A SOLID PHASE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE ANTIGENIC DETECTION OF *LEGIONELLA PNEUMOPHILA* (SEROGROUP 1): A COMPLIMENT FOR THE SPACE STATION DIAGNOSTIC CAPABILITY

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ABSTRACT

A SOLID PHASE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE ANTIGENIC DETECTION OF *LEGIONELLA PNEUMOPHILA* (SEROGROUP 1): A COMPLIMENT FOR THE SPACE STATION DIAGNOSTIC CAPABILITY

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It is necessary that an adequate microbiology capability be provided as part of the Health Maintenance Facility (HMF) to support expected microbial disease events and environmental monitoring during long periods of space flight. The applications of morphological and biochemical studies to confirm the presence of certain bacterial and fungal disease agents are currently available and under consideration. This confirmation would be facilitated through employment of serological methods to aid in the identification of bacterial, fungal, and viral agents.well. A number of serological approaches are currently being considered, including the use of Enzyme-Linked Immunosorbent Assay (ELISA) technology, which could be utilized during microgravity conditions.

A solid phase, membrane supported ELISA for the detection of *Legionella pneumophila* (Serogroup 1), an expected disease agent, was developed to show a potential model system that would meet the HMF requirements and specifications for the future space station. These studies demonstrate the capability of membrane supported ELISA systems for identification of expected microbial disease agents as part of the HMF.

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INTRODUCTION

The health and well being of individuals aboard a space station and possibly during future long space missions is of priority and must be assured. Certain expected clinical syndromes and diseases have been identified through an infectious disease conference conducted at the Johnson Space Center during October, 1985. Legionella pneumophila was identified as a possible problem due to its tendency to grow in filtration and water systems. Since 1976, 23 Legionella species comprising 37 serotypes have been identified. L. pneumophila, the most prevalent Legionella species in the United States, currently contains 12 serogroups, all which have been involved in pneumonia in humans (8). Previous spaceflight studies indicate a high probability of cross-contamination among crew members and microbial build up of space modules during long confinements, such as minimal (90 day) missions which are planned for the space station (7). Continual habitation, crowded conditions, possible immunosuppression, and other factors may create critical situations aboard the space craft. If a microbial disease is suspected, the major effort would be directed toward obtaining some indication of the specific kind of microorganism causing the problem. The exact nature of the etiological agent would determine the severity of the disease, treatment, prophylaxis, and subsequent health measures for the space station environment.

The diagnosis of a microbial disease currently rests upon one of a combination of clinical signs and symptoms, morphological and biochemical identification of isolates, and/or serological procedures. Special procedures such as cell culture may also be required. One problem with

limiting the scope of diagnosis to clinical signs and symptoms is that a particular microbe can sometimes produce infection having very different clinical characteristics and occurring in widely different areas of the body. For example, antibiotic-resistant Staphylococcus aureus may produce skin and subcutaneous tissue lesions as well as pneumonia, osteomyelitis, bacteremia, and acute membranous enterocolitis depending upon the means by which the organism gained entrance to the body, host resistance, antibiotic therapy, and other factors.

While the principle of one microorganism causing one clinical disease is often valid, there are many situations where this is not true. Indeed, pneumonias that are hardly separable clinically may be produced by several different kinds of bacteria and viruses. Correct diagnosis and treatment therefore heavily depend upon the abilities of the clinical laboratory.

Over the past few years, many new immunological methods have been developed which now provide the clinical laboratory with a large array of potentially valuable diagnostic tools. Antibodies and antigens labelled with radioisotopes or fluorescent dyes, or affixed to particulate materials, have been used extensively for immunodiagnosis over the past three decades. These methods do have disadvantages. Immunofluorescence, for example, usually depends upon a subjective assessment of end result, and the technique is frequently laborious. Radioimmunoassay requires expensive equipment and carries the risk of radioactive exposure and contamination. Moreover, the current methods for either technology are not applicable to microgravity. The concepts that antigen and antibody can be attached to a solid phase support yet retain immunological activity, and

that either can be coupled to an enzyme and the complex retain both immunological and enzymatic activity, led to the development of Enzyme-Linked Immunosorbent Assays (ELISAs). Antibodies and antigens have been shown to readily attach to plastic surfaces (such as polyvinyl or polystyrene) either by passive absorption or chemical conjugation, and still retain immunological activity. Antibodies and antigens have been linked to a variety of enzymes including glucose oxidase, peroxidase, and alkaline phosphatase. Table 1 indicates the positive factors for use of ELISA systems in diagnostic microbiology.

