Legionellosis

Volume II

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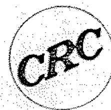

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Chapter 4

DIAGNOSIS: NEW CONCEPTS

Richard W. Gilpin

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INTRODUCTION

One exciting aspect of the laboratory diagnosis of Legionnaires' disease is the continuing search for better laboratory tests. The goal is to provide accurate, confirmatory information to physicians in the shortest amount of time with the least expense in terms of personnel time and cost per test.

The preceding chapters written by experts in the field covered methods currently used for diagnosis of legionellosis. Some of the new concepts will be reviewed in this chapter. This will include modifications of accepted tests as well as those which are of more recent origin. The chapter is divided into two sections: (1) tests that are used to detect a patient's immunological response to *Legionella* disease and (2) those designed to identify *Legionella* antigens or intact bacteria in patient specimens. The procedures are classified further by the types of equipment needed to perform the tests.

II. TESTS FOR ANTIBODY

A. Fluorescence Microscopy

Heat-killed instead of formalin-killed *Legionella* antigen seems to work best in the indirect

immunofluorescence assay (IFA) that was described in Chapter 3.[1] Patient sera are usually diluted in phosphate buffered saline (PBS). One modification was the use of an extract from *Escherichia coli* strain 013:K92:H4 for dilution of patient sera to remove nonspecific antibody reactions. Acute-phase titers, although lower, made it possible to detect seroconversions because convalescent titers were not reduced when *L. pneumophila* serogroup 1 antigen was used.[2] However, when polyvalent serogroup antigen was substituted, a loss of sensitivity was found. Therefore, the use of this immunosorbent may not be necessary in most situations. Comparisons between *Mycoplasma pneumoniae*, *Chlamydia psittaci* and *Legionella* spp. antigens with paired patient sera having concurrent titers to *Legionella* and either *Mycoplasma* or *Chlamydia* indicated that the IFA test did not produce nonspecific cross-reactions to either *Mycoplasma* or *Chlamydia*.[3] Also, adsorption of sera with *Legionella* spp. removed only antibody to *Legionella*.[3] The presence of cross-reactions between various species of *Legionella* may occur, however.

Determination of IFA serum titers with sheep anti-human IgM in addition to IgG may permit earlier detection of IgM antibody in patient serum before significant titers of IgG can be detected.[4] Therefore, anti-human IgM would be appropriate to incorporate into routine IFA testing for early presumptive diagnosis of legionellosis. Although the IFA test requires experienced personnel to interpret staining intensities, artifacts, and nonspecific background staining reactions, this technique remains the reference to which other antibody tests are compared.

B. Electron Microscopy

1. Reverse Immunoferritin Test (RIFEM)

Patient antibody was titered by floating carbon-coated grids containing formalin-killed *L. pneumophila* and normal yolk-sac membrane upside down on serial dilutions of patient serum for 30 min at 37°C.[5] The grids were washed and incubated on rabbit anti-human ferritin-conjugated globulin, washed, and examined for the presence of ferritin granules over the entire surface of the known *Legionella*. Comparisons of serum titers from 12 patients by the IFA and RIFEM tests were within one dilution of each other.[5] However, titration of patient sera by RIFEM is time and technique intensive, which makes it more appropriate for research rather than diagnostic applications.

C. Colorimetric/Fluorometric Tests

1. Enzyme-Linked Immunosorbant Assay (ELISA)

This test has been used to detect antibody in patient sera to heat-killed *L. pneumophila* serogroup 1 soluble antigen.[6] Wells in polystyrene micro-ELISA plates coated with Legionella antigen received serial dilutions of patient serum. After incubation for 1 hr at 37°C the wells were washed with buffered saline. An appropriate dilution of horseradish peroxidase-conjugated antihuman globulin was added and incubated for 1 hr at 37°C. The wells were again washed and orthophenylenediamine was added. The plates were incubated for an additional 30 to 60 min at 37°C and the reaction was stopped by adding H_2SO_4. The titer was the highest dilution of patient serum producing a color different from the negative control (no serum). More comparisons with the IFA procedure, which takes less time to perform, will indicate whether this procedure is superior. The advantage of the ELISA method is that results can be determined visually or by colorimetry.

2. Solid-Phase Immunofluorescence Test (FIAX)

This had previously been used for detection of antibody to viral and fungal antigens. The method was tested with immune sera from guinea pigs vaccinated with *Legionella*.[7] Sonicated, formalin-killed *L. pneumophila* were adsorbed to one side of a plastic paddle stick. The reverse side of the paddle contained no antigen (control). Paddles were placed in diluted guinea pig sera and shaken for 30 min at room temperature. The paddles were removed,

washed free of unbound serum, and incubated for 40 min with fluorescein isothiocyanate-conjugated rabbit anti-guinea pig IgG. After washing, the fluorescence on the paddles was measured with a special fluorometer. The fluorescence of the control side of each paddle was subtracted from the fluorescence of the side containing adsorbed *Legionella*. The method detected end points with a low coefficient of variation between assays. Although need for a UV light microscope was eliminated, a special fluorometer was used to measure fluorescence of the paddles.

A recent comparison was made between the IFA and FIAX tests using paired human serum from patients with legionellosis.[8] Both tests detected titers to *L. pneumophila* serogroup 1 within one twofold dilution of each other, but the IFA test was significantly more sensitive and used lower quantities of test reagents.

3. Immunofluorometric Assay (IFMA)

An assay originally developed to measure titers of antibody reagents and to screen for monoclonal antibody production was used to detect antibody to *Legionella* in patient sera.[9] *L. pneumophila* were formalin killed, washed, and incubated at 37°C with serial dilutions of patient sera for 1 hr. Suspensions were centrifuged and the resulting pellets were washed. Fluorescein-isothiocyanate-conjugated goat antihuman antibody was added to the tubes and incubated at 37°C for 1 hr. After washing, the fluorescent intensity of each *Legionella* suspension was measured in a fluorometer. Data analysis was performed by computer. The procedure detected antibody levels to *Legionella* in patient sera that were negative by the IFA assay. Although a fluorometer and appropriate computer software were needed, the procedure did not require the level of experience needed to perform the IFA assay. But, as with the FIAX assay, more reagents were needed to perform this assay. Further miniaturization may make these solid-phase immunofluorescent assays more economical.

D. Visible Agglutination or Precipitin Tests

These tests avoid the use of fluorescent or enzyme-conjugated antibodies for detection of antibody to *Legionella* test antigen.

