanol for 5 minutes and drained.

Staining technique using antibody coupled enzyme (enzyme covalently conjugated to antibody)

Streptococcus antibody was first conjugated to horseradish peroxidase. Two drops (approximately 50 μl) of the conjugated antibody is added to each dry smear, using a Pasteur pipette. Using separate applicator sticks or toothpicks, carefully spread the reagent is spread over each test area. The applicator stick can be held horizontally to catch the meniscus of the fluid in a manner that avoids scraping cells from the slide.

The slide is incubated at room temperature for about 20 minutes in a humid chamber using, for example, a slide box, or an inverted petri dish with moist paper towels fitted into or around the dish. The slide is then rinsed with 10 ml of tris-saline buffer dripped from a pipette and then washed by immersion in a staining dish of tris-saline buffer for 5 minutes. The slide is dried by tilting at an angle and placing an absorbent piece of paper (paper towels) at the edge of the inoculated area.

Two drops (approximately 50 microliters) of the staining solution is then added to each smear and allowed to react 15 minutes. The slide is rinsed with 10 ml distilled water dropped from a pipette and washed by immersion in a staining dish filled with distilled water. The slides are then examined for the presence of staining. At this point the slides may be stored for several months in a microscope slide box, at room temperature, with no noticeable decrease in staining intensity.

The slides are examined for staining under a light microscope using a 40× objective and 12.5× oculars (final magnification of 500×). The advantage of this procedure lies in its speed without the use of an expensive fluorescent microscope. The relative low magnification allows a larger area to be viewed when scanning the 1 cm. diameter circle on the slide, thus allowing greater detection limits.

The slide areas are scanned under a light microscope at a final magnification of 500×. The defined detection limits are not directly applicable when other objectives and/or magnification ranges are used with this procedure.

The positive control (Group A Streptococci) is clearly distinguished from the negative control (Group B Streptococci and Staphylococcus aureus) by a bright red color. The Group A Streptococci directly from the throat swab stain with the same intensity and possess the same single cell morphology as the positive control. Primary focus must be on intensity of color and on presence and extent of chained organisms.

**EXAMPLE II**

In a second embodiment of the invention as a variation of the techniques described in Example I, the pharyngeal mucosal swab is placed in 1 ml of Todd-Hewitt broth and incubated at 37°C for 2 to 5 hours, the broth is then centrifuged 5 minutes at about 2000 r.p.m. to pack the organisms. The supernate fluid is decanted and then the cells resuspended in 1 ml of saline and recentrifuged. The saline is decanted and the tube placed in a rack for 2 to 3 minutes to allow residual saline and organisms to collect in the bottom of the tube. The organisms are mixed thoroughly in the residual saline. Using a Pasteur pipette, some of the washed sediment is removed and placed within the inoculated area on a single slide.

The test areas are then allowed to air dry. The slide is fixed in absolute methanol for 5 minutes and allowed to drain dry. The smears are then processed as when the cells are eluted directly from the swab. All controls and data interpretations are the same.

It is also suitable to select with an inoculating needle some organisms from a colon grown on a blood agar plate, emulsify the colony in saline solution, and then transfer a drop to an inoculated slide and proceed as before.

What is claimed is:

1. A method of detecting the presence of Group A Streptococci in the presence of other Streptococci and Staphylococci organisms in an unknown sample comprising the steps of conjugating a Streptococcus antibody with a Group A Streptococcus by forming a complex of said antibody and Group A Streptococcus through means of a peroxidase enzyme coupling agent, preparing a series of at least three slide smears; one of such smears acting as a negative control comprising a suspension of Group G Streptococci and Staphylococcus aureus; a second positive control smear comprising a suspension of only Group A Streptococci and a third smear comprising a suspension of unknown organisms, adding the conjugate to each smear, incubating the treated smears, adding hydrogen peroxide and an electron donor dye to the incubated treated smears, and observing the thus treated slide smears under a microscope, said third unknown organism being compared with the positive control to determine the presence of Group A Streptococci.

2. The method of claim 1 wherein the smears prior to addition of conjugate are rinsed with a buffer solution.

3. A method of preparing a reagent useful in a method of detecting the presence of Group A Streptococci in the presence of other Streptococci and Staphylococci organisms in an unknown sample which comprises the step of conjugating a Streptococcus antibody with a Group A Streptococcus by forming a complex of said antibody and Group A Streptococcus through means of a peroxidase enzyme coupling agent, said resulting conjugate being the desired reagent.

**References Cited**

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