Table 1: Positive Factors for HMF Consideration of ELISA Systems.

- ✓ Low Cost
- ✓ Reagent stability
- ✓ Safety
- ✓ Sensitivity
- ✓ Specificity
- ✓ Reproducibility
- ✓ Ease of procedure
- ✓ Can be performed in poorly equipped laboratories
- ✓ No power requirements

It appears that the space station diagnostic capability will most likely require immunological testing applicable to the identification of microorganisms, particularly those that cannot be cultured or identified by standard laboratory techniques. In recent years, there has been increasing emphasis on accurate, reliable, and quick immunological procedures for the identification of many microorganisms and/or the immunological responses of

the host toward infection. Most current commercially available procedures have been developed for use in clinical laboratories and not designed for microgravity conditions which would be present in the space station environment (5). It appears and is reasonable that a number of some existing procedures, particularly solid phase immunoassays, could be modified in regard to uniformity and standardization for use aboard the space station. This project was designed to illustrate the concept of a solid phase, membrane supported ELISA for detection of L. pneumophila (Serogroup 1) to demonstrate the capability of ELISA systems for identification of expected microbial disease agents aboard the space station.

MATERIALS

Equipment: A 96 well Bio-Dot filtration apparatus (#170-6550) was obtained from Bio-Rad Laboratories, Richmond, Ca. 94801.

Buffers: A 20 mM Tris buffered saline (TBS), pH 7.5, was prepared by adding 4.84 g Tris (Bio-Rad) to 58.48 g NaCl, brought to a 2 liter volume with deionized water. The buffer was adjust to pH 7.5 with 1 M HCl.

Blocking Solution: Bovine serum albumin (BSA, #A-7030) was purchased from Sigma. A 3% BSA-TBS was prepared by adding 3 g of BSA to 100 ml of TBS.

Wash Solution: A wash solution containing 0.05% Tween-20 was prepared by adding 0.5 ml of Tween-20 (Bio-Rad) to 1 liter of TBS.

Antibodies: Antisera prepared in rabbits against L. pneumophila (strain Philadelphia 1 and OLDA) were obtained from Dr. Hazel Wilkinson, the Department of Health and Human Services, Center for Disease Control,

Atlanta, Ga. 30333. These antisera were pooled for coverage of all serogroup 1 subtypes (9). Horseradish perioxidase conjugated (HPR) goat anti-rabbit serum (#170-6500) was obtained from Bio-Rad Laboratories.

Nitrocellulose Membranes: Nitrocellulose membranes (#162-0117) with a pore size of 0.45 microns was purchased from Bio-Rad Laboratories.

Legionella Antigen: L. pneumophila antigen was prepared from an ATCC 3152 (serogroup 1) lyophilized culture vial (3). The lyophilized culture vial was broken and the lyophilized material was dissolved into 4 ml of Trypticase Soy Broth. Four 15 x 100 mm plates containing 25 ml of buffered charcoal yeast extract (BCYE) agar each of which was inoculated with 1 ml of the dissolved material. The plates were enclosed in a plastic container to prevent the agar from drying out and were incubated at 35 degrees centigrade for 48 hr. The cells were suspended from each agar surface in 3 ml of sterile 0.01 M phosphate buffered saline, pH 7.2, with a pasteur pipette into a 25 ml sterile conical tube. The conical tube containing the cell suspension was boiled for 1 hr to kill the cells. The killed cell suspension was centrifuged at 1600 x g for 30 min, the supernatant discarded, and the cells resuspended in 2 ml of 0.01 M phosphate buffer, pH 7.2, for each 0.1 ml of packed cells. One drop of a 1:1000 methiolate solution was added for each 2 ml of preparation. The stock solution was stored at 4 degrees centigrade for 10 days to allow for the release of soluble antigen from the cells. The suspension was centrifuged at 1600 x g and the supernatant used for assay development. The antigen preparation was subjected to the Lowry protein detection method indicating the antigen preparation concentration was approximately 0.025 mg/ml. Subsequent

