模なの臨床試験デザインでも考慮しなければならない。特に非臨床試験や早期臨床試験で、その様な兆候が認められ、がんワクチンの生物学的効果の発現には十分な時間経過が必要であることが非臨床試験等で示されている場合にはエンドポイントの判定に十分な注意が必要である。その点を十分配慮することが必要である。がんワクチンの効果に遅延性があることから、効果の遅延による有用性を示すための被験者集団サイズの増加と治験の達成に関する主要評価においてより注意深い解析が必要となる。

１０．その他

本文書ではがんワクチンの臨床評価について主としてペプチド／タンパク質を用いた製品を対象として議論されているが、がんワクチンの開発は、細胞治療、遺伝子治療、核酸医薬品など多様な製品が開発中である。これらの製品ではターゲットなるがん抗原が特定されている場合と、がん細胞全体を投与するといった抗原が必ずしも特定されていない製品もある。そのような多様な製品の評価では免疫応答性の評価や有効性の評価は製品の特性に応じたケースバイケースの判断をせざるを得ない場合も多いと想定される。本文書はそのような製品の評価において参考になる部分については利用されることが望ましい。

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RESEARCH LETTER

Genomic screening for *Chlamydophila pneumoniae*-specific antigens using serum samples from patients with primary infection

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Received 15 December 2011; accepted 31 January 2012.
Final version published online 24 February 2012.

DOI: 10.1111/j.1574-6968.2012.02520.x

Editor: Michael Galperin

Keywords
Chlamydophila pneumoniae; antigen screening; serological diagnosis.

Abstract

Chlamydophila pneumoniae, an obligate intracellular human pathogen, causes respiratory tract infections. The most common techniques used for the serological diagnosis of *C. pneumoniae* infections are microimmunofluorescence tests and commercial serological ELISA tests; these are based on the detection of antibodies against whole chlamydial elementary bodies and lipopolysaccharide/ outer membrane protein, respectively. Identification of more specific and highly immunodominant antigens is essential for the development of new serodiagnostic assays. To identify novel specific antigens from *C. pneumoniae*, we screened 455 genes with unknown function in the genome of *C. pneumoniae* J138. Extracts of *Saccharomyces cerevisiae* cells expressing GFP-tagged *C. pneumoniae* proteins were subjected to Western blot analysis using serum samples from *C. pneumoniae*-infected patients as the primary antibodies. From this comprehensive analysis, 58 clones expressing *C. pneumoniae* open reading frames, including hypothetical proteins, were identified as antigens. These results have provided useful information for the development of new serological tools for the diagnosis for *C. pneumoniae* infections and for the development of vaccines in future.

Introduction

Chlamydophila pneumoniae is an obligate intracellular human pathogen that causes community-acquired respiratory infections (Grayston *et al.*, 1990). Almost all humans face the possibility of contracting *C. pneumoniae* infections, at least once in their lifetime (Kuo *et al.*, 1995). Reinfections are very frequent, and the infections may turn chronic (Grayston, 2000). In addition, the organism can survive in the host cell following primary infection (Grayston *et al.*, 1990). These persistent bacteria are common in the respiratory tract or in atherosclerotic blood vessels, and therefore, they represent a potential risk factor for chronic inflammatory lung disease or atherosclerosis (Bunk *et al.*, 2008).

Several methods can be used for the specific diagnosis of *C. pneumoniae* infections, including microbiological culturing; for example, ELISA, a microimmunofluorescence (MIF) test, and PCR (Kuo *et al.*, 1995). The Centers for Disease Control recommend the MIF test as the gold standard for serodiagnosis of *C. pneumoniae* infections. The MIF test, an indirect fluorescent antibody technique, however, has certain limitations, including subjective interpretation, cross-reactivity between different *Chlamydia* species, and high intra- and inter-laboratory variation (Ozanne & Lefebvre, 1992). Highly trained personnel are necessary to perform the test, and it has not yet been adapted for routine use in diagnostic laboratories. Because of these limitations, ELISA tests are most commonly used for the clinical diagnosis of *C. pneumoniae*. In Japan, most clinicians and researchers use commercial serologic ELISA test kits from Hitachi Chemical, Co., (Japan) or Medac Diagnostika (Germany). The results obtained with these kits have accumulated over

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recent years and have exposed discrepancies between some kits with respect to false-positive and false-negative reactivity among asymptomatic subjects (Miyashita *et al.*, 2008). Therefore, identification of *C. pneumoniae*-specific antigens, which could be used for the development of new serodiagnostic assays, is of great importance.

