Preparation and evaluation of monoclonal antibodies against chlamydial protease-like activity factor to detect *Chlamydia pneumoniae* antigen in early pediatric pneumonia

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Abstract *Chlamydia pneumoniae* causes diseases in humans, including community-acquired pneumonia, bronchitis, and sinusitis. It is also associated with atherosclerosis, coronary heart disease, and hyperlipidemia. In this study, we investigated novel materials with which to develop a sensitive and specific method to identify early *C. pneumoniae* infection, to allow more effective clinical treatment and prevention. We prepared novel monoclonal antibodies (mAbs) against a recombinant protein equivalent to the immunodominant region of chlamydial protease-like activity factor (CPAF) from *C. pneumoniae*. The mAbs specifically reacted with the endogenous CPAF antigen of the *C. pneumoniae* type strain in immunoblotting and indirect immunofluorescence (IIF) assays, but did not react with *C. trachomatis* type strains or genital secretions from patients with acute *C. trachomatis* infection. The mAb with the highest titer was used to develop a new IIF assay and enzyme-linked immunosorbent assay (ELISA) to detect the *C. pneumoniae* antigen in clinical specimens from child patients suspected of pneumonia. The sensitivity, specificity, and concordance rate of the mAb-based IIF and ELISA tests were compared with those of polymerase chain reaction (PCR). Our results show that these mAbs have excellent specificity and may be used to develop new screening tools for the diagnosis of early pediatric pneumonia.

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Introduction

Chlamydia pneumoniae causes diseases in humans, accounting for approximately 10 % of community-acquired pneumonia and 5 % of bronchitis and sinusitis in adults and children [1, 2] *C. pneumoniae*-induced respiratory infections are often asymptomatic or mild, although severe pneumonia can develop in older patients and those with coexisting cardiopulmonary disease. *C. pneumoniae* may also be a risk factor for lung cancer [3]. Seroepidemiological studies have shown antibody prevalence of 50–70 %, suggesting a high frequency of previous infections. Nearly all humans can expect to be infected with *C. pneumoniae* at least once in their lifetimes. Reinfection is common, and persistence of the agent in the host after the primary infection is a potential risk factor for chronic infection. The associations between *C. pneumoniae* and atherosclerosis and coronary heart disease have attracted the attention of many investigators [4, 5]. The bacterium appears to be associated with hyperlipidemia and might, therefore, represent a further risk factor for cardiovascular disease in human immunodeficiency virus (HIV)-positive patients [6].

The laboratory methods currently used for the diagnosis of acute *C. pneumoniae* infection include culture, serological assays [including microimmunofluorescence (MIF) and enzyme-linked immunosorbent assay (ELISA)] and polymerase chain reaction (PCR), although the latter two are the most frequently used. However, it is still difficult to reliably diagnose *C. pneumoniae* infection because standardized and commercially available diagnostic tests that are both sensitive and specific are rare. Although infections with *C. pneumoniae* can be identified by the direct isolation of the agent, it is difficult to culture this chlamydial species in vitro, because the culture procedure for *C. pneumoniae* is laborious and time-consuming. The MIF test is currently considered the "gold standard" for the serodiagnosis of *C. pneumoniae* infection. However, the need for paired serum samples and the extended

persistence of immunoglobulin G (IgG) antibodies in some adult populations make this test retrospective in nature and unsuitable for an early diagnosis [7]. MIF testing is also hampered by poor specificity because of cross-reaction with other chlamydial species. ELISA is often used to diagnose *C. pneumoniae* infection because the results can be interpreted objectively, and it is convenient and rapid [8]. However, some cross-reactions occur among chlamydial species because the antigens mainly used are whole *C. pneumoniae* bacteria or antigens common to the genus *Chlamydia*, such as the major outer membrane protein (MOMP), outer membrane protein 2, and lipopolysaccharide [9, 10]. Molecular-analysis-based assays, such as PCR, have recently been developed for the rapid and sensitive detection of *C. pneumoniae*. However, the overall diagnostic utility of PCR-based assays is currently limited by the lack of type specimens, an appropriate nucleic acid extraction method, standardized amplification protocols, and the special instruments and trained professional personnel required [2].

