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EVALUATION OF AN INDIRECT HEMAGGLUTINATION TEST FOR DETECTION OF ANTIBODY TO SEROGROUPS 1-4 OF *Legionella pneumophila* AND ITS USE IN A SERUM DEMIOLOGIC SURVEY OF MICHIGAN RESIDENTS

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EVALUATION OF AN INDIRECT HEMAGGLUTINATION TEST FOR DETECTION OF ANTIBODY TO SEROGROUPS 1-4 OF *Legionella pneumophila* AND ITS USE IN A SEROEPIDEMIOLOGIC SURVEY OF MICHIGAN RESIDENTS.

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ABSTRACT

EVALUATION OF AN INDIRECT HEMAGGLUTINATION TEST FOR DETECTION OF ANTIBODY TO SEROGROUPS 1-4 OF *Legionella pneumophila* AND ITS USE IN A SEROEPIDEMIOLOGIC SURVEY OF MICHIGAN RESIDENTS.

By

Cheryl A. Yonke

Parallel testing of 895 sera by the indirect hemagglutination (IHA) and indirect fluorescent antibody (IFA) methods for legionellosis showed 97.3% agreement between the techniques. Although the IHA showed more cross reactivity between serogroups than the IFA, the etiological serogroup could easily be defined. Since the IHA was shown to detect both IgM and IgG class antibodies and was found to be rapid, simple, and inexpensive, it appears to be an excellent alternative to the IFA test for serodiagnosis and seroepidemiologic studies of legionellosis.

When employed in a survey of 1200 apparently healthy Michigan residents, the IHA showed serogroup 1 antibody the most prevalent; 71 (11.8%) of 600 sera collected during the winter (January-April, 1980) and 131 (21.8%) of 600 sera collected during the summer (July-September, 1980) demonstrated serogroup 1 titers. Prevalence of antibody to serogroups 2, 3, and 4 was significantly lower, all showed less than 1% prevalence regardless of season of the year.

ABSTRACT

EVALUATION OF AN INDIRECT HEMAGGLUTINATION TEST FOR SEROGROUPS 1-4 OF *Legionella pneumophila*.

By

Cheryl A. Yonke, Harlan E. Stiefel, David L. Wilson, and Berttina Wentworth

Parallel testing of 895 sera by indirect hemagglutination and indirect fluorescent antibody techniques showed 97.3% agreement. Although the IHA usually showed more cross reactivity between serogroups than the IFA using formalin fixed antigens, heterologous serogroup reactions were significantly lower than homologous serogroup titers and the etiological serogroup could easily be defined. The IHA showed no cross reactivity with a crude extract of *E. coli* 013:K92:H4. Since the IHA was shown to detect both IgM and IgG class antibodies and was found to be rapid, simple, and inexpensive, it appears to be an excellent alternative to indirect fluorescent antibody testing for serodiagnosis of legionellosis.

ABSTRACT

PREVALENCE OF ANTIBODY TO SEROGROUPS 1-4 OF *Legionella pneumophila*: A SEROEPIDEMIOLOGIC STUDY USING THE INDIRECT HEMAGGLUTINATION TEST

By

Cheryl A. Yonke, Harlan E. Stiefel, David L. Wilson, and Berttina Wentworth

An indirect hemagglutination test was used to determine the prevalence of antibody to serogroups 1-4 of *Legionella pneumophila* in sera from 1200 apparently healthy Michigan residents. Serogroup 1 antibody was the most prevalent; 71 (11.8%) of 600 sera collected during the winter months (January-April, 1980) and 131 (21.8%) of 600 sera collected during the summer period (July-September, 1980) demonstrated serogroup 1 titers. This seasonal difference was independent of sex and was statistically significant in four of six age groups studied. A trend towards decreasing prevalence in the 50-59 and 60 or older age groups was noted in the winter sample and was statistically significant in the summer study. Prevalence of antibody to serogroups 2, 3, and 4 was significantly lower; all showed less than 1% prevalence, regardless of season.

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Introduction

The American Legion Convention at Philadelphia's Bellevue Stratford Hotel in July, 1976, captured world-wide attention when an outbreak of pneumonia occurred among conventioneers. The pneumonia, subsequently designated "Legionnaires' disease," was diagnosed in 182 persons who had been in or near the hotel (35). There were 29 deaths, a 16% mortality rate overall. A number of possible etiologies were investigated, including those of toxic and infectious nature, but it was not until January, 1977, that the causative agent became known. At that time, McDade and associates (59) at the Center for Disease Control succeeded in isolating a gram-negative bacillus after intraperitoneal injection of lung tissue from four fatal cases into guinea pigs and subsequent passage of the infected guinea pig spleen into embryonated hens' eggs. Indirect fluorescent antibody (IFA) testing on sera from surviving patients clearly defined the etiologic role of this infectious agent.

Further investigation proved that neither the organism nor the disease had gone entirely unrecognized in the past; IFA testing on stored sera from four previous epidemics (40, 52, 76, 77) of pneumonia identified the Legionnaires' bacillus as the causative agent. In addition, an unclassified agent, OLDA, isolated by Jackson et al. (45) in 1947 was shown by DNA hybridization studies and G+C (guanine + cytosine) content to be identical to the Philadelphia isolates (58).

THE DISEASE

Clinical Manifestations

Legionellosis has been shown to manifest itself in one of two forms:

1. An acute pulmonary form (Legionnaires' disease) observed in several epidemics and a large number of sporadic cases, or

2. A milder, self-limiting form, termed "Pontiac fever," seen in only two outbreaks to date (34, 40).

The pneumonic form begins with malaise, myalgia, and a nonproductive cough, following a two to ten day incubation period. Within two to three days of onset, a high, unremitting fever develops, frequently accompanied by relative bradycardia and pleuritic pain. Radiographic studies reveal patchy infiltration early in the disease, with increasing consolidation as the disease progresses. Routine laboratory examinations commonly demonstrate a moderate leukocytosis with a left-shift, proteinuria, hyponatremia, hypophosphatemia, azotemia, increased aminotransferases, and an increased erythrocyte sedimentation rate. Although largely pulmonary, the disease features several extrapulmonary effects including gastrointestinal (1, 35, 49, 72), renal (48), and central nervous system anomalies (53). A classical gram-negative endotoxin has been proposed to explain these multisystem effects (23, 38). The organism caused gelatin of *Limulus* amebocyte lysates (44, 91), but showed only low pyrogenicity in rabbits (91). No investigation has clearly demonstrated the existence of such a toxin.