calculations were determined from this estimate.

Stock Chromogenic Substrate Stain Solution: Two substrates were utilized for comparison. O-phenylenediamine (OPD, Abbott Laboratories) was prepared by dissolving 12.8 mg into 5 ml of citrate phosphate buffer, pH 7.2, containing 0.02% hydrogen peroxide. 4-chloro-1-napthol (4C1N, Bio-Rad) was prepared by dissolving 60 mg of 4C1N into 20 ml of ice cold methanol. Immediately prior to use, 0.06 ml of ice cold 30% hydrogen peroxide was added to 100 ml of room temperature TBS. The two solutions were mixed just prior to use.

Preparations Obtained for Specificity Studies: All antigen preparations tested were obtained from Difco Laboratories. These preparations included Salmonella O Poly A-I and Vi (#2364-47-2) which contains antigens from Group A, B, C1, C2, D2, E1, E2, E4, F, G, H, I, and Vi; Shigella Group A (#2100-50-1), A1 (#2101-50-3), B (#2102-50-2), C (#2103-50-0), C1 (#2104-50-0), C2 (#2105-50-9), and D (#2106-50-8); Pseudomonas aeruginosa antigen set (#3082-32-7); Streptococcus antigen set containing Groups A, B, C, D, E, F, and G (#2368-32-4).

Immunofluorescent Assay Kit: A MonAbrite Legionella Polyscreen kit was obtained from Serono Diagnostics, Inc., 100 Longwater Circle, Norwell, MA 02061 and used for comparison with results obtained by the ELISA. This kit recognizes 21 species and 33 serogroups of Legionella including Serogroup 1.

METHODS

The ELISA system utilized the Bio-Dot apparatus with mounted nitrocellulose paper. The procedure for assembly of the apparatus and

preparation of the nitrocellulose paper was provided by Bio-Rad Laboratories (1). Figure 1 illustrates the basic components of the system. Nitrocellulose paper was first soaked in TBS to ensure uniform

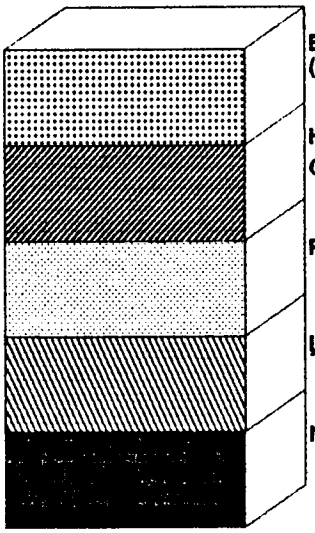

Figure 1: Major components of the ELISA for L. pneumophila.

protein bindings and low background absorption. The cleaned and dried Bio-Dot apparatus was assembled, and the nitrocellulose paper sheet wetted prior to being placed in the apparatus. The apparatus was appropriately tightened to insure that cross well contamination would not occur.

The flow valve was adjusted to allow the vacuum chamber to be exposed to the atmosphere and the appropriate wells to receive antigen preparations were inoculated with a 0.05 ml volume. The entire sample was allowed to filter through the membrane by gravity flow (approximately 30 min). Each well was filled with the same volume of sample solution to insure homogeneous filtration of all sample wells.

