ISSN 1021-366X

Department of Health
REPUBLIC OF CHINA

Vol. 12 No. 8/August 25, 1996

Epidemiology Bulletin

Testings for Legionellosis in the Taiwan Area — A Preliminary Report —

Introduction

In the summer of 1976 at the 200th Anniversary gathering of US veterans in Philadelphia, 221 participants were infected with acute pneumonia, and 34 of them died as a consequence(1). This incident shocked not only citizens of the entire country, but was also of great concern to the medical world. In 1977 Dr. McDade, of the US Centers for Disease Control and Prevention (CDC), identified the pathogen from pulmonary specimens, naming it *Legionella pneumophila*. But there had been at least four outbreaks even before 1976. As early as 1947, a rickettsia-like medium was isolated from infected guinea pigs. In 1965, the first outbreak had occurred in Washington, DC, with 81 patients and 15 deaths reported. Then, in 1974, another outbreak occurred in a hotel(2). Around 1,000 to 1,300 cases are reported to the CDC each year. In fact, many cases are not diagnosed and, therefore, the incidence of Legionellosis may well be much higher than the number of cases actually reported(3). Anyone is susceptible to the infection, although there have been very few patients under 20 years of age. Several outbreaks have occurred among hospitalised patients. In Taiwan, there have been cases of medical care personnel (including dentists and pathology technicians) infected with Legionellosis who have then required long-term hemodialysis as a result of renal failure(4). Even deaths had been reported in Taiwan as late as 1995 (IFA titer as high as 1:2048), when the matter attracted the attention of health and medical circles. Though many infections are not diagnosed, studies show that at least half of the Legionella infections are related to pneumonia. The older the patient (and many are older than 50 years), the more serious the condition. Smokers, diabetes patients, patients with chronic pulmonary diseases, kidney diseases or malignant neoplasms, patients with impaired immune functions and particularly patients on corticosteroid treatment or with organ transplants are more prone to Legionella infections. The male-female sex ratio is about 2.5:1, and the mortality rate is about 5-30%.

Legionella pneumophila is an aerobic, not easy to stain, gram-negative bacillus which is 2-20 μm long and 0.3-0.9 μm wide. Its biochemical characteristics are: Catalase (+), Gelatinase (+), Hippurate hydrolysis (+); its enzyme characteristics

are: Oxidase (−), Urease (−), Nitrate reductase (−). In buffered charcoal yeast extract medium (BCYE medium), grey to light blue colonies will develop in three to five days. The bacillus grows under 29-40°C in an optimum pH value of 6.9. It can survive under room temperature or up to 40°C for several months, and for one year in unsterilized tap water. L-cysteine and other nutrients are needed for its culture. Thus far, 14 different sero types of *Legionella pneumophila* have been identified; of these, type 1 induces infections most often (70-80%). Others often isolated from pneumonia patients with lowered immune function include *L. micdadei, L. bozemanii, L. longbeachae,* and *L. dumoffi.* Thus far, 34 kinds of Legionella bacilli in 50 serotypes have been identified.

The bacillus distributes widely in the natural world. It has been detected in rivers, lakes, swamps, well water, shower nozzles and in cooling-tower water. The bacillus survives in soil or water on amoebae as natural hosts; some examples are *Acanthamoeba, Echinamoeba, Naegleria, Tetrahymena* and *Chclidium.* As most outbreaks occur from summer to fall, it is speculated that infection is related to the use of air-conditioners and that the infection is caused by the inhalation of droplets of cooling-tower water[6-8].

Legionella pneumophila exists more often in water solutions. It has been detected in the hot water supply system, the cooling towers of air-conditioners and in vapor condensers. The bacillus has been isolated from cold and hot water, shower water, stream, pond and soil. It can survive in tap water or distilled water for months.

The disease is transmitted via aerosol-producing facilities such as cooling towers, vapor condensers, whirlpools, dehumidifiers, fountains, shower nozzles and faucets[3]. Since the late 1970's, cooling-tower and condensation facilities have been considered sources of infection of Legionellosis[5]. There have been several outbreaks in the United States recently. Epidemiologists have investigated the three most recent outbreaks, occurring in July and August of 1993. Findings of these investigations have improved understanding of the spread of the infection[2].

