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Serodiagnosis of *Chlamydia pneumoniae* Infection Using Three Inclusion Membrane Proteins

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The *Chlamydia pneumoniae* genome-encoded open reading frames Cpn0146, Cpn0147, and Cpn0308 were expressed as recombinant proteins for detecting *C. pneumoniae*-specific antibodies in samples from three groups of individuals including 183 with *C. pneumoniae*-associated respiratory infection (group I), 60 healthy blood donors (group II), and 32 with no known respiratory infection (group III). The recombinant Cpn0146 was recognized by 71 (38.8% positive recognition rate), 15 (25%) and 1 (3.1%), Cpn0147 by 75 (40.9%), 14 (23.3%), and 2 (6.3%), and Cpn0308 by 82 (44.8%), 16 (26.7%), and 0 (0%) samples from groups I, II, and III, respectively. The positive recognition rates with any of the three antigens were significantly higher in group I than those in groups II and III, suggesting that more individuals from group I were likely infected with *C. pneumoniae*. This conclusion was confirmed with a commercially available whole organism-based ELISA kit (Savyon Diagnostics Ltd., Ashdod, Israel), which detected *C. pneumoniae* antibodies in 98 (64.1%), 26 (43.3%), and 4 (12.5%) samples from group I, II, and III, respectively. Comparing to the commercial kit, the recombinant antigen-based detection assays displayed $>97\%$ of detection specificity and $>87\%$ of sensitivity, suggesting that these recombinant antigens can be considered alternative tools for aiding in serodiagnosis of *C. pneumoniae* infection. J. Clin. Lab. Anal. 24:55–61, 2010. © 2010 Wiley-Liss, Inc.

Key words: *Chlamydophila pneumoniae*; serodiagnosis; recombinant antigens; ELISA

INTRODUCTION

Chlamydophila (previously *Chlamydia*) *pneumoniae* (*C. pneumoniae*) is considered an important non-viral intracellular human respiratory pathogen, whose organism's role as a human pathogen was not defined until 1983, when the first respiratory isolate (AR-39) was obtained in Seattle, Washington, from a university student with pharyngitis (1). Its unique and complex reproductive cycle can enable the effective evasion of the host's defense mechanisms, leading to the epidemic and endemic respiratory tract infections in many areas of the world. Also, *C. pneumoniae* has been associated with bronchitis, pharyngitis, sinusitis, and otitis in all age groups and is most common among the 60 to 79-year old and teenagers. Antibodies have also been found frequently in human bodies in many countries worldwide (2,3). Reinfection and reactivation are common after a short period of immunity in adults, enhanced by immunosuppression. An intriguing association between seropositivity to *C. pneumoniae* and chronic human diseases, particularly atherosclerosis, has been observed, although causal effect has yet to be definitively demonstrated (4–6). Because of the potential impact that *C. pneumoniae* infection could have on public health practices, rapid diagnosis of infection is critical.

There are several laboratory methods for the specific diagnosis of *C. pneumoniae* infection, such as isolation in culture, microimmunofluorescence (MIF) methods and polymerase chain reaction (PCR). Isolation of *C. pneumoniae* by cell culture could provide more

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accurate diagnosis, but it is difficult, time-consuming, and relatively insensitive (7). *C. pneumoniae* PCR which is expensive and requires sophisticated facilities and trained personnel, has its own limitations, perhaps associated with low *C. pneumoniae* DNA copy numbers in clinical specimensis. The current "gold standard" for serodiagnosis of *C. pneumoniae* infection is the MIF test (8), although it is claimed to be species specific. Cross-reactions between Chlamydial species have been reported, and the MIF test is not readily adaptable for routine use in diagnostic clinical laboratories due to the requirement for highly trained personnel (9,10). Because of these limitations, immunological methods, such as enzyme linked immunosorbent assay (ELISA), which are much easier to perform than MIF and more suitable for large-scale testing, have been developed to facilitate the diagnosis of *C. pneumoniae* (11,12). This raises the question as what are the best antigens to use in ELISA-based serological diagnosis. Cpn0146, Cpn0147, and Cpn0308 are three hypothetical *C. pneumoniae* antigens that were identified as novel inclusion proteins and remained to be in the inclusion membrane throughout the infection (13,14).

