Rapid Identification and Serogrouping of *Legionella pneumophila* from the Environmental Cooling Towers by Fluorescence Activated Cell Sorter

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SUMMARY

The fluorescein labelled antibody/Fluorescence Activated Cell Sorter (Ab/FACS) assay was used for direct identification of *Legionella pneumophila* from the cooling tower samples around the Taipei city area, Taiwan during the period of 8/1998 to7/1999. For comparison, conventional *Legionella* culture method was also performed in parallel. Specimen harvested directly from the cooling towers were first concentrated by filtration through a millipore membrane, the bacteria in the suspension were then inoculated on BCYE-α (L-cysteine)/BAP plates for isolation and also identified directly by Ab/FACS assay. Latex agglutination and Ab/FACS with antibody specific to *L. pneumophila* serogroup 1 were subsequently performed for detection of the *L. pneumophila* serogroup-1 strains from the isolates. Of the 403 specimens, a total of 205 suspected *L. pneumophila* colonies were isolated. Further biochemical analysis identified 177 *L. pneumophila*. After latex agglutination, 105 strains were classified to be *L. pneumophila* serogroup 1, and 72 strains to be *L. pneumophila* non-serogroup 1. In parallel, the Ab/FACS assay showed good concordance with the traditional identification and classification method. Our study suggests that Ab/FACS appears to be a rapid and efficient technique for identification of *L. pneumophila* from the environment.

INTRODUCTION

Legionella pneumophila is recognized as an important pulmonary pathogen (McDade et al., 1977; Kirby et al., 1998; Fujii and Yoshida 1998; Vobel and Isberg 1999). Predominant clinical disease caused by this organism includes Pontiac fever (Cordes and Fraser 1980) and Legionnaires' disease (Fraser et al., 1977). Legionnaires' disease can be acquired by the inhalation of aerosols containing legionella or by microaspiration of water contaminated with *legionella* (Edelstein 1993).

The legionella could be isolated from the cooling towers, and the cooling towers were thought to be an important aerosol-generating system that was linked to the disease transmission (Addis et al., 1989; Woo et al., 1992; Garbe et al., 1993; Miyamoto et al., 1997; Castellani Pastoris et al., 1997; Brown et al., 1999). Based on this, surveillance of legionellae in the cooling towers and preventive measures against the contamination are needed. A sensitive and practical procedure for legionellae surveillance in cooling tower water should be established to prevent outbreaks of legionellosis.

The diagnosis of *L. pneumophila* infections mainly relies on the ability to grow these organisms in the laboratory. Growth on BCYE-α (L-cysteine) agar (Mast Diagnostics, Merseyside, U. K.) with no growth on blood agar is one of the most useful presumptive clues that an isolate could be a species of *Legionella*. For confirmation, the biochemical reaction such as sodium hippurate hydrolysis test (Pratt-Ripplin and Pezzlo 1992) which is positive for *L. pneumophila* and negative for the majority of other *Legionella* species, and the slide latex agglutination assay or direct and indirect immunofluorescence assay (Koide et al., 1996; Maiwald et al., 1998) were used for distinguishing the *L. pneumophila* from other species.

For the purpose of rapid diagnosis of *L. pneumophia* by molecular methods (for review, see Maiwald et al., 1998), some other identification systems such as hybridization with specific nucleic acid probes (Brenner, 1986; Grimm et al., 1998), PCR (Koide et al., 1996; Villari et al., 1998; Weir et al., 1998), *legionella* urine antigen test (Plouffe et al., 1995; Dominguez et al., 1998), lipopolysaccharide antigen pattern (Jurgens and Fehrenbach 1997) and cellular fatty acid composition and isoprenoid quinone content analysis (Lasmbert and Moss 1989) have been used for identification of the *Legionella* species.

