Molecular Detection and Seroepidemiology of the *Chlamydia pneumoniae* Bacteriophage (ΦCpn1)

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Recent whole-genome analysis has demonstrated limited genetic variation in *Chlamydia pneumoniae*, with one strain (AR39) containing a 4,524 nucleotide single-stranded DNA bacteriophage, ΦCpn1. Using PCR, reverse transcription (RT)-PCR, and Western blotting, we confirmed the presence and functional expression of ΦCpn1 in *C. pneumoniae* strain AR39 and its absence in strain CWL029. Six additional epidemiologically distinct clinical isolates of *C. pneumoniae* also did not contain ΦCpn1. We generated recombinant viral protein 1 (Vp1) from ΦCpn1 in *Escherichia coli* and showed that Vp1 antigen is highly immunogenic in mice and that murine antiserum readily recognizes native Vp1 from *C. pneumoniae* strain AR39 elementary bodies (EB). We developed an enzyme-linked immunosorbent assay (ELISA) to measure antibodies to recombinant Vp1 in human sera collected from 32 patients with abdominal aortic aneurysm (AAA) and 40 controls. Among the 72 subjects, 61 had *C. pneumoniae* EB antibodies shown by ELISA. Antibodies to Vp1 were found in 39 of the 61 (64%) seropositive individuals and were significantly correlated with AAA (adjusted odds ratio, 13.9; 95% confidence interval, 1.1 to 175). Our studies indicate that phage-containing strains of *C. pneumoniae* are uncommonly found by isolation but may commonly infect individuals with vascular disease.

There is growing evidence linking infection with *Chlamydia pneumoniae* with vascular diseases, such as atherosclerosis and abdominal aortic aneurysm (AAA) (3, 5). However, the data remain inconclusive, and the clinical and public health importance of *C. pneumoniae* as a vasculopathic organism is unclear. Skeptics have suggested that *C. pneumoniae* is simply an “innocent bystander” that more readily infects diseased arteries (8) or that its association with vascular disease is confounded by its association with other atherosclerotic risk factors (13). Since seroepidemiological studies indicate that most people are infected by *C. pneumoniae* by the age at which the clinical manifestations of atherosclerosis usually appear (11), it is likely that if *C. pneumoniae* is a causal agent for vascular disease, there is variability in the characteristics of the organism, the host response to infection, or both. Variability in host characteristics could explain why some people might be more prone to develop vascular disease when infected with *C. pneumoniae*, and it is well known that family history is a strong risk factor for AAA. There may also be important variations in the vasalectrophilisms and pathogenicities of different *C. pneumoniae* strains. In this regard, recent genomic studies have shown genetic variation in *C. pneumoniae* (16, 24, 29). One of the most prominent genetic differences among the three genetically characterized strains of *C. pneumoniae* was that one strain (AR39) contained a 4,524-nucleotide single-stranded DNA bacteriophage, ΦCpn1 (24). Though three phylogenetically related phages were identified in *Chlamydia psittaci* (14, 20, 26), ΦCpn1 was the first phage to be identified in *C. pneumoniae*. ΦCpn1 was predicted to contain up to eight open reading frames (ORFs), with ORFs 1 to 3, by analogy to the avian *C. psittaci* phage Chp1, encoding the viral structural proteins viral protein 1 (Vp1), Vp2, and Vp3, respectively (24). In a recent report of comparative analysis of chlamydia phages, the well-conserved Vp1 protein was predicted to contain a potential receptor binding site (25).

Phage-bearing strains of other bacteria are often more pathogenic than phage-free strains (6), and it may be that phage-containing strains of *C. pneumoniae* are more strongly correlated with vascular disease. However, the role played by the phage ΦCpn1 in the disease pathogenesis of *C. pneumoniae* is not obvious, since ΦCpn1 does not carry any known virulence genes, unlike other phages associated with pathogenic bacteria. The development of methodologies to detect this interesting phage is therefore necessary in order to carry out detailed epidemiologic studies of the association of the phage with *C. pneumoniae*-associated vascular diseases.

The purpose of the present study was to develop molecular methods to differentiate *C. pneumoniae* strains containing the phage ΦCpn1 from other strains that lack the phage based on the *vpl* gene encoding Vp1 and to develop an enzyme-linked immunosorbent assay (ELISA) to detect Vp1 antibodies in order to determine whether exposure to *C. pneumoniae* strains containing the phage is associated with AAA.