Pontiac fever presents as a milder, non-pneumonic form of the disease with a significantly shorter incubation period (mean 36 hours). Fever, myalgia, and headache characterize the disease. Cough, diarrhea, vomiting, chest pain, and a sore throat were described in a small number

of patients, but did not appear to be prominent features of the syndrome. Hepatic and renal involvement or fatality has not been observed.

Pathology

The only consistent pathological feature in fatal cases of Legionnaires' disease is the finding of lesions in the lungs. The pneumonia is lobar, with no lobes preferentially involved (90). Consolidation is usually bilateral. Only recently has the Legionnaires' disease organism been demonstrated outside the thoracic cavity (86).

Microscopic examination of lung tissues from several Legionnaires' disease patients has revealed an acute fibrinopurulent pneumonia (4). Alveolar spaces contain an exudate composed of neutrophils, macrophages, and large amounts of fibrin. Lysis of these inflammatory cells has been associated with an increased number of the Legionnaires' bacillus (4). Coagulative necrosis has been observed in a few cases (90).

The bacterium stains poorly with routine histologic stains, including hematoxylin-eosin, Brown-Brenn, Brown-Hopps, and McCallum-Goodpasture; visualization of the organism in tissue sections or imprints is best achieved with the modified Dieterle (13, 84) or other silver impregnation stains (26).

Diagnosis

Diagnostic criteria for legionellosis include:

1. Direct *in vitro* isolation of the organism. The first direct *in vitro* isolation of the Legionnaires' bacillus was made in 1976 by Dumoff (21). The organism, recovered from pleural fluid, was cultured on GC base chocolate agar containing 1% hemoglobin and 1% Isovitalex.

Since that time, a number of primary isolation media have been developed, including Mueller-Hinton-Isovitalex-Hemoglobin (MH-IH) (31), Feeley-Gorman (F-G) (31), and charcoal yeast extract (CYE) agars (29). F-G and CYE are supplemented with ferric pyrophosphate and L-cysteine hydrochloride, the cysteine being an essential growth requirement and the iron serving as a stimulatory nutrient (85). Legionella pneumophila has been isolated from blood (23, 56), pleural fluid (21), transtracheal aspirates (22, 51), and lung tissue. On the whole, direct in vitro culture has not been highly successful, probably due to the fastidiousness of the organism, overgrowth of contaminant organisms, antibiotic inhibition, or loss of viability.

2. Staining of the organism by the direct fluorescent antibody technique (9, 14, 15). This is, at present, the only rapid diagnostic technique available to the physician. The test appears to be highly specific. Cherry and associates (15) tested 374 strains of bacteria representing 25 genera and 59 species by the direct immunofluorescent technique; only one strain of Pseudomonas fluorescens was found to cross react with Legionella specific confugate. A strain of Pseudomonas alcaligenes has since been reported to cross react in the test (9).

3. Detection of antibody by the indirect immunofluorescent test (IFA). The indirect fluorescent antibody test has been used to diagnose the majority of legionellosis cases. A four-fold rise in titer on paired sera or a single serum titer of at least 1:128 is considered diagnostic (59). The IFA is apparently quite specific; however, four-fold rises in titer in human cases subsequently diagnosed as leptospirosis, plague, and tularemia have been reported (83). The test is disadvantageous in that diagnosis can be made only retrospectively,

since significant titers do not generally develop until at least 14 days after onset of the disease (75).

Antimicrobial therapy

Present recommendations for antibiotic therapy are based largely on a review of the effectiveness of these agents in treating past cases of Legionnaires' disease. Fraser et al. (35) observed that case fatality rates were highest for those treated with cephalosporins, intermediate in those treated with aminoglycosides, chloramphenicol, ampicillin, and penicillin, and lowest in those treated with tetracycline and erythromycin. In one of the earliest susceptibility trials, Nash and associates (64) found erythromycin and minocycline hydrochloride effective in an in vivo guinea pig model. Fraser and co-workers (36) showed erythromycin and rifampin effective using a similar guinea pig model, while penicillin, chloramphenicol, tetracycline and gentamicin showed no significant effect. Lewis et al. (55), using an embryonated hens' egg model, showed rifampin and gentamicin to have the highest prophylactic effectiveness, followed by streptomycin, erythromycin, sulfadiazine, chloramphenicol, cephalothin, oxytetracycline, and chlortetracycline, in decreasing order.

In vitro studies do not correlate well with in vivo results. A study by Thornsberry, Baker, and Kirven (79) showed Legionella pneumophila susceptible to rifampin, cefoxitin, erythromycin, the aminoglycosides, minocycline, doxycycline, chloramphenicol, ampicillin, penicillin G, carbenicillin, colistin, and sulfamethoxazole-trimethoprim, in that order. In the same study, L. pneumophila showed intermediate susceptibility to tetracycline, methicillin, cephalothin, cephamandole, and clindamycin and was resistant to vancomycin as measured by minimum inhibitory

concentration endpoints. Saravolatz and colleagues (74) found little difference in *in vitro* susceptibility among serogroups 1-4, the only notable differences being an increased susceptibility of serogroup 2 to penicillin and serogroups 3 and 4 to sulfamethoxazole-trimethoprim.

Thus relatively little is certain in terms of the antimicrobial susceptibility of *Legionella pneumophila*. The lack of correlation between *in vitro* and *in vivo* studies may be explained by relative differences in intracellular bactericidal action on *Legionella*-laden macrophages (80). Cephalosporin ineffectiveness is undoubtedly due to a recently discovered β lactamase (81) which is unlike that of either the *Enterobacteriaceae* or *Pseudomonas* (37).

Although limited, existing data suggest erythromycin and rifampin to be the best available therapeutics for treatment of Legionnaires' disease. Erythromycin is considered the drug of choice, with rifampin as a reserve for patients unresponsive to erythromycin, as widespread use of rifampin may result in the emergence of resistant tubercle bacilli.

THE ORGANISM

Taxonomic classification

The organism isolated by McDade in 1977 (59) had growth and staining patterns as well as biochemical characteristics far different than any previously described species. DNA homology studies convinced Brenner and co-workers (7) that they were indeed dealing with a previously unrecognized species. The Legionnaires' bacillus was found to have a genome size of 2.5×10^9 daltons and a G + C content of 39%. When Brenner and Steigerwalt (7) compared the Legionnaires' agent with 20

bacterial species having comparable G + C ratios, none showed more than 3% DNA relatedness. They felt there was sufficient evidence to declare the organism a new species. Legionella pneumophila sp. nov. has been proposed as the type species of the genus Legionella.