Although *C. pneumoniae*-specific antibody responses have been characterized by immunoblotting, only few major surface proteins (MOMP, Omp2, and CrpA; Iijima *et al.*, 1994; Klein *et al.*, 2003; Mygind *et al.*, 1998) and some Inc proteins (Cpj0146, Cpj0147, and Cpj0308) have been detected (Hongliang *et al.*, 2010). However, these antigens have yielded variable results with respect to the consistency and accuracy of *C. pneumoniae* identification. Taken together, very little information is available regarding specific detection of *C. pneumoniae.*

We determined the sequence of the whole genome of *C. pneumoniae* J138 isolated in Japan (Shirai *et al.*, 2000) and found that this strain features putative protein coding from its 1069 open reading frames (ORFs). A comprehensive bioinformatics approach was applied for annotation taxonomy, and about half of the predicted genes were found to encode proteins without any known functions. To identify novel specific antigens from *C. pneumoniae*, we screened 455 genes without any known functions. A fusion protein expression library of *C. pneumoniae* was constructed in *Saccharomyces cerevisiae*. Protein extracts of the recombinant yeast cells expressing the green fluorescent protein (GFP)-tagged *C. pneumoniae* proteins were subjected to Western blot analysis using serum samples from *C. pneumoniae*-infected patients as the primary antibodies. This study sought to identify specific and highly immunodominant antigens, which are required for the development of new serodiagnostic assays, and hopefully, vaccines, in the future.

Materials and methods

Patients and sera

Thirteen serum samples were collected from eight patients (age: range, 4–11 years; Table 1), who had been clinically diagnosed with primary acute *C. pneumoniae* infection. The levels of *C. pneumoniae*-specific immunoglobulin (Ig) IgA, IgG, and IgM in these patients were evaluated using two different ELISA kits: (1) HITAZYME *C. pneumoniae* kits for IgA, IgG, and IgM that utilize the soluble elementary body (EB)-outer membrane complex, without the lipopolysaccharide, as the antigen (Hitachi Chemical, Japan) and (2) *C. pneumoniae*-ELISA *plus* Medac kits for IgA and IgG and *C. pneumoniae*-sELISA Medac kit for IgM, which utilize the purified cell wall membrane proteins as the antigen (Medac Diagnostika, Germany). Eight serum samples from 0-year-old healthy children were used as negative controls.

Cloning of the chlamydial genes and expression of the GFP-tagged *C. pneumoniae* ORFs

Chlamydophila pneumoniae genomic DNA was obtained from the EBs of the *C. pneumoniae* J138-infected HEp-2 cells (Miura *et al.*, 2001). We used a gene expression system controlled by a Tet-off promoter in *S. cerevisiae.* The ORFs of 455 genes from *C. pneumoniae* J138, including genes of unknown function (Supporting Information, Table S1), were cloned into a pMT830 vector, which was constructed as previously described (Tabuchi *et al.*, 2009). This vector system allows a protein of interest to be expressed with GFP fused to the C-terminus. The vector was transformed into the *S. cerevisiae* strain MTY483, protein expression was studied, and proteins were extracted, as previously described (Tabuchi *et al.*, 2009).

Western blot analysis

Ten micrograms of total protein was separated by 10% SDS-PAGE. The gels were electroblotted onto PVDF membrane (pore size, 0.45 μm) and incubated with human serum (1 : 200 dilution) as primary antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-human IgA + IgG + IgM immunoglobulin (KPL, MD) and goat anti-human IgA (Monosan, Netherland), IgG (Invitrogen, CA), and IgM (Invitrogen) were used at a dilution of 1 : 3000 as the secondary antibodies. Immunoreactive bands were visualized by Immobilon Western (Millipore, MA) with an LAS-1000 imaging system. The membranes were reprobed with anti-GFP antibody (1 : 5000 dilution; Tabuchi *et al.*, 2010) and HRP-labeled anti-rabbit IgG (1 : 5000 dilution: Cell Signaling Technology, MA).

Results

Characterization of patient sera

Thirteen serum samples from eight patients were tested with the commercially available HITAZYME and Medac ELISA kits (Table 1). All samples tested positive for at least one anti-*C. pneumoniae* antibody. However, some discrepancies were observed between the HITAZYME and Medac kits.