This disease was previously little known to Taiwan medical circles, and patients were often treated for pneumonia. Many could have been mis-diagnosed. Professor M.H. Chen-Kuo of the National Yangming Medical College is the only person who ever studied the antibody titer, using specimens of blood of 200 healthy young men of 20 to 30 years of age[9]. Otherwise, Legionellosis was relatively unknown to professionals in Taiwan. In 1989, realizing the importance of this disease, the National Institute of Preventive Medicine brought for the first time, from Japan and the United States, laboratory testing methods for the Legionella bacillus and started to study the disease in Taiwan. With laboratory testing conducted by the National Institute of Preventive Medicine and an increased understanding of this disease among medical professionals, specimens of suspected patients are now laboratory-tested immediately to detect the actual causes of any infections in order to expedite prompt treatment. Lives of many patients in Iintensive Care Units have thus been saved.

Preliminary findings from laboratory testing of both human and environmental

specimens, as conducted by the Republic of China's National Institute of Preventive Medicine in the three -year period between 1993 and 1995 follow:

Materials and Methods

A. Collecting clinical specimens of *Legionella pneumophila*

Clinical specimens are collected from some major hospitals throughout the country. Specimens include sera and respiratory discharge such as transtracheal aspiration, sputum, bronchoalveolar lavage, bronchoscopic washing, pleural effusion, lung tissue and tracheal suction.

1) sera specimens: sera in the early stage of disease onset and four weeks later or in the recovery stage are collected. If they cannot be tested immediately, they are placed under -20°C and tested within one week.

2) Human specimens are collected as soon as possible in the early stage of onset before medication and placed in airtight containers to avoid outflowing and drying. If they cannot be cultured within three days, they are placed under 4°C; and −70°C storage is used for any period of more than seven days.

B. Testing clinical specimens of *Legionella pneumophila*

1) sera specimens: to test for the sera titer of patients by the indirect fluorescent antibody test (IFA) method in the following ways:

a. preparing antigen slides
   (1) on fluorescent stain slides, place 10 μL of antigens (Zeus Scientific Inc. production) in each hole;
   (2) dry for 30 minutes under natural conditions;
   (3) stabilize with heat;
   (4) stabilize with ether.

b. testing for antibody titer
   (1) dilute serum with phosphate-buffered saline (PBS) to 1:128 and 1:256;
   (2) add the diluted serum to the slide;
   (3) conduct positive, negative and PBS contrast; do not mix different sera;
   (4) place in wet box under 37°C for 30 minutes;
   (5) remove slide, wash it gently with PBS, and place in PBS for 10 minutes;
   (6) wash with water, dry under natural conditions;
   (7) add one drop of fluorescent conjugate of human antibody in each hole;
   (8) place in wet box under 37°C for 30 minutes;
   (9) remove slide, wash gently with PBS, and place in PBS for 10 minutes;
   (10) wash with water, dry under natural conditions;
   (11) add one drop of mounting medium, cover the slide;

(12) examine under fluorescent microscope (Nikon, Model UFX-IIA)

c. criteria for reading
4+ bacilli displaying dazzling yellow-green fluorescent light;
3+ bright yellow-green fluorescent light;
2+ clear but dim fluorescent light;
1+ slight fluorescent light;
Neg: no yellow-green reaction though possibly yellow-brown reaction.

2) Culturing sputum and other discharges from the respiratory tract

a. Transtracheal aspiration, pleural effusion and lung tissue require no acid treatment as they often are not contaminated by other bacteria. They may be inoculated directly on BCYE and PAV media containing polymycin B, anisomycin and vancomycin; if the quantity of microorganisms is not sufficient, centrifuge the specimens for higher density. Components of media on the market are shown in Table 1. The present study used a product from MAST Diagnostics Limited.

Table 1. Components of Culture Media on Market for *Legionella pneumophila*

Components	MAST	BBL	OXOID	DIFCO
Buffered charcoal	1.5	2.0	2.0	1.5
Yeast extract	10.0	10.0	10.0	10.0
Moderator	6.0	10.0	1.0	6.0
Iron pyrophosphate	0.25	0.25	0.25	0.25
α-ketogluterate	1.0	1.0	0.2	1.0
Agar	12.0	15.0	13.0	17.0
L-cysteine	0.4	0.4	0.08	0.4
Postassium hydroxide				1.5

(Sources: product brochures)

b. Other respiratory tract discharges may be contaminated. They should be homogenized and then treated with acid.

(1) homogenization: add an equal amount of sterilized deionized water, add a few sterilized glass bead, shake for 30 seconds, and inoculate 0.01 mL each on BCYE and PAV media.

(2) treatment with acid: add 4.5 mL of 0.2 M HCl-KCl solution to 0.5 mL

of specimen for treatment for 3-4 minutes, inoculate 0.01 mL each on BCYE and PAV media, complete the process in 5 minutes.