The fluochrome labelled antibody/flow cytometry assay has been used frequently in many clinical areas such as differentiation of T cell/B cell subpopulations (Bomberger et al., 1998; Maruoka et al., 1998). Application of similar assays were also reported in the differentiation of bacterial lipopolysaccharides (Evans et al., 1990), separation of bacterial cells expressing cell wall bound antibody fragments (Fuchs et al. 1996),

quantification of bacterial cell surface receptors (Anderoni et al. 1997), detection of *Cryptocporidium oocysts* from human stool samples (Valdez et al., 1997), direct quantification of cytomegalovirus antigenemia (Ramani et al., 1997) and identification of antibodies in milk (Iannelli et al., 1998). Recently, a series of studies using flow cytometry for rapid drug susceptibility test of bacteria and virus were also reported (Bownds et al., 1996; Kirk et al., 1997, 1998; McSharry 1999; Kesson et al., 1998; Kesson et al., 1999). These results add rapid drug susceptibility test to the rapidly expanding list of rapid bacterial diagnosis by flow cytometry.

The purpose of this study was, based on our previous report which used Ab/FACS for direct identification of *Mycobacterium* species from clinical samples (Yi et al., 1998), to further test whether this assay is applicable for rapid identification of *Legionella* species from the environmental samples such as those from cooling towers in the public buildings around Taipei city area. A total of ** cooling towers of ** public buildings were tested. For comparison, a culture method followed by serogrouping by latex agglutination was also performed ( ). After optimization for the experimental conditions, our results showed that this method has the advantages of relatively low complexity, high sensitivity, ease of bacterial cell number quantification, time-saving and safty. Thus this detection system shows its potential of being applied in the rapid diagnosis of *L. pneumophila* from the environment.

MATERIALS AND METHODS

Bacterial strains. Standard *L. pneumophila* (ATCC43661 need serogroup1), *Pseudomonas aeruginosa*, *Mycobacterium fortuitum*, *Staphyloccus aureus* and *Candida albicans* were used as the positive controls.

Bacteriologic methods. *L. pneumophila* were grown at 37℃ on buffered charcoal yeast extract α (BCYE-α) agar media containing supplements of L-cysteine and *Legionella* selectatab (Mast diagnostics) in 5% CO_2 and saturated humidity. For decreasing the contamination of other microorganisms from the cooling towers, CCVC [cephalosporin (4 μg/ml), colistin (16 μg/ml), vancomycin (0.5μg/ml) and cycloheximide (80 μg/ml)] were added into the isolation media (Bopp et al., 1981). Growth on blood agar plate (BAP) was used as the control (Bornstein et al., 1989).

Processing of cooling tower samples. The water samples were taken from the cooling towers of buildings around Taipei city area, including train station, shopping centers, Universities, Hospitals, Mansions, and others during the period of 8/1998 to 7/1999. $Na_2S_2O_3$ (final concentration 100 μg/ml) was first added to the water sample (up to 2 liter). After mild shaking for 3 minutes, the water was passed through 0.22 μm nitrate cellulose filters (Millipore, MA, U. S. A.), followed by washing with 5 ml pH 2.2, 0.2N KCl-HCl solution. The bacterial sample was then washed again by the sterile filtrate to neutralize the pH of the sample. BCYE-α (L-cysteine) broth containing CCVC 2.5 ml was added onto the filter membrane and 1/3 of the bacterial suspension was dropped onto the BCYE-α (L-cysteine) agar plates containing CCVC for isolation of single bacterial colonies. The remaining bacterial suspension was collected, washed and dissolved in Phosphate buffered saline (PBS, pH 7.5) for flow cytometry analysis.

Identification of *L. pneumophila* by conventional method. Identification of *L. pneumophila* was followed the procedures described by Koneman et al., (1997) with some modifications. After inoculation, BCYE-α (L-cysteine) /CCVC plates were incubated at 35℃ and 2.5% CO_2 under humid condition for up to 14 days. Usually suspected colonies were observed after 2-4 days of culture. The bacteria which grew on BCYE-α (L-cysteine) agar but did not grow on BAP were selected and subjected to hippurate hydrolysis test (Pratt-Ripplin et al., 1992). The bacteria which showed positive hippurate hydrolysis test reaction were identified to be *L. pneumophila*. These bacterial strains were further grouped by Prolex Latex agglutination assay (Pro-Lab Diagnostics, U.S.A.) using monoclonal antibody specific to sero-group 1 *L. pneumophila*.