Immunoscreening for novel *C. pneumoniae* antigens

To identify novel *C. pneumoniae* antigens, we expressed 455 unique GFP-tagged ORFs encoded by the *C. pneumoniae*

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Table 1. Clinical information of the eight subjects with *Chlamydophila pneumoniae* infections

			HITAZYME			Medac		
Group	Sample No.	Age/sex	IgA (index)	IgG (index)	IgM (index)	IgA (AU mL^{-1})	IgG (AU mL^{-1})	IgM (index)
Patients	1*	9/M	2.65+	3.35+	4.73+	102.6+	330.5+	4.7+
	2*	11/M	ND	3.49+	3.00+	49.3+	434.9+	1.3+
	3-1	5/F	0.07−	0.03−	1.13+	1.9−	0.8−	0.5−
	3-2*		ND	0.02−	1.51+	2.2−	1.3−	0.4−
	4-1	11/M	0.94±	0.03−	6.99+	10.5−	45.6+	5.4+
	4-2		1.05±	1.25+	5.13+	12.4−	34.6+	5.2+
	4-3*		0.89−	2.19+	5.36+	16.4−	144.2+	4.8+
	5-1	7/F	0.73−	0.14−	3.70+	2.4−	5.7−	0.8−
	5-2*		1.31+	0.63−	6.15+	7.7−	16.6−	2.9+
	6*	4/M	1.31+	2.94+	0.56−	42.9+	586.6+	0.1−
	7-1*	9/F	2.10+	1.45+	7.20+	40.3+	73.9+	2.1+
	7-2		0.75−	1.71+	7.44+	6.8−	125.0+	2.6+
	8*	8/M	1.90+	4.08+	0.63−	67.3+	878.0+	0.1−

M, Male; F, Female; ND, not detected; −, negative (HITAZYME: index < 0.9, Medac: IgA and IgG < 22, IgM < 0.9); +, positive (HITAZYME: index > 1.1, Medac: IgA and IgG > 28, IgM > 1.1); ±, false positive (HITAZYME: index = 0.9–1.09, Medac: IgA and IgG = 22–28, IgM = 0.9–1.1).
*Serum sample used for Western blot assays of Fig. 3.

J138 genome (Table S1). Of these clones, the expression of 398 clones was recognized by anti-GFP antibody, although the levels of expression varied in each yeast clone (Fig. 1a). The expression of the remaining 57 clones was undetectable by anti-GFP antibody for unclear reasons.

We attempted to comprehensively identify the antigens by Western blot analysis using a pool of 13 serum samples as the primary antibody and four different immunoglobulins as the secondary antibodies. As an example, the expression of eight ORFs of *C. pneumoniae* genes is shown in Fig. 1. The serum samples from these patients did not contain significant anti-*S. cerevisiae* antibodies that would have produced a high-level background on the Western blots. Therefore, we were able to specifically detect the *C. pneumoniae* antigens recognized by human anti-*C. pneumoniae* antibodies under conditions of low-level background.

Positive signals were detected in the yeast clones expressing Cpj1056 and Cpj1070 ORFs when anti-human IgA + IgG + IgM immunoglobulin and anti-human IgG were used as secondary antibodies (Fig. 1b and d). The recombinant proteins derived from the ORFs Cpj1056 and Cpj1070 were estimated to be 55 and 81 kDa, respectively, which were matched well with the molecular weights predicted from the sequences of *C. pneumoniae* when they were fused with GFP. The other six ORFs were not detected on these blots and remained 'negative' throughout this investigation.

Among the 398 recombinant ORF clones, 58 clones gave positive signals on Western blots when probed with the pool of 13 serum samples (Fig. 2). The ORF clones that gave positive signals varied with each type of secondary antibody. Twenty-nine ORFs were detected by anti-human IgA + IgG + IgM immunoglobulin; 12, by anti-human IgA; 41, by anti-human IgG; and 24, by anti-human IgM.

