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MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES

CE

NOTE: Changes highlighted

PRODUCT AVAILABILITY

The following Organism Antigen Substrate Slides are available individually from Bion Enterprises, Ltd. (Bion):

Antigen Substrate Slide	Code No. REF
Mycoplasma pneumoniae	MP-1212
Σ Number of Tests	12-Well

INTENDED USE

The Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES may be used as the antigenic substrate in indirect fluorescent antibody assays for the qualitative and/or semi-quantitative determination of Mycoplasma pneumoniae antibodies in human serum. The Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES are intended for use as an aid in the diagnosis of active infection and as a determination of immunological experience with Mycoplasma pneumoniae.

SUMMARY AND EXPLANATION

Mycoplasma pneumoniae was the first mycoplasma for which an etiologic role in human disease was demonstrated. The first descriptions of Primary Atypical Pneumonia (PAP) as a clinical syndrome appeared in the late 1930's, with the recognition that some cases of pneumonia failed to respond to therapy with sulfonamides or penicillin. The infectious nature of PAP was demonstrated by Eaton and colleagues and the offending organism came to be called Eaton's agent.[1]

Most bacterial pneumonias (typical pneumonias) have an abrupt, often rigorous onset. Mycoplasma (atypical) pneumonia is characterized by flu-like symptoms, including a dry cough. Symptoms are typically chronic in both onset and recovery. The nondescript and somewhat chronic nature of the disease prompted the nickname "walking pneumonia". M. pneumoniae is the leading cause of pneumonia in older children and young adults, causing up to 20% of all cases of pneumonia.[2] It is a common cause of a wide range of upper and lower respiratory tract infections. While the majority of these infections appear to be relatively mild cases of pneumonia, more severe infections, such as pneumonia requiring hospitalization or lung abscess can also occur.[3] This disease may also be found with non-respiratory complications in other organ systems, and the subsequent infection may be life-threatening.[4]

Mycoplasmal and viral pneumonia, pharyngitis, and tracheobronchitis are often clinically indistinguishable. It is important to differentiate mycoplasmal and viral infections because mycoplasmal infections are readily treatable with tetracycline and its derivatives and with erythromycin, while viral infections do not respond to antibiotics.[2] The organism remains in the respiratory tract for prolonged periods, even after symptoms subside, and this feature may contribute to the endemic nature of the disease. Unlike other respiratory diseases, M. pneumoniae infections are not seasonal.[5] The disease spreads slowly in families and transmission seems to require close contact between family members.[5]

Diagnosis of mycoplasmal infections by isolation of the organism is difficult because mycoplasmas grow slowly and require complex media for isolation. Therefore, diagnosis of Mycoplasma pneumoniae infections has relied heavily upon serological methods such as complement fixation (CF) tests. However, due to the fact that the lipid antigen of the organism is found broadly distributed in nature, this has resulted in clearly false positive assay results using this test method.[7] For this reason, other assays have been developed in an attempt to overcome the problem of nonspecific lipid antigen. Enzyme linked immunosorbent assays (ELISA)[8] and whole organism immunofluorescence assays (IFA)[9,10,11] appear suitable for the detection of infection caused by Mycoplasma organisms. In IFA, the original problem of fixing the organism to glass slides was alleviated by use of cell cultures, which provide both a plane of focus and a matrix for adherence of mycoplasmas. Both ELISA and IFA can be used to detect IgG, IgA or IgM antibodies in serum and IgA antibodies in secretions.[6]

PRINCIPLE OF THE IFA PROCEDURE

The Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES may be utilized in the indirect fluorescent antibody assay method first described by Weller and Coons[12] and further developed by Riggs, et al.[13] The procedure is carried out in two basic reaction steps:

Step 1 - Human serum is reacted with the antigen substrate. Antibodies, if present, will bind to the antigen forming stable antigen-antibody complexes. If no antibodies are present, the complexes will not be formed and serum components will be washed away.