1. Microagglutination Test (MA)

This procedure was applied to detection of IgM antibody to *L. pneumophila* in patient sera.[6] Several authors have used this procedure for serologic surveys because several sera can be screened on one microtiter plate. Heat-killed *Legionella* stained with safranin were added to microtiter wells containing serially diluted patient serum. After incubation at room temperature for 16 hr followed by 2 hr at 4°C, visible agglutination end points were determined to be the highest dilution of serum that did not result in formation of a distinct button at the bottom of a microtiter well.[6] This procedure was used to evaluate the major F-1 antigen of *L. pneumophila*.[10] The MA test did show serogroup specificity against serogroups 1 to 4 *Legionella*.[11] Minor modifications of the MA test involved different serum dilutions and different methods for preparing the *Legionella* antigen.[12]

A modified microagglutination test that could be performed in 30 min instead of the usual overnight incubation was recently reported.[13] *L. pneumophila* were incubated overnight at 37°C in 2% formalin buffered saline (FBS), pH 7.0. An equal volume of phosphate buffered saline (PBS), pH 6.4, containing 0.005% (w/v) safranin was added; the mixture was incubated for 1 hr at room temperature, and centrifuged at 3000 × g for 15 min. The pellet was then suspended in PBS, pH 6.4, for use as antigen. Doubling dilutions of patient serum were placed in microtiter wells, antigen was added, and the plates were sealed and shaken for 10 sec. After 10-min incubation at room temperature, the plates were centrifuged at 300 × g for 4 min, inclined at a 70° angle for 10 min, and scored as negative (tear-drop pattern) or positive (clear button). Tests of patient sera produced a 97% agreement with the IFA test.[13]

The shorter incubation times possible with the modified MA technique justify further evaluation in diagnostic laboratories.

One drawback of the MA tests is that IgM is more reactive than IgG antibody. Therefore, other methods are needed to detect IgG. One advantage of the MA test is that equipment such as an UV light microscope or fluorometer are not needed. One disadvantage is occasional difficulty reading end point titers, but this may be avoided if the antigen is dispersed uniformly.

2. *Indirect Hemagglutination Test (IHA)*

This procedure was also performed in microtiter plates.[14] Turkey erythrocytes containing absorbed *L. pneumophila* soluble antigens were added to microtiter wells containing patient sera diluted 1:16 and incubated at room temperature for 2 hr. Sera which caused agglutination of the sensitized erythrocytes were then serially diluted and retested to determine their antibody titer to *Legionella*. Turkey erythrocytes without *Legionella* antigen served as the control. Agreement with the IFA test was 95% but IHA titers were somewhat lower. Another study evaluated the IHA test with *L. pneumophila* serogroup 1 to 4 antigens.[15] Agreement with the IFA test was 97% overall, but in many instances one test was positive when the other test was negative.[15] Sera fractionated on sucrose gradients showed that the IHA test detected IgM antibody that would not be picked up by the IFA procedure unless fluorescein-conjugated anti-IgM antibody was used. Occasionally, the IHA test did not detect IgG antibody. For screening of sera this technique appeared to be suitable and slightly more sensitive than the MA test. Another study indicated that screening sera with polyvalent *Legionella* antigen produced lower titers, although agreement with the IFA test remained at 97%.[16]

3. *Immune Adherence Hemagglutination Test (IAHA)*

Heat-killed *L. pneumophila* test antigen was suspended in PBS, pH 7.4, and stored at −80°C. Patient sera were diluted 1:4, complement was heat inactivated at 56°C, and then serially diluted in microtiter wells.[17] Antigen was added and the plates were incugated at 37°C for 1 hr. Diluted guinea pig complement was added and incubation was continued for 40 min at 37°C. After the addition of ethylenediaminetetraacetate and dithiothreitol, a suspension of human type O erythrocytes (RBC) were added and incubation was continued for 1 hr at room temperature. Complete agglutination of RBC was considered positive for the presence of antigen-antibody-complement immune complexes. Patient sera that were IFA positive were also positive in the test (25 of 26 sera). Although IAHA titers were often lower, they were specific for *Legionella* IgM and IgG antibody in patient sera.

4. *Immunodiffusion Test (ID)*

Soluble test antigen was obtained from the supernatant fluid after centrifugation of heat-killed suspensions of *L. pneumophila*.[18] Antigen was placed in the center well of a small petri dish containing 1% agar and four wells, 4 mm from the central well, were filled with patient sera to test for antibody to *Legionella* soluble antigen. During incubation at 37°C for 72 hr, the presence of precipitin bands was noted. One to two bands appeared after incubation in many of the positive patient sera. Test results correlated with IgM antibody. Sera with high IgG titers and low titers of IgM antibody may not produce a positive ID test, however.

5. *Card Agglutination Titer (CAT)*

A card agglutination test was developed to provide a facile method to determine the antibody titer to *Legionella* in patient serum.[19] The rapid plasma reagin card test for syphilis was modified for this purpose. Serogroup 1, *L. pneumophila* grown on charcoal-yeast extract (CYE) agar were suspended in PBS and steamed for 60 min. After washing, the diluted

suspension of *Legionella* antigen was mixed with crystal violet (0.01% final concentration). Patient sera (50 μℓ) were serially diluted up to 1:512 with buffered saline directly within each ring on the card. The diluted sera were spread over the rings and crystal-violet-stained *Legionella* were added. The card was rotated by hand for 15 to 20 sec. If no agglutination was observed, the card was placed on a card test rotator for 3 to 4 min at 100 rotations per minute. The end point titer was the highest dilution of patient serum producing agglutination. Although the card that was not as sensitive as the IFA test, the results were specific. Therefore, the method could be used for rapid screening in laboratories not equipped to do fluorescent microscopy.

6. Counterimmunoelectrophoresis (CIE)

Diagnosis of legionellosis by detection of antibody in patient serum was performed by using soluble *Legionella* antigen.[20] Heat-killed *Legionella* were disrupted by sonication and used without removing the large particles. Opposing wells were cut in 1% agarose-covered microscope slides. Diluted patient test serum was placed in a well toward the anode and soluble antigen was placed in the cathode well. Electrophoresis at 10 mA per slide was performed at room temperature for 90 min. After incubation at 4°C for 30 min, the slides were washed and examined for the presence of precipitin bands. Slides were preserved by drying and staining with naphthalene black. As might be expected, the serogroup-specific serum titers by CIE were lower than the IFA titers.

E. Cell-Mediated Immunity Tests

1. Lymphocyte Blastogenic Response

Lymphocytes were isolated from patients with culture-positive or IFA-confirmed legionellosis and those with other bacterial pneumonias. The lymphocytes were cultured in the presence of sonicated, formalin-killed *L. pneumophila* for 6 days. ^{3}H-Thymidine was added to the culture 6 hr before harvest to measure blastogenesis by incorporation of thymidine. Among a small group of patients tested, the assay appeared to detect an immune response to *Legionella* disease before positive titers were detected by IFA.[21,22] More studies would be needed to confirm these results. Although this test is difficult to perform, the ability to make a laboratory diagnosis before the primary humoral response can be detected by IFA would be quite useful.

2. Skin Test

A study of guinea pigs sensitized with *L. pneumophila* indicated that intracutaneous injection of antigen produced a delayed hypersensitivity reaction within 24 to 48 hr.[23] Control animals did not produce this reaction. In another study, *L. pneumophila* serogroup 1 to 4 bacteria, or antigens extracted with lithium acetate, were used to sensitize guinea pigs.[24] Four different procedures were used. Animals sensitized by heat-killed and live *Legionella* produced delayed hypersensitivity reactions within 48 hr when skin tested with cross-reactive antigens. Skin tests of immunized guinea pigs given a challenge dose of live *Legionella* were also positive. Skin tests of normal control animals were negative. Therefore, the diagnostic use of a skin test deserves further study.