**MATERIALS AND METHODS**

Strains of *C. pneumoniae*, growth, and purification. The *C. pneumoniae* strains used in this study are described in Table 1. The human HL-60 cell line was used for growth and propagation of the *C. pneumoniae* strains. Elementary bodies (EBs) were purified on discontinuous gradients of Renografin-76 (Squibb Canada, ...
TABLE 1. C. pneumoniae strains evaluated for phase

<table>
<thead>
<tr>
<th>Strain</th>
<th>C. pneumoniae CP0543 sequence</th>
<th>ϕCpn1-specific sequences</th>
<th>Source and/or reference</th>
</tr>
</thead>
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<tr>
<td>AR39</td>
<td>Yes</td>
<td>Yes</td>
<td>Laboratory strain (12)</td>
</tr>
<tr>
<td>CWLJ029</td>
<td>Yes</td>
<td>No</td>
<td>Laboratory strain (17)</td>
</tr>
<tr>
<td>CM1</td>
<td>Yes</td>
<td>No</td>
<td>Laboratory strain (2)</td>
</tr>
<tr>
<td>Kajaani7</td>
<td>Yes</td>
<td>No</td>
<td>Kajaani, Finland (P. Saikku)</td>
</tr>
<tr>
<td>Bay 13</td>
<td>No</td>
<td>No</td>
<td>Spartanburg, S.C. (M. Hammerschlag)</td>
</tr>
<tr>
<td>Bay 16</td>
<td>No</td>
<td>No</td>
<td>Toledo, Ohio (M. Hammerschlag)</td>
</tr>
<tr>
<td>Bay 19</td>
<td>No</td>
<td>No</td>
<td>Baltimore, Md. (M. Hammerschlag)</td>
</tr>
<tr>
<td>Bay 20</td>
<td>No</td>
<td>No</td>
<td>Springfield, Mo. (M. Hammerschlag)</td>
</tr>
<tr>
<td>Bay 21</td>
<td>Yes</td>
<td>No</td>
<td>Mesa, Az. (M. Hammerschlag)</td>
</tr>
</tbody>
</table>

Montreal, Canada) as previously described (28). The purified EBs were resuspended in isotonic sucrose-phosphate-glutamate buffer and stored at −80°C.

PCR analysis. PCR was performed using a PTC-200 Peltier thermal cycler (MJ Research). The reaction mixture contained 1.5 mM MgCl₂, 200 μM deoxyribonucleoside triphosphates, 5 U of Taq DNA polymerase enzyme, and 25 pmol of oligonucleotide primers (for the ϕ1 gene, forward [5'-CGTCTCTTAGTGGGGGATTTACTGA-3'] and reverse [5'-CACAGCTGGTACCCATAATGGCT-3']; for the 16S RNA gene, forward [5'-CGTCTCTTAGTGGGGGATTTACTGA-3'] and reverse [5'-AACAGGAGGTCAGTACCAGCTAC-3']; for the C. pneumoniae CM1, forward [5'-CTGTAAGGTGAAAAGTTTTTGA-3'] and reverse [5'-CGTCTCTTAGTGGGGGATTTACTGA-3'] in a total volume of 50 μl. The PCR cycling conditions were as follows: one cycle of 95°C for 3 min and 35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. This was followed by strand elongation for 10 min at 72°C. The sizes of the amplimers were as follows: ϕ1, 925 bp; 16S RNA, 430 bp; CP0543, 266 bp.

RT-PCR. Total RNA from HL cells infected with C. pneumoniae strains AR39 or CWLJ029 was isolated using Trizol (Life Technologies) and treated with RNase-free DNase (Roche). These RNA samples were used as templates for reverse transcription (RT) in a 20-μl reaction mixture containing 2 μg of random hexamers (Roche), 1 μl of 10 μM deoxyribonucleoside triphosphates, 2 μl of 0.1 M dithiothreitol, 1 μl of 10 mM dNTPs (Promega), and 200 U of Superscript II (Life Technologies). RT was carried out at 42°C for 50 min. PCR was performed with a PTC-200 Peltier Thermal Cycler. The reaction mixture contained 1.5 mM MgCl₂, 200 μM deoxyribonucleoside triphosphates, 5 U of Taq DNA polymerase enzyme, and 25 pmol of each oligonucleotide primers specific for the ϕ1 gene or the 16S RNA gene (see above), and 2 μl of RT product in a total volume of 50 μl. The PCR cycling conditions were as follows: one cycle of 95°C for 3 min and 35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min. This was followed by strand elongation for 10 min at 72°C.

Molecular cloning, expression, and purification of recombinant ϕ1. The ϕ1 DNA fragments were generated by PCR using genomic DNA isolated from C. pneumoniae AR39 as the template. In order to subclone the ϕ1 product as NcoI and XhoI fragments into the pET30b(+) vector (Novagen), forward 5'-AAGGGAAAGCCATGTTAGG-3' and reverse 5'-ACGACTCTGAGAATGTTTA-3' and reverse 5'-ACGTTAATACGGAGGGTGCAGTAC-3' (for the C. pneumoniae CM1) and reverse 5'-AAGGGAAAGCCATGTTAGG-3' and reverse 5'-ACGACTCTGAGAATGTTTA-3' and reverse 5'-ACGTTAATACGGAGGGTGCAGTAC-3' in a total volume of 50 μl. The PCR cycling conditions were as follows: one cycle of 95°C for 3 min and 35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min. This was followed by strand elongation for 10 min at 72°C.