In general, methods that detect pathogenic antigens are more direct and rapid than serological diagnostic methods in identifying the causes of infections, allowing early and effective treatment or disease prevention. Therefore, it is extremely important to identify new *C. pneumoniae* antigens, especially potentially immunogenic peptides, that can be used to develop highly sensitive and specific assays for the early diagnosis of *C. pneumoniae* infections.

Chlamydial proteasome/protease-like activity factor (CPAF), encoded in the chlamydial genome, is secreted into the host cell cytosol and has proteolytic activity that is important during chlamydial infection [11]. CPAF is immunogenic during human infection with live chlamydial bacteria. CPAF-specific antibodies are produced during CPAF synthesis, which requires the replication of live chlamydial organisms in chlamydia-infected cells. Humans infected with chlamydia produce a dominant antibody response to CPAF [12]. Therefore, CPAF can be used as an effective immunodominant antigen with which to diagnose early *C. pneumoniae* infection. Zhong et al. previously reported that a chlamydia-secreted protein, designated CPAF, is present in the cytosol of chlamydia-infected cells. They reported that CPAF does not preexist in chlamydial organisms and that the replication of the live organism is required for CPAF synthesis in cultured cells [11]. Mice inoculated with live chlamydial organisms produced a strong antibody response to CPAF, whereas mice inoculated with dead chlamydial organisms did not, and CPAF-specific antibody production correlated with CPAF synthesis in the animals. That study showed that the levels of CPAF antibodies were higher than antibodies directed against either chlamydial MOMP or heat shock protein 60 (HSP60) in humans infected with chlamydia. Specific anti-CPAF antibodies in human sera can neutralize endogenous CPAF and its glutathione S-transferase (GST) fusion

protein [12], suggesting that CPAF is both produced and immunogenic during human chlamydial infection. All known chlamydial organisms produce CPAF, and the *CPAF* gene is highly conserved among chlamydial strains, although it has no significant overall homology with any other known gene. The intraspecies identity of the CPAF protein is as high as 99 % and the interspecies identity is as low as 46 % [13]. Zhong et al. demonstrated that CPAF produced by *C. pneumoniae* consists of 619 amino acids, and its middle region, covering residues 200–338, is the immunodominant region, which is predominantly recognized by human neutralizing antibodies [14].

It is well known that a monoclonal antibody (mAb) directed against an antigen can distinguish and specifically combine with that antigen. Therefore, mAbs have become a new focus of research into the prevention and diagnosis of pathogenic infections [15, 16]. We produced mAbs directed against a recombinant protein containing the immunodominant region epitopes of CPAF (designated "CPAFm") from *C. pneumoniae*. The mAb with the highest titer was selected to develop a new indirect immunofluorescence (IIF) assay and indirect ELISA. These assays were used to detect the *C. pneumoniae* type strain and to analyze sputum pharyngeal swabs from pediatric patients suspected of *C. pneumoniae* infection, respectively. We also assessed the diagnostic value of the new IIF assay and ELISA by comparing their results with the detection of *C. pneumoniae* by PCR in the same samples. We thus established several novel materials to develop a method that is both sensitive and specific for the rapid and specific identification of early *C. pneumoniae* infections.

Materials and methods

Bacterial strains and clinical samples

The *C. pneumoniae* type strain CWL029 and its genome template and the *C. trachomatis* D, F, and I type strains were gifts from Prof. G. M. Zhong at the Department of Microbiology and Immunology, University of Texas Health Science Center, San Antonio, TX, USA. HeLa cells, *Escherichia coli* JM109 and BL21, mouse myeloma cells (SP2/0), and the expression vector pGEX6p-2 are stored in our laboratory. Clinical sputum pharyngeal swab samples (*n*=300) were obtained from symptomatic pediatric patients who had a persistent dry cough, moderate or slight fever, even slight bronchial rale, and X-ray-confirmed pneumonia between January, 2010 and May, 2013 in the pediatric departments of four affiliated hospitals in China. No patients had serological evidence of *C. trachomatis* infection. Normal sputum pharyngeal swab samples (*n*=120) were collected from children with no reported illness or symptoms, who had undergone a medical examination in the physical examination center. Forty genital

secretion samples were collected from patients with acute *C. trachomatis* infection, who had positive results on urethral or endocervical *C. trachomatis* DNA amplification with the AMPLICOR test (Roche Diagnostic Systems, USA). None of these patients had serological evidence of *C. pneumoniae* infection. Parents/guardians of pediatric patients gave their informed consent.

