Evaluation of a Direct Fluorescent Antibody Assay for Detection of *Chlamydia pneumoniae*

Naoyuki MIYASHITA¹, Akira MATSUMOTO², Rinzo SOEJIMA³, Yoshifumi KUBOTA¹, Toshio KISHIMOTO¹, Masamitsu NAKAJIMA¹, Yoshihito NIKI¹ and Toshiharu MATSUSHIMA¹

Division of Respiratory Diseases, Department of Medicine¹ and Department of Microbiology², Kawasaki Medical School, and Division of Welfare, Kawasaki University of Medical Welfare³

(Received : November 10, 1996)

(Accepted: December 27, 1996)

Key words: *Chlamydia pneumoniae*, direct fluorescent antibody assay, monoclonal antibody

**Abstract**

We examined the utility of the direct immunofluorescent antibody test kit, IMAGEN® (DAKO Diagnostic Co., Ltd.). The stainability of inclusions and purified elementary bodies (EBs) of all the *Chlamydia pneumoniae* strains used in this study was highly specific. Immunoelectron microscopy and light microscopy of stained EBs revealed the target antigen of IMAGEN® to be located on the surface of the EB outer membrane. In a clinical study, we tested oropharyngeal swab specimens obtained from 41 patients (45 specimens). The results were compared with those obtained by isolation in cell culture, the indirect immunofluorescent antibody (IFA) assay and serum antibody titration. *C. pneumoniae* was isolated from six specimens (13.3%), and the organisms were detected in 11 specimens (24.4%) with IMAGEN® and nine specimens (20%) with IFA. A diagnosis of *C. pneumoniae* infection was made in six patients (13.3%) by the serological examination. Six specimens positive in isolation were positive in the IMAGEN® but there were three cases which seemed to be false-positive by the IMAGEN®. We conclude that the use of IMAGEN® is an easy, rapid and sensitive method for detecting *C. pneumoniae* when there is a large amount of chlamydial antigen in the clinical specimen and when identification of the chlamydial species forming inclusions in cell cultures is required.

**Introduction**

*Chlamydia pneumoniae* is well established as a major respiratory pathogen¹. The organism is a common cause of pneumonia, bronchitis, sinusitis and pharyngitis, as well as being responsible for up to 10% of the cases of community-acquired pneumonia²,³. In addition, recent investigations have suggested a possible association between *C. pneumoniae* infection and atherosclerotic cardiovascular disorders⁴,⁵.

There are several techniques for detecting chlamydial organisms in urogenital, ocular and pharyngeal specimens. These are isolation in cell cultures, direct or indirect fluorescent antibody staining (DFA or IFA) using monoclonal antibody (MAb), enzyme immunoassays, and nucleic acid detectin, for example, use of a DNA probe, and nucleic acid amplification techniques, such as the polymerase chain reaction (PCR) and ligase chain reaction. Although isolation of the organisms from...
clinical specimens has been considered the “gold standard” for diagnostic testing, the procedures are labor intensive and time consuming. C. pneumoniae specific staining with a MAb, RR-402 (Washington Research Foundation)\(^6\), has been commonly used for identification of clinical isolates. Recently, a DFA staining kit, IMAGEN\(^R\) (DAKO Diagnostic Co., Ltd.), became commercially available. In this study, we examined the utility of the IMAGEN\(^R\).

