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APPLICATION NOTE

Biomarker Candidates of *Chlamydophila pneumoniae* Proteins and Protein Fragments Identified by Affinity-Proteomics Using FTICR-MS and LC-MS/MS

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Abstract

We report here an affinity-proteomics approach that combines 2D-gel electrophoresis and immunoblotting with high performance mass spectrometry to the identification of both full length protein antigens and antigenic fragments of *Chlamydophila pneumoniae (C. pneumoniae)*. The present affinity-mass spectrometry approach effectively utilized high resolution FTICR mass spectrometry and LC-tandem-MS for protein identification, and enabled the identification of several new highly antigenic *C. pneumoniae* proteins that were not hitherto reported or previously detected only in other *Chlamydia* species, such as *Chlamydia trachomatis*. Moreover, high resolution affinity-MS provided the identification of several neo-antigenic protein fragments containing N- and C-terminal, and central domains such as fragments of the membrane protein Pmp21 and the secreted chlamydial proteasome-like factor (Cpaf), representing specific biomarker candidates.

Keywords: Affinity-proteomics, *Chlamydia pneumoniae*, Protein biomarker candidates, High resolution mass spectrometry, LC- tandem mass spectrometry, Neoantigenic protein fragments

Introduction

C. pneumoniae is an important human respiratory pathogen that causes approximately 5% of all cases of bronchitis and is believed to be responsible for about 10% of community-acquired pneumonia cases [1]. *C. pneumoniae* has a two-phase development cycle alternating between a metabolically inactive, but infectious form, elementary bodies (EB), and a noninfectious active form, reticulate bodies (RB) [1]. The bacterium uses human host cells for replication to which EB adhere and are phagocytosed 72 to 96 h post-infection in which time EB develop into RB [2]. *C. pneumoniae* persisting in the host following primary infection has been associated with atherosclerosis leading to cardiovascular diseases [3]. Albeit investigations for more than 15 y, the role of *C. pneumoniae* infection still remains controversial, with the lack of reproducible serodiagnosis being a major drawback [4].

In previous studies, only limited molecular knowledge has been obtained about the immunodominant antigens in

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Table 1. Antigenic proteins identified by MALDI-FTICR- MS (A) and LC-tandem mass spectrometry (B). Numbers of gel spots are as in Supplementary Figure 1

Protein name/acc. no.[a]	Spot no.	Mass (kDa)	pI	MS method
Omp2/P23700	3[b]	61.63	6.0	A
DnaK/P27542	7[c]	71.42	5.0	A
CpB1072/Q9Z6M7	16	21.87	5.6	A, B
Pmp6/Q9Z899	35	145.97[d]	5.3[d]	A
GAPDH/Q9Z7T0	47	37.22	6.3	A, B
ClpP_1/Q9Z832	80	21.06	5.6	A, B
CpB0756/Q9Z7H7	89	68.18	4.9	A, B

[a] Accession numbers are from SWISS-PROT or TrEMBL database.
[b] Omp2 was identified in protein spots 3 and 1.
[c] DnaK was identified in protein spots 7 and 6.
[d] Spot 35 represents a fragment of Pmp6 protein, which is separated at smaller molecular mass (~60 kDa) and slightly basic pH (~7).

C. pneumoniae. The microimmunofluorescence (MIF) test, based on whole EB, has been employed as a standard for serology but presents several disadvantages such as cross-reactivity between different *Chlamydia* species and high interlaboratory variations [5]. Moreover, the MIF test does not correlate with the presence of *C. pneumoniae* in the host, nor differentiate between past and persistent infections [6].

A recent study on *Chlamydia trachomatis* by Sanchez-Campillo *et al.* employing an immunoproteomics approach, *N*-terminal sequencing and homology search provided the identification of a few immunoreactive proteins [7]. In a previous study, an affinity-proteomics approach combined with FTICR-MS with high specificity and reproducibility has been developed and applied for the identification of *C. pneumoniae* antigenic structures relevant for serodiagnosis [8]. The combination of 2-DE immunoproteomics with FTICR-MS and LC-MS/MS is shown here to be highly successful in identifying several hitherto unknown antigens and, particularly, the identification of several neo-antigenic protein fragments. These results provide new and additional evidence for the specific description of biomarker candidates and design of possible therapeutic lead structures.

Table 2. Antigenic protein fragments identified by MALDI-FTICR- MS (A) and LC-tandem mass spectrometry (B). Spot numbers are according to Supplementary Figure 1

Protein name/acc. no.[a]	Spot no.	Mass (kDa)	pI	MS method
Cpaf-c/Q9Z6P3	26	69.34[b]	5.5[b]	A, B
Cpaf-n/Q9Z6P3	44	69.34[b]	5.5	A, B
Pmp21-m/Q9Z6U5	9	169.41[c]	4.8	A, B
Pmp21-c/Q9Z6U5	10	169.41[c]	4.8	A, B
Pmp21-n/Q9Z6U5	36	169.41[c]	4.8	A

[a] Accession numbers are from SWISS-PROT or TrEMBL database.
[b] Cpaf-n fragment has ca. 24 kDa and Cpaf-c approximately 40 kDa and a pI of 5.
[c] The molecular mass and the pI are for the full length Pmp 21 protein. The N-terminal part has ca. 70 kDa (spot 36), the middle part ~55 kDa (spot 9) and the C-terminal domain ~45 kDa (spot 10).