Antigen recognition by individual patients

Because antigen recognition may vary greatly among patients, we examined in detail the reactivity of individual serum samples to each antigen. For this analysis, we selected the clones for the 58 ORFs of *C. pneumoniae* that exhibited positive signals in the initial immunoscreening; the serum samples that contained the highest titers in the ELISA assays were used as primary antibodies. The selected serum samples are indicated by an asterisk in Table 1. A great variability was noted in the number of antigens detected using various combinations of individual serum samples as the primary antibody and isotype-specific anti-human immunoglobulins as the secondary antibodies (Fig. 3). Among the 58 ORFs tested, positive signals were detected for the antigens in a total of 39 ORFs by the combination of at least one patient's serum sample as the primary antibody and one of the isotype-specific anti-IgA, anti-IgG, or anti-IgM as the secondary antibody. Although anti-*C. pneumoniae* IgA in No. 4-3 serum, anti-*C. pneumoniae* IgG in No. 3-2 and 5-2, and anti-*C. pneumoniae* IgM in No. 6 and 8 produced negative results in both the ELISA tests, some ORFs were clearly recognized as antigens. These

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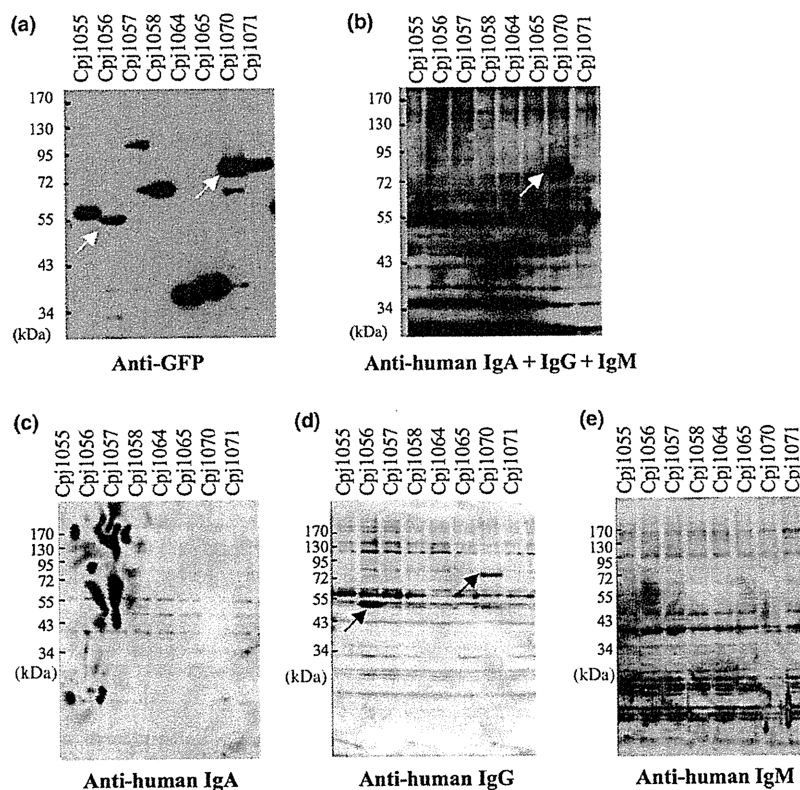

Fig. 1. Screening of *Chlamydophila pneumoniae* antigens by Western blot assays using pooled serum samples from patients with *C. pneumonia* infections. Of the 455 recombinant ORF clones from *C. pneumoniae*, eight are shown as examples of the first screening by Western blotting. The primary antibodies were as follows: (a) anti-GFP antibody and (b–e) human immunoglobulin in the 13 pooled serum samples (1 : 200 dilution of each serum sample) from eight patients with primary *C. pneumoniae* infection. Secondary antibodies were as follows: (a) HRP-labeled anti-rabbit IgG, (b) HRP-conjugated goat anti-human IgA + IgG + IgM, (c) HRP-conjugated goat anti-human IgA, (d) HRP-conjugated goat anti-human IgG, and (e) HRP-conjugated goat anti-human IgM.

results indicated that the serum sample definitely contains IgA, IgG, and IgM antibodies against the proteins encoded by some ORFs.

We summarized the data for positive ORFs and have listed their orthologs and homologs from *C. trachomatis* in Fig. 3b. Among the 39 ORFs, we identified 11 ORFs as antigens (Cpj0147, Cpj0159, Cpj0178, Cpj0186, Cpj0268, Cpj0308, Cpj0472, Cpj0677, Cpj0678, Cpj1056, and Cpj1070) that do not have orthologs in the *C. trachomatis* genome. Among the other 19 ORFs, which were not detected by any individual serum sample (Fig. 3a and b), but were detected by pooled serum sample (Fig. 2), nine ORFs (Cpj0067, Cpj0181, Cpj0214, Cpj0224, Cpj0225, Cpj0339, Cpj0355, Cpj0356, and Cpj0457) do not have orthologs in the *C. trachomatis* genome (Fig. 3b). We believe that these 20 ORFs without orthologs in the *C. trachomatis* genome represent strongly immunogenic antigens that are highly specific to *C. pneumoniae*.