(3) culturing: culture for 7 to 14 days under 37°C and relative humidity 60-90%; observe on the first day and every other day; select suspected colonies for L-cysteine requirement test; if bacilli grow on BYCE, but not on culture media without L-cysteine, they are most likely to be *Legionella pneumophila*.

3) appraisal of colonies

a. biochemical characteristics
(1) L-cysteine requirement
(2) oxidase test
(3) catalase test
(4) gelatine hydrolysis
(5) β-lactmase

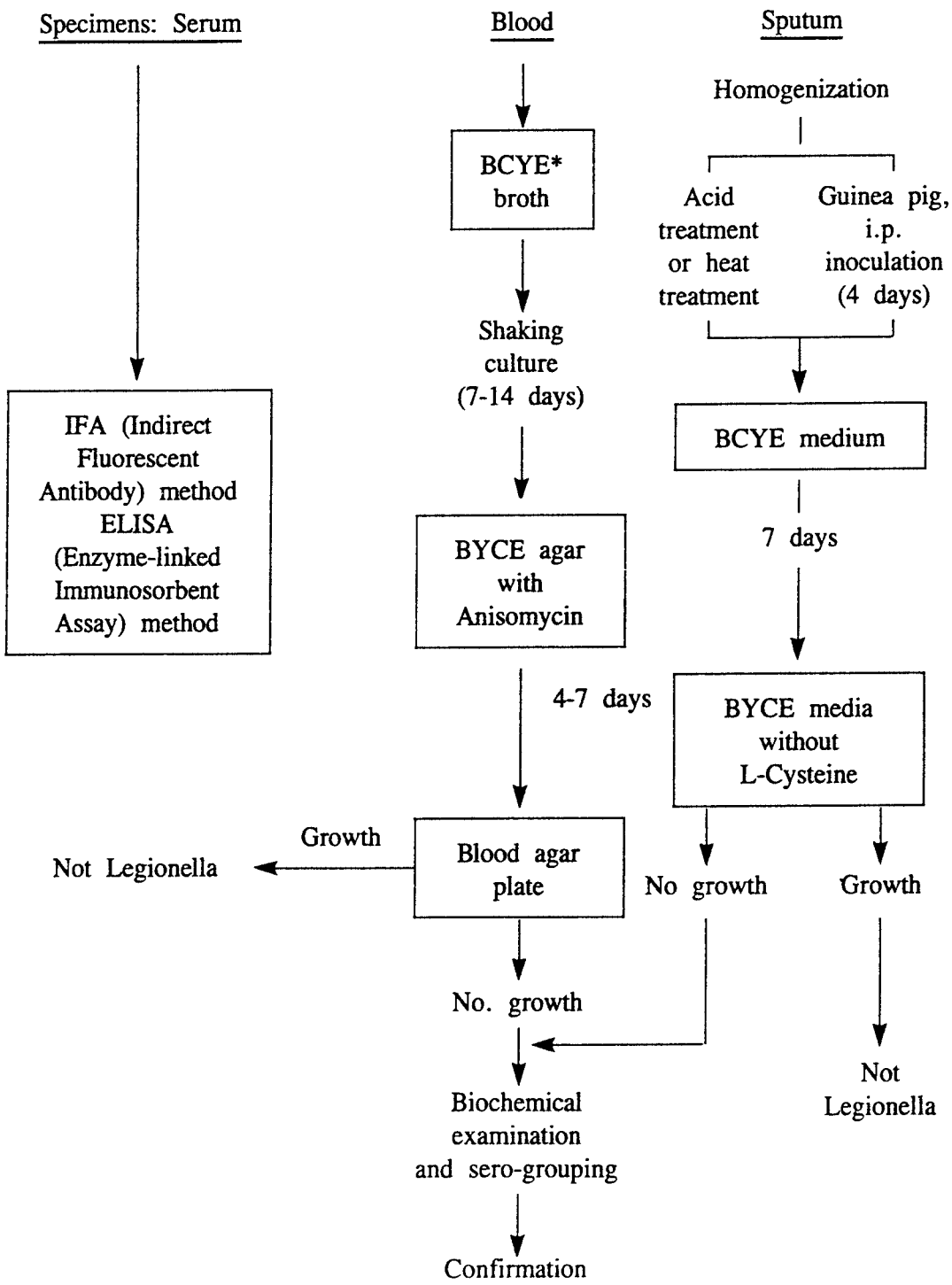
b. Direct Immuno-Fluorescent Antibody Test (DFA)
(1) preparing slides
((a)) add some 48-hour cultured bacilli in 1% neutral formalin to make a McFarland No.1 suspension;
((b)) on fluorescent stain slide, add 10 μL of the suspension into each hole;
((c)) dry under natural conditions for 30 minutes;
((d)) stabilize by heat.