F. Conclusions

Some tests to detect the presence of antibody or cellular antibody response are quite sophisticated and require specific equipment and/or training of laboratory personnel. The IFA test remains the baseline to which other serological tests are compared. Although this test does require an experienced observer to interpret end point titers, the IFA seems to be somewhat more sensitive than agglutination and precipitin tests described in this section if fluorescein-conjugated antibody to both human IgG and IgM are used. Many of the other methods require the use of more reagents, thus making each test more expensive.

The ability to make a diagnosis of legionellosis before specific humoral antibody can be detected in patient sera would make serological tests less retrospective. The method described to detect sensitization of lymphocytes or other similar procedures may be of more use to the diagnostician than tests for humoral antibody. The relevance of this and the skin test may become more apparent as further studies are performed.

III. TESTS FOR *LEGIONELLA* ANTIGEN

A. Electron Microscopy

1. Immunoperoxidase-Labeled Antibody

Visualization of surface antigens can be enhanced with specific antibody coupled to an enzyme. Peroxidase-conjugated anti-*Legionella* F-1 antibody was used to locate a high-molecular-weight surface antigen.[25] Saponin-treated suspensions of *L. pneumophila* were fixed with paraformaldehyde and gluteraldehyde.[26] After washing, the cells were preincubated with 5% bovine serum albumin (BSA) followed by peroxidase-conjugated rabbit antibody to F-1 antigens.[25] After incubation for 45 min at room temperature and washing, the stain reaction mixture[26] was added. After 15-min incubation the bacteria were washed, post-fixed in osmium, embedded in epon, sectioned, and observed with an electron microscope. Thin sections showed the surface location of the F-1 antigen on *L. pneumophila*. The staining reaction was serogroup specific. Application of this method to locating *Legionella* in tissue specimens may be of immediate interest to researchers.

The immunoperoxidase method was also used to study the antigens on *Legionella* flagella.[27] Flagellin recovered from agar cultures of *L. pneumophila*, serogroup 1, was used to immunize rabbits. Rabbit antibody to flagellin as well as to F-1 antigen were used for indirect immunoperoxidase assays. Glutaraldehyde-fixed *Legionella* were adsorbed onto carbon-coated grids, washed, and incubated with normal mouse serum for 20 min. After subsequent washing and incubation with normal mouse serum, the grids were incubated for 20 min with the rabbit antiserum. Washed grids were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for the same time period. After washing, diaminobenzidine tetrahydrochloride and hydrogen peroxide were added. The grids were then washed and stained with phosphotungstic acid for examination. Thin sections were also made using bacterial suspensions instead of bacteria adsorbed to grids. Flagella were easily observed with this indirect immunoperoxidase staining procedure. The anti-flagellin antibody was specific for flagella on all three serogroups of *L. pneumophila* that were investigated.

2. Immunoferritin-Labeled Antibody

Electron microscopy was used to identify Legionella in formalin-fixed and unfixed lung tissue.[5,28] Suspensions of bacteria from culture were also identified by this method. Ground tissue or culture suspensions were placed on formvar-carbon-coated grids and floated upside down on diluted rabbit antiserum prepared by immunizing a rabbit with *L. pneumophila*, serogroup 1. After incubation at 37°C for 30 min the grids were washed and floated on ferritin-conjugated anti-rabbit globulin diluted in buffer containing a wetting agent to reduce nonspecific reactions. After washing, the grids were dried and examined. Ferritin granules were observed on the surface of *Legionella* but not on other bacteria tested.

Serogroup-specific antigen was visualized on the surface of *L. pneumophila* using a similar technique.[29] Bacterial suspensions were fixed in glutaraldehyde, washed, and suspended in diluted rabbit anti-*Legionella* serum. After incubation at 23°C for 30 min, the suspensions were washed by centrifugation and incubated with ferritin-conjugated goat anti-rabbit IgG antibody for 30 min at 4°C. After washing, the suspensions were fixed, sectioned, and observed with an electron microscope. The ferritin granules were found on the surface of intact *Legionella*.

The immunoperoxidase and immunoferritin procedures are powerful research tools. Although antigens can be identified at specific locations on the bacteria, this may not be suitable for diagnostic purposes because the methods are time and cost intensive.

B. Light Microscopy

1. Bacterial Stains

Modified Gram stain — *Legionella* are often not stained by Gram's method.[30] This is particularly true with tissue specimens. However, peritoneal fluid samples from guinea pigs infected with *Legionella* were visible after Gram staining if neutral red was substituted for safranin.[31] This counter stain was left on the smear for 15 to 20 min instead of the usual 1 min. Although this procedure is not as sensitive as the direct fluorescent antibody method, it may be useful for screening clinical specimens from sites which do not have a resident normal flora.

Giemsa stain — A Giemsa stain has been used to demonstrate *Legionella* in smears of tissue scrapings, cultures, and embryonated egg-yolk-sac fluid.[32] However, the method may not permit visualization of the bacteria in tissue sections.

Silver stains — Silver impregnation stains have been successfully used to stain *Legionella* in tissue sections. The Dieterle stain[33] permitted visualization of *Legionella in situ*, but the Warthin-Starry silver stain appeared to work well and was much easier to perform.[30,34] This stain and the Geimsa stain have been used to screen tissue sections for bacteria such as *Legionella*.[35]

2. Modified Fluorescent Antibody Procedures

The direct fluorescent antibody (DFA) method is both sensitive and specific. However, background autofluorescence can make interpretation of results difficult. This may be encountered with lung tissue imprints or deparaffinized lung tissue sections from patients with extensive fibrosis. One method to reduce background fluorescence was to add crystal violet to the smear prior to performing the fluorescent antibody procedure.[36] Deparaffinized lung tissue sections on microscope slides were dipped quickly in diluted Gram's crystal violet, immediately washed in distilled water, air-dried, and stained by the FA procedure. If too much crystal violet remained on the smear, it was decolorized with alcohol before the FA stain was applied.

The presence of *L. pneumophila* in lung tissue was enhanced by an indirect fluorescent antibody (IFA) test.[37] Formalin-fixed lung tissue from one legionellosis patient was post-fixed in 1% osmium tetroxide for 1 hr, dehydrated with alcohol and propylene oxide, embedded, and polymerized overnight at 60°C in epoxy resin. Sections 1 μm thick were placed on slides, treated to remove the resin, washed in alcohol, and placed in water. After trypsinization for 5 min at 37°C and washing, rabbit anti-*Legionella* antibody was added to the slides and incubated for 30 min at room temperature. After washing, fluorescein-conjugated anti-rabbit globulin was added and the slides were incubated as before. After washing, the sections were mounted with glycerol and examined with a fluorescent microscope. Although this method may permit better visualization of *Legionella in situ* than the 5 min paraffin embedding technique, this method is available only to those laboratories that have experience with epoxy embedding and sectioning.

Flow cytometry reportedly can be used to detect *Legionella* in samples that contain at least 10^4 bacteria per milliliter.[38] Formalinized *L. pneumophila*, washed in buffered saline and adjusted to a density of about 10^8/mℓ were incubated for 4 hr at room temperature with fluorescein-conjugated rabbit anti-*Legionella* globulin. After centrifugation, pelleted *Legionella* were suspended in saline to a density of 10^7/mℓ and analyzed with a laser cell sorter (Coulter Electronics, Hialeah, Fla.) interfaced to a minicomputer. *Legionella* incubated with fluorescein-conjugated normal rabbit globulin served as controls. Specific fluorescent antibody-treated bacteria produced a characteristic broad peak of high-intensity fluorescence.