Discussion

In this study, we intended to identify novel *C. pneumoniae*-specific antigens by screening the *C. pneumoniae* genome. We applied a bioinformatics approach for annotation taxonomy that allowed us to concentrate on a subset of proteins with unknown functions. To identify the antigens recognized by the antibodies in the patients with primary *C. pneumoniae* infection, we designed a screening system to use patients' serum samples as immunological probes for the genomic screening of a *C. pneumoniae*-ORF expression library. We measured the titers of the isotype-specific immunoglobulins using the commercially available ELISA kits HITAZYME and Medac. These kits gave both negative and positive results for antibody titers of IgA, IgG, and IgM. However, even in cases where negative results were noted by ELISA, a number of ORFs could still be detected by Western blot analysis. These variations may to be due to the differences in the

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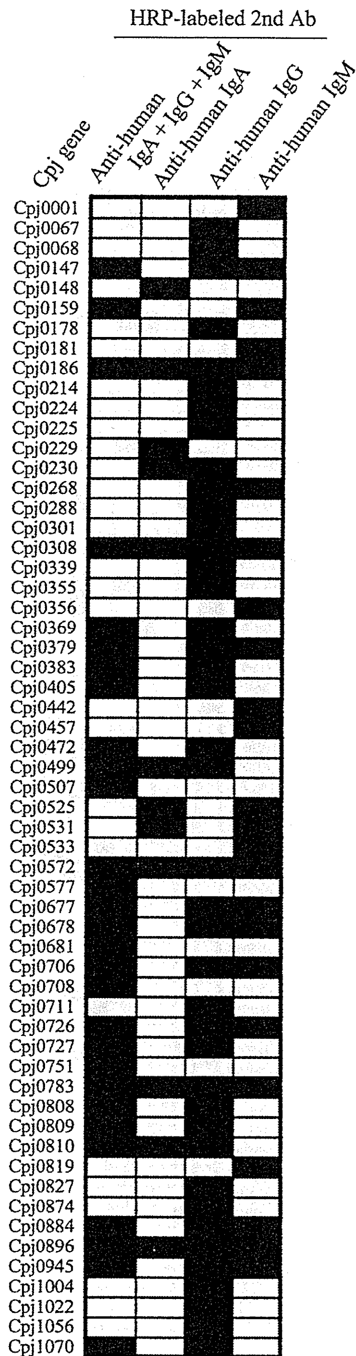

Fig. 2. List of *Chlamydophila pneumoniae* antigens identified by Western blot assays using serum samples pooled from patients with *C. pneumoniae* infections. In the 455 ORFs from *C. pneumoniae* screened by Western blot assays, about 58 *C. pneumoniae* antigens were recognized by human immunoglobulin in the 13 serum samples (1 : 200 dilution of each serum sample) pooled from eight patients with *C. pneumoniae* infections. The black boxes represent the antigens according to the four kinds of HRP-labeled secondary antibodies used in these assays.

antigens employed in each of the ELISA kits; HITAZYME is derived from the soluble EB-outer membrane complex, and Medac is purified from numerous cell wall membrane proteins. Biochemically, these antigens are not well characterized in the literature. Patients whose serum scores negatively for anti-*C. pneumoniae* immunoglobulins according to one of these ELISA tests may not be clinically diagnosed with *C. pneumoniae* infection. Therefore, it is of great importance to provide more sensitive and accurate methods for the diagnosis of *C. pneumoniae.*

We made an expression library of 455 ORFs with *S. cerevisiae* as the host. Expression libraries for recombinant proteins are usually made with *Escherchia coli* as the host, but because the human serum contains a large amount of antibodies against *E. coli* proteins, this method could easily produce high-level background in immunoassays, and thereby disturb the identification process. This issue was avoided using a eukaryotic host cell, *S. cerevisiae*, to express the recombinant proteins. Using a pool of 13 serum samples from eight patients as the primary antibody for Western blotting, the low level of the background indicated that these sera did not contain significant amounts of antibodies against *S. cerevisiae* proteins. This confirmed that Western blot analysis of recombinant yeast proteins can be a powerful tool for identifying specific antigens via genomic screening.