This study was aimed to determine the serological levels of immunoglobulinG (IgG) against each of the recombinant antigens, and compare the value of these recombinant proteins on serodiagnosis of *C. pneumoniae*. Therefore, we have developed ELISA tests by using three different *C. pneumoniae* recombinant antigens, each of which was purified in native conditions. It is hoped that the study will facilitate the development of a serological test for diagnosis of *C. pneumoniae* infection.

MATERIALS AND METHODS

Bacterial Strains and Genomic DNA

C. pneumoniae strain AR-39 and genomic DNA from *C. pneumoniae* were obtained from GM. Zhong, University of Texas Health Science Center, San Antonio, *E. coli XL-1 Blue* (Novagen, Madison, Wisconsin) for the over expression of the recombinant fragments of Cpn0146, Cpn0147, and Cpn0308.

Patients and Sera samples

Human sera used in this study were collected from independent clinic patients and inpatients from hospitals in Hengyang and Xiangtan, two major cities in Hunan, China, in 2007–2008. The study subjects were categorized into one of the three following groups: Group I patients ($n = 183$) with acute respiratory infections, diagnosed on the basis of an infectious syndrome accompanied by productive coughing, increased mucoid or mucopurulent expectorations, tachypnea, and slight bronchial rale; Group II subjects ($n = 60$) were healthy blood donors visiting the local blood donor center, with serum samples supplied anonymously; Group III consisting of 32 sera from children aged 1–6 year, which were negative for *C. pneumoniae* infection, as judged by *C. pneumoniae* MIF analyses. Serum samples were stored at -70°C until processed.

The median ages (in years), age range, and percentages of female subjects are given in Table 1.

Recombinant Proteins

Genes coding for the hypothetical open reading frame (ORF) Cpn0146, Cpn0147, and Cpn0308 were amplified from *C. pneumoniae* AR-39 genomic DNA by PCR using Pfu polymerase (Takara, Dalian, China) and oligonucleotide primers. The primer design was based on *C. pneumoniae* sequences obtained from the published data banks, and the primers used for amplification of each of these genes are listed as follows: Cpn0146, 5′-CGCGGGATCCATGAG-CAGTTCGGAAGTTGTTT-3′ and 5′-TTTTCC-TTTTGCGGCCGCTCAATCATCTGACATCTGAT-CG-3′; Cpn0147, 5′-CGCGGATCCATGGCTGTT-CAATCTATAAAAGA-3′ and 5′-TTTTCCTTTTGC-GGCCGCCTAACTTCCCGCCCCTGAA-3′; Cpn0308, 5′-CGCGGATCCATGGCTACAGTAGCACAAACA-3′ and 5′-TTTTCCTTTTGCGG CCGCTTATTTAGAG-GAGTAACGATCC-3′. After digestion of the PCR products with the indicated restriction enzymes, DNA fragments were cloned into pGEX vector and expressed as fusion proteins with glutathione S-transferase (GST) fused to the N-Terminus of the chlamydial protein. The nucleotide sequence of the entire DNA insert was identified by Sangon Biological Engineering Technology & Services Co., Ltd. Recombinant fusion proteins were then expressed. That is, first, recombinant clones were grown in Luria–Bertani medium (500 ml) containing 100 μg of ampicillin/ml at 37°C until an optical density at 600 nm (OD_{600}) of 0.4 to 0.8 was reached.

TABLE 1. Characteristics of the Patient Groups Examined

Sera group	Subjects and analysis	No. of patients	Median age in years (range)	% Female
Group I	*C. pneumoniae*-associated respiratory infection	183	51 (32–65)	28
Group II	Healthy blood donors	60	29 (19–48)	32
Group III	Negative controls	32	4 (2–6)	47

Overexpression of the fusion proteins was then induced by the addition of 0.2 mM IPTG (Isopropyl β-D-Thiogalactoside). After a 3 hr incubation at 30°C, cells were harvested and lysed via sonication in a Triton X-100 lysis (1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 75 U/ml aprotinin). After a high-speed centrifugation to remove debris, the fusion proteins were purified by affinity chromatography using Glutathione Sepharose 4B from ameshame pharmacia (Zkcy, BJ) for the subsequent experiment, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining.