After the antigen samples completely drained from the apparatus, 0.2 ml of a 3% BSA/TBS blocking solution was applied to each well. Gravity filtration was allowed to occur until the blocking solution completely drained from each well (approximately 30 min).

The flow valve was adjusted to vacuum and 0.4 ml of wash solution (TBS with 0.05% tween) was added to each well. The wash solution was allowed to completely drain from all wells. This process was repeated. Following the wash step, the flow valve was opened to the atmosphere and 0.1 ml of the rabbit anti-Legionella antibody solution was added to each of the wells. The solution was allowed to completely drain from the wells, and another wash step performed.

With the vacuum off and the flow valve to the atmosphere, 0.1 ml of horseradish peroxidase labelled goat anti-rabbit solution was added to each well. The solution was allowed to completely drain from the wells.

Following the second antibody step, the vacuum was turned on and a wash step performed. Immediately, 0.2 ml of a color development solution, either OPD or 4C1N was applied to each well. A positive ELISA reaction was shown as color development depending upon the substrate utilized. The reactions were stopped by the addition of 0.3 ml of distilled deionized water to each well.

The MonAbrite Legionella Polyscreen (Serono Diagnostics, Inc) Immunofluroescent Assay Kit was accomplished by the procedure which accompanied the package. Briefly, test specimens and controls were applied as 0.05 ml aliquots to slides and heat fixed. Rabbit anti-Legionella was applied to each specimen with the exception of the control wells. After a

30 min incubation period, a wash procedure was performed. The slides were air dried and FITC-labelled goat anti-rabbit serum was applied to each specimen. Slides were washed, air dried, and examined for immunofluorescence utilizing a Nikon Episcopic-Fluorescence microscope with a B-1A filter cube.

RESULTS

Assay sensitivity: Dose response curves utilizing decreasing concentrations of L. pneumophila (Serogroup 1) antigen applied to the nitrocellulose membrane and titrated with increasing dilutions of L. pneumophila (Serogroup 1) antibody are shown in Figure 2. The reaction intensity was measured by eye from 0 to 4+, 4+ graded as the most intense of the color outcome. O-phenylenediamine was the substrate used in these experiments. As the antibody was diluted, the sensitivity of the ELISA was shown to decrease. Dilutions of antibody tested between 1:10 and 1:100 allowed the detection of L. pneumophila antigen to 0.0626 ng (62.6 pg); whereas, antibody dilutions of 1:500, 1:1000, 1:5000, and 1:10000 reduced the sensitivity of the ELISA to 0.3442, 0.626, 6.26, and 34.43 ng, respectively. Antibody dilutions including and exceeding 1:50000 did not allow for detection of L. pneumophila antigen. The optimal dilution of antibody for used in the ELISA was determined to be 1:100.

Assay specificity: Available antigen preparations from other bacterial groups were applied to the nitrocellulose membrane to test for cross reactivity with the anti-L. pneumophila serum. As indicated in Table 2, no cross-reactivity was detectable between the various groups or types of Salmonella, Shigella, Pseudomonas, or Streptococcus when these

