Letters

Development and Evaluation of a MAb-Based ELISA for Detection of *Chlamydophila pneumoniae* Infection with Variable Domain 2 and 3 of the Major Outer Membrane protein*

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Abstract

Objective This paper aims to develop a monoclonal antibodies (MAbs)- based ELISA for detecting *Chlamydophila pneumoniae (C. pneumoniae)* antigens in humans with the variable domains (VD) 2 and 3 of the major outer membrane protein ($MOMP_{VD2\text{-}VD3}$) and to assess its sensitivity and specificity by comparing with a widely used MAb that is able to recognize the elementary bodies of *C. pneumoniae*.

Methods $MOMP_{VD2\text{-}VD3}$ were overexpressed in *Escherichia coli* and purified by affinity chromatography. Mice were immunized with the recombinant antigen, and hybridomas secreting MAbs were screened. Three stable hybridomas clones were selected and named 5D6, 7G3, and 8C9. The MAbs-based ELISA was scrutinized for species-specific recognition with a number of human throat swab samples from Group I (156 patients with typical respiratory illness clinically confirmed before) and Group II (57 healthy donors).

Results In Group I, 55 positive cases were detected by anti-EB MAb-based ELISA, 51 cases were positive by MAbs 5D6-based ELISA, and 33 and 38 cases were positive by MAb 8C9 and 7G3-based ELISA respectively. Of the 57 samples from Group II "healthy donors", 5 were positive and 52 were negative with both anti-EB and 5D6-based tests, while 2 and 3 positive cases were identified by the other two MAb-based ELISAs respectively.

Conclusion The novel $MOMP_{VD2\text{-}VD3}$ MAb-based assay may have higher specificity than the anti-EB MAb, which may possibly be used as an alternative tool for the diagnosis of *C. pneumoniae* infection.

Key words: *Chlamydophila pneumoniae*; Major outer membrane protein; Monoclonal antibody; ELISA

Biomed Environ Sci, 2012; 25(6):690-696 *doi: 10.3967/0895-3988.2012.06.011* *ISSN:0895-3988*

www.besjournal.com(full text) *CN: 11-2816/Q*

INTRODUCTION

Chlamydophila pneumoniae is an important human pathogen that causes respiratory tract infections such as pneumoniae, bronchitis, pharyngitis, and sinusitis[1-3]. It is considered a potential risk factor involved in the development of atherosclerosis, asthma, and other chronic or destructive diseases[4-8]. Seroepidemiological investigations have revealed

*This project was supported by grants from the National Natural Science Foundation of China (Grant No. 30901352), Innovative Research Team in University of Hunan Province (Number: [2008] 51), Hunan Provincial Innovation Foundation for Postgraduate and Hunan Provincial Training and Innovation Base for Post-graduate.

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Received: August 21, 2011; Accepted: May 31, 2012

that *C. pneumoniae*-specific antibody prevalence in the adult population ranges from 50% to 70%[9-12]. Many infected individuals are not treated due to asymptomatic presentation and result in chronic or persistent infection. Thus, accurate diagnosis of infections with *C. pneumoniae* may guide the treatment for preventing its further aggravation and potential development of serious complications.

There are several approaches to detecting *C. pneumoniae* infection, such as live organism isolation, antigen capturing, nucleic acid detection, and antibody titration. Although isolating live organisms can help define a causative relationship, it is often unachievable[13-14]. It is also difficult to detect *C. pneumoniae* antigens in the affected tissues. PCR has dramatically increased the detection sensitivity, but its specificity is always a concern for there are no uniform standards for performing PCR[15]. Enzyme-linked immunosorbent assay (ELISA) has been developed for routine diagnosis[16-21], but it strongly depends on preparations of the antigens, which explains the inherent problems concerning cross reactions between Chlamydia species. Thus, it requires improvements in sensitivity and specificity before it can be applied for clinical diagnostic use[22-23]. MOMP, an immunodominant antigen, shows moderate to strong reactivity with all sera from patients infected with *C. pneumoniae*, and the VD2 and the VD3 regions of the MOMP of *C. pneumoniae* are only recognized by *C. pneumoniae*-positive sera, suggesting that the VD2-VD3 domains may be of value for the improvement of the *C. pneumoniae*-specific diagnostic assay. Importantly, antigens-based ELISAs can be useful for accurate and retrospective diagnosis, and MAbs-based ELISAs can detect the living organism infection effectively.