Selection of antibody against *L. pneumophila*. After a series of antibody tests with antibodies from different sources, two kinds of mouse primary monoclonal antibodies

were used. The first one (original concentration of 0.1 mg/ml) was specific to all *L. pneumophila* serogroups (1~14)(Cortex, SanLeandro, CA, U. S. A.), and the other (original concentration of 3 mg/ml) was specific to *L. pneumophila* serogroup 1 (Biogenesis, Poole, England, U. K.). Goat-anti-mouse secondary IgG antibody (1.0 mg/ml) was FITC labelled.

Antibody binding. For identification of *L. pneumophila*, the bacterial cells in the sample were washed twice in 500μl PBS (pH 7.5), pelleted by centrifugation at 12600 x g for 5 min, and finally dissolved in 100μl PBS in eppendorf tubes. Primary antibody dissolved in PBS solution, with the final concentration of 10ng/μl and volume of 200μl, was added into the bacterial suspension followed by mixing and incubation at 4℃ for 30 min. The suspension was subsequently washed with PBS twice. Secondary antibody 200μl which was diluted either 1:10, 1:50, 1:100, or 1:200 by PBS was then added. After incubation at 4℃ for 30 min, the samples were then washed twice with PBS and dissolved in 1 ml PBS before FACS analysis. After repeated tests, the titration experiments established that the maximal specific fluorescence was obtained at the dilution factor of 1:100 for secondary antibody (final concentration of 10 ng/μl). Such an antibody suspension was used for all subsequent assays.

For identification of *L. pneumophila* serogroup 1, similar procedures were performed as mentioned above, except that primary antibody with the final concentration of 30ng/μl was used.

FACS identification and data analysis. The procedures for FACS operation and construction of the histogram followed the operator's guide supplied by the manufacturer (Coulter, U. S. A.). Basically, the fluorescence and the light scatter of the cells were measured with an EPICS XL-MCL (Coulter, U. S. A.), equipped with a 15-mW, air cooled, 488-nm-wavelength Argon laser light. For each sample, signals from 3,000-10,000 events were collected in list mode unless mentioned specifically in the sensitivity assay. No gates were set around the particles. Results were presented as the mean channel of fluorescence for the treated sample minus the mean channel of fluorescence for the control tubes (incubated with PBS). Logarithmic units ($\log_{10}$ U) were transformed into linear channels (LC) by using the following formula LC= total number of channels/number of log decades x $\log_{10}$U. The total number of channels and the number of log decades of the instrument were 1,024 and 4, respectively.

Histograms were constructed with the signals from single cells selected by gating the FLS and PLS fluorescence parameters. Red fluorescence was measured with a logarithmic amplifier. Mean linear fluorescence intensities were calculated from the histograms as the sum of the product of the linearized channel number times the number of events per channel, divided by the total number of events. Channel 1 on the

log scale was taken to be 1.0 arbitrary unit on the linear scale.

RESULTS

Isolation and identification of *Legionella pneumophila* by conventional culture method