Morphology

Legionella pneumophila is a gram-negative bacillus measuring 0.3 - 0.9 μm by 2.0 μm or greater in length (12). Filamentous forms are observed; length, however, is largely dependent on environment. Both "roller pin" and "spindle-shaped" forms of the organism have been reported, though their frequency and significance is not clear (65). Transmission electron microscopy studies (11, 12) reveal well-defined ribosomes, a double envelope enclosure composed of a triple-unit membrane, and a pinching type of division, all typical of gram-negative bacilli. The detection of diaminopimelic acid in low concentration in the cell wall further substantiates its gram-negative character (41). The bacillus contains large vacuoles which stain readily with Sudan Black B (11, 12). The ultrastructural appearance of these granules suggest that they are poly β hydroxybutyrate in nature. Although Legionella was originally thought to be nonmotile, flagella have recently been demonstrated on strains of serogroups 1-4 (69,78).

Colonial morphology of L. pneumophila varies somewhat with the medium of isolation. Colonies of GC base agar are described as gray and glistening (21). Colonies on CYE, F-G, and MH-IH agars usually develop after 3-5 days of incubation and have a ground-glass appearance (30, 87). A brown pigment is observed on MH-IH and other tyrosine-containing media, possibly due to L-phenylalanine hydroxylase activity (3). Colonies exhibit a yellow fluorescence under long wave ultraviolet light (366nm) (30).

Biochemistry and Physiology

Legionella pneumophila is a strict aerobe, and, in fact, is sensitive to excesses of oxygen (67). The organism can grow under microaerophilic conditions, but a 2.5% CO_2 atmosphere is superior. Acid pH is preferred, with a pH of 6.9 as optimum. Oleic acid is inhibitory and selenium stimulatory to growth (46). Serine and threonine serve as the primary energy sources (39). An investigation by Tison et al. (82) indicated that the temperature, pH, and nutritional requirements of L. pneumophila in nature may not be as strict as those observed when the organism is cultured on complex media. When grown in association with cyanobacteria, Legionella pneumophila grew well over wide pH and temperature ranges. This association, the investigators felt, might account for the wide distribution of L. pneumophila in nature.

Biochemically, the organism is relatively inert. It is weakly oxidase positive, liquifies gelatin, and hydrolyzes starch. Nitrates are not reduced, carbohydrates not utilized, and urea not degraded. Furthermore, L. pneumophila lacks lysine and ornithine decarboxylases and arginine dehydrolase. Thus, laboratory identification based on routine biochemical tests alone would be virtually impossible. The cellular fatty acid composition of L. pneumophila is unique and serves an an aid to identification. Gas-liquid chromatography and mass spectrometry indicate the cellular fatty acids are largely (81-90%) branched chain. The iso C16:0 acid is predominant, followed by antesio C15:0, antesio C17:0, iso C14:0, and iso C16:1, in decreasing order (62, 63).

Serology

Six serogroups of Legionella pneumophila have been described to date by the direct fluorescent antibody technique. The Knoxville 1 strain

serves as the prototype strain for serogroup 1. This serogroup contains the original Philadelphia isolates and the vast majority of clinical strains, as well as nine of eleven Michigan isolates. Togus 1 serves as the prototype of serogroup 2, a group which includes patient isolates from the Togus, Maine outbreak and two environmental isolates from the Atlanta, Georgia outbreak of 1978 (60). For many months, serogroup 3 contained only the Bloomington, Indiana creek water isolate (61), the prototype strain, but recently human serogroup 3 isolates have been recovered (32), including one from Michigan (Saravolatz, Chang, and Wentworth, unpublished). Serogroup 4 includes Los Angeles 1 (60), the prototype strain, as well as several isolates from the Wadsworth V.A. Hospital epidemic. Serogroup 5 was identified after an outbreak of legionellosis at a Veterans of Foreign Wars convention in Dallas, Texas in the fall of 1978 (25). The prototype strain, Dallas 1E, was recovered from water samples from a nearby cooling tower. Serotype 6 of L. pneumophila was recovered from pleural fluid or lung tissue from three renal transplant patients in Chicago (16). Isolates were also recovered from 3 of 6 shower heads in the ward where these patients had stayed.

It has recently been proposed that three Legionella-like bacteria be placed in the genus Legionella: WIGA, first recovered by Bozeman (5) in 1959, is to be designated L. bozemanii (6); TATLOCK (74), which has been found to be identical to the Pittsburgh pneumonia agent (66), is to be designated Legionella micdadei (43). Legionella dumoffii (6) is the proposed name of the NY 23 strain (19).

It appears that the Legionnaires' disease patient can develop an immunological response against serogroup specific antigenic determinants as well as against determinants common to serogroups 1-4 (89). We are

only beginning to characterize these antigenic determinants. Wong et al. (92) isolated a serospecific antigenic fraction consisting of a lipid-protein-carbohydrate complex which protected guinea pigs and mice against challenge with lethal doses of L. pneumophila. SDS acrylamide gel electrophoresis resolved this into 4 protein and 1 glycoprotein band. Johnson and co-workers (47) identified soluble high molecular weight complexes of serogroups 1-4, designated Fraction-1 (F-1). Immunodiffusion studies demonstrated serospecificity; F-1 showed single precipitin lines with homologous serogroup antisera and no precipitin lines with heterologous serogroup antisera. Both the complexes of Wong and Johnson elute in the void volume of Sepharose 6B columns and contain carbohydrate, but their exact relationship has not been assessed.

EPIDEMIOLOGY

A review of epidemic, sporadic, and nosocomial cases of Legionnaires' disease reveals some salient epidemiologic features of the malady (2, 8). Infection is greater in males than females by a ratio of approximately 2.4 to 1. Cigarette smoking and alcohol abuse appear to be high risk factors. Immunocompromised and immunosuppressed patients seem to be especially susceptible to the disease (42). A late summer-early fall seasonality is apparent, both in Legionnaires' disease cases and in antibody levels in a healthy population (24).

The mode of transmission in legionellosis is uncertain, but compelling evidence points to an airborne route. A 1965 epidemic of Legionnaires' disease at St. Elizabeth's Hospital in Washington, D.C., the earliest documented outbreak, indicated wind-blown dust as the source of infection (77). Sporadic cases of Legionnaires' disease have also been associated

with excavation and construction sites (2). The organism has been isolated from evaporative condensers and air cooling towers in at least nine epidemics (17, 18, 20, 25, 34, 40, 42, 57, 68).

Person-to-person spread has not appeared to be an important means of transmission. There were no secondary cases of legionellosis among roommates and family members of the Philadelphia conventioneers (35). In contrast, the increased prevalence of antibody to L. pneumophila in hospital employees is consistent with this mode of dissemination (70). Person-to-person transmission appeared likely in the 1974 case of a Scottish physician who acquired Legionnaires' disease after treating a patient with this disease (10); this has not been unequivocally proven.