The aim of our study is to develop ELISA tests by using a novel monoclonal antibody (MAb) in detecting *C. pneumoniae* antigens from throat swab samples. The variable domain (VD) 2 and 3 of the major outer membrane protein was expressed as recombinant protein, and the MAbs were produced and applied to detect the samples from healthy subjects and patients with acute respiratory tract infections. We compared the value of the produced $MOMP_{VD2\text{-}VD3}$ MAb with the anti-EB MAb in clinical diagnosis of *C. pneumoniae*. We expected that this method would be useful in the detection of *C. pneumoniae* infection.

MATERIALS AND METHODS

Organism and Cell Culture

C. pneumoniae AR-39 and its genomic DNA were obtained from GM. Zhong, University of Texas, Health Science Center, San Antonio. Monolayer cells HEp-2 (Cell Bank of Chinese Academy of Sciences, Shanghai) were cultured on coverslips and infected with *C. pneumoniae*. *E. coli* BL21 (Novagen, Madison, Wisconsin) were used for the overexpression of the recombinant fragments of $MOMP_{VD2\text{-}VD3}$.

Throat Swab Samples

Samples used in this study were collected from clinical patients and healthy donors in hospitals in Hengyang from 2007 to 2008. The study subjects were categorized into two groups: Group I with 156 patients of respiratory tract infections who were diagnosed as the infectious syndrome such as persistent coughing, rhinocleisis, and increased mucoid, and had not received any anti-chlamydial therapy during the acute stage of illness; Group II with 57 healthy donors who were asymptomatic. Throat swabs were collected as follows: the posterior wall of the throat was rubbed three times and then, the cotton-tipped swabs were mixed in 1 mL chlamydia transport medium SPG (75 g of sucrose, 0.52 g of KH_2PO_4, 1.22 g of Na_2HPO_4, 0.72 g of glutamic acid, distilled water to 1 liter, pH 7.4), vortexed with glass beads for 1 min, and then collected and kept at -80 °C until they were used.

Production of MAb, Indirect ELISA for Hybridoma Screening and Western Blot Assay

$MOMP_{VD2\text{-}VD3}$ recombinant protein was expressed and purified as described previously[24]. Eight 6-week-old BALB/c mice (purchased from Beijing Vital River Laboratory Animal Technology Co. Beijing, China) were immunized by intraperitoneal injection of the immunogen mixed with the same volume of Freund's complete adjuvant (Sigma). The mice received twice injections including immunogen and Freund incomplete adjuvant over a period of 3 weeks. After that, they were boosted with a final injection of the immunogen without adjuvant. In 3 days later, the mice were sacrificed and their spleen cells were fused with SP2/0 myeloma cells (American Type Culture Collection, Manassas, VA) following a standard protocol[25].

Plastic 96-well ELISA microtiter plates (Costar) were coated with 100 μL of the immunogen (3

μg/mL of protein in PBS) and incubated overnight at 4 °C. After nonspecific binding sites were blocked with 100 μL of PBS containing 1% skim milk for 1 h at room temperature, 100 μL of each hybridoma clone culture supernatant was added to each well in duplicate. The plates were incubated at 37 °C for 2 h, washed five times with PBS containing 0.05% Tween 20 (washing buffer), and further incubated with 100 μL of PBS containing 0.1% skim milk (dilution buffer) and peroxidase-conjugated anti-mouse immunoglobulin G (Sigma; with dilution done according to the protocol) at 37 °C for 1 h. After five additional washings, 50 μL of chromogen (TMB)/substrate solution (H_2O_2) was added and the color development was terminated in 15 min by using 100 μL H_2SO_4 (Jingmei Biotechnology, Inc.). The $MOMP_{VD2\text{-}VD3}$ positive hybridomas were further confirmed by Western blot with purified $MOMP_{VD2\text{-}VD3}$ proteins as the enveloped antigen, with anti-HSP60 pAb as negative control (where rabbits were immunized with purified CHSP60 protein to produce the pAb conserved by our laboratory).