SDS-PAGE and Western Blotting

Proteins were separated on 12% acrylamide slabs by SDS-PAGE. The gels were electroblotted onto nitrocellulose membranes (0.45 μm) by using semidry Trans-Blot SD (15), and then the membrane was blocked with blocking buffer (1 × phosphate-buffered saline (PBS), Tween20, 5% milk) for 2 hr at 37°C. After being washed in washing buffer (20 mM Tris base, 500 mM NaCl, 0.05% Tween 20), the membrane was incubated with human serum (diluted 1:100) overnight at room temperature. The secondary antibodies used were HRP-conjugated goat anti-human IgG diluted 1:12,000 and incubated with the membrane for another hour. Finally, Bands were visualized by DAB (3,3′-diaminobenzidine tetrahydrochloride)/H_2O_2 (Sigma-Aldrich, Shanghai, China).

ELISAs

The plate sensitization was done by coating polystyrene, and microtiter 96-well ELISA plates (MBB, Jianshu, China) were coated with 100 μl/well of the antigen diluted in carbonate bicarbonate buffer (0.1 M) (pH 9.6) at a concentration of 5 μg/ml for the three recombinant antigens. After incubation overnight at 4°C, the plates were washed three times with 200 μl/well of PBS (0.1 M) containing 0.05% Tween 20 (PBS-T) and post-coated with 200 μl of 3% BSA per well in PBS for at 37°C 2 hr. After washing, 100 μl of serum diluted 1:100 in 3% BSA in PBS was added to each well, followed by incubation at room temperature for 1 hr, respectively. Wells were then washed five times with the same buffer and incubated with 100 μl/well of horseradish peroxidase-conjugated goat anti-human IgG secondary antibody (Beyotime, Co., China) diluted 1:10,000 with PBS-T for 1 hr at room temperature. After washing with PBS-T (200 μl/well), the reactions were visualized with 50 μl TMB substrate (Beyotime, Co.) per well and incubated at room temperature for 30 min. Afterward, the enzyme reaction was stopped by the addition of 50 μl of 0.25 M H_2SO_4, and the OD_{450} value was read in a Labsystems Multiskan ELISA reader, with each serum tested in duplicate both in antigen-coated and in non-coated wells.

In all data presented in this article, the background OD_{450} value measured for each serum upon binding to plates without antigen was subtracted from the OD_{450} value measured when antigen was used in the ELISA, and this background was in the order of OD_{450} 0.0 to 0.1. The cut-off value for each antigen was derived on the basis of the mean absorbance obtained with negative controls sera, which was defined as the mean OD_{450} value obtained with 32 negative control samples plus three standard deviations.

Commercially Available ELISA

The ELISA kits used to detect the IgG anti-*C. pneumoniae* elementary body (EB) was provided by Savyon Diagnostics Ltd., (Ashdod, Israel) and purified EBs of *C. pneumoniae* were used as antigens to detect the antibody response in humans in this assay. The assays and calculations were performed in accordance with the manufacturers' instructions.

Day-to-day Variations

In order to determine the within- and between-subject variations, three sera (one with $OD < 0.2$, one with $0.2 < OD < 0.8$, one with $OD > 0.8$) were examined for anti-Cpn0146, Cpn0147, Cpn0308 IgG antibodies by the ELISA three times a day for four consecutive days (16). Day-to-day variations were determined by estimating the percent coefficient of variation (CV = (SD/mean) × 100%). The experiments were carried out by the same person who performed the analyses for the main study.

Statistical Analysis

Data were analyzed by the *t* test and chi-square test wherever appropriate. Probability values (with $P < 0.05$) were taken as significant, and the results were analyzed for statistical significance with SPSS 13.0.