Escherichia coli and *Saccharomyces cerevisiae* did not produce this characteristic peak. Although this procedure may find application in certain situations, such as concentrated urine samples, greater sensitivity may be needed for diagnostic use with patient specimens.

3. Immunoassays

Glucose oxidase immunoassay — Another method to detect Legionella in deparaffinized tissue sections involved the use of a four-layer immunological sandwich technique.[39] Tissue sections were incubated for 30 min at room temperature with 3% normal goat serum. This was followed by incubation for 30 min with *Legionella*-specific rabbit antibody diluted in 1% normal goat serum. Sections were then incubated with dilute rabbit antiserum for 16 hr at 4°C. After washing, goat anti-rabbit IgG serum diluted in 1% normal goat serum was added to the smears and incubated for 15 min at room temperature. The smears were washed and incubated for 30 min in a solution containing soluble glucose oxidase complexed with rabbit anti-glucose oxidase antibody diluted in 1% goat serum. After washing, nitro blue tetrazolium was added. After incubation for 5 min at room temperature, the sections were washed in 4% buffered formaldehyde and stained with nuclear fast red plus phloxine. The method was reported to be specific for intact *Legionella* and also permitted visualization of antigen.[39] This four-layer sandwich technique did require several steps. Whether this method would be useful for rapid diagnosis has not been determined.

Peroxidase immunoassay — Lung sections, sputum samples, and cultures have been evaluated by a peroxidase-conjugated antibody technique. The method was briefly described, but no data were presented.[40] Formalin-killed *Legionella*, serogroups 1 to 4, were used to immunize mice. The IgG fraction of mouse ascites fluid was conjugated to horseradish peroxidase and used to detect *Legionella* in smears. After washing, bound peroxidase-conjugated IgG antibody was detected by adding the substrate, diaminobenzidine, followed by incubation, washing, and counterstaining with fast green. This method could detect *Legionella* which had been added to sputum samples. Mention was also made of an indirect immunoassay using rabbit anti-*Legionella* antibody followed by peroxidase-conjugated goat, anti-rabbit antibody. The sensitivity and specificity of these methods were not documented.

4. Tetrazolium Dye Reduction Test (INT)

The *Legionella* electron transport system reduced the dye, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride (INT), to red formazan crystals which were observed with a light microscope.[41] This method, which detected metabolically active *Legionella*, was combined with the direct fluorescent antibody test to locate respiring *Legionella* in water samples. Tests were performed with cultures to determine the baseline reactivity of the INT test and its relationship to viable counts and culture optical density. INT was added to samples at a final concentration of 0.02 or 0.002% for cultures and water samples, respectively. After a 60-min incubation period, the reaction was stopped by adding formaldehyde at 0.1% final concentration. Samples were then placed on slides, stained by the FA technique, and observed with a combination of bright-field tungsten light and epi-illumination UV light. This is a very interesting technique and may find wide application for testing water samples. However, its usefulness with patient specimens may be somewhat limited because the INT method may also detect election transport activity in phagocytic or tissue cells.

5. Sandwich Immunofluorescence Assay (SIA)

A novel method to detect *Legionella* soluble antigen was recently described.[42] The solid support used to detect antigen was prepared as follows. *Staphylococcus aureus*, Cowan 1, bacteria were suspended in 0.5% formalin PBS and held at room temperature for 3 hr. After washing, the suspension was incubated at 80°C for 5 min and cooled for 5 min in an ice bath. The staphylococci were then washed and suspended in buffered saline. In order to detect soluble *Legionella* antigen, protein A on the surface of the staphylococci was coupled

to the Fc end of anti-*Legionella* IgG antibody. The staphylocci were placed on a microscope slide and heat fixed. Diluted antiserum produced by immunization of rabbits with heat-killed *Legionella* vaccine was placed on the smear and incubated at 37°C for 30 min. After the slide was washed and air-dried, soluble test antigen was placed on the slide and incubated as before. After washing, fluorescein-conjugated antibody to *Legionella* was added to the slide and incubated for 30 min. The slide was washed to remove unbound antibody and then observed with UV light microscope as in the standard FA procedure. If the test antigen corresponded to the serogroup specificity of the rabbit antiserum attached to the protein A on the staphylococci, the cocci fluoresced. This method detected soluble antigen with 100-fold more sensitivity than a similar agglutination test. Untreated urine collected from healthy volunteers mixed with soluble antigen from *Legionella* produced a positive reaction in the SIA test. This method may be quite useful for detection of soluble antigen in some patient samples.

C. Colorimetric and Radioimmunoassays

1. Enzyme-Linked Immunosorbent Assay (ELISA)

A direct ELISA was used to detect soluble *Legionella* antigen in patient specimens such as urine.[43] This colorimetric procedure was performed in polystyrene test tubes. Test tubes were coated with diluted rabbit anti-*Legionella* IgG by adding antiserum to the tubes and incubating for 1 hr at 37°C. The antibody was removed by aspiration, the tubes were rinsed, and 5% BSA was added. After incubation for 1 hr, the albumin was aspirated, the tubes were rinsed, and test urine sample was added to each tube and incubated as before. After aspirating the test urine and rinsing, dilute alkaline-phosphatase-conjugated anti-*Legionella* IgG antibody was added to each tube and incubated overnight at 4°C. After each tube was washed, *p*-nitrophenylphosphate and buffer were added. After incubation at 37°C for an appropriate period of time, the color reaction was stopped by adding sodium hydroxide. Color development was quantitated by spectrophotometry at a wavelength of 400 nm. Tests of urine specimens from 39 to 47 patients with legionellosis were positive. Urine from three patients with other bacterial diseases changed from positive to negative if their urine was heated to 100°C for 5 min before testing. Heating urine samples from legionellosis patients did not change the results of the ELISA tests. This was not the first application of the ELISA for detection of *Legionella* antigen; studies were performed previously with urine[44,45] and sputum[46] with mixed results. The assay system described here does seem to be specific and may have diagnostic applications.

2. Radioimmunoassay (RIA)

Test procedures similar to the ELISA described above used radionuclide-labeled antibody instead of enzyme-conjugated antibody as the indicator system. Detection of soluble *Legionella* antigen in patient urine by RIA used a procedure similar to that described above for ELISA.[47] After test urine was incubated for 1 hr at 37°C in polystyrene test tubes coated with rabbit, *Legionella*-specific IgG, the urine was aspirated and the tubes were rinsed. Iodinated ^{125}I-anti-*Legionella* IgG was added to the tubes and incubated for 1 hr at 37°C. The antibody was removed, the tubes were washed, and radioactivity was measured with a gamma-radiation spectrophotometer. Heat-treated RIA-positive urine samples remained positive. All urine specimens from patients with confirmed legionellosis were positive with this test if the patients had not been on antibiotic therapy for more than 2 days. However, use of higher-titered anti-*Legionella* rabbit IgG to coat the tubes may permit detection of urine antigen for periods greater than 2 days.[4] Only one false positive urine sample was reported. RIA tests of patient urine from 26 of 34 culture, DFA-, and/or IFA-confirmed legionellosis patients were reportedly positive.[48] The best RIA positive urine antigen results (93%) were obtained from patients that were culture positive. The RIA procedure may be a useful supplement to other tests for diagnostic laboratories that have a gamma counter.