(2) fluorescent staining
((a)) add polyvalent conjugate in hole; a negative control should be prepared for each specimen;
((b)) place in wet box for 20 minutes under room temperature; do not mix different specimens and conjugates;
((c)) remove slide, wash gently with PBS, and place in PBS for 10 minutes;
((d)) wash with water, dry under natural conditions;
((e)) add one drop of mounting medium, cover the slide;
((f)) examine under fluorescent microscope (Nikon, Model UFX-II A).

(3) criteria for reading
((a)) 4+ bacilli displaying dazzling yellow-green fluorescent light;
((b)) 3+ bright yellow-green fluorescent light;
((c)) 2+ clear but dim fluorescent light;
((d)) 1+ slight fluorescent light;
((e)) Neg: no yellow-green reaction.

The laboratory procedures for *Legionella pneumophila* employed by the National Institute of Preventive Medicine are shown in Figure 1. Abdominal injection of guinea pigs was practiced at one time, but no longer.

Figure 1. Testing Procedures for *Legionella pneumophila*



* BCYE medium: Buffered Charcoal Yeast Extract medium

C. Collecting environmental specimens

1) collection of cooling-tower specimens and water tower specimens:

Two samples from each spot: one water sample of 50 mL straight from the cock at the bottom; another water sample of 50 mL collected after the water has run for 20 seconds.

2) collection of specimens from faucets and nozzles

Use cotton swab to collect sediment on the wall, swab at least four times; add 0.5 mL of water; collect other 15 mL of running water.

3) for others, collect 100 mL of water.

D. testing environmental specimens for *Legionella pneumophila*

1) cooling-tower and other water specimens: centrifugate at 4,000 xg for 30 minutes; collect 1 mL of the lower fluid; add 2 mL of 0.2 M HCl-KCl buffer solution for 3-4 minues; inoculate 0.01 mL of the above solution on BCYE and MWY (Modified Wadowsky and Yee) media.

2) water specimens from faucet and nozzle: add 2 mL of 0.2 M HCl-KCl buffer solution for treatment for 3 minutes; inoculate 0.01 mL each on BCYE and MWY media.

3) culturing: culture for 7 to 14 days under 37°C and relative humidity of 60-90%; observe on the first day and every other day; select suspected colonies for L-cysteine requirement test; If bacilli grow on BCYE, but not on media without L-cysteine, they are most likely *Legionella pneumophila.*

4) appraisal method same as B. Testing Clinical Specimens for *Legionella pneumophila.*

Results

A. Findings of the testing of human specimens for *Legionella pneumophila*

The number of serum specimens and positive cases of the testings for *Legionella pneumophila* conducted by the National Institute of Preventive Medicine between 1993 and 1995 is shown in Table 2. Of 1,511 specimens collected over a three-year period, 87 were serologically confirmed positive. This knowledge should help hospitals to more accurate diagnosis of patients with pneumonia-like symptoms. In March 1994, in the sputum specimens of patients, serotype 1 *Legionella pneumophila* was isolated (total specimens referred for testing for the years 1993, 1994 and 1995 were 41, 100 and

135, respectively). This was the first strain isolated in Taiwan. The strain was assessed by L-cysteine requirement, latex agglutination, direct immuno-fluorescent antibody test and polymerase chain reaction (PCR).

As shown in Table 3, of the 87 positive cases, 65 (75%) were male, and 58 (67%) were aged over 50. As shown in Table 4, of the specimens referred for testing in the first two years, all came from the northern part of Taiwan with the exception of those from the the Tzu-chi Buddhist Hospital in Hualien, on the east coast. No specimens were referred for testing by hospitals in either the central or southern parts of Taiwan, possibly because there was as yet little awareness in those areas of the presence in Taiwan of the disease. In 1995, however, through the efforts of the health agencies concerned, specimens were referred for testing from all over the Island. Though the number of specimens referred by each individual hospital under "Others" was still few, the rate of reporting in fact increased from 10 to 34%. With more awareness and effort, the testing and control of Legionellosis on Taiwan should improve.