Production, purification, and identification of recombinant protein GST–CPAFm

The recombinant and purification of recombinant protein GST–CPAFm were as according to Zheng et al. [17]. The gene sequence encoding the CPAFm immunodominant epitopes of CPAF (amino acids 181–400) (gi: 15617929; bp 1, 167,582–1,168,241) was selected, and amplification primers were designed. The sense primer sequence was 5′-CCGGGA TCCGATGCGGTTCCTTCAG-3′, and the antisense primer sequence was 5′-TTGCGGCCGCTTAGACTTCATCCTGA GTG-3′. The purified PCR products were subcloned into the expression vector pGEX6p-2 and transferred into the bacterium *E. coli* BL21. The expression products were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The induced product from whole bacteria and the purified protein were both identified with SDS-PAGE and immunoblotting assays. *C. pneumoniae* IgG antiserum from patients was used as the primary antibody (confirmed with commercially available reagents for *C. pneumoniae* MIF from EUROIMMUN Medizinische Labordiagnostika AG, Germany). Coomassie Brilliant Blue solution and the enhanced chemiluminescence detection system were used to visualize specific straps.

Production of mAbs

To produce the mAbs, 4-week-old BALB/c mice were subcutaneously immunized with 50 mg of the prepared recombinant protein in complete Freund's adjuvant. The production of mAbs was according to Fazekas de St Groth and Scheidegger [18]. The cutoff value (COV), defined as the mean A_{450} value (mean = 0.10) plus two standard deviations ($s = 0.051$) for the 120 donor sera, was calculated to be 0.202. The A_{450} per well was determined three times and the mean value calculated. The supernatants of the growing hybrids were considered positive if the mean A_{450} value was more than the COV, which implied that the supernatants contained GST–CPAFm antibodies. If $A_{450} >$ COV, the result was negative and the sample contained no anti-GST–CPAFm antibodies. The purification effect was determined with an immunoblotting analysis with the *C. pneumoniae* IgG antiserum described above as the primary antibody.

Biological characteristics of mAbs

The isotype of each antibody was determined with the Mouse Monoclonal Antibody Isotyping Kit (Sigma Chemical Co.). The specificity of each antibody was identified with an IIF test and an immunoblotting analysis based on the *C. pneumoniae* type strain. The affinity constant was determined with the method described by Beatty et al. [19]. The Ultramicro Analytical System was slightly modified. The Ultramicro ELISA plates were sensitized with four concentrations of GST–CPAFm: 0.5, 1, 2, and 4 mg/mL. Every mAb was used at the highest and lowest dilutions, ranging from 2 to 0.007 mg/mL.

Identification of mAb specificity and construction of a new IIF test

HeLa cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum (Sigma Chemical Co.) and infected with the *C. pneumoniae* type strain CWL029, as described above. The supernatants were collected from the infected HeLa cells lysed with SDS cell lysis buffer (Beyotime Institute of Biotechnology) and diluted to use as the antigens. One of the mAbs was used as the primary antibody.

To determine the specificity of the mAbs, a new indirect IIF assay was developed as described below. Briefly, the membranes of infected HeLa cells were disrupted with 2 % bovine serum albumin (BSA; Gibco BRL, Rockville, MD) solution containing 0.1 % Triton X-100. The supernatants, as antigens, were added to clean microscope slides (15 holes). In one group, each of the four mAbs (dilutions ranging from 1:100 to 1:5,000) was used as the primary antibody and added to 12 parallel antigen samples (each mAb was tested in triplicate), and incubated for 30 min in a moist chamber at 37 °C. Another group of samples, in which a specific mouse mAb directed against *C. pneumoniae* MOMP (diluted 1:2,000; Sigma) was used as the primary antibody, was the positive control. PBS was used as the negative control, and no mAb was added to another three parallel samples as the blank control in the procedure described above. After the slides were washed three times with PBS, the mouse mAbs were visualized with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Sigma, 1:5,000). The mAb produced with the strongest response was selected as the primary antibody to detect the pathogen in clinical samples.