### Materials and Methods

#### Chlamydia strains

The chlamydial strains used were as follows: C. pneumoniae TW-183, AR-39, AR-388\(^7\), Kajaani-6\(^8\), YK-41\(^9\), KKpn-15\(^10\), IOL-207\(^7\), Kajaani-6\(^8\), YK-41\(^9\), KKpn-15\(^10\) and 14 clinical isolates; C. psittaci Prt/GCP-1\(^11\), Or/B577\(^12\), Frt-Hu/Cal 10\(^13\), Bud/budgerigar-1\(^14\), Bud/IZAWA-1\(^15\), Hu/30A\(^16\) and 3 clinical isolates; C. trachomatis D/ UW-3/Cx, E/UW-5/Cx\(^17\), L2/434/Bu\(^18\) and 20 clinical isolates; and C. pecorum Bo/E58\(^19\) strain. Strains TW-183, AR-39 and AR-388 were purchased from the Washington Research Foundation, Seattle, WA, U.S.A. Strains IOL-207 and Kajaani-6 were supplied by P. Saikku, University of Helsinki, Finland. Strain YK-41 was supplied by Y. Kanamoto, Hiroshima Prefectural Institute of Public Health, Japan. KKpn-15 and other clinical isolates including the C. pneumoniae, C. psittaci and C. trachomatis strains were isolated in our laboratory. All the C. pneumoniae strains were grown in HeLa 229 or HL cell cultures and harvested on day 3 postinoculation. Strains GCP-1, B577 and E58 were supplied by K. Hirai, Faculty of Agriculture, Gifu University. The C. psittaci budgerigar-1 and C. trachomatis D, E and L2 strains were supplied by S. Yamasaki, National Institute of Health, Japan. The C. psittaci Cal 10 strain has been maintained in our laboratory for more than 20 years. The C. psittaci IZAWA-1 strain was isolated from a budgerigar in our laboratory. All strains of C. trachomatis and C. psittaci were grown in L929 suspension or monolayer cultures and HeLa 229 cell cultures and harvested on day 2 or 3 postinoculation. The E58 strain of C. pecorum was cultured continuously in the MDBK cell line.

#### Monoclonal antibodies

The MAbs used for DFA were from commercially available kits; IMAGEN\(^R\), MicroTrak\(^R\) (Syva Co.) and Cultureset\(^R\) (Ortho Diagnostic Systems Inc.). For comparison with the stainability by the commercial DFA kits, three C. pneumoniae-specific MAbs, RR-402 (Washington Research Foundation)\(^6\), SCP-53\(^20\) and XC-60\(^10\) were also used in IFA staining.

#### Preparation of intact EBs, outer membranes and inner components

The elementary bodies (EBs) of the C. psittaci and C. pecorum strains were purified by the method of Tamura & Higashi\(^21\), but trypsin treatment was omitted to prevent any possible change in the antigenic properties of the EB surface. The EBs of the C. pneumoniae and C. trachomatis strains were purified by a method described previously\(^22\). Outer membranes were prepared from the purified EBs by treatment with 0.125% sodium dodecyl sulphate as described previously\(^9\). The inner structural component was prepared from the purified EBs by a method described previously\(^9\).

#### Immunoelectron microscopy

To determine the location of antigens reactive with dach C. pneumoniae-specific MAb, immunogold labeling was performed by the method of Birkelund et al.\(^23\). Briefly, the specimen mounted on a specimen grid was treated with the MAb, washed with phosphate-buffered-saline and then mixed with a suspension of protein A-gold particles (15-nm in diameter, Amersham Co.). After several washings, the specimen was examined with an electron microscope.

#### Patients and clinical specimens

All patients studied were admitted to the Kawasaki Medical School Hospital between February 1994 and January 1995. The acute respiratory tract infections diagnosed included 15 cases of...
pneumonia and four of bronchitis. Exacerbation of chronic obstructive pulmonary diseases included one case of diffuse panbronchiolitis, five of pulmonary emphysema and 16 of bronchial asthma. The 41 patients (28 males and 13 females) ranged in age from 32 to 69 years (mean, 54.2 for both sexes).

Oropharyngeal swab specimens were obtained from all patients (45 specimens including five collected from one patient) and used for three different procedures, isolation in cell culture, DFA and IFA. Sera were obtained from all patients in the acute and convalescent stages of their illness. The swab specimens were placed in sucrose-phosphate-glutamate (SPG) medium and stored at −70°C until used.

**Isolation in cell culture**

Each specimen in SPG medium was sonicated and briefly centrifuged (900 × g for 10 min) and then the supernatant was overlayed on HEp-2 cells grown on a round coverslip (14-mm in diameter) set in a 24-well cell culture plate. The plate was centrifuged at 900 × g for 60 min at room temperature and then incubated in minimum essential medium containing cycloheximide (1 μg/ml) and supplemented with 10% heat-inactivated fetal calf serum at 35°C in an atmosphere of 5% CO2. At 72 hr postinoculation, the cells on the coverslip were stained with the MAbs and then examined with a Nikon epifluorescence microscope at 100 or 400× magnification. If no inclusions were detected, culture was repeated at least twice.