Experimental

2D- Gel Electrophoresis and Immunoblotting

Sera from 39 human donors were tested for antibodies against *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* using the MIF assay (Savyon Diagnostics, St. Ashdod, Israel) [8]. One serum tested positive for *C. trachomatis* and was excluded from the study. *C. pneumoniae* TW-183 were cultured as previously described [8].

Chlamydia samples were purified using the 2D Clean-up Kit (Amersham Biosciences, Uppsala, Sweden) and applied overnight on 17 cm IPG strips (pH range 3–10 NL) using a passive in-gel rehydration method, as described [8]. Following separation, proteins were visualized with sensitive colloidal Coomassie staining [9], and gels were scanned with a Bio-Rad (Laboratories GmbH, München, Germany) GS-710 imaging densitometer. All separations were performed in pairs of two gels, each run involving two electrophoretic cells, with three gels used for immunoblotting and one gel for mass spectrometric identification of antigenic proteins.

Following electrophoretic separation 2D gels were electroblotted for 2 h at 60 V onto nitrocellulose membranes using a WEB-M tank blotter (PEQLAB, Erlangen, Germany). Immunoblotting was performed as previously described [8], and electronic image files matched with the 2DE maps of the Coomassie-stained gels. For the analysis of the immunoblots, the maximum intensity of each spot was determined using the AIDA software package (Raytest/Fuji, Straubenhardt, Germany) [8].

Mass Spectrometry

Spots were excised from the gels, subjected to tryptic in-gel digestion according to [10], and elution fractions desalted with C18 OMIX Pipette Tips (Varian, Walnut Creek, CA, USA.). MALDI-FTICR-MS peptide mapping was performed with a Bruker APEX II FTICR spectrometer equipped with an actively shielded 7T superconducting magnet (Bruker Daltonik, Bremen, Germany) as previously described [8]. Monoisotopic masses of singly charged ions generated by XMASS were directly used for database search [8] (http://www.matrixscience.com; and http://prowl.rockefeller.edu/).

LC-MS/MS analysis was performed on an Esquire 3000+ ion trap instrument (Bruker Daltonik) equipped with an atmospheric pressure ESI source, using nitrogen as both the nebulizing and drying gas. The instrument was operated in the data-dependent mode with MSMS acquisition upon a predetermined threshold; six microscans were collected at automatic gain control for each full MS scan and 20 microscans for each MS/MS scan, with a maximum accumulation time of 200 ms. An Agilent-1100 HPLC was operated at 50 µL/min, using binary elution of (A) (0.2% aqueous formic acid) to 55% (B) (0.2% formic acid in acetonitrile) over 90 min, with a 10 cm × 1 mm Discovery Bio Wide Pore C18 column (Sigma-Aldrich Chemie GmbH, München, Germany). MS data were externally calibrated and analysed using the Bruker Data Analysis