Application of the new IIF test

The IIF method developed was used to analyze 120 normal sputum pharyngeal swab samples and 300 clinical sputum pharyngeal swab samples lysed with SDS cell lysis buffer (Beyotime Institute of Biotechnology). The GST–CPAFm-

specific mouse mAb with the highest titer was selected as the primary antibody.

A commercially available PCR-based assay (DaAn Gene Technology Corp., Sun Yat-Sen University, China) was used to isolate and detect *C. pneumoniae* DNA in the clinical sputum pharyngeal swab samples, according to the manufacturer's instructions. This real-time fluorescent method for the quantitative detection of *C. pneumoniae* and the kit are based on the amplification of the conserved region of the *MOMP* gene of *C. pneumoniae*, followed by the colorimetric detection of the amplification products on microplates coated with specific probes. The regions targeted by the primers and probes are in the gene encoding the MOMP protein of *C. pneumoniae*. The forward primer sequence was 5′-gctactggaacaaagtctgcga-3′ and the reverse primer sequence was 5′-cattgtactccaatgtatggcacta-3′. The probe sequence is 5′-ttatcatgaatggcaagtaggagcctctctatctt-3′. The diagnostic specificity of this PCR assay is highly consistent with that of the "gold standard" MIF test, according to the specifications supplied by the manufacturer.

The sensitivity and specificity of the IIF method developed here were then calculated from the experimental results. The differences between the two methods (IIF and PCR) were measured with a χ^2 analysis and $p<0.05$ was considered significant. The prevalence of patients infected with *C. pneumoniae* was analyzed.

Construction of a new indirect ELISA

The membranes of HeLa cells infected with *C. pneumoniae* strain CWL029 were broken, and the supernatant containing the antigen to be detected was used as the positive control. Normal sputum pharyngeal swab samples were used as the negative controls. The GST–CPAFm-specific mouse mAb with the highest titer was used as the primary antibody. The collected supernatants were diluted as the antigen to be detected and used to coat enzyme immunoassay microtiter plates (Costar, Cambridge, MA) for 2 h at 37 °C or at 4 °C overnight. The wells were then washed with phosphate-buffered saline with Tween 20 (PBST). After the binding sites of other antigens were blocked with PBST containing 5 % BSA (100 μL; Gibco), the mAb (diluted 1:100) was then added to the wells. The mixtures were incubated for 2 h at 37 °C with shaking, and the wells were then washed with PBST. Horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Sigma) was diluted 1:3,000 in PBST containing 3 % BSA (Gibco) and added to each well. After incubation for 1 h, the wells were washed with PBST, and then 100 μL of the substrate solution was added. The substrate solution contained 0.4 % TMB and 0.02 % hydrogen peroxide in phosphate citrate buffer (pH 5.0). After incubation for 10 min, the reaction was terminated by the addition of 100 μL of 2-M H_2SO_4 solution and the A_{450} was measured.

Each sample was tested in triplicate. The COV used to assign the results was defined as the mean + 2SD of the absorbance of 120 normal samples.

Application of the new indirect ELISA

The PCR-based assay described above was used as the reference test. The newly developed ELISA based on the GST–CPAFm-specific mouse mAb was used to test 300 sputum pharyngeal swab samples. These samples were decomposed in 0.2-M NaHCO₃ (pH 9.6) solution, as described previously [20], and then adsorbed onto enzyme immunoassay microtiter plates (Costar) for 2 h at 37 °C. The sensitivity, specificity, and concordance rate of the indirect ELISA were calculated from the test results. The differences between the two methods were measured with the χ^2 test and $p<0.05$ was considered significant. The incidence rates of patients infected with *C. pneumoniae* were analyzed.

Cross-reaction with *C. trachomatis*

The *C. trachomatis* D, F, and I type strains and 40 genital secretions from patients with acute *C. trachomatis* infection were tested with the new IIF assay and ELISA to detect *C. pneumoniae* antigen. A positive result implied that the test cross-reacted with *C. trachomatis*.