Isotyping and Purification of MAb

The precipitate containing MAbs was purified by adding ammonium sulfate to ascites to a concentration of 50% of its saturation. Isotyping of the MAbs was performed with a commercial kit (Sigma, Mouse Monoclonal Antibody Isotyping Kit) according to the manufacturer's instructions. The affinity constant determination was performed as described previously and the result was calculated according to the formula $A_0/(A_0\text{-}A)=1+K/a_0$. MAb was purified by the procedures described before[26].

Application of the MAb for Detecting Cpn Ag with MIF

HEp-2 cells at a density of 5.0×10^5 cells/mL were seeded onto 12 mm^2 cover slips in 24 well culture plates and grew for 24 h at 37 °C. Then cells were infected with *C. pneumoniae* by adding DEAE or by centrifuging at 25 °C, 2000 rpm for 30 min. The MIF was performed as described previously[27] with some modifications. The specific MAb clone 5D6 was selected to undergo the MIF procedure to be able to produce high levels of titers. Briefly, after 72 h of infection, cells on coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature. After washing and blocking, the cells were incubated by using the novel $MOMP_{VD2\text{-}VD3}$ MAb and the anti-*C. pneumoniae* EB MAb [abcam (1691)] at 1:40 dilution for 1 h at 37 °C and with goat anti-mouse IgG secondary antibody conjugated with Cy2 (Jackson Immuno-Research Laboratories) and Hoechst (Sigma). The immunofluorescence images were taken with Nikon Eclipse TE 2000-S fluorescence microscope.

Development of Novel MAb-based ELISA for Detecting Clinical Specimens

To apply MAbs-based ELISA for the detection of the antigens from throat swabs, 3 novel $MOMP_{VD2\text{-}VD3}$ MAb and the anti-EB MAb used as control were bound to the microplate, and the anti-EB MAb was conjugated with HRP, which was performed commercially (CW Bio-Tech Co., Ltd.). With 100 μL per well, the purified novel MAb and the anti-EB MAb diluted in TBS at a concentration of 100 ng/mL were added into the 96 microplate to be coated at 4 °C overnight, and the plate was blocked with 1% BSA (diluted in TBS) for 30 min at room temperature. Fifty microliter clinical specimens were added per well and incubated at 37 °C for 1 h, and then 100 μL HRP-labeled anti-EB MAb at 1:2000 was added and incubated at 37 °C for 30 min. Washing was done 5 times between every two steps. The substrate reaction was stopped with 50 μL sulfuric acid. The absorbance was measured at 450 nm with an ELISA plate reader (Thermo Multiskan MK3). 213 human throat swab samples were examined with the ELISA and the results were compared.

Statistical Analysis

Statistical analyses of the results obtained were made with SPSS for Windows, Version 10.1. To assess the agreement among the different tests, we used χ^2 test to compare the results of different MAbs-based tests, and a P value <0.05 was considered statistically significant.

RESULTS

Production and Characterization of Anti-$MOMP_{VD2\text{-}VD3}$ MAbs

Indirect ELISA which used $MOMP_{VD2\text{-}VD3}$ protein as the coating antigen, identified specific antibody producing cells, and three stable hybridomas clones were selected and named 5D6, 7G3, and 8C9. The three positive clones were then accumulated and the titer and the specificity of the antibodies were identified with ELISA (data not shown). Isotyping of the MAbs showed that all MAbs belonged to the

IgG1 subclass and all of them had a kappa light chain. With SDS-PAGE, the three MAbs 5D6, 8C9, 7G3 were shown to have a heavy chain (52 kDa) and a light chain (24 kDa) (Figure 1A). After migration on SDS-PAGE and electrotransferring, purified proteins reacting with the three $MOMP_{VD2-VD3}$ MAbs showed a band corresponding in molecular mass 24 kDa (Figure 1B). The affinities of MAbs strains 5D6, 8C9, and 7G3 are shown in Table 1.