A comparison of RIA and ELISA for detection of soluble antigen in urine indicated that the sensitivity of both procedures was similar.[43] However, the antigen incubation step for ELISA was overnight vs. 1 hr for RIA. Also, the ELISA did produce a few more false positive results than RIA unless test urine was heated. Depending on the equipment available to a diagnostic laboratory, ELISA or RIA may be used to screen for soluble antigen in patient urine samples. Of course, the ELISA avoids the procedures required for handling and disposing of radioactive nuclides.

D. Visible Agglutination or Precipitin Tests

1. Slide Agglutination Test (SAT)

Slide tests are rapid and do not require expertise to interpret, provided that the antibody used to identify the visible test antigen is specific. A slide test to identify the serogroup of *L. pneumophila* colonies from agar plates was recently published.[49] Serogroup-specific antiserum was produced by vaccinating rabbits with boiled *L. pneumophila*. Cultures to be tested were suspended in 10% neutral formalin. The agglutination test was performed by mixing one drop of test antigen with one drop of rabbit antiserum on a microscope slide and rocking for 1 min. Agglutination was scored from strong to barely visible. The test was serogroup-specific for *L. pneumophila* serogroups 1 to 4. The slide agglutination test has also been evaluated with four other species of *Legionella*.[42] The only reported departure from the previous protocol was to rock the mixture of undiluted rabbit antiserum and culture suspension for 15 sec instead of 1 min. A positive test was visible agglutination within 15 sec. Again, the tests were species and serogroup specific.

This test has also been used to study flagellated or nonflagellated live *Legionella*.[27] Two drops of bacteria were mixed with one drop of rabbit anti-flagellin from serogroup 1, *L. pneumophila*. Positive reactions showed visible agglutination within 15 min. The anti-flagellin serum agglutinated flagellated but not nonflagellated live *Legionella*. Heat-killed bacteria did not agglutinate with the serum.

2. Card Agglutination Test

This procedure was developed for titration of patient sera using known *Legionella* antigen.[19] It may be possible to reverse the protocol and test *Legionella* culture suspensions with diluted hyperimmune rabbit serum. Comparisons with the slide agglutination test could be performed to determine whether this is a usable procedure.

3. Slide Coagglutination Test (coSAT)

A coagglutination test was developed as a modification of the slide agglutination test to detect the presence of soluble *Legionella* antigen.[42] *Staphylococcus aureus*, Cowan 1 strain, with protein A on the surface was used as an amplification system so that reactions between soluble antigen and specific antibody produced visible agglutination of the staphylococci. Cultures of *Legionella* on CYE agar were suspended in 10% neutral formalin when tests were performed with particulate antigen. Soluble antigen was prepared by suspending cultures in tubes of distilled water and heating in a boiling water bath for 15 min. *Legionella*-specific antisera were obtained by immunization of rabbits with heat-killed vaccines. The staphylococci were cultured in broth medium, washed by centrifugation, suspended in 0.5% formalin in PBS, and incubated at room temperature for 3 hr. After washing, the staphylococci were suspended in buffered saline, heated for 5 min in an 80°C water bath, cooled for 5 min in an ice bath, washed, and suspended in saline. The coSAT test was performed by adding appropriately diluted anti-*Legionella* serum to the staphylococcal reagent and mixing one drop of this with one drop of particulate or soluble test antigen on a slide. Visible agglutination after 15 sec was considered a positive test. Tests with whole cells or soluble antigens were species and serogroup specific. The advantages of the coSAT over the direct slide agglutination test were use of less antiserum and the ability to detect *Legionella* soluble

antigen. Normal urine seeded with soluble antigen produced a positive coagglutination reaction if the urine was first heated for 10 min in a boiling water bath. This procedure is less complicated than RIA or ELISA and can be performed without the need for sophisticated equipment.

4. Immunodiffusion Test

Legionella soluble antigens can be identified by an immunodiffusion test using wells cut in 1% agarose-coated slides.[50] Soluble antigen was isolated by suspending *Legionella* cultures in PBS and heating for 30 min in a boiling water bath. Soluble antigen was removed from the supernatant fluid after centrifugation of the boiled suspension. Anti-*Legionella* serum produced by vaccination of rabbits was placed in a central well and soluble antigens were placed in each of six surrounding wells. Precipitin bands were checked after 1, 2, and 3 days for identity, nonidentity, and partial identity. The method was also used to identify *Legionella* envelope antigen.[51] The test may be useful for studies of antigen identity between several isolates of *Legionella*. It is not known whether this test could be used to detect soluble antigen in patient specimens such as urine.

5. Counterimmunoelectrophoresis (CIE)

This test has been used to detect bacterial soluble antigen in cerebrospinal fluid. This method has been applied to detection of *Legionella* antibody in patient serum[20] as discussed previously, and identification of *Legionella* soluble antigens.[51] Loosely associated surface antigens from *L. pneumophila* serogroups 1 and 2 were isolated, partially purified, and analyzed by CIE.[51] Antiserum was produced by immunization of rabbits with crude surface antigen. Microscope slides were coated with 0.75% agarose, pH 8.6, two wells were punched across from each other, soluble test antigen was added to each well, and the slides were electrophoresed at 7 V/cm for 60 min at room temperature. Antiserum was placed in wells cut on each side of each antigen well and the slides were incubated at 37°C for 18 hr in a moist chamber. A major antigenic component migrated toward the cathode but a minor antigen migrated toward the anode. Later experiments were performed by placing antiserum in a well on the cathode side of the antigen well. For some unknown reason, the CIE test was less sensitive in this application than CIE diagnostic tests for other bacteria.

6. Two-Dimensional Crossed Immunoelectrophoresis

This test, along with immunoelectrophoresis, was used to study *Legionella* group-specific and common antigens.[52] Cultures grown in chemically defined medium were harvested by centrifugation, washed, and suspended in 0.25% formalin plus saline. After incubation overnight at 4°C, soluble test antigen was prepared by disruption in a Braun homogenizer in the presence of sodium deoxycholate. After concentration by pressure dialysis, soluble antigens were recovered in the supernatant fluids after centrifugation at 40,000 × g for 30 min. Before use, soluble antigens were mixed with Triton X-100 (5% final concentration). Anti-*Legionella* sera were produced by vaccination of rabbits. Two-dimensional crossed immunoelectrophoresis was performed on 4.3-cm square glass slides coated with 0.8% agarose. Antigen was placed in a well cut in the agarose and electrophoresed at 8 V/cm for 45 min. A layer of agarose containing diluted anti-*Legionella* serum was placed on the slide and electrophoresed at room temperature in the second direction for 16 hr at 1.5 V/cm. The slides were washed and stained with coomassie brilliant blue. *L. pneumophila* serogroups 1 to 4 produced 8 to 11 precipitin lines against homologous antisera. Both common antigens and serogroup-specific antigens were resolved by this method, but the latter were more clearly defined. There were no cross-reactions between serogroup-specific antigens. This procedure may be too rigorous for use as a diagnostic tool, but it does permit more detailed evaluation of *Legionella* antigens than heretofore possible.