Results

Expression and purification of recombinant protein

The recombinant plasmid constructed to express the target protein was confirmed with PCR and restriction enzyme analysis to contain the *CPAF*m gene. The specific fragment was observed with 1 % agarose gel electrophoresis. The size of the PCR amplification product was about 700 bp, consistent with the anticipated fragment size (Fig. 1a). A BLAST search demonstrated that the DNA sequence of the inserted fragment was identical to the target gene registered in GenBank.

The analysis of *E. coli* BL21 cells containing the recombinant plasmid clearly showed a new protein band on a Coomassie Brilliant Blue-stained gel compared with the control cells (Fig. 1b). Its molecular weight was about 51.3 kDa (about 25 kDa for CPAFm and 26 kDa for GST) and was identical to the calculated molecular weight. The expression product formed inclusion bodies in *E. coli* at 37 °C and 24 °C. After purification, the expressed protein showed only a clear 51.3-kDa band, with no other band visible, and a purity of 95 % on 10 % SDS-PAGE (Fig. 1c). The protein concentration was 1.365 mg/mL, calculated with the A_{280} UV absorbance method.
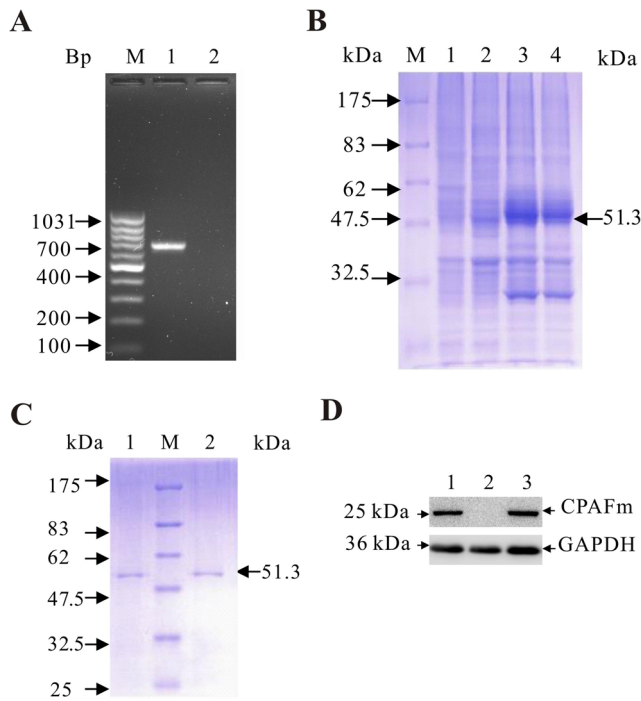
Fig. 1 Expression and purification of recombinant protein. **a** 1.0 % agarose gel electrophoresis analysis of the PCR product: *M* 100-bp DNA marker; *1* PCR product (714 bp); *2* negative control. **b** Expression of GST–CPAFm at various induction temperatures: *M* protein marker; *1* induced *E. coli* BL21(DE3); *2* uninduced *E. coli* BL21(DE3) with pGEX6p-2/CPAFm; *3* induced *E. coli* BL21(DE3) with pGEX6p-2/CPAFm at 37 °C; *4* induced *E. coli* BL21(DE3) with pGEX6p-2/CPAFm at 24 °C. **c** SDS-PAGE analysis of purified GST–CPAFm: *1, 2* purified GST–CPAFm; *M* protein marker. **d** Immunoblotting analysis of recombinant GST–CPAFm protein: *1* induced *E. coli* BL21(DE3) cells with pGEX6p-2/CPAFm; *2* uninduced *E. coli* BL21(DE3) cells with pGEX6p-2/CPAFm; *3* purified recombinant protein GST–CPAFm

An immunoblotting assay demonstrated that the expressed recombinant GST–CPAFm protein reacted with human anti-*C. pneumoniae* antiserum (Fig. 1d). Only one specific immunoreactive band of CPAFm protein (molecular weight ~25 kDa) was clearly visible, but was not observed in the negative control.