We identified a total of 58 ORFs in the *C. pneumoniae* genome that were recognized as antigens by immunoscreening. Out of the 58 ORFs, Cpj0507, Cpj0577, Cpj0681, and Cpj0751 were detected by isotype-nonspecific anti-human immunoglobulins as the secondary antibodies, but were not detected by isotype-specific anti-human immunoglobulins (Fig. 2). It was not clear which isotype of antibody against these four clones was produced in patients. However, three of these clones (not Cpj0681) were recognized by 1–3 isotypes of immunoglobulins in the sera of selected individual patients (Fig. 3). The precise reason for this variation is unclear, but it may be due to the variations in the affinity of the secondary antibodies toward the human immunoglobulins used in this study. Of the 58 ORFs that tested positive in the screening, 19 were not detected by selected individual sera (Fig. 3b). However, these clones were positive in the pool of the 13 serum samples (Fig. 2). Each serum sample was diluted 200-fold in the reaction solution throughout the study. For the initial screening, the 13 serum samples were combined, and the reaction solution contained each sample at a 200-fold dilution. This means that the serum concentration was 13-fold higher in the reaction solution of the first screening, as compared to later experiments where the serum of selected individuals was used. Of the 13 serum samples used in the initial screening, we selected eight samples with relatively high titers for subsequent analysis of individuals. It is unlikely that the other five samples, which

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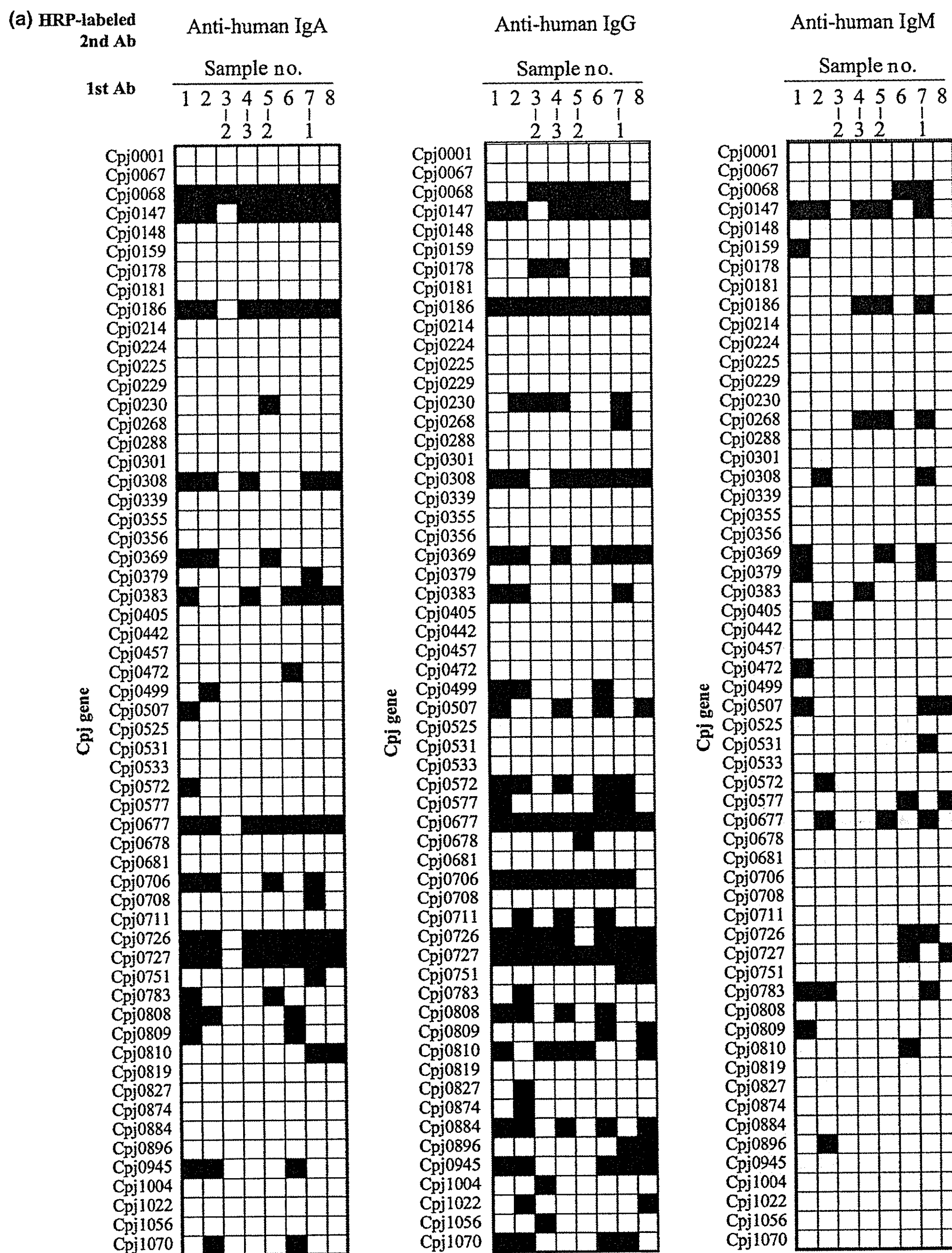