Step 2 - Fluorescein labeled antihuman IgG (or IgM) antibody is added to the reaction site which binds with the complexes formed in step one. This results in a positive reaction of bright apple-green fluorescence when viewed with a properly equipped fluorescence microscope. If no complexes are formed in step one, the fluorescein labeled antibody will be washed away, exhibiting a negative result.

REAGENTS

Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES are individually foil-wrapped twelve well slides with Mycoplasma pneumoniae (FH Strain) infected HEp-2 cells fixed onto each well. Each reaction well will contain a cell monolayer along with microorganisms and colonies of the Mycoplasma pneumoniae antigen.

STORAGE AND STABILITY

The Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES are stable in sealed foil pouches at 8°C or lower until labeled expiration date.

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic use. Thus, only staff trained in methods of in vitro diagnsotics may perform the test. IVD
2. Substrate slides are for single use only and must not be used more than once.
3. Use with non-Bion reagents could result in erroneous results.
4. Care should be taken when handling substrate slides due to sharp edges.
5. The antigenic substrates have been fixed and contain no detectable live mycoplasmal agents. However, they should be handled and disposed of as any potentially biohazardous laboratory material.
6. Do not remove slides from pouches until ready for testing. Do not use if pouch has been punctured, as indicated by a flat pouch.
7. Antigen substrate slides should be brought to room temperature (20-25°C) prior to use.
8. Abnormal test results may be seen if the antigen substrate slides are allowed to dry during the staining procedure.
9. Refrigeration (2-8°C) of antigen substrate slides immediately upon arrival will insure stability until labeled expiration date.
10. Antigen substrate slides should not be used beyond stated expiration date.
11. Avoid microbial contamination of all reagents involved in the testing procedure or incorrect results may occur.
12. Incubation times or temperatures other than those specified may give erroneous results.
13. Reusable glassware must be washed and thoroughly rinsed free of detergents.
14. Care should be taken to avoid splashing or generation of aerosols.
15. Previously frozen specimens after thawing should be thoroughly mixed prior to testing. It is recommended that sera is freeze thawed no more than one time. If repeated testing is required, it is suggested that specimen be aliquoted.
16. Patient samples, as well as all materials coming into contact with them, should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/NIH manual "Biosafety in Microbiological and Biomedical Laboratories", 1984 Edition. Never pipette by mouth. Avoid contact with skin and mucous membrane.

SPECIMEN COLLECTION

Blood should be collected fasting or at least one hour after meals to avoid lipemic serum, as excess lipids may produce a "film" over the substrate. Aseptically collect 5-8 ml of blood by venipuncture. Allow the blood to clot at room temperature (20-25°C) before separating serum to avoid hemolysis which could interfere with test results. Specimens should be stored refrigerated at 2-8°C and tested within one week of collection. Long term storage should be at -20°C in aliquots to avoid repeated freezing and thawing. Do not store in self-defrosting freezer.

Avoid using contaminated sera as they may contain proteolytic enzymes which will digest the substrate. It is unnecessary to heat inactivate serum specimens prior to testing; however, sera that have been heat inactivated may be used.

When testing paired samples to look for evidence of recent infection, the acute specimen should be obtained as soon as possible after onset of illness and the convalescent specimen obtained 7-14 days later. Acute and convalescent specimens must be tested simultaneously, in the same assay, looking for a significant change in antibody titer between the paired sera. If the first specimen is obtained too late during the course of the infection, a significant rise in the antibody titer may not be detected.