7. Reversed Passive Hemagglutination Test (RPHA)

Detection of *Legionella* soluble antigen in urine from one patient with suspected legionellosis was accomplished with this test.[53] After the erythrocytes had been tanned with dilute tannic acid for 15 min at 37°C, they were incubated with diluted goat anti-*Legionella* globulin for 15 min at 37°C, washed, and suspended in buffered saline containing 1% normal rabbit serum. Test antigen samples were diluted eightfold in buffered saline containing normal rabbit serum, heated for 30 min at 60°C to destroy complement, and adsorbed with sheep red blood cells for 30 min to reduce nonspecific reactions. The test samples were serially diluted in microtiter wells and antibody-containing sensitized sheep red blood cells were added to each well. After incubation at room temperature for 2 to 3 hr, agglutination at a titer greater than 1:16 was considered positive for *Legionella* antigen. Multiple urine samples from one patient with confirmed legionellosis produced positive agglutination reactions. It is not known whether this procedure was more sensitive than some of the other tests described for detection of soluble antigen, but a detection limit of 4.3 ng protein per milliliter was reported.

8. Lectin Agglutination Test

Plant agglutinins were used to evaluate the agglutination patterns of *Legionella*.[54] Cultures from CYE agar were suspended in PBS, washed, and mixed with plant agglutinin in microtiter wells. After incubation at 4°C overnight, agglutination patterns were scored. The positive reactions were not serogroup-specific with *L. pneumophila* isolates or other species of *Legionella*. Two plant agglutinins, from *Aloe arborescens* and *Persia americana*, did react with most isolates of *L. pneumophila*. Several other lectins and plant agglutinins did not react. The authors suggested that *Legionella* may not have accessible carbohydrate groups on their surface because the two reactive agglutinins seem to bind protein rather than carbohydrate. At this time, plant agglutinin reactions do not have any diagnostic significance.

E. Conclusions

Tests for *Legionella* antigens range from simple slide agglutination tests to highly sophisticated techniques involving electron microscopy. The primary objective of a diagnostic laboratory is to have a rapid, specific, and sensitive assay for antigen in body fluids. At this time, there have not been enough comparisons of each procedure with the same patient specimens to provide an answer to which test works best. In terms of simplicity and quantity of reagents needed, the slide coagglutination test would appear to be quite useful. Equivocal reactions may be further evaluated by using the sandwich immunofluorescence assay. Once *Legionella* have been isolated on CYE agar, several of the visible agglutination or precipitin tests could be used for screening purposes.

IV. TESTS FOR *LEGIONELLA* ENZYMES

Most species of *Legionella* have been characterized by biochemical tests commonly available to some diagnostic microbiology laboratories.[55,56] Tests for β-lactamase, catalase, gelatin liquefaction, hippurate hydrolysis, nitrate reduction, and oxidase are discussed in Chapter 2. Although the above tests provide useful information, serological tests such as DFA are more specific. Therefore, tests for other biochemical activities have been investigated in an effort to find a rapid *Legionella*-specific procedure that would provide diagnostic laboratories with an alternative to serological tests.

A. Fluorogenic Substrate Cleavage

These tests rely on the hydrolysis of substrates that produce products that fluoresce under long-wavelength UV light. Five species of *Legionella* cleaved diacetylfluorescein, whereas *Staphylococci* and most Gram-negative bacteria, with the exception of *Proteus rettgeri*, did

not.[55] This test was also positive with *L. jordanis*.[57] Briefly, suspensions of *Legionella* were incubated with substrate for 5 min at 20°C. A positive test suspension produced a yellow-green fluorescence under a long-wavelength UV lamp. Other fluorogenic substrates were tested, but they were positive with both *Legionella* and other Gram-negative bacteria.[55] Therefore, diacetylfluorescein may have potential application in the diagnostic laboratory.

B. Colorimetric and Other Enzyme Tests

The API ZYM system (Analytab Products, Plainview, N.Y.) containing several chromogenic substrates has been tested with several *Legionella* species.[58,59] *Legionella* agar cultures were suspended in saline and used to fill each capsule on the API ZYM strip. After incubation for 4 hr at 37°C in air supplemented with 5% CO_2, two color development reagents were added. After 5 min, color development was scored on a scale from 0 to 5. All six *Legionella* species tested produced color changes suggesting alkaline and acid phosphatase, leucine aminopeptidase, butyrate and caprylate esterase, and phosphoamidase. However, *L. bozemanii* was caprylate esterase negative. All carbohydrate substrates were negative.[59] Comparisons with other Gram-negative bacteria were not reported, but other genera do have some of these enzyme activities. Further studies will indicate whether this system can be used to identify *Legionella*.

A chromogenic assay with four para-nitroanilide (p-NA) conjugated peptides (KABI Diagnostica, Stockholm) has been used to analyze peptidase activity of five *Legionella* species.[60,61] Culture supernatants were dialyzed, concentrated by ultrafiltration, and placed in microtiter wells to assay enzyme activity.[60] Alternatively, a sonicated extract of *L. pneumophila* was used.[61] Buffer and conjugated substrates were added to each well and incubated at 37°C for up to 30 min. Release of yellow-colored para-nitroanilide due to peptidase activity was measured at 410 nm with a spectrophotometer. The assay was pH and crude enzyme protein dependent. Culture supernatants of *L. pneumophila* serogroups 1 to 6 were moderately active on the Bz-Ile-Glu-Gly-Arg-pNA substrate; the other species had lower levels of activity. Activity on three other substrates was considerably less.[60] Experiments with sonicated of *L. pneumophila* reportedly had no activity.[61] Concentrated culture supernatants from *L. pneumophila*, *L. bozemanii*, *L. dumoffi*, and *L. gormanii* were most active on SucOMe-Arg-Pro-Tyr-pNA substrate. *L. micdadei* was inactive on 18 different peptides.[61] The authors concluded that chymotrypsin-like activity predominated. At this time, there is not enough data to make conclusions about use of these assays for identification of *Legionella* species.

Other tests for proteolytic activity have been reported.[56,62,63] *L. pneumophila* serogroups 1 to 6 degraded denatured casein, skim milk, gelatin, and Remzol brilliant blue conjugated hide powder.[62] Bacteria were cultured in liquid medium and removed by centrifugation. Culture supernatants were filter sterilized and used directly for enzyme assays. Skim milk, gelatin, and casein assays were performed with culture plates. Hide powder protease reactions, the main subject of this report, were done in test tubes.[62] All substrate incubations were at 37°C for various time intervals. The hide powder proteolytic activity was temperature and pH dependent. The authors concluded that *L. pneumophila* produced a neutral protease with an approximate molecular weight of 40,000. Again, more studies will be needed to determine the relevance of these findings to laboratory identification of *Legionella*.