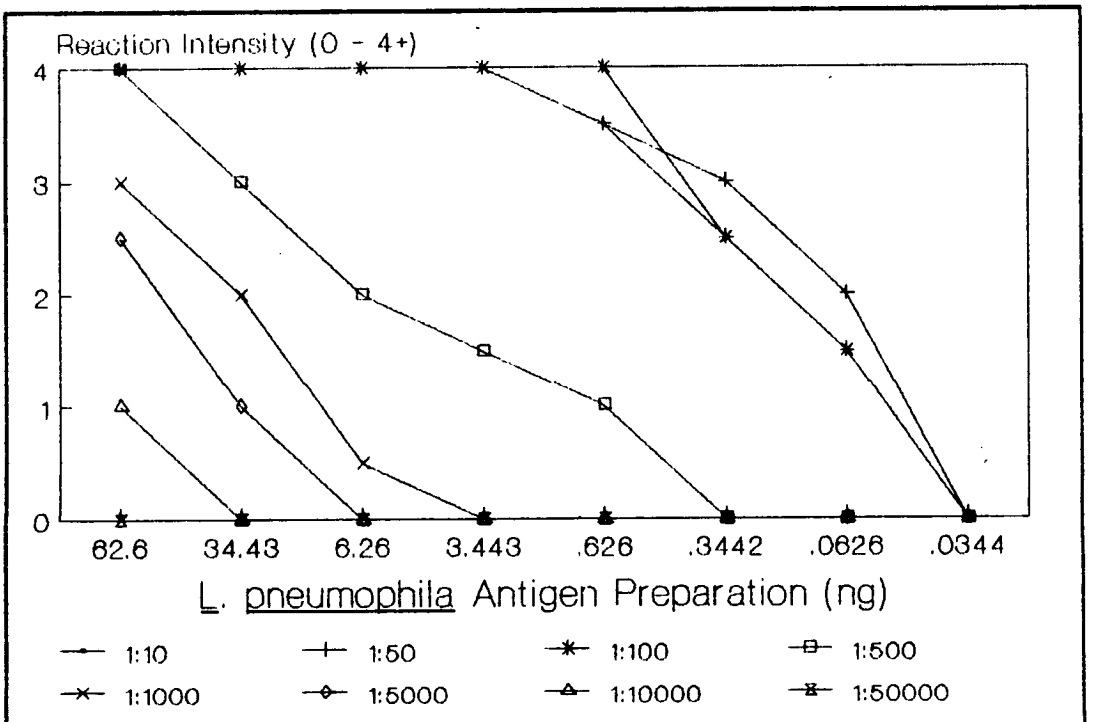

Figure 2: Titration of L. pneumophila (Serogroup 1) antigen and antibody preparations by ELISA methods. (Note: intervals are not equal units)

preparations were applied to the nitrocellulose membrane and tested by the ELISA. Each of the preparations were tested as 10x dilutions from the stock to a final dilution of 1:1000. Five urine, blood plasma, and two sputum samples were applied to the nitrocellulose membrane and were negative when tested by the ELISA. Dose response curves were not affected by either biological fluidwhen L. pneumophila was seeded into these specimens and applied to the nitrocellulose membrane.

Comparison of sensitivity between ELISA and IFA methods: The L. pneumophila antigen preparation was titrated by the MonAbrite Legionella Polyscreen kit (Serono Diagnostics, Inc.) and compared to that achieved by ELISA. The IFA results were based upon the brightness of field (0 to 4+)

Table 2: Antigens and Biological Fluids Testing Negative in the ELISA.

- Salmonella
  Groups A,B,C1,C2,D2,E1,E2,E4,F,G,H,I,Vi
- Shigella
  Groups A,A1,B,C,C1,C2,D
- Pseudomonas
  Types 1-17
- Stretococcus
  Groups A,B,C,D,E,F,G
- Urine
- Blood plasma
- Sputum

with relationship to the diluted antigen preparation. As indicated in Figure 3, ELISA results are similar to that indicated in Figure 2 for the standard dose response curve with minimum antigen detection between 0.0626 and 0.0344 ng. IFA results indicate a minimum detection limit of 0.626 ng of antigen, an approximate 10 fold reduction in sensitivity when compared with the results of the ELISA. A dilution curve of the polyvalent antigen control in the IFA kit was titrated by dilution using both ELISA and IFA techniques. In these determinations the ELISA was capable of detecting the control specimen to a 1:50 dilution; whereas, the IFA was readable to a 1:10 dilution. Since the Serono IFA kit is polyvalent and not specific toward Serogroup 1, strict interpretation of the comparsion of the