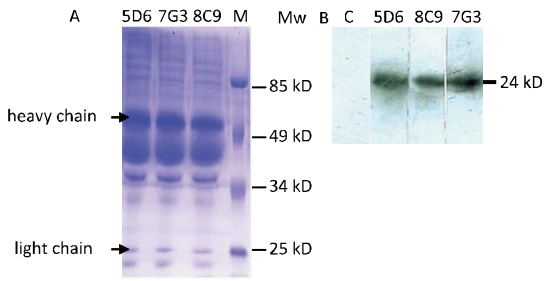

Figure 1. (A) SDS-PAGE analysis of three MAbs strains (ascites). The figure displayed the separated results of the 3 MAbs strains on SDS-PAGE, followed by coomassie blue staining. The 1st, 2nd, and 3rd lines were shown for 5D6, 7G3, and 8C9 ascites respectively; the 4th line was a reference marker. SDS-PAGE, sulfate-polyacrylamide gel electrophoresis. (B) *C. pneumoniae* specific Western-blot analysis of the three MAbs strains (ascites). The figure displayed the immunoreaction of $MOMP_{VD2-VD3}$ antigens with 3 positive produced MAbs by Western-blot, and anti-*C. pneumoniae* HSP60 pAb was used as negative control. (1) Negative control; (2) 5D6; (3) 8C9; (4) 7G3.

Table 1. Affinity Constants of MAbs

Monoclonal Antibodies	*Kaff* (μg/mL)*
5D6	52
8C9	48
7G3	47

Note. *The affinity constants (Kaff) were calculated with the formula $A_0/(A_0-A)=1+K/a_0$, A_0, absorbance at 450 nm without any protein; A, absorbance at 450 nm with different diluted antigan; K, affinity constant; a_0, the antigen gross.

Application of MAb-based Test System for Detecting C. Pneumoniae Antigens

To determine the predictive value of the novel MAb for the *C. pneumoniae* antigens with the gold standard test MIF, HEp-2 cells infected with *C. pneumoniae* were stained with either MAb 5D6 or anti-EB MAb. As shown in Figure 2, $MOMP_{VD2-VD3}$ MAb 5D6 are highly specific to *C. pneumoniae* strain. It can produce a fluorescent lighting with *C. pneumoniae* inclusion as good as anti-EB MAb, at the dilution of 1:40.

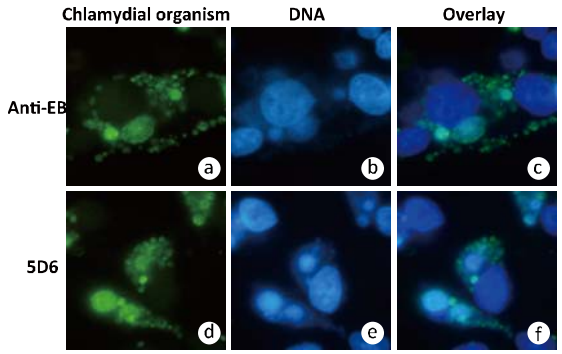

Figure 2. Specificity of 5D6 and anti-EB MAbs to *C. pneumoniae* by IFA. The inclusion body of *C. pneumoniae* was tested by using 5D6 and anti-EB MAbs. *C. pneumoniae* infected HEp-2 cells were stained with anti-EB MAb (a-c), and 5D6 MAbs produced (d-f). Green: inclusion body of *C. pneumoniae*; Blue: DNA.