The specificity of *L. pneumophila* serogroup 1 caseinate precipitating enzyme was studied by using anti-proteinase antibody produced by rabbit vaccination.[64] Enzyme was isolated from the supernatant of broth cultures incubated at 37°C for 14 hr or at 22°C for 80 hr. Supernatant fluid was filter sterilized, dialyzed overnight, concentrated by ultrafiltration, and fractionated on a Sephadex G-100 chromatography column. Column fractions containing caseinate-precipitating activity were pooled, concentrated by ultrafiltration, and used to vaccinate rabbits. Assays were performed with agar plates containing 1% sodium casenate and 0.004% $MgCl_2$ at pH 6.2. Serial dilutions of caseinate-precipitating enzyme were added

to paper strips and the strips were placed on the surface of the agar and incubated at 37°C for 4 to 18 hr. The highest dilution of enzyme producing visible precipitation was considered one unit. Specific antibody inhibition of caseinate precipitation was performed by placing strips containing antibody that had been subjected to paper electrophoresis on the surface of the agar and incubating for 3 hr at 37°C. The antibody strips were then removed from the agar and replaced by strips containing enzyme. *L. pneumophila* produced significant levels of caseinate-precipitating enzymes. Three other species of *Legionella* were much less active; *L. micdadei* had no activity. Antibody made against *L. pneumophila* caseinate-precipitating enzyme prevented enzyme activity of all *Legionella* species tested. Antibodies to proteinase enzymes isolated from several other Gram-positive and Gram-negative bacteria did not prevent *Legionella* caseinate-precipitating enzyme activity. Sera from patients recovering from legionellosis were tested for specific, anti-proteinase antibody to *Legionella* caseinate-precipitating enzyme but no inhibition was observed.[64]

Proteolytic activity on human serum proteins was determined by incubating a suspension of *L. pneumophila* with normal human serum for 4 hr at 37°C.[63] The serum was subjected to immunoelectrophoresis for 3 hr on slides containing 2% agar in dietylbarbiturate acetate buffer, pH 8.2. After electrophoresis, rabbit antihuman serum was added to determine if there were changes in the location of serum protein precipitin bands. Five serum proteins were affected; α_1-acid glycoprotein, α_1-antichymotrypsin, β-lipoprotein, β_{1E}-globulin, and β_2-glycoprotein-I.[63]

Toxin produced by *L. pneumophila, L. micdadei, L. bozemanii, L. dumoffii*, and *L. gormanii* reportedly have antigenic relatedness that can be identified by a precipitin test, according to a recent review.[65] Other tests of toxin activity included mouse macrophage viability assays, Chinese hamster ovary cell cytotoxicity tests and stimulation of polymorphonuclear leukocyte (PMNL) chemiluminescence. *Legionella* toxin may also impair human PMNL superoxide production.[66] These in vitro laboratory assays are usually not available in diagnostic laboratories, however. It is possible that further investigations of extracellular enzymes and/or toxins produced by *Legionella* species may result in development of a diagnostic test that could be used to screen patient sera for anti-enzyme antibody, or to identify *Legionella* colonies on agar plates.

V. CULTURE METHODS

Culture is routinely used for diagnosis of legionellosis. The basic agar culture method plus embryonated chicken egg yolk sac and guinea pig procedures were discussed in Chapter 3. This section will cover recent modifications of the standard agar culture technique. *Legionella* have been cultured from sputum, tracheal aspirate, bronchial wash, bronchial brushing, pleural fluid, lung, spleen and liver tissue, hemodialysis fluid and blood. Some specimens contain normal flora that inhibit the growth of *Legionella*.[67] Therefore, the basic BCYE agar developed by Feeley et al.[68] as modified by Pasculle et al.[69] has been supplemented with various antibiotics and/or dyes to enhance recovery of *Legionella*.

A. Antibiotic-Supplemented CYE

Antibiotics have been added to inhibit the growth of oropharyngeal flora while permitting growth of *L. pneumophila*. The first reported medium contained polymyxin B (40 units/mℓ) and vancomycin (0.5 μg/mℓ).[70] This medium was tested with three lung specimens contaminated with one or more of *Citrobacter, Klebsiella, Enterobacter, Staphylococcus*, and *Candida*. Contaminants in two of three specimens did not grow on the antibiotic-containing CYE medium. *Candida* was not inhibited but the addition of anisomycin (80 μg/mℓ) was reportedly effective.

A survey of various combinations of five antibiotics plus α-ketoglutarate and sodium selenate found that BCYE agar supplemented with α-ketoglutarate, anisomycin (80 μg/mℓ),

polymyxin B (80 units/mℓ), and cefamandole (4 μg/mℓ) produced the best results.[71] Twelve patient specimens yielded significantly better recoveries of *L. pneumophila* on this antibiotic-containing medium, but *Pseudomonas* and group D streptococci were not inhibited.

B. Other Media

1. Diphasic Medium

Liquid specimens from body sites without a normal flora, such as blood or pleural fluid, may produce greater recovery rates of *Legionella* if diphasic culture medium is substituted for agar medium. Although such patient specimens have not been tested, this method deserves further investigation. Diphasic medium containing 50% (v/v) solidified CYE agar below an equal volume of liquid medium containing either corn starch instead of charcoal[72] or filter-sterilized yeast extract medium without charcoal or starch[73] have reportedly been useful.

2. Enriched Blood Agar

Medium containing 5% horse blood, blood agar base, L-cysteine, and ferric pyrophosphate, was reported to be useful for isolation of *L. pneumophila* from patient specimens.[74] However, more parallel comparisons with BCYE medium will be needed to confirm this observation.

3. Transparent Culture Medium

Charcoal in BCYE agar makes the medium opaque so that colonies cannot be easily observed with transmitted light under a culture plate. A clear agar medium (YPH) containing yeast extract, monosodium glutamate, potassium phosphate, soluble starch, L-cysteine, ferric pyrophosphate, and hemin reportedly produced better growth of *Legionella* than CYE agar.[75] The plating efficiency or recovery of numbers of colonies on CYE vs. YPH was similar. Again, parallel studies with primary patient specimens will indicate the usefulness of this medium for routine culture of *Legionella*.

4. Antibody Agar Medium

Colonies of *L. pneumophila* serogroups 1 to 6 and four other *Legionella* species developed precipitin rings when cultured on modified medium containing appropriate levels of anti-*Legionella* antibody.[76] Antiserum was produced by vaccination of rabbits with formalinized suspensions of *Legionella*. Liquid medium containing yeast extract, L-cysteine and ferric pyrophosphate was filter sterilized and mixed with 1% agarose (w/v final concentration) that had been sterilized by autoclaving. The medium was then mixed with anti-*Legionella* serum (5% v/v, final concentration) and allowed to solidify in a petri dish. Tissue samples from *Legionella* infected guinea pigs, water samples seeded with *L. pneumophila,* and culture suspensions all produced visible precipitin rings around individual *Legionella* colonies when the agar contained the appropriate antiserum. No precipitin rings were found around colonies of other Gram-negative bacteria. This procedure may facilitate diagnosis of legionellosis by culture if further studies indicate that similar results are obtained with patient specimens. However, anti-*Legionella* rabbit serum would be needed in larger quantities and antibiotic supplementation of the agar medium may still be required for isolation of *Legionella* from contaminated specimens.

C. Selective/Differential Culture Techniques

Isolation of *Legionella* from environmental samples has proven to be difficult because of the presence of high numbers of other bacteria which either inhibit the growth of *Legionella* or overgrow *Legionella* colonies. Methods that have been applied to isolation of environmental *Legionella* will be discussed because some methods may become applicable to patient samples contaminated with other bacteria.