Statistical analyses. For all analyses, the presence of antibodies to C. pneumoniae strain AR39 EB and ϕCpn1 ϕ1 in sera was arbitrarily dichotomized.
such that those with measured levels of ≥0.5 OD units were classified as seropositive. This cutoff was chosen because visual inspection of the distribution of OD values to Vp1 antigen showed that an OD value of less than or greater than 0.5 separated the study subjects into two distinct populations. Odds ratios (OR), 95% confidence intervals (CI), and chi-square analyses were used to assess the relationship between these variables and AAA. Unconditional logistic regression was used to compute adjusted OR and 95% CI for the association between AAA and seropositivity to C. pneumoniae EB and Vp1. Other variables included in the logistic regression model were age, gender, pack years of smoking, the presence of hypertension, and the presence of hypercholesterolemia.

RESULTS

Differentiation of C. pneumoniae strains. We developed molecular methods to identify C. pneumoniae strains that contain the phage ΦCpn1. Genome sequencing revealed the strain AR39 as a phage-containing strain and CWL029 as a phage-free strain (16, 24). The DNA sequence of the phage was originally found during the genome sequencing of strain AR39 (24), and no functional studies have been performed so far on this phage. We performed PCR, RT-PCR, and Western blot analysis to determine the presence and expression of the ΦCpn1 vp1 gene in C. pneumoniae strain AR39 in comparison to strain CWL029, which is known to lack the phage (16). As expected, results from PCR (Fig. 1A), RT-PCR (Fig. 1B), and Western blot (Fig. 1C) analyses confirm the presence and expression of the phage vp1 gene in C. pneumoniae strain AR39 and its absence in strain CWL029.

Distribution of ΦCpn1 among C. pneumoniae strains. Among the three C. pneumoniae genomes sequenced so far, only one (AR39) contained the phage ΦCpn1 (16, 24, 29). It is of interest to know the distribution of ΦCpn1 among C. pneumoniae clinical isolates, and therefore we performed PCR analysis to determine the presence of the ΦCpn1 vp1 gene in six clinical isolates of C. pneumoniae in addition to the laboratory strains AR39 and CWL029. Our results demonstrate that none of the isolates other than AR39 contains phage ΦCpn1 (Table 1 and Fig. 2).

ELISA design and specificity. In order to determine whether the ΦCpn1 Vp1 protein can be used as an antigen to detect the presence of the phage antibodies in sera collected from controls and patients with AAA, we raised antibodies in mice using recombinant Vp1 protein generated in E. coli and showed that Vp1 antigen is highly immunogenic in mice and that antibodies thus generated readily recognize the Vp1 protein in C. pneumoniae EBs (Fig. 1C). Antiserum from a non-immunized mouse did not give any bands for C. pneumoniae EBs (data not shown).

We next used ELISA to measure human serum antibodies in 32 AAA patients and 40 controls. There were no significant differences in age, gender distribution, smoking history, or history of hypertension between the original study participants (98 patients and 102 controls) previously reported (4) and the subset analyzed for this study. Using a cutoff value of 0.5 OD units for both C. pneumoniae EB and ΦCpn1 Vp1 antibody levels, 61 (84.7%) of the 72 participating subjects were seropositive for C. pneumoniae EB and 42 (58.3%) were seropositive for ΦCpn1 Vp1. Of the 61 who were seropositive for C. pneumoniae EB, 39 (63.9%) were also positive for ΦCpn1 Vp1. There were no consistent variations by age, gender, or smoking history for either C. pneumoniae EB or ΦCpn1 Vp1 seropositivity.