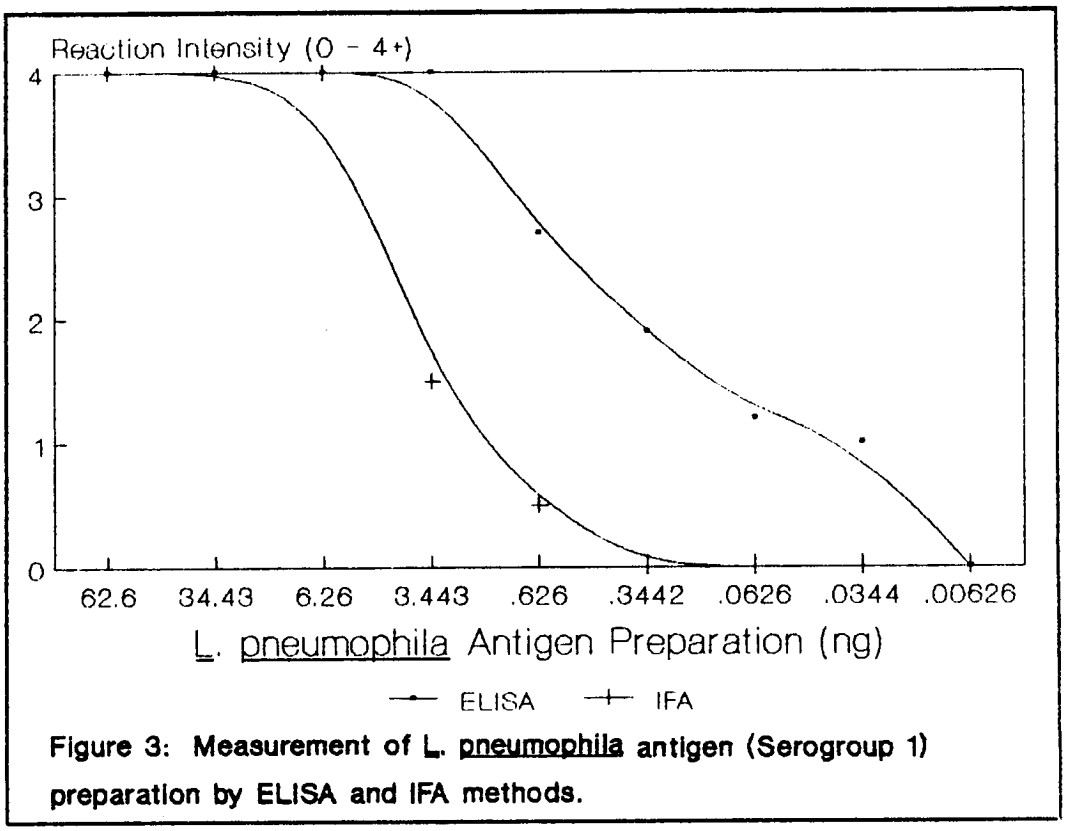

Figure 3: Measurement of L. pneumophila antigen (Serogroup 1) preparation by ELISA and IFA methods.

companion polyvalent antigen control between the two methods is not valid. However, in these determinations the ELISA was capable capable of detecting the control specimen to a 1:50 dilution; whereas, the IFA was readable to a 1:10 dilution.

Comparison of OPD and 4C1N as enzyme substrates: OPD (0-phenylenediamine) and 4C1N (4-chloro-1-napthol) were compared in the ELISA for their performance to detecting L. pneumophila antigen. As shown in Figure 4, the OPD was capable of detecting lesser amounts of antigen than 4C1N. Only 34.43 ng was detectable with 4C1N.