Accumulating data show an association between infection and reservoirs of the Legionnaires disease bacillus in the inanimate environment and indicate that L. pneumophila may be a common inhabitant of our surroundings. The organism was isolated from water and soil of a nearby stream after one outbreak of legionellosis (61). Fliermans and associates (33) showed fluorescing cells of L. pneumophila in 90% of water samples collected from 23 lakes in Georgia and South Carolina. Although fastidious in the laboratory, Legionella seems to survive well in stringent environmental conditions; strains of the organism survived 139 days in distilled water and 369 days in tap water (73).

With the rapidly-expanding genus of Legionella, serodiagnosis by IFA is becoming exceedingly complex. A test which is rapid and inexpensive, yet sensitive and specific, is needed. Microagglutination (28), immune adherence (54), card agglutination (50), and ELISA (27, 28) techniques are currently under development. It was the purpose of this study to define more clearly the sensitivity and specificity of a microhemagglutination

test for legionellosis and to evaluate further its usefulness in sero-epidemiologic studies.

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Evaluation of an Indirect Hemagglutination Test for Serogroups 1-4 of *Legionella pneumophila*

By

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INTRODUCTION

The indirect fluorescent antibody (IFA) test for legionellosis (9, 14) has become an important diagnostic tool in the three years since its introduction. Performing the test on a routine basis, however, is becoming an increasingly complex task; with the recent addition of the agents TATLOCK (12), WIGA (1), and NY 23 (4) to the genus Legionella (proposed designations L. micdadei (8), L. bozemanii, and L. dumoffii (2), respectively), proper serodiagnosis of Legionnaires' disease (LD) involves the use of nine separate antigens. The use of polyvalent antigens (7, 14) can simplify testing somewhat, but even this does not discount some inherent disadvantages of the technique, i.e. that it is technically difficult and expensive to perform. As a practical alternative, Edson and associates (5) introduced an indirect hemagglutination technique (IHA) for legionellosis. The test is rapid, inexpensive, simple to perform, and showed a 94.9% agreement with IFA sergroup 1 (S1) results. The IHA test was recently expanded to include serogroups 2, 3, and 4 (S2, S3, and S4) of Legionella pneumophila, and this study was made to evaluate the sensitivity and specificity of the method.

MATERIALS AND METHODS

Sera for parallel testing of IHA and IFA techniques

Sera were received at the Michigan Department of Public Health for routine IFA legionellosis testing between March and October, 1979. All sera received during this period were included in the study except for those where the quantity of serum was insufficient for testing by both techniques. The sample tested included a total of 895 sera. There were single serum specimens from 483 patients and paired or serial sera from 188 patients.

Sera selected for comparison of IHA and IFA

Sera from 55 patients with IFA confirmed (four-fold or greater rise in titer) or presumptive (titer $\geq$1:128) legionellosis were used. Single serum specimens were available from 8 patients and paired or serial sera from 47. These cases represent 41% of the 134 cases of legionellosis diagnosed by serology between October, 1977, and August, 1980. The only additional criterion for inclusion in the study was sufficient serum volume for testing.

Sera for cross absorption and blocking fluid studies

The thirteen sera with serogroup 1 titers used were from IFA confirmed or presumptive cases. Of the twelve sera with *E. coli* titers, four were from LD patients and eight from patients without legionellosis.

Sera for sucrose density gradient fractionation

Twelve sera selected from the 55 patients described above were used. An additional three sera, which showed four-fold or greater rises, or titers $\geq$1:128, by IHA, which were IFA negative, were fractionated for

analysis.

Bacterial strains

Bacterial strains were grown in Roux bottles on enriched chocolate agar (GC medium base with heated sheep blood and vitamin supplements). Flint 1, Flint 2, and Detroit 1 strains of Legionella pneumophila were maintained as stock cultures at the Michigan Department of Public Health. Strains of Knoxville 1, Togus 1, Bloomington 2, Los Angeles 1, and E. coli 013: K92:H4 were obtained from the Center for Disease Control (Atlanta, Georgia).

IFA antigen preparation

Bacterial growth was washed from the surface of the Roux bottle after 72 hours of incubation with 0.4% formalinized phosphate buffered saline (FPBS), filtered through a fine mesh wire screen to remove agar debris, then refrigerated (4° C) for one week. The suspension was then washed three times in FPBS and the opacity standardized to 300 International Opacity Units per ml (IOU/ml) by comparison to W.H.O. opacity standards. Suspensions of formalinized whole organisms, adjusted to 2 IOU/ml, served as antigen.

IHA antigen preparation

IHA antigens consisted of turkey erythrocytes stabilized by glutaraldehyde treatment and sensitized with boiled, sonicated antigen from agar-grown isolates by the bis-diazotized benzidine technique. Antigens were prepared from the following strains: S1 antigen as a mixture of Flint 1, Flint 2, and Detroit 1; S2 from Togus 1; S3 from Bloomington 2; and S4 from Los Angeles 1. The polyvalent antigen was made from a mixture

of Knoxville 1, Togus 1, Bloomington 2, and Los Angeles 1. All antigens were supplied as experimental antigens by Difco Laboratories, Inc. (Detroit, Michigan).

IFA technique

A standard microtiter technique using 0.005 ml antigen amounts on 12 well acetone-resistant glass microscope slides was employed. Conjugates were diluted in phosphate buffered saline (PBS) with 2% Tween 80. Rabbit anti-human globulin conjugate was purchased from Beckman Instruments, Inc. (Fullerton, California). Cappel (Cochranville, Pennsylvania) anti-rabbit globulin conjugate was used to determine titers on hyperimmune rabbit antisera. Anti-human IgM conjugate was supplied by Burroughs Wellcome Co. (Research Triangle Park, North Carolina).

IHA technique

The IHA technique was similar to that of Edson et al. (5). Unless otherwise noted, sera were screened at an initial 1:16 dilution against polyvalent sensitized cells and unsensitized control cells. Any serum showing a reaction with the test, but not control, cells was subsequently screened with the four monovalent (S1-S4) antigens and corresponding control cells. Sera were titrated to endpoint in serial two-fold dilutions with appropriate monovalent antigens.

Any serum showing agglutination of both antigen sensitized and unsensitized control cells was treated in the following manner: 0.02 ml of packed, unsensitized (control) cells were added to 0.12 ml of serum. After overnight incubation at 4^0 C, the cell suspension was centrifuged at 3,000 rpm for 15 min., the absorbed serum removed, and the IHA test repeated. Sera with high titers of anti-cell antibody required two or

three successive absorptions for complete removal of these agglutinins.

Cross absorption

For absorption, 0.1 ml of serum was added to approximately 3.7×10^{10} organisms which had been previously washed three times in PBS to remove formalin. The absorption mixture was incubated overnight at 4° C, then centrifuged at 3,000 rpm for 15 min., and the absorbed serum removed.