Of the 156 samples from the "respiratory tract infection patients" in Group I, 55 were detected as positive by anti-EB MAb-based ELISA, with the positive rate of 35.26% (55/156); 51 were detected as positive by MAbs 5D6-based ELISA with the positive rate of 32.69% (51/156); 33 and 38 were tested as positive by MAb 8C9 and 7G3-based ELISA with the positive rates of 21% and 24% respectively (Table 2). Of the 57 samples from "healthy donors" in Group II, 5 were positive and 52 were negative with both anti-EB and 5D6-based tests (with the positive rate of 8.77%), and the other two MAb-based ELISA identified 2 and 3 positive cases with positive rates of 3.51% and 5.26% respectively. The sensitivities of 5D6, 8C9, 7G3 MAbs were 91.67%, 73.33%, and 86.67% respectively and their specificities were 99.35%, 100%, and 100% in relation to the anti-EB MAb-based ELISA. Among all the novel MAbs-based ELSIAs, the highest sensitivity was obtained from 5D6 (32.69%).

A total of 213 samples from throat swabs were collected and tested by ELISA and with the three different MAbs 5D6, 8C9, and 7G3 as the coating antigens. It seems that patients in Group I had higher *C. pneumoniae* infection rates than healthy donors

(P<0.05), as shown in Table 3. Moreover, significant difference was observed in detection of antigens with 5D6 MAb compared with the other two novel MAbs (Table 4).

Table 2. Antigen Recognition by the Anti-$MOMP_{VD2-VD3}$ and Anti-EBs MAbs

ELISA	Anti-EB MAb		5D6		8C9		7G3	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Group I (156)	55	101	51	105	42	114	49	107
Group II (57)	5	52	5	52	2	55	3	54

Table 3. Antigens of *C. pneumoniae* from 2 Groups Detected by 3 Produced MAbs and Anti-EB Mabs

Monoclonal Antibody Used	Antigen Tested	% of Patients with Acute Respiratory Infection Detected (n=156)	% of Healthy Donors Detected (n=57)	Statistical Analysis
Anti-EB	EB	35.26% (55/156)	8.77% (5/57)	X^2=14.472, P<0.001
5D6	$MOMP_{VD2-VD3}$	32.69% (51/156)	8.77% (5/57)	X^2=12.326, P<0.001
8C9	$MOMP_{VD2-VD3}$	26.92% (42/156)	3.51% (2/57)	X^2=13.964, P<0.001
7G3	$MOMP_{VD2-VD3}$	31.41% (42/156)	5.26% (3/57)	X^2=15.467, P<0.001

Table 4. The Specificity and Sensitivity of the Novel MAbs-based Detection Assays

Produced MAbs	Sensitivity (%)	Specificity (%)
5D6	91.67%	99.35%
8C9	73.33%	100%
7G3	86.67%	100%

DISCUSSION

There has been an increasing interest in developing a diagnostic test for *C. pneumoniae* infection with recombinant protein and MAb technology. MAb technology has facilitated the development of sensitive and specific tests for the detection of antigens in clinical specimens. MOMP with molecular weight around 40 kDa is a surface-exposed protein, representing 60% of the outer membrane-complex[28], and it is an important immunodominant that elicits the specific humoral immune response[29]. Furthermore, unlike other *Chlamydophila* spp., the gene encoding *C. pneumoniae* MOMP (ompA) has a high homology between different strains[30]. Jantos and his colleagues reported in their study that[31] all sera had moderate to strong reactivity with the MOMP of all *C. pneumoniae* strains. Thus, MOMP can be a breakthrough in diagnosis, as it is easy to substitute the whole antigen in detecting *C. pneumoniae* infection. Meanwhile, the VD2 and VD3 regions from MOMP of *C. pneumoniae* can only be recognized by *C. pneumoniae*-positive sera, suggesting the existence of species-specific epitope and confirming the value of the two regions for improving the *C. pneumoniae*-specific diagnostic assay. The antigen applied herein including multi-immunodominant epitopes was analyzed by ExPASy Proteomics Server and selected attentively, so the reliability of the experiment had been confirmed in the first step.