RESULTS

Preparation and Identification of the Purified Proteins

Cpn0146, Cpn0147, and Cpn0308 genes were successfully amplified from the *C. pneumoniae* strain AR-39 genome (Fig. 1), and then cloned into *E. coli XL-1 Blue* expression vector pGEX. The nucleotide sequences of cloned genes were found to have 100% identity to the GeneBank accession number nucleotide sequence by analyzing with BLAST, respectively. After the induction

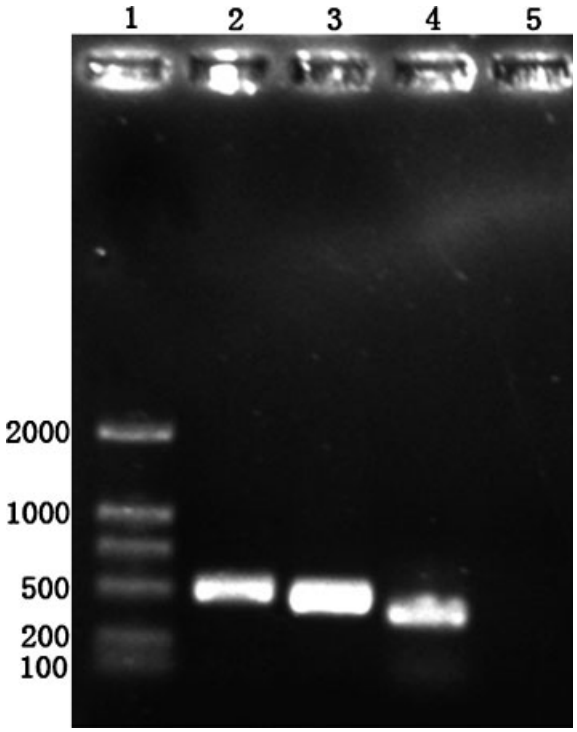

Fig. 1. Amplification of Cpn0146, Cpn0147, Cpn0308 genes from the *C. pneumoniae* strain AR-39 genome. (1) DNA Marker; (2) PCR product of Cpn0146 gene; (3) PCR product of Cpn0147 gene; (4) PCR product of Cpn0147 gene; and (5) Negative control.

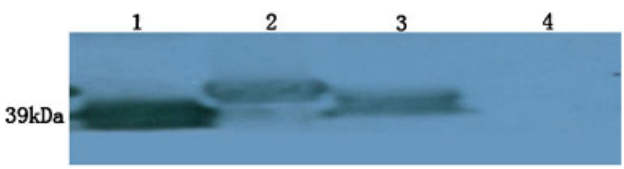

Fig. 2. IgG-specific immunoblot of GST-Cpn0146, Cpn0147, Cpn0308 with *C. pneumoniae*-infected patient sera. (1) Cpn0308; (2) Cpn0146; (3) Cpn0147; and (4) Negative control.

with IPTG, both of these recombinant proteins were found to be present mainly as soluble fractions. Glutathione Sepharose 4 B affinity chromatographic columns were used to purify recombinants proteins, which were analyzed by SDS-PAGE and Coomassie blue staining. And the GST-Cpn0146, Cpn0147, Cpn0308 fusion proteins were obtained with a correct molecular mass 42, 41, 39 kDa and high purity, respectively. Western blot assays were performed to evaluate the immunoreactivity of the purified GST-Cpn0146, Cpn0147, Cpn0308 fusion proteins. The result showed that the recombinant proteins of *C. pneumoniae* could be recognized positively by IgG antibody in one of *C. pneumoniae*-infected patient sera tested (Fig. 2).

Detection of Sera by Recombinant Protein-based ELISA

A total of 275 sera samples were collected and tested by ELISA using three different antigens: the GST-Cpn0146, Cpn0147, Cpn0308 antigens. The mean OD was the highest with Cpn0308 antigen compared with that for Cpn0146, Cpn0147 antigens. The controls, on the other hand, gave the lowest mean OD with Cpn0308 antigen. The Cpn0147 antigen gave a significantly higher mean OD ($P<0.05$) compared with the Cpn0146 antigen in Group I subjects, but the OD was not significantly different in the control group.

