Detection of *Legionella pneumophila* antigens in patients' sera using monoclonal antibodies

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Abstract

Three monoclonal antibodies (McAb) were produced against soluble antigens of *Legionella pneumophila* serogroup 1 which was cultured on BCYE agar. The McAbs were all of the IgM isotype. The McAbs were used in the McAb-based ELISA for detection of circulating *L.pneumophila* antigens in 186 sera collected from patients with symptoms and signs suggestive of atypical pneumonia. The normal reference optical (OD) density value of each of the McAbs was determined using 44 sera collected from healthy blood donors. The antigen positivity rates for the McAbs 1C7.2B, 2B2.10F and 2B2.11E were 11.3%, 7.7% and 22.2% respectively. Antigen positivity of the McAb 2B2.10F was significantly higher in the younger age group ($p<0.05$). There is no significant association between the antigen positivity with age and sex for all the McAbs. There was no cross-reaction demonstrated between the McAbs with other bacterial antigens.

Key words: Legionellosis, *L.pneumophila*, antigen detection, monoclonal antibodies, immunology, serum laboratory diagnosis, ELISA.

INTRODUCTION

Legionella pneumophila infections in man can be manifested as two different clinical presentations, flu-like illness (Pontiac fever) and pneumonia (Legionnaire's disease). Most infections are in individuals with compromised immune status or pulmonary function. Laboratory diagnosis of legionellosis in the acute stage of the illness requires culture of the organism from appropriate specimens or demonstrationof antigens by immunofluorescent technique. However, isolation of this organism by culture is difficult and time consuming due to its fastidious nature. Morever, it has been shown that a few *Legionella* strains may be viable but cannot be cultured.[1] Due to a delay in detecting a rising titre of *Legionella* antibody with respect to the onset of the illness, diagnosis should not rely mainly on antibody detection. Therefore, newer techniques including antigen detection in serum and urine had been developed to improve the diagnosis. In this study, we have developed a monoclonal antibody-based ELISA (McAb-ELISA) for the detection of circulating *L.pneumophila* antigens in serum.

MATERIALS AND METHODS

Preparation of bacterial antigen

L. pneumophila serogroup 1 was harvested from buffered charcoal yeast extract (BCYE) agar plate. Pure colonies of the bacteria were solubilized by homogenization and sonication on ice. The suspension was centrifuged after overnight incubation at 4°C and the supematant recovered as soluble antigen. Other bacterial species namely *Haemophilus influenzae, Klebsiella pneumoniae, Sreptococcus pneumoniae, Staphylococcus aureus, Helicobacter pylori* and *Capnocytophaga canimorsus* were cultured on appropriate agar plates and processed similarly to obtain their soluble antigens for use in the cross-reactivity study of the monoclonal antibodies.

Production of monoclonal antibodies

BALB/c mice were immunized subcutaneously with 100 ug/ml of *L. pneumophila* soluble antigens in equal volume of Freund complete adjuvant. Antibody response of the mice was measured by indirect ELISA. Immunization was

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repeated twice at two weeks interval. When the immune response was strong (ELISA optical density was greater than 1.00), a booster dose of 50-100 ug/ml of the soluble antigen was administered. Three to four days after the booster, the spleen cells of the mice were harvested for fusion procedure. Fusion of the spleen cells with myeloma cells (NSI) was carried out following the method described by Kohler and Milstein[2] with some modification. The fused cells were cultured in 96-well plate (Costar, MA, USA) which was laid with macrophages obtained from mouse peritoneal cavity. Selection of the hybrids was carried out by addition of HAT solution, i.e. 1 ml of 100x hypoxanthine, aminopterin and thymidine solution (Gibco, BRL, USA) in complete culture medium (RPMI 1640 supplemented with 1000 IU/ml penicillin and 100 ug/ml streptomycin and 20% fetal calf serum (FCS) in 100 ml of medium). After 10 days, HT medium (hypoxanthine and thymidine solution) (Gibco, BRL, USA) was used followed by complete culture medium. Antibody productionof the hybrids was screened by ELISA and positive hybrids were propagated and cloned by limiting dilution technique. The subclass of the monoclonal antibodies was determined by ISO strip™, mouse monoclonal antibody isotyping kit (Boehringer Mannheim, Germany). The positive clones were then grown in large quantities in culture flasks and subsequently inoculated into peritoneal cavities of pristane-primed mice for production of ascites.

Monoclonal antibody-based ELISA (mAb-ELISA) for antigen detection

Detection of antigen using these monoclonal antibodies was carried out following the technique of Zheng ***et al***.[3]

Patients' sera

A total of 186 sera from patients admitted to hospitals with respiratory symptoms and fever suggestive of atypical pneumonia were tested for circulating L. ***pneumophila*** antigens using the McAb-ELISA. This comprised 111 (59.7%) Malays, 31 (16.7%) Chinese and 34 (18.3%) Indians and 10 (5.4%) of other races. Their ages ranged from 0.2-91 years with a mean age of 37.6 years. One hundred and three (55.4%) were males while 83 (44.6%) were females.