Preparation of hyperimmune sera

Rabbits, greater than 2 kg in weight, were immunized with an initial intramuscular injection of 1 ml of antigen mixed with an equal volume of Freund's complete adjuvant, followed by five intravenous boosters of 1 ml of antigen at days 22, 24, 26, 28 and 30. Rabbits were exsanguinated nine days after the last booster.

Comparison of immune sera prepared against IFA and IHA antigens

Rabbits were divided into four groups of two rabbits each and immunized as described above. Groups 1 and 3 received injections of IFA formalinized antigens at 16 IOU/ml. Groups 2 and 4 received injections of IHA antigen (boiled, sonicated) at 16 IOU/ml. Groups 1 and 2 were inoculated with the Flint 1 strain Legionella pneumophila. Groups 3 and 4 were immunized with Detroit 1 strain of serogroup 1. Sera from two rabbits in each immunization group were pooled, then IFA and IHA titers determined on each of the pools.

Blocking fluid preparation

Blocking fluids of Flint 1 and E. coli 013:K92:H4 were prepared in the manner described by Wilkinson et al. (13). Sera were initially

diluted in IHA diluent or PBS as a control and in both blocking fluids. Subsequent dilutions were made in diluent or PBS and IHA or IFA titers determined.

Sucrose density gradients

Sera were fractionated on 10-50% sucrose density gradients (11) by centrifugation at 33,500 rpm for 17 hours in a Spinco ultracentrifuge with a SW 50.1 rotor. Ten fractions of 0.5 ml were collected for analysis.

Immunodiffusion

The presence of IgM and/or IgG in each of the fractions was confirmed with Meloy (Springfield, Virginia) radial immunodiffusion kits. These kits employ the Fahey (6) technique to measure immunoglobulins.

RESULTS

Parallel testing of 895 sera by the IHA and IFA techniques

Results of parallel testing of 895 sera by the IHA and IFA techniques are shown in Table 1. There were 796 nonreactive and 46 reactive sera by both techniques in serogroup 1. There were 14 sera reactive in the IFA which were nonreactive in the IHA at a 1:16 dilution. There were 39 sera reactive in the IHA that failed to react in the IFA at a 1:16 dilution. Discrepancies in serogroups 2, 3, and 4 were due largely to the cross reactive nature of S1 antibody and not to sera with specific S2, S3, or S4 antibody. Agreement (% sera positive by both techniques plus % sera negative by both techniques) was 94.1%, 98.3%, 99.4%, and 97.5% for S1, S2, S3, and S4, respectively. There was an overall

TABLE 1 : Results of parallel testing by IHA and IFA on 895 sera

No. of sera that were:	S1	S2	S3	S4
IHA-/IFA-	796	879	885	858
IHA+/IFA+	46	1	5	15
IHA-/IFA+	14	0	2	2
IHA+/IFA-	39	15	3	20
TOTAL	895	895	895	895

(- = titer $<$1:16, + = titer $\geq$1:16)

agreement for serogroups 1-4 of 97.3%.

Among the 188 paired or serial sera, there were nine instances of four-fold or greater increases in titer by both IHA and IFA techniques and four discrepancies. One patient with a four-fold rise in IFA titer showed a two-fold rise by IHA and one patient had the reverse (i.e., a four-fold rise by IHA and a two-fold rise by IFA). Another patient with a greater than four-fold increase in IFA had a high stable titer (1:2048) by IHA. Finally, serum from a patient with successive IFA titers of 1:256 and 1:128 in S1 was IHA negative.

Some of the diverse reactivity between serogroups of the human antibody response noted by Wilkinson et al. (14) in the IFA was observed with the IHA. However, the IFA, using formalin fixed antigen, did not appear to be so cross reactive in this study. Representative results are shown in Table 2.

Where this cross reactivity was seen, it was found that the etiological serogroup could best be defined by cross absorption. Absorption of a serum with a serogroup specific antigen removed the cross reacting as well as homologous antibody. Absorption with a heterologous serogroup antigen removed only the cross reacting antibody, but did not decrease the serogroup specific titer. Ten cases of serogroup 1 and one case of serogroup 4 were identified by cross absorption. Two examples are shown in Table 3. Patient 1 appears to have S1 legionellosis and patient 2 to have LD of S4 etiology.

Comparison of IHA and IFA on selected sera from 55 patients

Of the 55 patients with IFA confirmed or presumptive legionellosis, 54 were identified as serogroup 1 and the other as serogroup 3. The IFA detected four-fold or greater rises in titer in 42 patients (41 S1, 1 S3),

TABLE 2 : Diverse serogroup reactivity of the human antibody response as measured by the IHA and IFA tests

	Titer of convalescent phase sera by:					
Legionella pneumophila antigen:						
Serogroup	**IFA A**	**IHA A**	**IFA B**	**IHA B**	**IFA C**	**IHA C**
1	1:64	1:512	1:64	1:16	1:128	1:2048
2	< 1:8	<1:16	< 1:8	1:256	<1:8	1:32
3	< 1:8	< 1:16	1:512	1:512	< 1:8	<1:16
4	< 1:8	1:32	1:16	1:128	1:8	1:128

TABLE 3 : Use of the cross absorption technique to define the etiological serogroup

Patient	Serum	IHA titer of convalescent phase serum with: S1	S2	S3	S4
	Unabsorbed	1:256	<1:16	<1:16	1:64
1	Absorbed with Flint 1	<1:16	<1:16	<1:16	<1:16
	Absorbed with Los Angeles 1	1:256	<1:16	<1:16	<1:16
	Unabsorbed	1:256	<1:16	<1:16	1:2048
2	Absorbed with Flint 1	<1:16	<1:16	<1:16	1:2048
	Absorbed with Los Angeles 1	<1:16	<1:16	<1:16	<1:16

37 (36 S1, 1 S3) of whom also showed significant rises by IHA (Table 4). In three of the remaining five patients, the IHA showed a rise from 1:16 to 1:16 and in two cases the IHA was nonreactive. Seven of 13 patients with presumptive legionellosis showed IHA titers $\geq$1:128, one showed a rise from $<$1:16 to 1:16, two showed titers between 1:16 and 1:128, and three were nonreactive in the IHA (Table 4).

It was considered that the differences between IHA and IFA results might be explained by: 1. measurement of two different antigen-antibody systems; 2. reactions with cross reacting antigen(s); or 3. differences in immunoglobulin specificity. To explore each of these possibilities, the following studies were conducted:

1. Comparison of immune sera prepared against IFA and IHA antigens

Results of IFA and IHA testing of hyperimmune rabbit sera, prepared with IFA and IHA antigens, are shown in Table 5. If two different antigen-antibody systems are involved, titers with homologous antigens might be expected to be higher than those with heterologous antigens. However, there were no significant differences in titers with IFA and IHA antigens, regardless of the antigen used to prepare the antisera.