Table 2. No. of Serum Specimens Tested for Legionellosis and No. of Positive Cases, 1993-1995

Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	
1993:														
No. sent	6	3	9	9	6	6	7	5	14	14	26	31	136	
No. (+)	0	0	0	0	0	0	0	1	0	2	8	3	14	(10.3%)
1994:														
No. sent	34	31	52	57	46	21	25	53	34	42	40	64	499	
No. (+)	3	2	3	7	0	2	5	1	0	2	2	1	28	(5.6%)
1995:														
No. sent	42	51	86	62	96	81	92	85	75	75	67	64	876	
No. (+)	0	0	3	3	4	6	9	5	8	2	0	5	45	(5.1%)

Table 3. Positive Cases of Legionellosis by Sex and Age

Year	20-50	51-60	61-70	71-99	Male	Female	Total
1993	2(14.3%)	4(28.6%)	3(21.4%)	5(35.7%)	11(78.6%)	3(21.4%)	14
1994	9(32.1%)	2 (7.1%)	7(25.0%)	10(35.7%)	21(75.0%)	7(25.0%)	28
1995	18(40.0%)	6(13.3%)	7(15.6%)	14(31.1%)	33(73.3%)	12(26.7%)	45
Total	29(33.3%)	12(13.8%)	17(19.5%)	29(33.3%)	65(74.7%)	22(25.3%)	87

Table 4. No. of Specimens Referred by Hospital

Hospital	1993	1994	1995
NTU Hospital	91(67%)	228(46%)	164(19%)
Mackay Memorial Hospital	0 (0%)	55(11%)	99(11%)
Air Force General Hospital	0 (0%)	23 (5%)	87(10%)
Po-ai Hospital	0 (0%)	20 (4%)	76 (9%)
Cathay General Hospital	11 (8%)	47 (9%)	33 (4%)
Provincial Taoyuan Hospital	1 (1%)	33 (7%)	34 (4%)
Tzu-chi BuddhistHospital	9 (7%)	22 (4%)	30 (3%)
Tri-service General Hospital	5 (4%)	18 (4%)	36 (4%)
Municipal Jenai Hospital	7 (5%)	23 (5%)	21 (2%)
Others	12 (9%)	30 (6%)	296(34%)

2. Findings of the testings of the environmental specimens for *Legionella pneumophila*

The Division of Bacteriology of the National Institute of Preventive Medicine conducted initial testings of 97 randomly selected environmental specimens between 1989 and 1991. From two specimens, as shown in Table 5, *Legionella pneumophila* was isolated.

Table 5. *Legionella pneumophila* in Water Specimens Taipei Area, 1989-1991

Water Source	No. examined	No. bacillus isoilated
Cooling tower water	50	2*
Lake	10	0
Pond	10	0
Drinking fountain	12	0
Faucet	10	0
Toilet	3	0
Soil on roof	2	0
Total	97	2

* isolated *Legionella pneumophila* strains confirmed by Professor Saito of Okinawa University, Japan

In March 1994, two serotype 1 strains of *Legionella pneumophila* were isolated from 12 cooling towers and the cooling-tower water specimens. The strains were assessed with L-cysteine requirement, latex agglutination and direct immuno-fluorescent antibody method. In August, using the same method, 6 serotype 1 strains were isolated from 13 cooling- tower water specimens (Table 6).

The existence in the environment of *Legionella pneumophila* may bring about Legionellosis. Testing of *Legionella pneumophila* in the environment is , therefore, most important. The National Institute of Preventive Medicine of the Department of Health has been conducting a large-scale survey to establish a basic information database on *Legionella pneumophila* for reference in the formation of disease prevention policies and measures.

Table 6. Testings of Water Specimens for *Pegionella pneumophila* in Taipei Area, 1994

Month	Source of Specimen	No.	No. (+)	Assessment Method
March	Water tower and cooling water tower	12	2	L-cysteine requirement Latex agglutination DFA
August	Cooling water tower	13	6	L-cysteine requirement Latex agglutination DFA PCR

Discussion

1. Laboratory Testing Methods

US CDC regulations state that any diagnosis of Legionellosis should be done based on the following: 1) the pathogen is isolated; 2) the pathogen is positive to direct immuno-fluorescent antibody reaction; 3) the IFA titer of serum in the recovery period (from three to six weeks) is four times higher than, or equal to, that at the early stage of disease onset, and higher than 128 (this is the serial serum antibody titer method); 4) the single serum antibody titer is larger than 256; 5) the urine antibody titer (the radioactive immuno-analysis method) is positive. To test Legionellosis with urine antigen, generally speaking, is relatively sensitive, with high specificity and speed. However, the specimens have to be collected within one week of disease onset. The serial serum antibody titer method gives results only in a few weeks. The reading of the findings

of the single serum antibody titer is not easy, and the diagnosis at times other than outbreaks has less meaning[2].