The cooling towers from a total of 403 private and public buildings composed of hospitals, schools, theaters, and supermarkets around Taipei city area were chosen for study on the prevalence rate of *L. pneumophila* during the period of 7/1998 to 6/1999. Water samples were $Na_2S_2O_3$ treated, and concentrated by filtration through filter membrane as described in the Materials and Methods. Isolation and identification of *L. pneumophila* were achieved by inoculation of 1/3 volume of the sample suspensions on BCYE-α (L-cysteine)/CCVC plates, followed by inoculation of suspected colonies on BAP. The bacteria which grew well on BCYE-α (L-cysteine)/CCVC, but not on BAP were selected for confirmation of *L. pneumophila* by hippurate hydrolysis test (Pratt-Ripplin 1992). A total of 205 suspected *L. pneumophila* colonies were isolated from the BCYE-α (L-cysteine)/CCVC and BAP plate assay. After hippurate hydrolysis test, 177 bacterial strains (86.3%) were identified to be *L. pneumophila*. The remaining 28 strains were classified to be non *L. pneumophila*. The *L. pneumophila* strains were further subjected to serogroup analysis by Prolex latex agglutination test (specific to *L. pneumophila* serogroup 1; Pro-Lab Diagnostics, U. S. A.). The result showed that 105 bacterial strains (59.3% out of the *L. pneumophila*) were identified to be *L. pneumophila* serogroup 1, and 72 strains were negative in the assay and were classified to be *L. pneumophila* non-serogroup 1 (belonging to groups 2~14). The remaining 28 strains which showed negative hippurate hydrolysis test results were also latex agglutination test negative.

The environmental temperature had significant effect on the isolation of *L. pneumophila*. Among the 618 cooling towers analyzed, the lowest temperature detected was at 7℃ (12/1997), and the highest temperature detected was at 37℃ (6/1998). Our results **(Table 1)** showed that the isolation rate was at its highest when the environmental temperature ranged between 25~30℃, a total of 30.1% isolation rate was obtained. The next was the temperature between 20~25℃, with the isolation rate of 27.8%. Further analysis showed that when analyzing, the temperature of 362 out of the 618 cooling towers (58.9% of the total cooling towers) was shown to be between 25~35℃, and the isolation rate for *L. pneumophila* was 29.6%. These data suggested that *L. pneumophila* were easier detected during the summer period. Indeed, the months with the highest positive isolation rate during this period was 8/1997 and 9/1997 (temperature ranged between 25~35℃), with 31 strains being isolated each month. The month with the lowest isolation rate was in the 3/1998 (temperature

ranged between 15~20℃), with only 7 strains being isolated.

Specificity of Ab/FACS in the detection of standard *Legionella pneumophila*

Before performing the Ab/FACS assay, the specificity and sensitivity limit was evaluated. For evaluation of the specificity, a mouse monoclonal antibody against *L. pneumophila* serogroup 1 (Cortex, SanLeandro, CA, U. S. A.) together with a fluorescein (FITC) labelled secondary antibody were used to identify *L. pneumophila* through flow cytometry as described in the Materials and Methods section. Combinations of a series of primary and secondary antibody dilutions were tested in the assay for a better experimental condition and the result showed that use of the primary antibody (1:100 dilution)(100 ng/ml) and secondary antibody (1:10 dilution)(10 ng/ml) gave the best result. The same experimental format was used for the subsequent study.

A cultured standard *L. pneumophila* strain was first tested for the evaluating the applicability of this assay. For negative control, *Candida albicans*, *Mycobacterium fortuitum*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and were used. The results in **Fig. 1** show a clear difference in the peak of fluorescence intensity between *L. pneumophila* and negative controls, implying that this assay bears the characteristic of high specificity. For confirmation of the results, fluorescence microscopy was performed and the results show that compared with negative controls, fluorescein labelled *L. pneumophila* can be observed clearly (data not show), allowing the clear distinction of *L. pneumophila* from the negative controls.

Sensitivity of Ab/FACS in the detection of standard *Legionella pneumophila*

To test the sensitivity of this assay, *L. pneumophila* suspensions with known optical density (A_{600}) were 10x serially diluted. The actual bacterial particle number detected was counted by FACS. The results in **Fig. 2** show that the significant luminescence intensity peak of *L. pneumophila* was obtained even when bacterial cells were diluted to be about 1000 particles per sample. This result shows that the sensitivity of this assay is quite high. Samples containing as few as 1000 bacterial cells could be detected by this method.