Fig. 3. Identification and characterization of the *Chlamydophila pneumoniae* antigens recognized by individual patients. A total of 58 *C. pneumoniae* antigens were analyzed by Western blot assays, using individual serum samples from eight patients with primary *C. pneumoniae* infections as the primary antibody, and HRP-labeled anti-human IgA, IgG, or IgM, as the secondary antibody. (a) The black boxes represent the antigens recognized by each serum sample and each secondary antibody. The isotype of the secondary antibody is presented at the top of the column. (b) The orthologous genes of *C. trachomatis*. The number of antigens recognized by the serum sample of each patient is listed in the right column. The graph shows the frequency that was observed in the Western blot assays (%).

were not analyzed individually, include antibodies against the 19 ORFs. Thus, the reason why these 19 ORFs were not detected in individual serum samples could be the differences in the concentration and affinity of the antibodies against the *C. pneumoniae* antigens in the selected individual serum samples.

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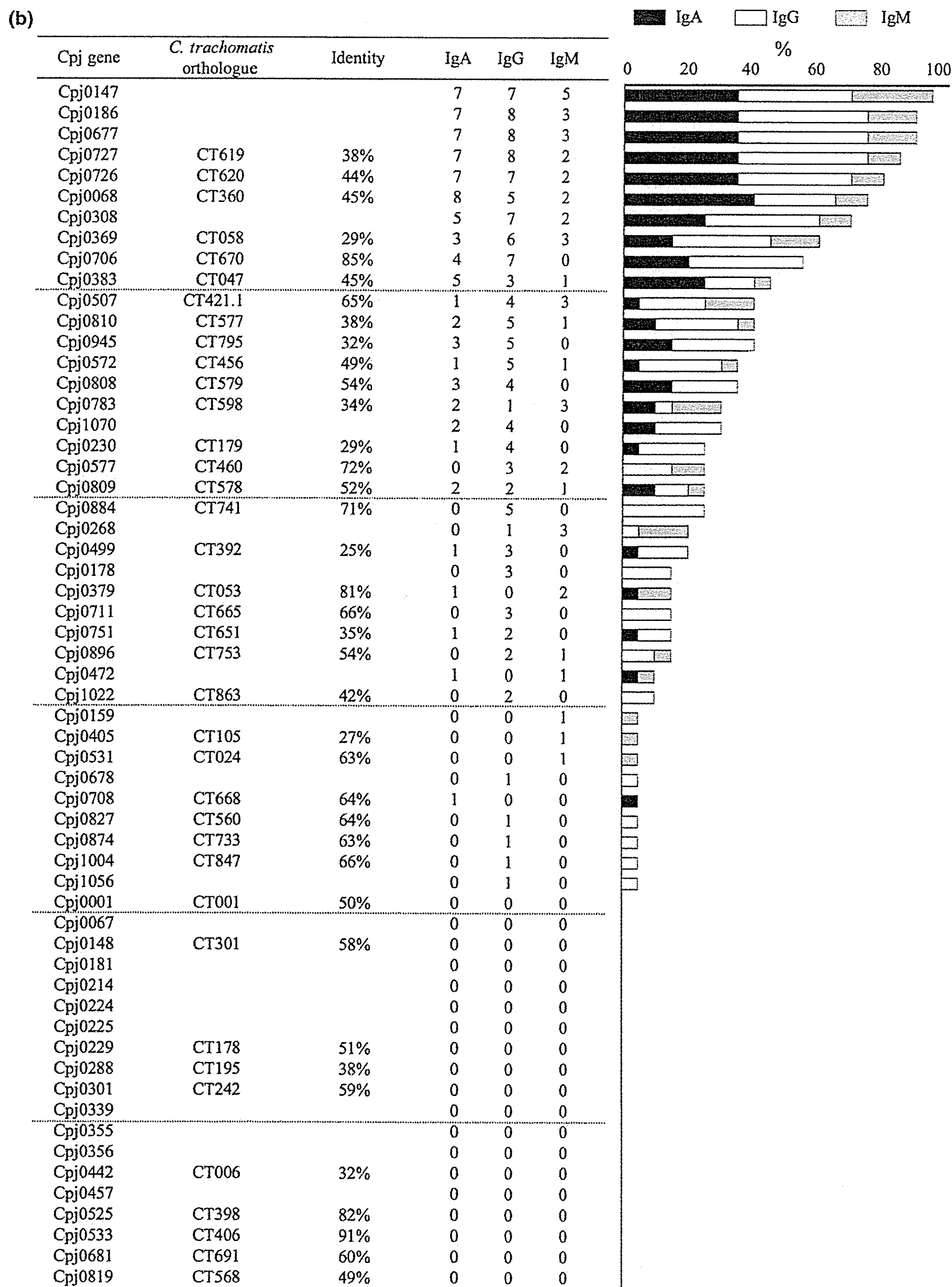