PROCEDURE

Detailed descriptions of indirect immunofluorescence techniques may be found in the references listed in the bibliography.[14,15,16]

MATERIALS PROVIDED

Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES. Lot Number provided on label. LOT

MATERIALS AVAILABLE FROM Bion

1. Fluorescent Antibody Conjugate with 0.01% Evans Blue counterstain
2. Mycoplasma pneumoniae Positive Human Control Serum
3. Mycoplasma pneumoniae Negative Human Control Serum
4. Phosphate Buffered Saline (PBS)
5. Mounting Medium
6. IgG Binding Reagent

MATERIALS REQUIRED BUT NOT PROVIDED

1. Disposable test tubes (12 x 75 mm or comparable) and rack
2. Disposable serological pipettes
3. Calibrated pipettes to deliver 50 µl, 100 µl and 200 µl with disposable pipette tips
4. Pasteur pipettes and bulbs
5. Moist chambers
6. Plastic squeeze wash bottle
7. Coplin jars or staining dishes with slide racks
8. 24 x 60 mm #1 coverslips
9. Felt tip marking pen
10. Fluorescence microscope equipped with a mercury or tungsten-halogen light source, a 390-490 nm excitation filter and 515-520 nm barrier filter, and optics to give a total magnification of 200X or 250X. The excitation wavelength of FITC is 490 nm and the emission wavelength is 520 nm.

TEST PROCEDURE

1. **SPECIMEN PREPARATION**

Screening:

Each laboratory should establish its own protocol for the preparation of serum screening dilutions. Most indirect fluorescent antibody staining procedures utilize a 1:10 dilution of each patient's serum which is prepared by adding 0.05 ml (50 µl) of the patient's serum to 0.45 ml of PBS. However, due to the large number of low titers seen in the population, it is suggested that a 1:160 IgG screening dilution is utilized.

NOTE: If testing for IgM specific antibodies using an IgM specific fluorochrome conjugate, each patient serum specimen must be pre-treated to remove any IgG interference by separating the IgM from the IgG, and then running the screening test on the IgM eluate. Suggested methodologies are ion exchange chromatography[17] or IgG immunoprecipitation.[18,19]

IgG Binding Reagent Catalog No. GBR-9982 available from BION Enterprises, Ltd.

Semi-quantitation:

Serum dilutions are utilized to measure antibody titer. Each laboratory should establish its own titering protocol. The selection of either twofold or fourfold dilution procedures depends upon the experience level and training of the individual(s) reading the fluorescent antibody assay.

The following fourfold serial titration is suggested for IgG testing:

a. Prepare a 1:10 dilution of each patient's serum by adding 0.05 ml (50 µl) of patient's serum to 0.45 ml of PBS in tube #1.

b. Add 0.3 ml PBS to tubes #2, #3, #4, #5 and #6.

c. Using a 100 µl pipette, transfer 0.1 ml (100 µl) from tube #1 to tube #2. Mix. Using a new tip for each dilution, transfer 0.1 ml (100 µl) from the second tube to the third, from the third tube to the fourth, from the fourth tube to the fifth, and from the fifth tube to the sixth mixing after each transfer.

d. Do not use tube #1 & #2. Starting with tube #3, apply to substrate slide.

The following twofold titration is suggested for IgM testing:

a. Prepare a 1:10 dilution of each patient's serum using one of the treatment methodologies mentioned in the "Screening NOTE" above. This will be designated as tube #1.

b. Add 0.2 ml PBS to tubes #2, #3, #4, and #5.

c. Using a 200 µl pipette, transfer 0.2 ml (200 µl) from tube #1 to tube #2. Mix. Using a new tip for each dilution, transfer 0.2 ml (200 µl) from the second tube to the third, from the third tube to the fourth, and from the fourth tube to the fifth, mixing after each transfer.

d. Do not use tube #1. Starting with tube #2, apply to substrate slide.