Detection of *Legionella pneumophila* from cooling tower samples by Ab/FACS

The bacterial suspensions prepared from 403 cooling tower samples were subjected to species identification by Ab/FACS analysis after treatment. The result of identification was compared with those obtained using conventional culture methods. For each sample, a total of about 1000 legionella particles were counted and clear fluorescence peaks appearing at a different position from the control could be

observed. The results shown in **Fig. 3** indicate that *L. pneumophila* can be identified by Ab/FACS assay. A total of *** samples were identified to contain *L. pneumophila* (**Table 1**). Our method showed high sensitivity (100 %) and high specificity (90 %) compared with the conventional culture method.

Serogrouping of *L. pneumophila* serogroup 1 by Ab/FACS. The 177 *L. pneumophila* strains identified by culture/biochemical method were serogrouped by Ab/FACS. The cultured bacterial cells and a monoclonal Ab specific to *L. pneumophila* serogroup 1 (Biogenesis, Poole, England, U. K.) were used. A standard *L. pneumophila* serogroup 1 strain was used as the positive control. *L. pneumophila* non-serogroup 1 strains such as *L. pneumophila* strains were used as the negative controls. The results shown in Fig. 4a confirmed the specificity of this assay. The detection peak from that of *L. pneumophila* serogroup 1 can be clearly distinguished from those of the other strains of different serogroups. Based on this test result, the 177 *L. pneumophila* strains were subject to Ab/FACS assay. Some Ab/FACS results of the strains that were identified to be sero-group 1 were shown in Fig 4b.

The Ab/FACS serogrouping results were compared with those from latex agglutination assay. **Table 2.** showed that 100 out of 177 *L. pneumophila* strains (** %) were grouped to be serogroup 1. Compared with Latex agglutination test, the Ab/FACS method showed sensitivity of 90 %, and specificity of 88% in the serogrouping of *L. pneumophila* strains.

DISCUSSION

One of the objectives of the present study was to validate whether the single cytometric test has the capability to rapidly detect the presence of *L. pneumophila* from environmental samples such as those from cooling towers without the procedures of cell culture and identification by biochemical test. Besides detection, we also hope to see whether this assay can also be used for rapid serogrouping of *L. pneumophila*. For these purposes, the results from Ab/FACS assay were compared with those obtained by conventional cell culture isolation and latex agglutination methods. This comparison established that flow cytometry was a useful test for use in the identification of environmentally fastidious bacteria. While flow cytometry has been widely used clinically for the separation of subpopulations of blood cells and in research in other related fields, this assay is just started to be considered for use in clinical bacteriology. Some fluorescence based methods using fluochromes as the light-emission source are used in the preliminary quantification of microbial pathogens (Fang 1996; Valdez et al., 1997; Maiwald et al., 1998). These assays either lack specificity for identification (without using antibodies), or use UV fluorescence microscopy instead of FACS in the identification process, which are rather laborious and time-consuming.

Our data show that the Ab/FACS assay has several advantages over existing methods for the diagnosis of *L. pneumophila*: 1) It is more rapid than cell culture method. The sensitivity of the technique (to about 1000 bacteria per specimen) is especially useful for samples that contain few *L. pneumophila*. 2) The technique is easy to perform. Most of the processes in this assay are performed by automation and this saves quite a lot of time and labor. Because this method does not amplify DNA fragments, the possibility of contamination which quite often occurs in PCR assay is low. 3) The capability of FACS to quantify cell number allows easy quantification of the bacterial cell number without culture. 4) It shows low background compared with using fluorescein labelled oligonucleotide DNA as the probe for Fluorescence *In Situ* Hybridization (FISH) assay (Yi et al., unpublished data). Indeed, a previous study from our laboratory which used similar assay for rapid identification of Mycobacterial species from sputum samples also showed that this assay was useful for diagnosis of bacteria (Yi et al., 1998).

Although there are many advantages, we still have to notice the possibility that phase-variable expression of bacterial surface macromolecules such as lipopolysaccharide, which was usually used as the antigen for induction of antibody production, occurs frequently on *L. pneumophila* (Luneberg et al., 1998). This might cause inconsistency during the identification process, which remains to be elucidated.