2. Blocking fluid and cross absorption studies

Wilkinson and associates reported that a crude extract of E. coli 013:K92:H4 could block 97% of IFA positive reactions with a variety of gram-negative bacterial species, and blocked 6% of Legionella pneumophila titers (13). This strain of E. coli was obtained from Dr. Wilkinson and blocking fluids of this and Flint 1 strain were prepared.

Twelve sera with anti-serogroup 1 IHA titers were tested as shown in Table 6. Dilution in E. coli blocking fluid had no significant effect on any of the 12 sera tested, while Flint 1 blocking fluid caused a

TABLE 4 : Comparison of IFA and IHA results on 55 patients with IFA confirmed or presumptive legionellosis

	IHA					
	Confirmed* Legion-ellosis	Presumptive** Legion-ellosis	Rise in Titer <1:16 to 1:16	Titer >1:16 but <1:128	Titer <1:16	Total
IFA						
Confirmed* Legion-ellosis	37	0	3	0	2	42
Presumptive** Legion-ellosis	0	7	1	2	3	13
						55

* Four-fold or greater increase in Titer

** Titer ≥1:128

TABLE 5 : Comparison of immune sera prepared against IFA and IHA antigens

Immunization Group	Immunizing Antigen	Titer by* IHA	IFA
1	Flint 1, IFA	1:4096	1:2048**
2	Flint 1, IHA	1:4096	1:4096**
3	Detroit 1, IFA	1:4096	1:2048***
4	Detroit 1, IHA	1:8192	1:4096***

* Results shown are the average value of triplicate determinations

** Flint 1 served as the test antigen

*** Detroit 1 served as the test antigen

TABLE 6 : Results of blocking fluid and cross absorption studies in the IHA and IFA tests

Test	Initial Serum Titer to	Blocking Fluid or Absorbing Antigen Used	Effect on Titer: No Significant Decrease in Titer*	Effect on Titer: Significant Decrease in Titer**
IHA	Serogroup 1	Flint 1 B.F.	0/12	12/12
"	"	*E. coli* B.F.	12/12	0/12
"	"	Flint 1 Abs.	0/11	11/11
"	"	*E. coli* Abs.	11/11	0/11
IFA	Flint 1	Flint 1 B.F.	0/13	13/13
"	"	*E. coli* B.F.	13/13	0/13
"	"	Flint 1 Abs.	0/9	9/9
"	"	*E. coli* Abs.	9/9	0/9
IFA	*E. coli*	Flint 1 B.F.	12/12	0/12
"	"	*E. coli* B.F.	0/12	12/12
"	"	Flint 1 Abs.	7/8	1/8
"	"	*E. coli* Abs.	0/8	8/8

* No change in titer or two-fold decrease in titer

** Four-fold or greater decrease in titer

B.F. = Blocking fluid used for initial dilution of serum

Abs. = Whole cell antigen used to absorb serum

four-fold or greater decrease in all.

Blocking fluid studies in the IFA showed similar results. E. coli blocking fluid had no effect on the 13 sera tested. Dilution in Flint 1 blocking fluid reduced serogroup 1 titers by at least a four-fold decrease in 100% of the cases tested. Twelve sera with initial IFA E. coli titers were similarly tested (Table 6). Flint 1 blocking fluid caused no significant decreases in titer in any of the 12 sera, while E. coli blocking fluid significantly decreased all titers. There was one patient with a concurrent four-fold rise in titer to both E. coli and Flint 1 in which blocking was specific, i.e. Flint 1 blocking fluid removed only the Flint 1 titer and E. coli blocking fluid reduced only the E. coli titer. Another patient who showed a four-fold rise in S1 titer to 1:128 by IFA and a maximum IHA titer of 1:16 was blocked by Flint 1 but not E. coli blocking fluids.

Cross absorption was felt to be a more conclusive test for common antigens than the use of crude blocking fluid extract. Absorption of 11 sera with S1 IHA titers with whole cell E. coli antigen caused no significant decrease in any S1 titers, while absorption with whole cell Flint 1 caused a four-fold or greater decrease in all (Table 6). Similarly, in the IFA, absorption with whole cell E. coli did not change Flint 1 titers on 9 sera, while absorption with Flint 1 organism significantly decreased all. Cross absorption studies on 8 sera with E. coli IFA titers proved specific on all but one serum. This serum had an initial E. coli titer of 1:32, and absorption with E. coli organism decreased the titer to $<1:8$. Absorption with whole cell Flint 1 decreased the titer four-fold to 1:8.

3. Fractionation of sera on sucrose density gradients to determine the immunoglobulin class reactive in the IHA and IFA tests

A total of 15 sera were fractionated by the sucrose density gradient technique and the fractions analyzed by both methods. Results of IFA and IHA determinations are summarized in Table 7. Three sera from cases diagnosed by IHA but not IFA showed reactivity only in the IgM fractions. Ten sera showed reactivity in the IgM and IgG fractions in the IHA test, but showed reactivity only in the IgG fractions by IFA. Another serum showed reactivity in the IgG fractions by both techniques. The remaining serum showed reactivity in the IgG fractions with the IFA, but was non-reactive in all fractions with the IHA. This serum had an initial IFA titer of 1:128 (S1) and was nonreactive in the IHA. It appeared that the standard antiglobulin conjugate used in the IFA did not detect IgM. To test this hypothesis, the fractions of 10 sera which showed the presence of IgM by IHA and not IFA were re-examined by IFA with an IgM specific conjugate. All sera now showed reactivity in the IgM fractions. Typical results are shown in Table 8.