LIMITATIONS OF THE PROCEDURE (continued)

11. Endpoint reactions may vary between laboratories due to differences in type or condition of fluorescence microscope employed, diluting apparatus, IgG/IgM separation methods, as well as the experience level of personnel performing the assay.
12. If both the positive and negative control substrate cells are not visible when viewed using the fluorescence microscope, it may be necessary to replace or realign the light source and check the specific filters.
13. Cell culture substrate slides may exhibit nonspecific fluorescence due to contamination of antibodies or PBS rinse-wash solutions with bacteria or fungi. It is very important that personnel reading the staining results have experience in fluorescence microscopy.
14. If testing for IgM specific antibodies, the presence of Rheumatoid Factor (RF) in serum may cause a false positive reaction if pathogen specific IgG is also present. Routine RF tests may not be sensitive enough to detect small amounts of RF which exist within the normal range, but are sufficient to cause a false positive reaction in the more sensitive IFA technique.[24] Therefore, all sera should be treated by ion exchange chromatography[17] or IgG immunoprecipitation[18,19] before testing to eliminate possible RF interference.
15. If testing for IgM specific antibodies, high titers of specific IgG when present in the patient serum may compete with the pathogen specific IgM for the antigen sites resulting in a false negative IgM reaction.[24] Therefore, all sera should be treated by ion exchange chromatography[17] or IgG immunoprecipitation[18,19] before testing to avoid this possible problem.
16. Two methods such as immunoprecipitation and ion exchange chromatography have been commonly used for neutralizing or removing possibly interfering IgG antibodies prior to testing for specific IgM antibodies in IFA tests. Immunoprecipitation neutralizes all classes of IgG while not affecting the IgM levels; however, high levels of IgG may need to be treated with proportionally increased amounts of the precipitating reagent. Ion exchange chromatography will only eliminate IgG subclasses 1, 2 and 3 with subclass 4 (usually less than 5% of the total IgG) remaining in the fraction with the IgM. Also, only a portion of the IgM antibodies can be recovered.[27]
17. IgM serology performed by IFA is very attractive since it combines specificity with sensitivity and in most cases only a single serum sample is required. However, the need for careful interpretation of the significance of positive IgM tests in relationship to patient's clinical situations must be emphasized. It is essential to have an awareness of understanding of the many problems associated with IgM testing to avoid the many pitfalls that can trap the most experienced of workers.
    IgM results must always be interpreted with caution.[27]

SPECIFIC LIMITATIONS OF THE M. PNEUMONIAE ASSAY

1. One specific Mycoplasma organism, Mycoplasma genitalium, is known to resemble Mycoplasma pneumoniae. It has demonstrated serological cross-reactivity with Mycoplasma pneumoniae.[25]
2. A fourfold rise in IgG titer is diagnostic; however, because the symptoms of M. pneumoniae are so insidious and mild, many patients do not initially seek medical care but wait until the disease fails to improve. Thus, the acute phase sera are not readily available. Testing for specific IgM antibodies can be beneficial in these cases.
3. The level of IgG antibodies normally rises much later than the IgM level. Therefore, early in the disease, serology tests will be negative if only testing for IgG.[28]
4. Many older patients demonstrate high titers of IgG but do not produce IgM antibodies. This is probably a result of reinfection.[28]
5. Because antibodies persist long after infection, the presence of IgG antibody cannot be used as an indicator of recent infection; however, high levels of antibody are suggestive and indicate the need to test for IgM and/or look for a rise in IgG titer between acute and convalescent sera.[6]

EXPECTED VALUES

Mycoplasma-specific immunoglobulin titers are known to peak during, or shortly after acute infection, and then decline. The systemic antibody response to M. pneumoniae demonstrates an initial rise of IgM titer, usually seven to ten days following infection with the microorganism, and then the development of a Mycoplasma IgG titer.[11] The time interval between the development of IgM and IgG antibodies is quite brief, and it is not common to find a serum specimen from an acutely ill patient that is positive for only IgM immunoglobulins.[11] During convalescence the Mycoplasma IgM titer drops and eventually becomes undetectable by IFA. The time period of this antibody drop varies with the individual patient and, in a low percentage of patients, IgM may persist at low levels for an extended period of time. For this reason, it is suggested that IgM antibody levels be titered out to look for a drop in titer as evidence of convalescence. IgG persists indefinitely. It has been established that immunity to M. pneumoniae is not complete, and recurrent infections have been reported.[26]