When the percentage of individuals with serum antibodies to recombinant antigens was examined, Group I patients ($n=183$) had significantly more IgG to Cpn0146, Cpn0147, Cpn0308, and EBs antigens than healthy blood donors. The most significant difference was observed for IgG responses to Cpn0308 antigen ($P<0.05$) compared with other antigens (Table 2).

As shown in Tables 3 and 4, when the number of individuals with serum IgG antibodies to recombinant antigens was considered, the recombinant Cpn0146 was recognized by 71 (38.8% positive recognition rate), 15 (25%) and 1 (3.1%), Cpn0147 by 75 (40.9%), 14 (23.3%), and 2 (6.3%), and Cpn0308 by 82 (44.8%), 16 (26.7%), and 0 (0%) samples from groups I, II, and III, respectively. And the sensitivities of anti-Cpn0146, Cpn0147, Cpn0308 IgG antibodies were 87.5, 88.2, 96.5% and the specificities were 100, 100, 97.5%, respectively in relation to EBs ELISA. The highest sensitivity was obtained for IgG anti-EBs but with a low specificity. The best results were obtained for IgG anti-Cpn0308 (sensitivity, 96.5%; specificity, 97.5%).

From ELISA results, 71 of the 183 sera from Group I (Table 3) had absorbency readings indicative of a positive result with each recombinant antigen. The degree of the antibody reactivity within the same serum differed for each antigen. In order to differentiate sera from different groups, a cut-off value for each recombinant antigen was established as described above. It was interesting that several sera from group I and group II that were negative when tested with Cpn0146, Cpn0147, Cpn0308 recombinant antigen, respectively, were, however, positive when tested with EBs antigen in the ELISAs assays.

Analysis of the Day-to-Day Variations in ELISAs

From the results of the three sera used in the within-assays analysis, it seemed that generally the greater the amount of IgG antibodies in a serum, the smaller the coefficient of variation of the ELISA. The coefficients of variation for the four sera for each ELISA are shown in Table 5. The between-assay analysis of precision gave

TABLE 2. Individuals with Serum Antibodies Recognizing *C. pneumoniae* Whole Organisms or Fusion Proteins

Antigen	Serum antibody response	% of patient with acute respiratory infections ($n = 183$)	% of healthy blood donors ($n = 60$)	Statistical analysis (χ^2 test) between both groups
EBs (Savyon Diagnostics Ltd.)	IgG	53.5	38.3	$\chi^2 = 4.19$, $0.01 < P < 0.05$
Cpn0146	IgG	38.8	25.0	$\chi^2 = 3.84$, $0.01 < P < 0.05$
Cpn0147	IgG	40.9	23.3	$\chi^2 = 3.95$, $0.01 < P < 0.05$
Cpn0308	IgG	44.8	26.6	$\chi^2 = 5.08$, $0.01 < P < 0.05$

TABLE 3. Recognition of Three *C. pneumoniae* Recombinant Antigens and EBs by Three Groups of Human Sera

	Cpn0146		Cpn0147		Cpn0308		EBs (savyon)	
ELISA results	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Group I	71	112	75	108	82	101	98	85
Group II	15	45	14	46	16	44	26	34
Group III	1	31	2	31	0	32	4	28

TABLE 4. The Specificity and Sensitivity of Recombinant Antigen-Based Detection Assays

Recombinant antigen	Specificity (%)	Sensitivity (%)
Cpn 0146	100.0	87.5
Cpn 0147	100.0	90.7
Cpn 0308	97.5	96.7

TABLE 5. Comparison of Coefficients of Variation of Three ELISAs for the Detection of Ig G Antibodies on Four Three Sera Tested Nine Times in One Assay

	Coefficient of variation (%) of serum no.		
ELISAs	(OD < 0.2)	(02 < OD < 06)	(OD > 1.0)
Cpn0146	7.64	3.58	2.69
Cpn0147	8.05	5.30	7.28
Cpn0308	6.82	3.18	2.43

coefficients of variation as follows: Cpn0146-ELISA, 6.32%; Cpn0147-ELISA, 7.63 %; Cpn0308-ELISA, 5.88%.