Another problem encountered is the specificity of the monoclonal antibodies. We have screened more than 10 different sources of monoclonal or polyclonal antibodies for applicability of the assay before the large scale test. High cross-reaction with other bacterial genus was usually observed. It is thus essential that monoclonal antibodies with high specificity be used for the assay. Monoclonal antibodies with narrow strain specificity are being developed (Baranov et al., 1998). Once more specific antibodies are available, specifically rapid identification of *L. pneumophila* will be readily achieved. For subsequent serogrouping, Helbig et al., (1997) reported that 98% of the human and environmental *L. pneumophila* isolates could be grouped by serotyping. If the antibody system was well developed, by using Ab/FACS assay, most of the *L. pneumophila* could be serogrouped easily.

Another limitation of the Ab/FACS assay is the high cost of the flow cytometer. However, this instrument, in view of its versatility, is expected to soon become widely used in many fields which are involved in the process of antibody-antigen interaction. Besides clinical serology, clinical and industrial microbiology are also expected to find applications (Iannelli et al. 1997; McSharry 1999; Boer and Beumer 1999). The availability of visible-wavelength diode lasers of low price, high efficiency, and long lifetime is expected to promote broader applications of flow cytometry and, to reduce the cost of equipment. In addition, such a technique can be easily adapted to identify pathogens other than *Legionella* species, such as the *Mycobacterium* species as reported (Yi et al., 1998). It can also be adapted to detect the concentration of antibodies in the serum specimen. Detection of clinically significant *L. pneumophila* either in bacteriology or in serology is now being performed in our laboratory due to the availability of species-specific antibody. Preliminary data showed a strong potential in clinical application (Yi et al. unpublished data).

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Legends

FIG 1. Use of Ab/FACS for identification of cultured mycobacterial spp. A rabbit polyclonal Ab was used and the results were analyzed by flow cytometry. The arrow indicates the negative control: *Candida albican* (a, b, c, d) and *Aeromonas hydrophila* (e, f, g, h). The result of the assay from *M. fortuitum* shows relative count of RPE labelled cells 61.8% (a, e); *M. scrofulaceum* 98.0% (b, f); *M. cheloneiss* 86.7% (c, g); *M. tuberculosis* 71.4% (d, h). Data presented are the means of three determinations (SEM<5%).

FIG 2. Sensitivity of Ab/FACS in detection of mycobacterial spp. Bacterial cells of original concentration about 10^6/ml determined by counting the numbers of CFU after plating aliquots of the same suspensions were diluted 1:10, 1:100 and 1:1000. The actual cells measured (10^5,10^4 and 10^3 for each sample) were determined by flow cytometry. The relative number of RPE labelled cells show (a) *M. fortuitum* (98.4%, 95.6%, 90.8%, 86.2%), (b) *M. tuberculosis* (80.2%, 76.0%, 75.4%, 71.9%), (c) *M. cheloneiss* (80.9%, 76.7%, 71.4%, 72.3%), (d) *M. scrofulaceum* (98.0%, 97.8%, 85.0%, 79.6%), (e) *C. albicans* (3%, 1%, 1%, 0%), (f) *A. hydrophila* (20.3%, 9.9%, 3.5%, 2.5%). Data are the means of three independent determinations (SEM<5%).

FIG 3. .Detection of mycobacterial species directly form clinical specimens by Ab/FACS. The *A. hydrophila* was used as the negative control. (a) *M. tuberculosis* B46091, the relative count of RPE-labelled cells is 72.8%, (b) *M. tuberculosis* B46092 (70.6%), (c) *M. avium* B46042 (68.7%), (d) *M fortuitum* B46043 (86.7%), (e) *M. tuberculosis* B46045 (56.0%), (f) *M. tuberculosis* B46046 (60.4%), (g) *M. tuberculosis* B46048 (71.5%), (h) *M. tuberculosis* B46051 (62.5%), (i) *M. avium* B46052 (72.6%). Data are means of three independent determinations (SEM<5%).