A study was performed on 60 normal blood donors from the Midwestern United States using the Bion MYCOPLASMA PNEUMONIAE-G ANTIBODY TEST SYSTEM to determine the range of normal adult IgG antibody titers to Mycoplasma pneumoniae. In this study, titers range from 1:40 to 1:2560 with a mean titer of 1:160 (63%). [27]

SPECIFIC PERFORMANCE CHARACTERISTICS

Relative specificity and sensitivity evaluations using Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES were conducted in comparison with two other commercially available IFA test systems for the presence of IgG antibodies to Mycoplasma pneumoniae. A panel consisting of 62 specimens with titers ranging from less than 1:20 to 1:2560 was compared qualitatively and semi-quantitatively. Qualitatively there was an overall agreement in 20 of 62 specimens (32%) and 34 of 62 specimens (55%) between the BION Test System and the other two commercially available test sytems respectively. The relative sensitivity was 100% and 93.3% respectively; whereas, the relative specificity was 16% and 19% respectively. This appeared to be due to the fact that when compared semi-quantitatively for IgG antibodies, BION titers were consistantly several fold higher than the two other commercially available test systems. **TABLE 1** summarizes comparative results from 12 serum specimens ranging in titer from less than 1:20 to 1:5120 indicating an increase in relative sensitivity with the BION Test System. As further evidence of sensitivity, for the 60 normal blood donors tested, BION's calculated IgG mean titer was 1:218, whereas the other test systems' calculated mean titers were 1:68 and 1:37 respectively.[27]

Relative specificity and sensitivity evaluations using the Bion MYCOPLASMA ANTIGEN SUBSTRATE SLIDES were conducted in comparison with two other commercially available IFA test systems for the presence of IgM antibodies to Mycoplasma pneumoniae. A panel consisting of 58 specimens was compared qualitatively with the two other commercially available test systems. Positive Complement Fixation titer ($\geq$1:64) were present in 46 specimens with positive cold agglutinin results ($\geq$1:32) present in 30 specimens. There was an overall agreement in 57 of 58 specimens (98%) on the three systems. *One specimen was negative in one test system but positive in the other two. Twelve serum specimens ranging in titer from less than 1:20 to 1:2560 were compared semi-quantitatively. Three were negative for IgG and IgM, four were positive for IgG and negative for IgM and five were positive for both IgG and IgM. Included are Complement Fixation (CF) and Cold Agglutinin (CA) results if available. Whereas the IgG test results indicate an increase in relative sensitivity with the BION System, the IgM test results showed that the three systems were essentially equivalent. These results are summarized in **TABLE 2.**[27]

TABLE 1 - Summary of Relative IgG Sensitivity Testing

Spec #	BION	1st Other	2nd Other
1	<1:20	<1:20	<1:20
2	<1:20	<1:20	<1:20
3	<1:20	<1:20	<1:20
4	1:20	1:20	<1:20
5	1:80	1:40	1:40
6	1:80	1:40	<1:40 QNS
7	1:160	1:40	1:40
8	1:160	1:40	1:40
9	1:160	1:40	1:20
10	1:640	1:80	1:40
11	1:2560-5120	1:1280	1:1280
12	1:2560	1:160-1:320	1:80

TABLE 2- Summary of Relative IgM Sensitivity Testing

Spec #	BION	1st Other	2nd Other	CF Titers	Cold Agg. Titers
1	<1:20	<1:20	<1:20	ND	ND
2	<1:20	<1:20	<1:20	ND	ND
3	<1:20	<1:20	<1:20	ND	ND
4	<1:20	<1:20	<1:20	ND	ND
5	<1:20	<1:20	<1:20	ND	ND
6	<1:20	<1:20	<1:20	ND	ND
7 Acute	1:40	1:40	1:20	ND	ND
8 Conv.	1:2560	1:2560	1:1280	1:2048	1:16
9 Acute	<1:20	<1:20	<1:20	<1:8	1:64
10 Conv.	1:2560	1:640	1:640	1:512	1:64
11 Acute	1:40	1:40	1:40	1:256	1:32
12 Conv.	1:2560	1:640	1:640	>1:1024	1:256