DISCUSSION

It appears logical that the methods selected for the diagnostic

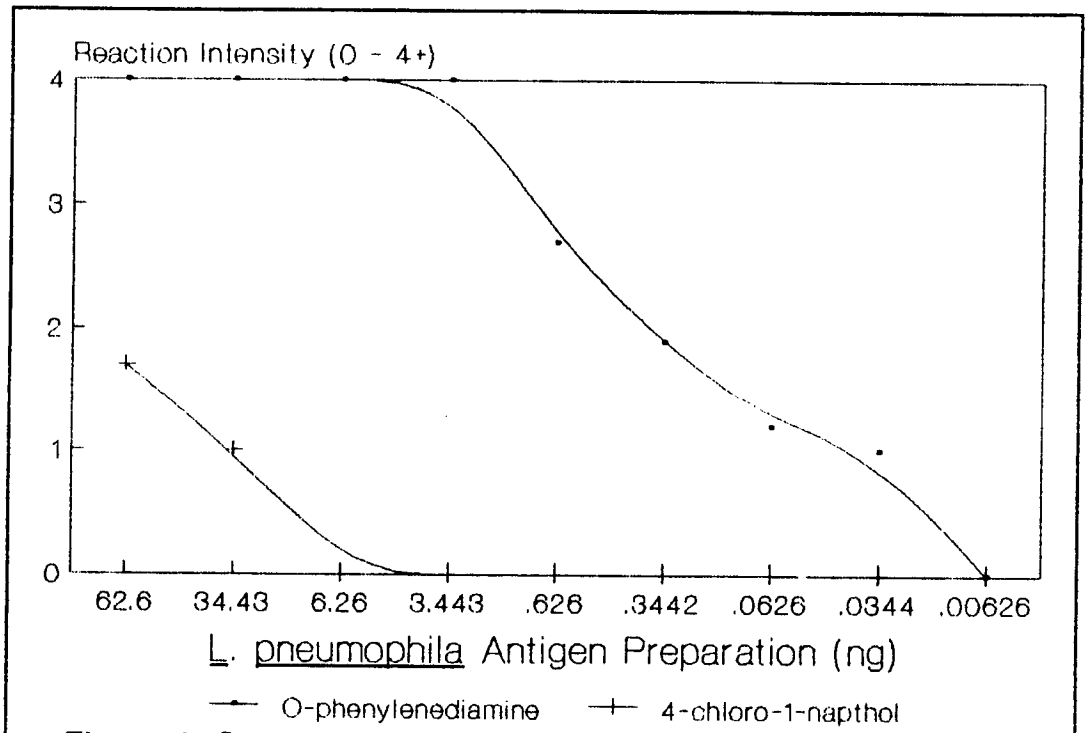

Figure 4: Comparison of OPD and 4C1N as Substrates in the ELISA.

systems Health Maintenance Facility (HMF) as well as microbiological environmental monitoring of space stations and vehicles will need to support identification of microorganisms that pose a health threat to the astronauts. A closed environmental system makes its necessary to monitor surfaces, water, air, waste systems, and possibly food for contamination with microorganisms. The growth of microorganisms in a closed recycled environment increases the potential for cross-contamination of crew members particularly when missions of 3 months and possible missions to Mars (1-3 years) are being considered. Depending upon the humidity and organic debris build-up in this environment, microorganisms may grow uninhibited. It is evident that the HMF and environmental monitoring systems must be in

place for microbial detection since quick return trips will be impossible. Thus, detection of a microbial problem could result in methods applied to eliminate that situation before more serious problems develop. Illnesses caused by microorganisms vary significantly in regards to symptoms, severity, prophylaxis, and treatment. As experienced during Apollo 13, a urinary tract infection with Pseudomonas aeruginosa could have resulted in a serious situation if the crew member had not been able to receive immediate health care (4). The potential for cross-contamination of crew members has been documented by chamber tests and experiences aboard the Skylab missions (2). These situations provide evidence that adequate microbiological diagnostic systems be in place aboard space vehicles or stations prior to their habitation for long time periods. There is currently considerable debate of on the effects of microgravity, radiation, temperature, and pressure on man, particularly immunosuppressive effects. If immunosuppression is indeed a reality during long space missions, the HMF must have the capability to not only provide identification of a microbial problem, but also medication against the invading microorganisms. During the past decade, numerous immunoassays have gained wide acceptance as the method of choice in the diagnosis of numerous disease states (6). The ideal considerations of a diagnostic test particularly for the NASA space program include speed, sensitivity, specificity, accuracy, safety, inexpensive reagents, potential for automation, long reagent shelf life, and broad applicability. Moreover, they must function in microgravity and not contain biologicals which would themselves threaten the health of the crew members. Although