Control sera

Negative reference value for each of the monoclonals was determined by running the test on 44 sera collected from blood donors.

Cross-reactivity study

Cross-reactivity of the McAbs was assessed against different species of bacteria known to cause respiratory infections or reported to cross-react with L. ***pneumophila.*** These organisms include H. ***influenzae,*** K. ***pneumoniae, S. pneumoniae, S. aureus, H. pylori*** and ***C. canimorsus.***

Statistical analysis

Data were entered into a database and analysed using a statistical software SPSS version 6.1. Significance test was carried out using a Chi-square and correlation test and a P value of < 0.05 was taken as significant.

RESULTS

Three McAbs designated as 1C7.2B, 2B2.10F and 2B2.11E were produced. The normal reference McAb-ELISA optical density (OD) values for the McAbs were 0.381, 0.540 and 0.271 respectively. Using the McAb 1C7.2B, 21 (11.3%) were positive for L. ***pneumophila*** circulating antigen with the mean OD of 1.88. The McAb 2B2.10F detected antigen positively in 14 (7.7%) patients, while 2B2.11E gave positive results in 42 (22.6%) suspected cases. There was no significant association between antigen positivity with race and sex by the three McAbs. There was also no significant correlation between antigen positivity with age for McAb 1C7.2B and 2B2.11E but antigen positivity for McAb 2B2.10F was observed to be higher in the younger age group ($p<0.05$). The OD values of the McAb-ELISA between the McAbs with antigens of different species of bacteria are shown in Table 1. There is no cross-reactivity between the three McAbs with ***S. pneumoniae,*** S. ***aureus, K. pneumoniae, H. influenzae, H. pylori and C. canimorsus.***

DISCUSSION

The diagnosis of Legionellosis is practically made on clinical grounds. Most often the diagnosis is supported by culture and serological techniques. Unfortunately, culture of the organism takes a longer time and false negatives may occur.[1,4,5] Seroconversion in most patients may not occur for three to six weeks[4] therefore demonstration of antibody is not of much assistance in the diagnosis of acute infection.

TABLE 1: Cross-reactivity of McAbs raised against *Legionella pneumophila* to other bacteria (ELISA OD VALUES)

McAbs	Control cut off	*S. pneumoniae*	*S. aureus*	*K. pneumo*	*H. influenzae*	*H. pylori*	*Capnocytophaga spp*
1C7.2B	0.381	0.214	0.276	0.195	0.259	0.115	0.149
2B2.11E	0.540	0.181	0.205	0.181	0.190	0.123	0.102
2B2.11E	0.271	0.079	0.093	0.086	0.146	0.092	0.091

Several studies indicate that detection of ***L. pneumophila*** urinary antigen is a promising tool for rapid diagnosis of the infection.[6-8]

Mangiafico ***et al***[9] examined serum specimens from patients with legionellosis using reverse passive haemagglutination test, but they were unable to detect antigen. Bibb ***et al***[10] demonstrated bacterial antigen in patients' sera using ELISA but the antigen level in serum was 30-100 fold less than in urine collected the same day. In this study, we have developed an in-house McAb-ELISA system using three monoclonal antibodies that were produced against ***L.pneumophila*** serogroup 1. Using the three McAbs 1C7.2B, 2B2.10F and 2B2.11E, we demonstrated circulating ***L.pneumophila*** antigens in 11.3%, 7.7% and 22.6% of patients with atypical pneumonia respectively. The difference in the sensitivity of the McAbs could be due to the difference in the affinity of the McAbs for their respective determinants or the difference in the number of determinants present.

It was shown that there was no significant difference between antigen positivity by the three McAbs with race and sex. There was also no significant difference between antigen positivity with age except for McAb 2B2.10F where antigen positivity was found to be higher in younger patients ($p<0.05$). Studies on the prevalence of antibody to ***L.pneumophila*** did not show significant difference between antibody titre distribution with age and sex.[11,12] Higher antigen positivity in younger patients observed in this study could be due to the presence of underlying predisposing factors in these patients such as underlying respiratory diseases, compromised immune status due to systemic illness and underdeveloped defence mechanism.

There was no cross-reaction observed between the McAbs with other bacteria that may cause respiratory diseases namely H. ***influenzae***, K. ***pneumoniae***, ***S. pneumoniae***, and ***S. aureus***. Studies have shown that there was serological cross-reaction between ***Legionella spp***. with ***H. pylori*** and ***Capnocytophaga spp***.[13,14] In this study, there was no cross-reaction detected between McAbs with these bacteria.

Although studies have shown that less antigen is detectable in the serum than in urine and therefore urine is the preferred specimen, there are instances when urine is difficult or impossible to obtain such as in cases of renal failure. In addition, serum specimens but not urine are often saved for antibody study, therefore the former again provides an alternative to urine as a diagnostic specimen. Although urinary antigen detection is reported to be sensitive, it has disadvantage in that it only reliably detects ***L.pneumophila*** serogroup 1 antigens, then serum is a useful specimen for other serogroups and species of ***Legionella***.[15]

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