Correlation between C. pneumoniae EB and ΦCpn1 Vp1 antibodies and AAA. Table 2 shows the relationship between AAA and C. pneumoniae EB and bacteriophage Vp1 seropositivity. Seropositivity (i.e., levels of ≥0.5 OD units) was more common among patients than controls for both C. pneumoniae EB (90.6 versus 80.0%) and ΦCpn1 Vp1 (68.8 versus 50.0%). There was a positive, but nonsignificant, association with AAA for both C. pneumoniae EB (crude OR, 2.4 [95% CI, 0.6 to 10.0]) and ΦCpn1 Vp1 (crude OR, 2.2 [95% CI, 0.8 to 5.8]) seropositivity. Multivariate analysis to control for age, gender, pack years of smoking, hypertension, and hypercholesterolemia showed a positive, but nonsignificant, association between AAA and C. pneumoniae EB seropositivity (adjusted OR, 2.3 [95% CI, 0.5 to 11.5]). Strikingly, multivariate adjustment resulted in a stronger and statistically significant association between AAA and ΦCpn1 Vp1 seropositivity (adjusted
This observation immediately raises the question of whether the chlamydia phage contribute to *C. pneumoniae* virulence and disease pathogenesis. As a step to answer this question, we developed methodologies to detect the presence of the phage *ΦCpn1* in clinical isolates of *C. pneumoniae* and of phage-related antibodies in sera from humans. We provide preliminary data supporting an association between AAA and infection with *C. pneumoniae* containing the phage *ΦCpn1*. We acknowledge that the results of this investigation cannot yet be used to draw firm conclusions with respect to a causal relationship between *C. pneumoniae* containing *ΦCpn1* and AAA due to a modest sample size and statistical imprecision. However, the data are sufficiently intriguing to warrant further microbiological and seroepidemiological study.

Our data indicate that a subset (64.5%) of persons who are seropositive to *C. pneumoniae* have serological evidence of infection with *ΦCpn1*. Our data also suggest that serological evidence of exposure to strains of *C. pneumoniae* containing phage correlate better with the presence of AAA than does seropositivity to *C. pneumoniae* in general. This is demonstrated by the finding of a persistent positive association between Vp1 seropositivity and AAA among all subjects who were seropositive to *C. pneumoniae* and a much stronger association when seropositivity to both was compared to seronegativity to both.

It is striking that only one isolate of *C. pneumoniae* contained the phage among the three *C. pneumoniae* strains whose genomes were completely sequenced and the six clinical strains analyzed by PCR in this study. Thus, phage infection of *C. pneumoniae* isolates appears uncommon. Nonetheless, the ELISA results indicate that among *C. pneumoniae* EB-seropositive individuals, nearly two-thirds have antibodies against the phage. This difference between the infrequency of detection of *C. pneumoniae* containing phage in clinical isolates and the frequency of chlamydia phage antibodies in humans may be explainable in a number of ways. (i) *C. pneumoniae* strains containing the phage (such as AR39) may be more capable of causing a persistent infection. We are in the process of performing experiments using animal models to see whether a *C. pneumoniae* strain containing the phage (AR39) persists longer in a mouse lung infection model than does a phage-free strain. (ii) Since the phage is likely to be lytic based on its sequence homology to other lytic phages (25), phage-bearing strains may be relatively difficult to isolate and propagate in cell culture. (iii) The number of *C. pneumoniae* isolates analyzed for the phage may still be too small to reach a definite conclusion.

**DISCUSSION**

*ΦCpn1* is the first bacteriophage to be identified as associated with a chlamydial strain that causes disease in humans.

### TABLE 2. Association between AAA and seropositivity to *C. pneumoniae* EBs and *ΦCpn1* Vp1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>AAA patients (n = 32)</th>
<th>Controls (n = 40)</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted ORa (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. pneumoniae</em> EBb</td>
<td>90.6</td>
<td>80.0</td>
<td>2.4 (0.6–10.0)</td>
<td>2.3 (0.5–11.5)</td>
</tr>
<tr>
<td><em>ΦCpn1</em> Vp1c</td>
<td>68.8</td>
<td>50.0</td>
<td>2.2 (0.83–5.8)</td>
<td>4.2 (1.2–14.4)</td>
</tr>
<tr>
<td>Both <em>C. pneumoniae</em> EB and <em>ΦCpn1</em> Vp1</td>
<td>62.5</td>
<td>47.5</td>
<td>7.4 (0.83–65.7)d</td>
<td>13.9 (1.1–175)e</td>
</tr>
</tbody>
</table>

a OR adjusted for age, sex, pack years of smoking, hypertension, and hypercholesterolemia using multiple logistic regression.
b Antibodies to *C. pneumoniae* AR39 EB.
c Antibodies to Vp1 of *ΦCpn1*.
d Compared to a reference category of seronegativity to both *C. pneumoniae* EB and bacteriophage Vp1.
Analysis of a larger collection of C. pneumoniae isolates for the phage is clearly needed, and analysis of strains directly isolated from atherosclerotic lesions for the presence of the phage will be particularly informative.

In summary, the molecular and serological methods developed in this study can be used to detect and analyze the ΦCpn1 phage in order to further understand its involvement in the pathogenesis of C. pneumoniae disease. The findings from ELISA analysis may have important implications regarding the association between C. pneumoniae and vascular disease. More research to investigate the association between specific strains of C. pneumoniae, including those carrying ΦCpn1, and various vascular disease outcomes is warranted.

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REFERENCES