DISCUSSION

Parallel testing of 895 sera showed the IHA and IFA to yield essentially the same results for serogroups 1-4. There was approximately 97% overall agreement between the two techniques and discrepancies were observed in both directions. IHA positive/IFA negative discrepancies were associated with the inability of the antiglobulin conugate used in this study to detect IgM. Nagington and associates (10) reported that 3 of 22 LD patients showed a rise in IgM antibody without concurrent rise

TABLE 7 : Immunoglobulin reactivity of 15 sera fractionated on sucrose density gradients as measured by IHA and IFA

Technique	Reactivity in IgM Fractions	Reactivity in IgG Fractions
IFA	0*	12
IHA	13	11

* Number of sera

TABLE 8 : Comparison of IHA and IFA results on ten fractions of a convalescent phase serum

	Titer by			Immunodiffusion***	
Fraction	IHA	IFA*	IFA**	IgM	IgG
1	1:8	< 1:1	< 1:1	-	-
2	1:32	< 1:1	1:2	+	-
3	1:512	< 1:1	1:32	+	-
4	1:128	< 1:1	1:8	+	-
5	1:32	1:4	< 1:1	-	+
6	1:32	1:16	< 1:1	-	+
7	1:4	1:32	< 1:1	-	+
8	< 1:4	1:2	< 1:1	-	+
9	< 1:4	< 1:1	< 1:1	-	-
10	< 1:4	< 1:1	< 1:1	-	-

*Anti-human globulin conjugate
**Anti-human IgM conjugate
***Used to confirm presence or absence of IgM and IgC in each fraction

in IgG. Such cases would go undetected with a conjugate of this type. Polyvalent immunoglobulin conjugates should be used for maximum sensitivity (10, 13). This poses no problem for the IHA, as it was conclusively shown to detect IgM and IgG class antibodies.

The problem of IFA positive/IHA negative sera is yet to be resolved. One such serum showed reactivity only in the IgG fractions. Continued fractionation of sera of this nature may provide an explanation of differences between the two techniques.

Correlation of clinical with laboratory data provides further insight into the sensitivity and specificity of the two techniques. Two cases of LD defined by the IHA which were undiagnosed by the IFA had clinical symptoms consistent with legionellosis and responded promptly to erythromycin therapy. Among 6 cases diagnosed by IFA but not IHA, three patients had clinical histories consistent with LD and responded to treatment with erythromycin; one patient had symptoms consistent with the disease but responded well to treatment with gentamicin and cephalothin, two antibiotics which are of low efficacy in the treatment of legionellosis. Clinical histories of two patients were not consistent with the diagnosis made by IFA serology. Clinical data were not available on the remaining discrepancies described in this study.

Even when cross absorption was used in addition to the cruder blocking technique, the IHA showed no significant cross reactivity with E. coli. The IFA showed evidence of cross reactivity with E. coli in only one instance, but it should be noted that this serum had a low initial titer. Studies with 12 common gram-negative bacterial antigens, including Hemophilus, showed no cross reactivity in the IFA with Legionella antigens (Dr. B. Wentworth, personal communication).

Cherry and associates (3) found one strain of Pseudomonas fluorescens antigenically related to serogroup 1 Legionella pneumophila among 400 bacterial strains tested.

Although the IHA shows more cross reaction between serogroups than the IFA with formalin fixed antigens, heterologous reactions are usually significantly lower than the homologous serogroup reactivity. The IHA adequately defined the etiologic serogroup in most cases. If there was any question as to the etiologic serogroup, cross absorption could be used to define it.

The IHA is rapid, simple, inexpensive, and well suited to large scale studies and routine use. It provides a suitable alternative to the use of more complicated and expensive IFA technique.

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Prevalence of Antibody to Serogroups 1-4 of *Legionella pneumophila*: A Seroepidemiologic Study Using the Indirect Hemagglutination Test

By

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INTRODUCTION

Much has been learned about the diagnosis and clinical characterization of legionellosis since the Philadelphia outbreak of 1976. Less is known, perhaps, of the epidemiology of the disease. Legionella pneumophila has been shown to reside in the soil (13) and in water (5, 13). Seroprevalence studies (4, 11) have suggested that the organism is widespread in nature, surprising in view of the fastidious nature of the organism in the laboratory. It is not clear whether the inanimate environment is the primary reservoir or merely an incidental source of the organism. Also unclear is the mode of transmission, although accumulating evidence points to an airborne route of infection (2, 3, 7, 8, 10, 14). Factors which result in overt diseases as opposed to subclinical infection, whether host, bacterial, environmental, or a combination of the three, are yet to be elucidated. Only through continued epidemiologic studies will such questions be answered and the true incidence of the disease as well as its importance as a cause of pneumonia be determined.

This study employed an indirect hemagglutination test to determine the prevalence of antibody to Legionella pneumophila serogroups 1-4, in an apparently healthy group of Michigan residents. Data was analyzed by age, sex, and season of the year.

MATERIALS AND METHODS

Sera

Sera from 1200 apparently healthy residents of the eastern lower peninsula of Michigan were selected from sera submitted to the Michigan Department of Public Health for routine premarital and pre-employment syphilis serology. All were nonreactive in the VDRL test. A total of 600 sera were examined for each of two seasons: winter (January-April, 1980) and summer (July-September, 1980). Sera with sufficient volume for testing were assigned to the study until there were 50 males and 50 females in each of the following age groups: 15-19, 20-29, 30-39, 40-49, 50-59, and 60 or greater years of age. Premarital sera constituted 59.3% of the female and 61.2% of the male sera tested. The remaining sera were sent as part of pre-employment physical examinations.

IHA Test

The indirect hemagglutination test (IHA) was that of Edson *et al.* (4), expanded to include serogroups 2, 3, and 4. IHA antigens, supplied by Difco Laboratories, Inc. (Detroit, Michigan) as experimental antigens, had been sensitized with the following agar-grown isolates: serogroup 1-- a mixture of Flint 1, Flint 2, and Detroit 1 strains; serogroup 2--Togus 1; serogroup 3--Bloomington 2; serogroup 4--Los Angeles 1. Polyvalent antigen was sensitized with a mixture of Knoxville 1, Togus 1, Bloomington 2, and Los Angeles 1. Sera were initially screened at a 1:16 dilution. Any serum reactive in the screen was further tested with monovalent 2, 3, and 4 antigens, then subsequently titrated to endpoint in serial two-fold dilutions with appropriate monovalent antigens. Reactive and nonreactive control sera were tested in parallel with the test sera.

Statistics

Data for both seasons were compiled by age and sex, then analyzed for significant differences by the Chi-square test (9).

RESULTS

Initially it was intended to screen sera with only a polyvalent antigen. Parallel testing of the polyvalent and four monovalent antigens, however, revealed a marked difference in reactivity of the serogroup 1 and polyvalent antigen. Further studies suggested this difference was due to the Detroit 1 strain, which is incorporated in the serogroup 1, but not in the polyvalent antigen. Although Flint 1, Knoxville 1, and Detroit 1 are members of the same serogroup, the Detroit 1 strain was not antigenically identical to either the Flint 1 or Knoxville 1 strains in fluorescent antibody testing (Wentworth and Chang, Abstracts APHA, October, 1980, Detroit, Michigan). Therefore, it was found necessary to include Detroit 1 in preliminary screen for maximum serogroup 1 sensitivity.