DISCUSSION

An accurate and reliable serological test for detecting *C. pneumoniae* IgG antibodies become more and more important because there is increasing concern that *C. pneumoniae* is invovled in chronic disease processes, especially cardiovascular disease (17). The micro-IF test remains the most frequently used method for serodiagnosis and the tool for seroepidemiologic studies of *C. pneumoniae* infections. However, it fails to detect antibodies in the majority of children with culture-documented *C. pneumoniae* infections and difficult to interpret the test results in interlaboratory variations. As a result, the use of this assay has been restricted to a relatively small number of qualified research laboratories. Several recent studies have been performed to identify novel *C. pneumoniae*-specific and immmunodominant antigens available for ELISAs that may facilitate the development of such serodiagnosis (18–20).

This study focused on three immunodominant proteins Cpn0146, Cpn0147, and Cpn0308 that were reported to be in the inclusion membrane throughout the infection course and suggested as putative candidates for the development of serodiagnostic testing. These three antigens were tested by ELISA in exactly the same way in order to determine the key immunodominant antigens. And the results presented in this study suggest that a more widely applicable enzyme immunoassay, based on recombinant Cpn0308 antigen, may be helpful in the serodiagnosis of *C. pneumoniae* infections.

The goal of this study was to develop a reliable diagnostic tool for future serological studies dealing with *C. pneumoniae*. Our first step was to identify and purify conserved antigens that could be used as serological markers of *C. pneumoniae* infections, and then we identified and succeeded in purifying three immunogenic proteins encoded by the genes Cpn0146, Cpn0147, and Cpn0308, which could be specifically recognized by antibodies in post-infection sera.

To evaluate the ELISA with these three antigens, 103 sera from different patient groups were selected and grouped (Table 1). Concerning all the antigens tested by ELISA, the highest sensitivity was observed for the IgG reactivity to EBs but this determination had a low specificity, which may be due to EBS as a genus-specific antigen. A high prevalence of anti-*C. pneumoniae* IgG antibodies in the population (38.3%) was observed in this study, which is a bit low compared with the results as judged by MIF (~50%). The reason may be a lower sensitivity of ELISA than that of MIF, as proposed by Hermann et al. Meanwhile, the prevalence of anti-*C. pneumoniae* IgG antibodies was significantly increased in acute respiratory infection patients (53.5%) against healthy controls (38.3%) when analyzed by the ELISA tests.

It was interesting that the significant variations in the reactivities of IgG antibodies were noted when sera from group I patients were tested in an ELISA with individual recombinant antigens. When the reactivities of IgG antibodies from each serum were analyzed, the differences were evident. For example, 11 sera reacted with Cpn0308, EBs, but not with Cpn0146 and Cpn0147; another 13 sera reacted only with EBs. What is more interesting, four sera from group III negative controls were positive when tested using the EBs-ELISA, and one, two sera for Cpn0146, Cpn0147-ELISA, respectively, but none of the sera were tested positive by using the Cpn0308-ELISA. The different reactivities observed for the four antigens are probably the result of their surface exposure, as bacterial infection usually induces serum antibody against surface-exposed components. Thus, the different reactivities observed in this study between the four antigens can be explained by its different immunoaccessibility (21,22).

Our data also demonstrate that a *C. pneumoniae*-specific antigen Cpn0308 was recognized by most of the samples (45.3% for IgG) from patients with acute respiratory infection and rarely (3.1%) by those from group III controls ($P<0.001$), (26.6%) by those from healthy blood donors ($P<0.005$), which could be of great diagnostic value, but its identity was not determined.

In conclusion, we have established a recombinant ELISA with good specificity and sensitivity, and the results presented in this study indicate that recombinant Cpn0308 antigens could be a valuable tool in the development of a standardized serological assay for the detection of *C. pneumoniae* infections. However, the Cpn0308-ELISA was less sensitive than EBs-ELISA, thus improvement in the sensitivity of such a serological test is imperative. Several studies have reported that combined use of this antigen with others may prove useful. Further studies are under way to develop a standardized, commercially available assay in the future.

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