Though the laboratory testing method for Legionellosis is listed in "The Manual for the Collection of Specimens for Disease Control" issued by the ROC Health Department, reporting by physicians was not made mandatory, and whether or not specimens should be sent for testing was at their discretion. At that earlier time, physicians knew little about Legionellosis. Thus the number of specimens tested was low, and most of the specimens were collected only after medication, making the isolation of strains difficult. Major hospitals are now familiar with this disease and have conducted studies on the isolation of strains. With the joint efforts of academic and research institutions, the low isolation rate of strains can be improved.

2. Prevention of Legionellosis

With improvement in living standards and an increase in the number of tall buildings, the use of central air conditioning systems has become quite common. How to prevent the hot water supply system and the cooling tower from becoming contaminated by *Legionella pneumophila* is an urgent public health issue.

Hot water supply systems maintain the water temperature by fast flow of the water. The temperature of the water at the out-going faucet should be kept at 80°C and above; water that returns to the heater should be kept at 60°C and above. The parts in the whole system where the temperature of the water cannot reach 60°C and above (for instance, the pipeline which is not in use, and the faucets less frequently used,) should be removed. If contamination by *Legionella pneumophila* is suspected, the water pipes should be washed daily for from two to five minutes, for seven days, using hot water of at least 60°C or above.

All cooling towers should be cleaned before use or when not in use. When not in use, the tower should be emptied of water. Disinfectants may be used to inhibit the growth of microbes, but a daily record should be kept of the amount of disinfectant applied. The application of disinfectants is thus far the only available and inexpensive method of prevention.

In the US, public places such as McDonald's Restaurants and and Hilton Hotels are inspected once every three months. In Taiwan, however, no organization has as yet been made responsible for the inspection of environmental specimens. *Legionella pneumophila* is considered by US professionals to be the third major pathogen of pneumonia. To prevent the spread of this disease in Taiwan, regular professional responsible inspections of public places are essential.

On 11 January 1995, the Department of Health announced that: all regional and other hospitals of higher levels must, upon having identified cases, immediately report those to the local health authorities, and then to the Department's National Quarantine Service. On 7 March 1995, the Department invited eight noted epidemiologists to discuss

preventive measures for Legionellosis. On 16 June 1995, local health authorities, military medical care institutions and medical associations were informed of the outcome of their discussions. With this continuing effort, the chances of infection from Legionellosis in Taiwan should be significantly reduced.

Prepared by: T.M. Pan, H.L. Yeh, C.B. Horng (National Institute of Preventive Medicine, the Department of Health)

References:

1. Yu VL. *Legionella pneumophila* (Legionnaires' disease). In Mandell/Douglas/Bennett, eds. Principles and Practice of Infectious Diseases. 3rd ed. New York: Churchill Livingstone. 1990: pp 1764-1772.
2. Benenson AS. Control of Communicable Diseases in Man. 15th ed. American Public Health Association. Washington, DC, 1990: pp 235-238.
3. Gecewicz TE, Lett SM, Kludt PE. Legionnaires' disease associated with cooling towers--Massachusetts, Michigan and Rhode Island, 1993, MMWR 1994; 43(27):491-493.
4. Lin SL, Chen HS, Yu CJ et al. Legionnaires' disease with acute renal failure: report of two cases. J Formosan Med Asso 1995; 94(3):123-126.
5. Winn WC. Legionella, in Murray PR, Baron EJ, Pfaller MA, et al., eds. Manual of Clinical Microbiology. 6th ed. Am Soc Microbil, Washington, DC. 1995: pp533-544.
6. Thornberry C. Legionella, Proceedings of the 2nd International Symposium. Am Soc Microbil, Washington, DC. 1984:212-215.
7. Koide M, Saito A, Kusano N, at al. Detection of Legionella spp. in cooling tower water by the polymerase chain reaction method. Appl Environ Microbiol 1993; 59:1943-1946.
8. Morton S, Barlett C, Bibby LF, et al. Outbreak of Legionnaires' disease from a cooling water system in a power station. Br J Indust Med 1986; 43:630-635.
9. Chen MSG, Yang YR, Shen HD. *Legionella pneumophila* antibodies as detected by IFA, Proc Natl Sci Counc. ROC(A) 1983; 7:249-254.