Cpj gene	*C. trachomatis* orthologue	Identity	IgA	IgG	IgM
Cpj0147			7	7	5
Cpj0186			7	8	3
Cpj0677			7	8	3
Cpj0727	CT619	38%	7	8	2
Cpj0726	CT620	44%	7	7	2
Cpj0068	CT360	45%	8	5	2
Cpj0308			5	7	2
Cpj0369	CT058	29%	3	6	3
Cpj0706	CT670	85%	4	7	0
Cpj0383	CT047	45%	5	3	1
Cpj0507	CT421.1	65%	1	4	3
Cpj0810	CT577	38%	2	5	1
Cpj0945	CT795	32%	3	5	0
Cpj0572	CT456	49%	1	5	1
Cpj0808	CT579	54%	3	4	0
Cpj0783	CT598	34%	2	1	3
Cpj1070			2	4	0
Cpj0230	CT179	29%	1	4	0
Cpj0577	CT460	72%	0	3	2
Cpj0809	CT578	52%	2	2	1
Cpj0884	CT741	71%	0	5	0
Cpj0268			0	1	3
Cpj0499	CT392	25%	1	3	0
Cpj0178			0	3	0
Cpj0379	CT053	81%	1	0	2
Cpj0711	CT665	66%	0	3	0
Cpj0751	CT651	35%	1	2	0
Cpj0896	CT753	54%	0	2	1
Cpj0472			1	0	1
Cpj1022	CT863	42%	0	2	0
Cpj0159			0	0	1
Cpj0405	CT105	27%	0	0	1
Cpj0531	CT024	63%	0	0	1
Cpj0678			0	1	0
Cpj0708	CT668	64%	1	0	0
Cpj0827	CT560	64%	0	1	0
Cpj0874	CT733	63%	0	1	0
Cpj1004	CT847	66%	0	1	0
Cpj1056			0	1	0
Cpj0001	CT001	50%	0	0	0
Cpj0067			0	0	0
Cpj0148	CT301	58%	0	0	0
Cpj0181			0	0	0
Cpj0214			0	0	0
Cpj0224			0	0	0
Cpj0225			0	0	0
Cpj0229	CT178	51%	0	0	0
Cpj0288	CT195	38%	0	0	0
Cpj0301	CT242	59%	0	0	0
Cpj0339			0	0	0
Cpj0355			0	0	0
Cpj0356			0	0	0
Cpj0442	CT006	32%	0	0	0
Cpj0457			0	0	0
Cpj0525	CT398	82%	0	0	0
Cpj0533	CT406	91%	0	0	0
Cpj0681	CT691	60%	0	0	0
Cpj0819	CT568	49%	0	0	0

Fig. 3. Continued.

Cpj0146, Cpj0147, and Cpj0308 were recently described as *C. pneumoniae* immunogenic proteins (Hongliang *et al.*, 2010). Cpj0147 and Cpj0308 were also recognized as antigens in our present study, demonstrating the validity of our screening system. Furthermore, we revealed that antibodies against Cpj0147 and Cpj0308 belong not only to the IgG isotype, but also to IgA and IgM. Although Cpj0146 was not recognized by the patient serum sample used in this study, it was recently reported that Cpj0146 has low recognition rates in the adult population com-

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