Biological characteristics of mAbs

The four hybridomas were cloned three times with the limiting dilution method and designated 3F8, 7B9, 8C4, and 11B5. The titers of the ascites produced by these four hybridomas were 1:28,000, 1:16,000, 1:38,000, and 1:12,000, respectively. The isotypes of the mAbs secreted by the 3F8 and 7B9 strains were IgG2b and those of the other two were IgG1. The affinities of these four mAbs are shown in Table 1; 8C4 showed higher affinity than the other mAbs ($Kaff$=4.98 × 10^8 M^{-1}).

Table 1 Affinity constant of the mAbs

Monoclonal antibodies	$Kaff$ (M^{-1})
3F8	4.26×10^7
7B9	2.65×10^8
8C4	4.98×10^8
11B5	3.73×10^7

Specificity of GST–CPAFm mouse mAb tested with immunoblotting and IIF assays

The reaction specificity of each mAb was examined with IIF based on the *C. pneumoniae* type strain CWL029, and the assay results are shown in Table 2. Compared with the positive and negative controls, these mAbs reacted strongly with the endogenous CPAF antigen from the type strain. Although the other mAbs displayed good immunoreactivity, 8C4 showed the highest titer and the best reactivity, so it was selected to test the clinical specimens.

An immunoblotting analysis was used to confirm the specificity of the mAbs produced. HeLa cells infected with the *C. pneumoniae* type strain CWL029 were used as the antigen to be detected and each of the mAbs was used individually as the primary antibody. The mAbs excreted by the four hybridomas all combined with the endogenous 25-kDa CPAFm protein and the specific reaction bands are shown in Fig. 2. The internal control for the primary antibody was glyceraldehyde 3-phosphate dehydrogenase.

Clinical tests of the new IIF method

When mAb 8C4 was used to probe normal sputum pharyngeal swab samples as the negative controls, the cells in these samples were not labeled (Fig. 3a). In contrast, HeLa cells infected with the type strain were clearly fluorescent when treated with the same antibody preparation (Fig. 3b). When the same mAb was used to test the sputum pharyngeal swab samples from patients possibly infected with *C. pneumoniae*, the cells in these samples were also clearly fluorescent (Fig. 3c). The prevalence rates of infection in patients with respiratory disease who were possibly infected with *C. pneumoniae* were analyzed in two cities, Shanghai and Hengyang, in China.

Table 2 Reactivity of each MAb, identified with the IIF assay

IIF results	MAbs			
	A: 3F8	B: 7B9	C: 8C4	D: 11B5
Positive level	3+	3+	4+	3+

IIF immunofluorescence

Fig. 2 Immunoblotting analysis of four mAbs (ascites): *1* negative control; *2* positive control; *3* 3F8; *4* 7B9; *5* 11B5; *6* 8C4
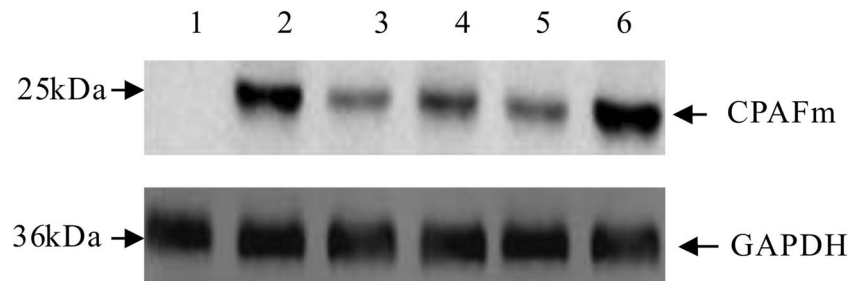
Three hundred specimens were tested for *C. pneumoniae* antigen simultaneously with PCR using commercial reagents and with the IIF test. The results are shown in Table 3. The sensitivity and specificity of the IIF test when compared with PCR were 88.89 % (48/54) and 90.24 % (222/246), respectively. When the differences between the two methods were measured with the χ^2 test, they were not significantly different ($p=0.088$). The prevalence rates for *C. pneumoniae* in the 300 patient specimens determined with IIF and PCR were 24 % (72/300) and 18 % (54/300), respectively. The concordance rate for the two methods in detecting *C. pneumoniae* antigen was 90.00 % [(48+222)/(72 + 228)].