many techniques have been recently developed for the immunological detection of antigens and/or antibodies, enzyme immunoassays such as the ELISAs are among the most popular both in research and clinical laboratories for the diagnosis of bacteria, protozoans, and viruses (5). In general, ELISAs are user-friendly, reliable, highly sensitive and specific, and require little time to run. Additional considerations include that no power source or instruments are required for the performance of the tests, little equipment is needed, reagents are stable, and results can be read by eye as intensive color changes.

The majority of commercially available ELISA systems are designed to detect antibody levels in blood plasma or other biological fluids such as urine and few have been developed for the detection of microbial antigens. At the present time, commercially available ELISA systems were not designed to be utilized in microgravity, and thus, little concern was given to designing these tests for those conditions. Only one ELISA, the Test Pack by Abbott Laboratories, released in June, 1986 for purchase, appears promising for space station use. This test detects the presence of Streptococcus pyogenes (Group A, beta-hemolytic streptococci) directly from throat swabs. The test performed well when tested aboard the KC 135 which simulates microgravity during parabolic curves.

Results from experiments conducted in this project and the existence of a commercially available kit paralleling these findings, provide a current technology to be considered for the HMF and environmental monitoring systems for space vehicles and stations. A major advantage to consider with the solid phase filter membrane systems is that the fluids

involved in the system can be retained resulting in little chance of spillage in the spacecraft environment. This project also presents an ELISA developed against Legionella pneumophila (Serogroup 1), the causative agent of Legionaires' disease. Because of its habitat, growth in filter and condensation units, this bacteria poses a possible health threat particularly in a closed recycled environment as that indicated aboard the space station and future space vehicles. The ELISA developed in this project could be utilized for monitoring and detecting its presence and levels in filters and condensates aboard these space crafts as well as to test a crew member for its presence in a respiratory disease syndrome.

REFERENCES

1. Bio-Dot Microfiltration Apparatus Instruction Manual. Bio-Rad Laboratories, Richmond, Ca., 1984.

2. Cintron, N.: JSC Microbiological and Toxicological Requirements for Space Station. Briefing Document, NASA/JSC Medical Sciences Division, Houston, Texas, August, 1985.

3. Farshly, C.E. and J.C. Feeley: ELISA for Legionaires' Disease - Antibody and Antigen Detection Systems: An Interim Report. In: "Legionaires'" the Disease, the Bacterium and Methodology. HEW Publication (CDC) #79-8375, 1979.

4. Furukawa, S.: Medical Support for Long Duration Space Missions. Applic. Space Technol., 3:203-209, 1983.

5. Hejtmancik, K.E.: Expansion of Space Station Diagnostic Capability to Include Serological Identification of Viral and Bacterial Infections. In: NASA/American Society for Engineering Education (ASEE) Summer Faculty Fellowship Program. B.C. McInnis and S.H. Goldstein (eds.), NASA Contractor Report #171984, June, 1987.

6. O'Beirne, A.J. and H.R. Cooper: Heterogeneous Enzyme Assay. J. Hist. Cyto., 27:1148-1157, 1979.

7. Pierson, D.L.: Microbiology Support Plan for Space Station. National Aeronautics and Space Administration Publication, JSC #32015, 1986.

8. Thacker, W.L., H.W. Wilkinson, R.F. Benson, and D.J. Brennex: Legionella pneumophila Serogroup 12 Isolated from Human and Environmental Sources. J. Clin. Microbiol., 25:569-572, 1987.

9. Wilkinson, H.W., B.J. Fikes, and D.D. Cruce: Indirect Immunofluoresence Test for Serodiagnosis of Legionaires' Disease: Evidence for Serogroup Diversity of Legionaires' Disease Bacterial Antigens and for Multiple Specificity of Human Antibodies. J. Clin. Micro. 9: 379-383, 1979.