Prevalence of Antibody to Serogroup 1

Two hundred and two of the 1200 sera tested had detectable serogroup 1 antibody. This is an overall prevalence of 16.8%. Seventy-one (11.8%) of the winter sample were positive, while 131 (21.8%) of the summer sera group showed serogroup 1 antibody. The overall seasonal difference is statistically significant ($P < 0.001$) and was independent of sex. This seasonal difference was statistically significant in 4 of 6 age groups (15-19, 30-39, 40-49, and 50-59) (Table 1).

In both winter and summer samples there was a trend toward decreasing prevalence in the fourth through sixth decades. Only the 50-59 and 60 or

TABLE 1 : Number and per cent of sera with IHA titers equal to or greater than 1:16 to serogroup 1 of Legionella pneumophila

	WINTER				SUMMER					
Age in	MALE		FEMALE		MALE		FEMALE		TOTAL	
years	No.	%	No.	%	No.	%	No.	%	No.	%
15-19	5	10	7	14	15	30	13	26	40	20.0
20-29	7	14	14	28	12	24	14	28	47	23.5
30-39	8	16	5	10	16	32	15	30	44	22.0
40-49	3	6	8	16	11	22	9	18	31	15.5
50-59	4	8	3	6	6	12	10	20	23	11.5
60+	3	6	4	8	3	6	7	14	17	8.5
Total	30	10	41	13.7	63	21	68	22.7	202	100.0

older groups in the summer samples were significantly different from prevalence at other ages ($P < 0.05$).

The 20-29 female group in the winter sample was significantly different from three other female groups in the winter (30-39, 50-59, 60 or older). This may be due to the small sample size in each group (n=50).

Eighty six (42%) of the sera with serogroup 1 titers showed reactivity with serogroups 2, 3, and 4 antigens. Past experience with the IHA suggests that these are cross reactions in the presence of S1 antibody and not the result of exposure to more than one serogroup.

Geometric mean titers (GMT) were calculated by age and season for those sera possessing serogroup 1 antibody. All but two of the summer groups had a higher GMT than the corresponding age groups in the winter sample (Table 2). The difference between the overall GMT for the two seasons was not statistically significant.

Most reactive sera (81%) had titers of 1:16 (Table 3), while only 19% had titers greater than or equal to 1:32. There was a statistically significant difference between the number of sera with titers $\geq$1:32 in winter (9) versus summer (31) samples. Only 2 sera (0.2%) showed titers $\geq$1:128, both in the summer sample.

Prevalence of Antibody to Serogroups 2, 3, and 4

There were only 4 sera that showed reactivity in serogroups 2, 3, and 4 in the absence of serogroup 1 reactivity among the winter sample. One was reactive to serogroup 2, and three showed serogroup 3 reactivity. One serogroup 3 in the 60 or older female group had a titer of 1:128, suggestive of recent infection.

TABLE 2 : Geometric mean titers to Legionella pneumophila, serogroup 1, by age and season

Age in years	Winter Geometric Mean Titer	Summer Geometric Mean Titer
15-19	20.3	17.7
20-29	16.5	23.9
30-39	17.8	19.1
40-49	16.0	24.3
50-59	16.0	18.2
60+	19.5	18.4
Total	17.5	20.2

TABLE 3 : Distribution of IHA titers to serogroup 1 of Legionella pneumophila by season and sex

	WINTER		SUMMER	
Titer	Male	Female	Male	Female
1:16	24	38	45	55
1:32	6	3	11	9
1:64	0	0	6	3
1:128	0	0	1	1
Total	30	41	63	68

Among the summer sera, only two showed reactivity with serogroup 2 antigens in the absence of serogroup 1 reactivity. There were no sera showing reactivity in serogroups 3 or 4 without reactivity in serogroup 1. This yields an overall prevalence of 0.3%, 0.2% and 0.0% for serogroups 2, 3, and 4, respectively. The antibody prevalence for each of these three serogroups is statistically different from that of serogroup 1. There appeared to be no predominance of serogroups 2, 3, or 4 titers in any one age group, or in either sex.

DISCUSSION

Analysis of the data from this study shows that titers of serogroup 1 of Legionella pneumophila are more prevalent than titers to serogroups 2, 3, and 4. These data closely parallel the relative importance of these serogroups as a cause of diagnosed legionellosis in Michigan. Of 134 cases of legionellosis diagnosed between October, 1977, and August, 1980, only four have been other than serogroup 1. Three cases appeared to be due to serogroup 3 and the fourth to serogroup 4 infection.

The overall prevalence of 16.8% of serogroup 1 antibody suggests that this serogroup is relatively widespread in nature. The lower prevalence of serogroups 2, 3, and 4 suggests that these serotypes are not as common in the environment. However, this may not be the case; the four serogroups may be equally prevalent, with serogroup 1 possessing greater infectious potential.

There has been a wide variance in reported prevalence of Legionella antibody in the normal population. Macrae and co-workers (12) reported that 1.5% of 2,023 persons tested possessed serogroup 1 antibody. Helms

and associates (11) found 13.2% of a rural Iowa population to have serogroup 1 titers $\geq$1:128. Such variations may be due to differences in geographic distribution or to the method employed for antibody determination.

The possibility that some of the antibody detected in this study is due to cross reacting antigens cannot be totally excluded. Cross absorption and blocking fluid studies with E. coli 013:K92:H4 in the IHA (in press) do not show the cross reactivity observed by others (15) in the IFA.

Edson and co-workers (4) suggested that the prevalence curve for legionellosis was similar to that of chickenpox (Varicella) and postulated that primary exposure to L. pneumophila occurred in children less than 15 years of age. Preliminary data (16) do not support this hypothesis. A survey of apparently healthy children less than 15 years old showed serogroup 1 antibody in only 2 (9.1%) of the sera. Only 3 (1.3%) of 238 children in this age group with pneumonia of undetermined etiology possessed serogroup 1 antibody. Only one of these children achieved a titer $\geq$1:128. In a study of 500 patients in a prepaid medical group in Seattle, Foy et al. (6) found no significant difference in the prevalence of antibody in children versus adults. Further studies are needed to ascertain the prevalence of antibody in healthy children and to determine the importance of Legionella sp. as a cause of pneumonia in children.

The seasonal variation in prevalence of serogroup 1 noted by Edson and associates (4) was observed in this study. This seasonal variation is also seen among sporadic cases of legionellosis (1). The overall prevalence and geometric mean titers reported here are significantly lower than those previously observed in Michigan. This may be the result of a

relatively mild winter in Michigan during the 1979-1980 season with a corresponding decrease in respiratory infections. It is possible too that there are yearly as well as seasonal peaks in prevalence of the disease, with corresponding peaks in antibody prevalence. Repeated testing will be needed to demonstrate periodic fluctuations in prevalence.

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