Clinical application of the new ELISA

In this study, the COV for the ELISA based on the mAb directed against CPAFm (8C4) was calculated from 120 normal samples to be 0.316. The results of the indirect ELISA revealed that all absorbances were less than 0.300. *C. pneumoniae* antigen was detected simultaneously in 300 specimens with the commercial PCR reagents used and the new ELISA method based on mAb 8C4. The results are shown in Table 4. The sensitivity and specificity of the ELISA compared with PCR were 87.0 % (47/54) and 94.71 % (233/246), respectively. When the difference between the two methods was measured with the χ^2 test, they were not significantly different ($p =0.603$). The prevalence rates for *C. pneumoniae* in the 300 patient specimens

Table 3 IIF and PCR detection of *C. pneumoniae* antigen in 300 samples

IIF results	PCR results		
	Positive	Negative	Total
Positive	48	24	72
Negative	6	222	228
Total	54	246	300

The sensitivity, specificity, and concordance of the IIF assay relative to PCR were 88.89, 90.24, and 90.00 %, respectively. The differences between the two methods were measured with a χ^2 test ($\chi^2 =1.33$, $p= 0.088$)

IIF immunofluorescence; *PCR* polymerase chain reaction

measured with the new indirect ELISA and PCR were 20 % (60/300) and 18 % (54/300), respectively. The concordance rate of the two methods in detecting *C. pneumoniae* antigen was 93.33 % [(47+233)/(60+240)].

Cross-reaction with *C. trachomatis*

The new IIF and ELISA methods for the detection of the endogenous CPAF antigen of *C. pneumoniae* based on mAb 8C4 were used to detect the *C. trachomatis* D, F, and I type strains and to test 40 genital secretion samples from *C. trachomatis*-infected patients. No result was positive, indicating that mAb 8C4 does not cross-react with *C. trachomatis*.

Discussion

The tests available for the serological diagnosis of *C. pneumoniae* infection are extensive and are frequently used. However, there is currently no perfectly satisfactory serological method for the diagnosis of *C. pneumoniae* infection. Detecting an antigen specific to the pathogen is a more direct and quicker method than serological diagnostic strategies to identify the cause of an infection. In general, culturing the pathogen or detecting an antigen of the live pathogen and its gene are used as direct evidence upon which to base the diagnosis of a human pathogenic infection. Therefore, the identification of new *C. pneumoniae* antigens should be useful and important for the development of highly sensitive and specific diagnostic methods for the detection of early *C. pneumoniae* infections. Based on the principle that an mAb directed against an antigen is highly specific, an mAb prepared against *C. pneumoniae* CPAF was used to diagnose infections caused by this pathogen to improve the specificity of the diagnostic method.

We successfully amplified the immunodominant region of the *CPAF* gene from *C. pneumoniae* and cloned it into the pGEX6p-2 vector. The recombinant protein GST–CPAFm expressed in induced *E. coli* BL21 cells and purified GST–CPAFm were identified with SDS-PAGE and immunoblotting assays. The results showed that GST–CPAFm reacted with *C. pneumoniae* IgG antisera from patients diagnosed with *C. pneumoniae* pneumonia, with excellent immunoreactivity.
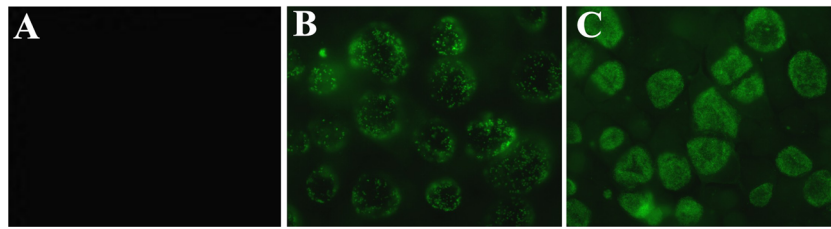
Fig. 3 IIF results with 8C4 mAb as the primary antibody (original magnification, ×400). **a** IIF staining of normal sputum pharyngeal swab samples. **b** IIF staining of HeLa cells infected with the *C. pneumoniae* type strain. **c** IIF staining of a sputum pharyngeal swab sample from a patient possibly infected with *C. pneumoniae*