In our study, three MAbs producing cells were identified by using indirect ELISA with the $MOMP_{VD2-VD3}$ antigen as coating antigen. The selected MAbs were all positive by three repeated ELISA tests, and it ensured the homogenicity of MAbs. After initial screening, these MAbs were found to belong to the IgG1 subclass and all of them had a kappa light chains. Specificities of MAbs were evaluated with *C. pneumoniae* $MOMP_{VD2-VD3}$ antigens. These MAbs have shown high specificity to the antigens.

Antigen capture was found to be a better way to measure the pathogen in acute infection and chronic *C. pneumoniae* infection without many limitations. The $MOMP_{VD2-VD3}$ antibody assay which was performed herein can be used to recognize antigens in *C. pneumoniae* infections. Cross-reaction tests were done with purified MAbs, at the same optimal working concentration of MAbs in *C. pneumoniae* immunofluorescence assay (IFA). Cross-reaction remained in IFA with *C. trachomatis* and *C. psittaci*. These results indicated that cross

reaction still existed in both novel MAbs and anti-EB MAbs assays, no matter how weak or strong it was. This suggested the existence of some species-specific epitopes in the $MOMP_{VD2-VD3}$ antigens, and that is the key problem for the establishment of a more specific ELISA for *C. pneumoniae*.

However, as compared with IFA in *C. trachomatis* and *C. psittaci*, fluorescent intensity of *C. pneumoniae* was relatively strong with 20 fold objective lens and oil immersion (data not shown). Among those 213 throat swab samples, 56 were 5D6 MAbs positive, 44 8C9 MAbs positive, and 52 7G3 MAbs positive, all lower than 60 that were positive for anti-EB MAb respectively. The results also indicated that the VD2 and VD3 fragments might be of value for the improvement of *C. pneumoniae*-specific diagnostic assay, and that cross-reactivity could be effectively avoided when VD2 and VD3 domains of MOMP were used.

In the study, we tested 213 samples with MAbs from *C. pneumoniae* in our laboratory. Among 156 samples from patients in Group I who suffered from acute respiratory tract infection, the positive rates were 35.26%, 32.69%, 26.92%, and 31.41% for anti-EB MAb, 5D6, 8C9, and 7G3 MAbs respectively, while in 57 samples from healthy donors in Group II, the positive rates were 8.77%, 8.77%, 3.51%, and 5.26% respectively. Though differences existed among the four MAbs, it was obvious that the people with acute respiratory tract infection had a higher infection rate of *C. pneumoniae* than the healthy donors. These results are consistent with the previous studies[32-33].

Among the samples collected from clinic patients and healthy donors, we did not distinguish the positive and the negative cases with *C. pneumoniae* infection in advance, for there was no wholly satisfactory method as the “standard”. But to make a control, we compared the novel MAbs-based ELISA with the commercial anti-EB MAbs which had been widely used in experiments. In Group I, we tested 156 throat swab samples with MAbs, and the results tested by 5D6, 8C9, and 7G3 showed 91.67%, 73.33%, and 86.67% sensitivity respectively when compared with anti-EB MAbs-based ELISA. It was interesting that significant difference was observed in the tests with different MAbs. One sample could react with 5D6 and 7G3, but was not able to react with anti-EB MAb and 8C9. Another 2 samples could react with 7G3, while failed to react with the other 3 MAbs. The different reactivity obtained was probably because of the surface exposure which MAbs recognized. However, the result has to be confirmed by further studies, which can collect more samples, apply different methods such as PCR and isolation, compare and analyze the cross-reactions of MAbs in different *Chlamydophila* spp.

In our study, we produced MAbs and established a MAbs-based ELISA specific for *C. pneumoniae*. The results presented herein indicated that $MOMP_{VD2-VD3}$ MAbs could be used as a valuable tool in detection of *C. pneumoniae* infections. However, there are several questions to be further addressed. Firstly, differentiating healthy people from the clinically infected patients can provide a preliminary screening test for the laboratory test. Secondly, in our study, the rate of infection with *C. pneumoniae* was not very high, which is a little different from what has been reported before[34]. This difference may be regional or age-related.

ACKNOWLEDGEMENT

We thank Dr. ZHONG Guang Ming for kindly providing *C. pneumoniae* organism and DNA template.

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