**DFA and IFA of clinical specimens**

Each swab specimen in SPG medium was sonicated and briefly centrifuged (900 × g for 10 min) and then the pellet was directly smeared onto a slide (8-mm wells). After fixation with acetone, the samples were stained with the IMAGEN® and SCP-53, which is specific for the 53-KDa protein of *C. pneumoniae* and then examined under a fluorescence microscope at 400× magnification. When three or more particles stained apple-green were seen, the specimen was judged as positive. Samples containing less than 10 columnar epithelial cells or one or two particles and those with excess mucus were scored as inconclusive. In such cases, swab specimens were collected again and examined by the identical procedures.

When a positive result was obtained in the FA test, the sample was examined by the PCR using a *C. pneumoniae*-specific primer to confirm the result.

**Serological test**

The microimmunofluorescence (micro-IF) test was used for titration of chlamydial antibodies with formalinized EBs of the *C. pneumoniae* KKpn-15, *C. trachomatis* L2/434/Bu and *C. psitaci* Cal 10 strains used as antigens. When a serum showed a fourfold or more increase or decrease in antibody titers, ≥512 in IgG titer or ≥16 in IgM titer, the patient was diagnosed as seropositive.

**Results**

**Determination of IMAGEN® specificity**

Without exception, inclusions and EBs of all the *C. pneumoniae* strains used were positively stained with IMAGEN®. In contrast, no inclusions or EBs of the *C. psittaci*, *C. trachomatis* and *C. pecorum* strains were stained. The results indicate that the IMAGEN® is highly specific for *C. pneumoniae*.

**Antigen location revealed with MAb**

When immunogold-labeling with RR-402 MAb was used, the surface of the EBs of all the *C. pneumoniae* strains was heavily labeled with protein A-gold particles (Fig. 1A). Similarly, the EB surface was labeled with SCP-53 MAb as reported previously. In contrast, when XC-60 MAb, which has been shown to be specific for the 60–KDa heat-shock protein contained within the EB, was used, the gold-labeling was seen only on the inner component (Fig. 1B). These results indicate that
Fig. 1 Immunolabeling on the surface of intact EBs and the inner component of ruptured EBs of the *C. pneumoniae* TW-183 strain. The samples were treated with MAbs RR-402 and XC-60, and then labeled with protein A-gold particles. (A) When the RR-402 MAb was reacted with EBs, the intact EB surfaces were heavily labeled with the protein A-gold particles. (B) When the XC-60 MAb was reacted with EBs, the intact EB surface was not labeled and the inner component released from a ruptured EB was heavily labeled with protein A-gold particles. Bar indicates 0.5 μm.

### Table 1 Staining properties of monoclonal antibodies

<table>
<thead>
<tr>
<th>MAb</th>
<th>Whole EB</th>
<th>Outer membrane</th>
<th>Inner component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultureset®</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>*MicroTrak®</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>RR-402</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>SCP-53</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>XC-60</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>IMAGEN®</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

*KKpn-15 and TW-183 strains were used in the test except for MicroTrak® staining. *C. trachomatis* L2/434/Bu was used as antigen for MicroTrak® staining.

The staining properties of the MAbs used in the present study are summarized in Table 1. Since the target antigens of the MicroTrak® and Cultureset® are the major outer membrane protein and lipopolysaccharide, respectively, the results of FA staining obtained with RR-402, SCP-53 and XC-60 agreed well with those of the immunogold-labeling test (Fig. 2).

**Clinical evaluation of the IMAGEN®**

*C. pneumoniae* was isolated from six specimens (13.3%) (five specimens were obtained from one patient and *C. pneumoniae* was continuously isolated), and the organisms were detected in 11 specimens (24.4%) with IMAGEN®, and in nine specimens (20%) by IFA with SCP-53. Six patients (13.3%) were diagnosed as having *C. pneumoniae* infection by the serological examination. Six specimens positive in isolation were positive in the DFA before cultivation. Furthermore, these six
Fig. 2 Direct fluorescent antibody staining with IMAGEN®.
(A) Outer membrane fraction prepared from purified EBs of *C. pneumoniae*
strain KKpn-15.
(B) Inner component fraction of *C. pneumoniae* strain KKpn-15.
The outer membranes were clearly stained apple-green but no reaction occurred with the inner components.