BALB/c mice were immunized with the purified GST–CPAFm protein and four clones were generated that stably secreted mAbs. An indirect ELISA based on the purified GST–CPAFm protein indicated that each of the mAbs reacted specifically with the recombinant GST–CPAFm protein. We used an immunoblotting analysis to test the reaction specificity of the produced mAbs. The results showed that each of the mAbs specifically reacted with the 25-kDa endogenous CPAFm protein. The combined results of the indirect ELISA and immunoblotting analysis of the *C. pneumoniae* type strain demonstrated that these mAbs have excellent reaction specificity. A new indirect IIF assay based on the four mAbs was also developed and used to detect the *C. pneumoniae* type strain CWL029. The mAbs also reacted with the endogenous CPAFm protein from the *C. pneumoniae* type strain. Thus, the mAbs produced display specific immunoreactivity for *C. pneumoniae* CPAFm.

The new IIF assay was used to detect the endogenous CPAFm antigen of *C. pneumoniae* in 300 clinical sputum pharyngeal swab samples from patients diagnosed with *C. pneumoniae* pneumonia. The CPAFm-specific mouse mAb 8C4, which had the highest titer, was selected as the primary antibody. Compared with the reference PCR test, the sensitivity and specificity of the IIF test were 88.89 and 90.24 %, respectively. The differences between the two methods were not statistically significant. The prevalence rates of *C. pneumoniae* in the 300 patient specimens tested with IIF and PCR were 24 and 18 %, respectively. The

agreement between the two methods in detecting the *C. pneumoniae* antigen was 90.00 %.

A new indirect ELISA based on CPAF-specific mouse mAb 8C4 was constructed and used to detect the endogenous CPAFm antigen of *C. pneumoniae* in 300 clinical sputum pharyngeal swab samples from patients diagnosed with *C. pneumoniae* pneumonia. The CPAFm-specific mouse mAb 8C4 was used as the primary antibody. Compared with the reference PCR test, the sensitivity and specificity of the ELISA were 89.36 and 92.89 %, respectively. The differences between the two methods were not statistically significant. The prevalence rates of *C. pneumoniae* in the 300 patient specimens tested with the ELISA and PCR were 20 and 15.67 %, respectively. The agreement of the two methods used to detect *C. pneumoniae* antigen was 92.33 %. The new IIF assay and ELISA based on mAb 8C4 were used to test the *C. trachomatis* D, F, and I type strains and 40 genital secretions from patients with acute *C. trachomatis* infection. The results implied that the new IIF assay and ELISA do not cross-react with other *Chlamydia* species when used to detect *C. pneumoniae*. Thus, we evaluated the performance of the mAb-based IIF assay and ELISA in the detection of the *C. pneumoniae* antigen in early pediatric pneumonia. We suggest that the novel mAbs produced here have excellent reaction specificity and may be useful in the development of screening tools for the early diagnosis of pediatric pneumonia.

Currently, there are few commercial IIF- or ELISA-based diagnostic kits to detect *C. pneumoniae* antigen in early bacterial infections. We have successfully prepared mAbs directed against a recombinant protein corresponding to the immunodominant region of CPAF from *C. pneumoniae*. Using these mAbs, we constructed a new IIF assay and indirect ELISA that can be used to detect the endogenous *C. pneumoniae* antigen in the early diagnosis of pediatric pneumonia, for both its prevention as well as clinical treatment. However, the process is a little time-consuming because CPAF-specific antibodies are produced during CPAF synthesis, which requires the replication of live chlamydial organisms in chlamydia-infected cells. Our findings also require confirmation with larger samples. In addition, our further research is to compare new IIF and ELISA tests against the "gold standard" MIF test.

Table 4 New ELISA and PCR detection of *C. pneumoniae* antigen in 300 samples

ELISA results	PCR results		
	Positive	Negative	Total
Positive	47	13	60
Negative	7	233	240
Total	54	246	300

The sensitivity, specificity, and concordance of the ELISA relative to PCR were 87.0, 94.71, and 92.33 %, respectively. The differences between the two methods were measured with a χ^2 test ($\chi^2 = 1.12$, $p = 0.603$)

ELISA enzyme-linked immunosorbent assay; *PCR* polymerase chain reaction

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