1. Dye-Containing Agar

BCYE agar containing 0.001% bromcresol purple and 0.001% bromthymol blue permitted visual differentiation by color between colonies of *L. pneumophila* (pale green to white), *L. micdadei;* (green), and *L. bozemanii* (blue-gray).[77] Other genera of bacteria may produce colored colonies on this medium so additional methods should be used to verify *Legionella*.

BCYE agar supplemented with 0.01% aniline blue permitted visual differentiation between colonies of *L. pneumophila* and four other species of *Legionella*.[78] *L. pneumophila* colonies were colorless but produced a yellow-green fluorescence in the medium when exposed to long-wavelength UV light. Conversely, colonies of *L. micdadei, L. dumoffii, L. bozemanii*, and *L. gormanii* incorporated the blue dye. *L. dumoffii* colonies were dark blue, *L. bozemanii* colonies were blue, and *L. micdadei* or *L. gormanii* were light blue. When exposed to UV light, colonies of *L. bozemanii* and *dumoffii* produced yellow-green fluorescence in the medium and blue-white fluorescent colonies. Therefore, *Legionella* colonies on this medium can be presumptively identified by colony color, presence of UV fluorescence in the medium, and UV fluorescence of the colonies. Growth on the dye-containing medium was reported to be similar to that observed on BCYE agar without aniline dye.[78]

2. Antibiotic Enrichment Before Plating

Water samples seeded with *L. pneumophila* serogroups 1 to 4 were mixed with equal amounts of tryptic soy broth containing final concentrations of colistimethate (75 μg/mℓ), nystatin (125 units/mℓ), and vancomycin (30 μg/mℓ).[79] After incubation at room temperature for 8 hr, the samples were plated on agar medium. Numbers of contaminating bacteria were dramatically reduced but not eliminated by this procedure. Further studies are required to determine the suitability of this method for patient specimens.

3. Low pH Treatment Before Plating

In order to reduce the contaminating bacterial flora, water samples containing *Legionella* were concentrated by centrifugation, the sediments were suspended in 1 mℓ of water and added to 9 mℓ of buffer containing 0.2 *M* KCl and HCl, pH 2.2.[80] After incubation for 5 min at room temperature the samples were placed on CYE agar alone or on agar containing cephalothin (4 μg/mℓ), colistin (16 μg/mℓ), vancomycin (0.5 μg/mℓ), and cycloheximide (80 μg/mℓ). A survey of 11 environmental samples indicated that acid treatment and plating on antibiotic-containing medium usually enhanced recovery of *Legionella*.

Another investigation of *Legionella* in potable water suggested that acid treatment did not enhance recovery over direct plating on BCYE agar containing α-ketoglutarate (0.1%), cefamandole (4 μg/mℓ), polymyxin B (80 units/mℓ), and anisomycin (80 μg/mℓ),[81] or on BCYE agar supplemented with the above components plus glycine (0.3%), vancomycin (1 μg/mℓ), bromthymol blue (10 μg/mℓ), and bromcresol purple (10 μg/mℓ).[82] The latter was superior to the former medium for isolation of *Legionella* from potable water.[82] Clearly, no one method has proven to be superior for isolation of *Legionella* from water samples. The number and types of contaminating bacteria vary with the source of water. For example, the mean of bacteria in potable water is much lower than in cooling tower water from air-conditioning systems.[89]

4. Heat Treatment Before Plating

Heating respiratory tract specimens was tested to determine whether recovery of *Legionella* would be enhanced.[83] Seven DFA-positive but culture-negative patient specimens were heated in a water bath at 60°C for 1, 2, and 3 min, placed in ice water for 15 sec, and held at room temperature for plating. Heated respiratory tract specimens plated on antibiotic-containing BCYE medium were culture positive in 17 of 28 specimens vs. of 13 of 28 without heat treatment. However, heat treatments did not always significantly reduce the

numbers of contaminating bacteria except for one specimen containing *P. aeruginosa*.[83] When all else fails, heat treatment should be considered.

5. Other Culture Modifications

L. pneumophila cultured from patient specimens and environmental sites were not inhibited by 0.3% glycine incorporated into BCYE agar medium.[84] However, this concentration of glycine did inhibit growth of other bacteria. Optimum recovery of *Legionella* was reported with BCYE agar containing 0.3% glycine, polymyxin B (100 units/mℓ), and vancomycin (5 μg/mℓ). However, a single isolate of *L. gormanii* did not grow on this medium. This medium has produced good isolation results from additional environmental samples.[85] Also, *Legionella* were isolated from environmental samples taken from potable water plumbing fixtures and plated on BCYE agar, or BCYE agar supplemented with polymxin B (50 units/mℓ), vancomycin (1 μg/mℓ), 0.001% bromthymol blue, and 0.001% bromcresol purple.[50]

D. Conclusions

There seems to be general agreement that BCYE agar medium is adequate for isolation of *Legionella* from patient specimens that do not contain other bacteria. However, the use of media supplemented with various antibiotics seem to produce variable isolation rates among the various laboratories where parallel tests have been performed. Further evaluation of antibiotics, medium without charcoal, antibody-containing medium, and dye-containing medium will be needed before definite conclusions can be reached on the applicability of these modifications to patient specimens.

Isolation of *Legionella* from environmental sources has been intensively investigated by several authors. Antibiotic or low pH treatments have increased recovery rates in some situations but not others. Antibiotic and/or amino acid supplementation of BCYE agar medium has been useful, especially for culture of potable water samples or associated plumbing fixtures. Culture media containing dyes have also permitted more rapid presumptive identification of species other then *L. pneumophila*. Although differential/selective culture media have been evaluated for over 5 years, most authors recommend that patient and environmental samples continue to be plated on BCYE agar medium in addition to one of the supplemented agar media.

VI. MAGNETOPHORETIC ENRICHMENT

Many of the new procedures for diagnosis of legionellosis have been adapted from methods that were successful with other bacteria. *Legionella* use amino acids instead of glucose as a source of carbon and energy.[86,87] *Legionella* are also antigenically and genetically different from other bacteria. However, these characteristics have not led to the development of a rapid laboratory test that will confirm the presence of low numbers of *Legionella* in patient specimens that are contaminated with other bacteria such as oropharyngeal flora. The DFA test, although specific, requires an average number of about 10^5 *Legionella* per milliliter of specimen before one fluorescent organism will be observed in each oil immersion field with a UV light microscope.

One approach to this situation has been development of magnetic particles,[88] smaller than bacteria, that contain anti-*Legionella* antibody on their surface. When such particles are placed in a sample containing *Legionella*, the particles attach to the surface of the bacteria, thus making them susceptible to a magnetic field. If the sample is placed near a strong magnetic gradient, *Legionella* will be pulled toward the magnet while other bacteria will not be affected. Nonmagnetic bacteria that do not have anti-*Legionella* magnetic particles bound to their surface can be removed, leaving behind a sample enriched for *Legionella*. The sample can then be plated on agar medium or tested by DFA or other rapid tests for

specific antigen. In practice, this method has permitted an enrichment of approximately 10^4-fold with some samples.[90]

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