Table 2 Prevalence of *C. pneumoniae* infections in 41 patients as determined by four different methods

<table>
<thead>
<tr>
<th>Test</th>
<th>No. positive (%)</th>
<th>No. confirmed-positive (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients (n=41)</td>
<td>Specimens (n=45)</td>
</tr>
<tr>
<td>Culture</td>
<td>2 (4.9)</td>
<td>6 (13.3)</td>
</tr>
<tr>
<td>DFA (IMAGEN&lt;sup&gt;®&lt;/sup&gt;)</td>
<td>7 (17.1)</td>
<td>11 (24.4)</td>
</tr>
<tr>
<td>IFA (SCP-53)</td>
<td>5 (12.2)</td>
<td>9 (20.0)</td>
</tr>
<tr>
<td>Serology&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4 (8.9)</td>
<td>6 (13.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Confirmed-positive means a case which was determined as positive by two or more different tests.

<sup>b</sup> Serological diagnosis of an acute infection was made when a fourfold rise or drop occurred in antibody titers, or there was an IgG titer of ≥512 or an IgM titer of ≥16.

Specimens were also positive by the PCR test. Three specimens were positive in all assays. Based on these findings, it is likely that positive results in two different examinations should enable us to diagnose *C. pneumoniae* infection. The incidence of *C. pneumoniae* infection determined by the methods used is summarized in Table 2. Table 3 shows the sensitivity, specificity and positive and negative predictive values determined by the different methods. The percentages in the table were calculated by the way as the positive cases determined by isolation and DFA and those in two different tests (confirmed-positive cases) were regarded as standard. Taking isolation-positive cases as the standard, both DFA and IFA were more sensitive than serodiagnosis. With confirmed-positive cases used as the standard, both DFA and IFA were more sensitive than isolation and serodiagnosis.

Table 4 shows the results of the four tests. Three of the 45 specimens were positive in all tests; four were positive in three tests; two were positive in two tests and four were positive in one test. Thirty-two specimens were negative in all tests. Two cases which were positive only in the serodiagnosis seemed to be *C. pneumoniae* acute infections because of a change in the antibody titer<sup>26</sup>. However, two DFA-positive cases were negative by both the PCR and nested PCR tests. Therefore, these cases seemed to be false-positive. When the PCR was done for the two cases positive in both DFA and IFA tests, one case was positive but the other was negative by the PCR and nested PCR.
Table 3: Sensitivity, specificity and positive and negative predictive values of the various methods compared to three different gold standards

<table>
<thead>
<tr>
<th>Gold standard</th>
<th>Method</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Predictive values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>Culture</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0% 100.0%</td>
</tr>
<tr>
<td></td>
<td>DFA</td>
<td>100.0%</td>
<td>87.2%</td>
<td>54.5% 100.0%</td>
</tr>
<tr>
<td></td>
<td>IFA</td>
<td>100.0%</td>
<td>92.3%</td>
<td>66.7% 100.0%</td>
</tr>
<tr>
<td></td>
<td>Serology</td>
<td>50.0%</td>
<td>92.3%</td>
<td>50.0% 92.3%</td>
</tr>
<tr>
<td>DFA</td>
<td>Culture</td>
<td>54.5%</td>
<td>100.0%</td>
<td>100.0% 87.2%</td>
</tr>
<tr>
<td></td>
<td>DFA</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0% 100.0%</td>
</tr>
<tr>
<td></td>
<td>IFA</td>
<td>81.8%</td>
<td>100.0%</td>
<td>100.0% 94.4%</td>
</tr>
<tr>
<td></td>
<td>Serology</td>
<td>36.4%</td>
<td>94.1%</td>
<td>66.7% 82.1%</td>
</tr>
<tr>
<td>Confirmed-positive cases</td>
<td>Culture</td>
<td>66.7%</td>
<td>100.0%</td>
<td>100.0% 92.3%</td>
</tr>
<tr>
<td></td>
<td>DFA</td>
<td>100.0%</td>
<td>94.4%</td>
<td>81.8% 100.0%</td>
</tr>
<tr>
<td></td>
<td>IFA</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0% 100.0%</td>
</tr>
<tr>
<td></td>
<td>Serology</td>
<td>44.4%</td>
<td>94.4%</td>
<td>66.7% 87.2%</td>
</tr>
</tbody>
</table>