Interlot and intralot precision using Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES was evaluated by testing 12 serum specimens for Mycoplasma pneumoniae IgG antibodies; 3 negative and 9 positive sera ranging in titer from less than 1:20 to 1:5120. They were tested on three different lots of substrate slides, and three times on one lot. In each instance there was no more than a twofold difference (+/-) in titer between any of the comparison testings, which is within the confidence limits of this methodology. None of the sera vacillated between a positive or negative result.[27]

Interlot and intralot precision of the Bion MYCOPLASMA PNEUMONIAE ANTIGEN SUBSTRATE SLIDES was evaluated by testing 12 serum specimens for Mycoplasma pneumoniae IgM antibodies; 7 negative and 5 positive sera ranging in titer from less than 1:20 to 1:2560. They were tested on three different lots, and three times on one lot. In each instance there was no more than a twofold difference (+/-) in titer between any of the comparison testings, which is within the confidence limits of this methodology. None of the sera vacillated between a positive or negative result.[27]

In addition, 20 specimens from 13 patients with serological diagnosis of Mycoplasma pneumoniae infection based on Complement Fixation (CF) and Cold Agglutinin (CA) test results were compared on all three IFA Test Systems. Eighteen specimens were positive on all three IFA systems; five had either the CF or the CA not done; and for two the CF and CA results weren't available. *One acute specimen was negative on all systems except the Cold Agglutinin test and ** one specimen was positive on all test systems except the second other commercially available IFA system. Positive IgM IFA test results may allow diagnosis to be made immediately on results from acute specimens without waiting for convalescent specimens to show a significant rise in titer.

TABLE 3 - Comparison of IgM Test Results for IFA, CF and CA

Patient No.	Specimens	BION Test System	1st Other Test System	2nd Other Test System	CF Titers	CA Titers
1	Acute Convalescent	=/>1:160 =/>1:160	1:80 =/>1:160	1:20 =/>1:160	1:32 1:1024	1:256 1:1024
2	Acute Convalescent	=/>1:160 =/>1:160	1:80 =/>1:160	1:20 =/>1:160	1:16 1:2048	Not Done
3	Acute Convalescent	=/>1:160 =/>1:160	1:80 =/>1:160	1:80 =/>1:160	1:256 =/>1:1024	1:32 1:256
4	Acute Convalescent	=/>1:160 =/>1:160	=/>1:160 =/>1:160	=/>1:160 =/>1:160	1:256 =/>1:1024	Not Done
5	Acute Convalescent	=/>1:160 =/>1:160	=/>1:160 =/>1:160	1:80 =/>1:160	1:128 1:1024	1:128 1:128
6 *	Acute Convalescent	<1:20 =/>1:160	<1:20 =/>1:160	<1:20 =/>1:160	<1:8 1:512	1:64 * 1:64
7	Acute Convalescent	=/>1:160 =/>1:160	=/>1:160 =/>1:160	=/>1:160 =/>1:160	Not Available	Not Available
8 **	Single Spec.	1:40	1:40	<1:20**	1:512	1:256
9	Single Spec.	=/>1:160	=/>1:160	=/>1:160	=/>1:512	=/>1:512
10	Single Spec.	=/>1:160	=/>1:160	=/>1:160	1:64	1:128
11	Single Spec.	=/>1:160	=/>1:160	=/>1:160	1:64	1:256
12	Single Spec.	=/>1:160	=/>1:160	=/>1:160	1:128	1:64
13	Single Spec.	=/>1:160	=/>1:160	=/>1:160	Not Done	1:512

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