Table 4: Results of culture, DFA, IFA, serology and PCR test for detection of C. pneumoniae

<table>
<thead>
<tr>
<th>No. of specimens</th>
<th>Culture</th>
<th>DFA</th>
<th>IFA</th>
<th>Serology</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D.: not done

tests. Therefore, this PCR-negative case appeared to be false-positive.

Discussion

The results obtained in the present study showed that the IMAGEN® was highly specific for C. pneumoniae and that it stained an antigen located on the surface of the organisms. However, the stainability by the IMAGEN® was lower than that by the Cultureset®. EBs stained with the IMAGEN® were apple-green, but the color rapidly faded into the reddish background. Consequently, the EBs appeared to be smaller than those stained with the Cultureset®. This weak point caused difficulty in detecting scattered EBs in clinical specimens smeared on the glass slide. We previously reported the sensitivity of MicroTrak® staining, by which only two EBs of C. trachomatis could be detected in the smear of a urethral swab specimen25). Such high sensitivity was not expected for the IMAGEN®. However, no marked difference in the staining effect between the IMAGEN® and other staining tests was seen when massive EBs and inclusions were stained. Therefore, the IMAGEN® seems to be quite applicable to the identification of isolated chlamydial species forming inclusions. Nevertheless, we could detect C. pneumoniae in 11 of 45 specimens (24.4%). This might be due to the degree of skill in examination, indicating that the efficiency of detection with the IMAGEN® depends on the experience of the observer. Since only a few specimens were examined in the present study,
the comparison between isolation and DFA seems to be inadequate for evaluation. Further comparative studies are therefore required.

Acknowledgement

This work was supported in part by a Grant-in-Aid (No. 06670634) for Scientific Research from the Ministry of Education, science and Culture, Japan and by a Project Research Grant (No. 6-506) from Kawasaki Medical School.

References

22) Miyashita N & Matsumoto A.: Establishment of a particle-counting method for purified elementary bodies of
Evaluation of C. pneumoniae Direct Fluorescent Antibody Assay


Chlamydia pneumoniae 検出用直接蛍光抗体法の検討

川崎医科大学呼吸器内科1), 同 微生物学教室2),
川崎医療福祉大学医療福祉学科3)

宮下 修行1) 松本 明2) 副島 林造3) 嵩田 好史1)
岸本 寿男1) 中島 正光1) 二木 芳人1) 松島 敏春1)

要 旨

FITC 標識 Chlamydia pneumoniae 特異モノクローナル抗体（IMAGEN®: DAKO Diagnostic Co. Ltd.）を使用した直接蛍光抗体法（DFA）の基礎的・臨床的有用性の検討を行った。本モノクローナル抗体（RR-402）は、今回の検討で、標的抗原が基本小体の外膜に存在すること、さらにその高い特異性が確認された。一方、臨床的検討として、呼吸器感染症患者41人、45検体を対象に培養法、間接蛍光抗体法（IFA）、抗体価測定法と比較検討を行った。C. pneumoniae は、6 株（13.3％）が分離され、またその陽性頻度は、IMAGEN® で11検体（24.4％）、IFA で9 検体（20％）であった。また、血清抗体価の推移より C. pneumoniae と診断されたものは6 検体（13.3％）であった。分離陽性 6 検体はいずれも IMAGEN® 陽性であったが、IMAGEN® 陰陽性と考えられるものも3例存在していた。以上、検体数が少なかったため、敏感度にあまり差はみられなかったものの、IMAGEN® の染色性が従来のDFA キットである Cultureset®より劣ることから、抗原量が多い場合や、種の同定が必要な場合には、本キットは簡便かつ迅速であることから有用であろうと考えられた。

平成 8年 3月 20日