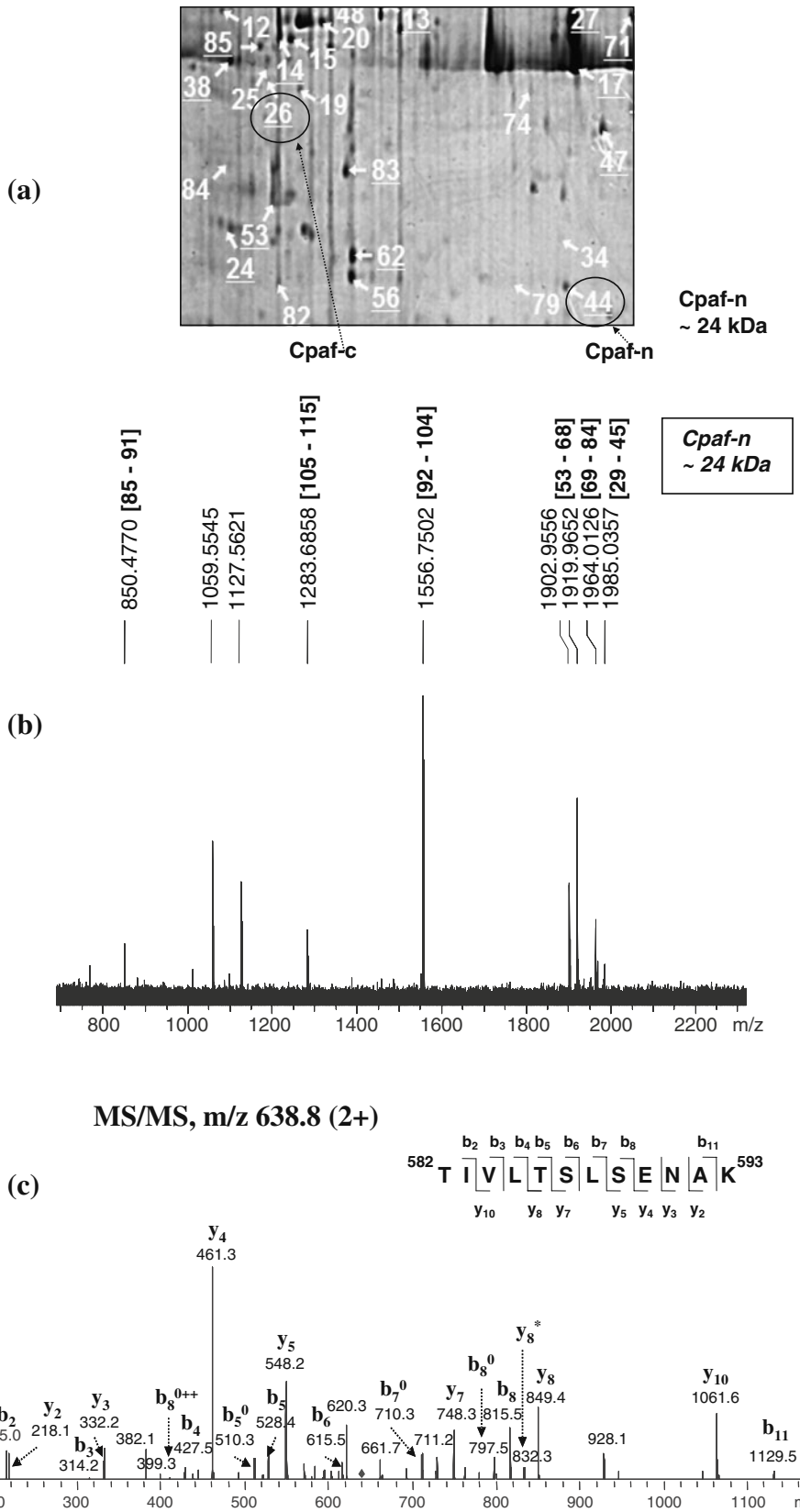

Figure 1. Identification of Cpaf (Chlamydial protease/proteasome like-activity factor) protein fragments. (**a**) 2D-gel section containing labeled spots 26 (Cpaf-c) and 44 (Cpaf-n). (**b**) MALDI-FTICR-MS of Cpaf-n tryptic peptides from spot 44; identified peptides are labeled, partial sequences are marked with brackets. (**c**) LC-MS/MS identification of the Cpaf-c fragment (spot 26); MS/MS of *m/z* 638.8 (2+), with y and b ions assigned (*: loss of NH_3; ^{0}: loss of H_2O)

software, and data saved in a *.mgf file and searched (among *Other Bacteria* sequence entries) against the NCBInr database.

Results and Discussion

Identification of C. pneumoniae Antigens by Immunoproteomics-Mass Spectrometry

C. pneumoniae antigens were detected in four gels, processed in each 2DE run under identical conditions and subjected to immunoblotting and mass spectrometry. Approximately 600 protein spots were separated within a m.w. range of ca. 15 to 130 kDa (Supplementary Figure 1). Among the proteins visualised, 89 spots were detected as immunoreactive, and reactivities estimated based on corresponding intensities among all sera, as previously described [8].

For identification of proteins, gel spots were excised, digested with trypsin, and the proteolytic peptides analyzed by MALDI-FTICR-MS and LC- tandem mass spectrometry. Initially, proteins from 42 spots showing consistent antigenic reactivity were examined. A total of 22 *C. pneumoniae* proteins (from 28 protein spots) and six human proteins were identified (Supplementary Table 1). Human proteins, also found in previously reported proteome maps [2], are likely resulting from contamination by the Hep-2 cells used in the *C. pneumoniae* culture. Eight *C. pneumoniae* proteins (from nine protein spots) were identified by both MALDI-FTICR-MS and LC-MS/MS analysis, and are underlined in Supplementary Figure 1.

New proteins identified (not found in [8]) of substantial antigenic reactivity are summarized in Table 1 (shown underlined and in red in the 2D-gel, Supplementary Figure 1). The application of LC-tandem- MS in the present study was found to effectively extend the previous MALDI-FTICR-MS data (Table 1). Protein spots 3, 7, 16, 35, 47, 80, 89 were identified as *C. pneumoniae* proteins, while spots 55, 62, 70, and 71 were human proteins. All identified *C. pneumoniae* proteins are summarized in Supplementary Table 1. For two of the eight proteins identified by LC-MS/MS (Pmp21-c and Cpaf-c), single peptide masses provided individual ion scores exceeding the identity or homology threshold for unambiguous identification.

Identification of Neo-antigenic Protein Fragments by High Resolution Affinity-Mass Spectrometry

In addition to several antigenic full length proteins, new, specific neo-antigenic protein fragments were identified in this study by affinity-proteomics using LC-MS/MS (Table 2). Two polymorphic membrane proteins, Pmp6 and Pmp21, that contain an autotransporter domain and thus can pass the outer bacterial membrane, were identified both as full length proteins and as specific truncation forms. Protein spot 45 of ca. 130 kDa was identified as full length Pmp6 ([8]), and in the present study as a 60 kDa C-terminal fragment (spot 35) with high antigenic reactivity. For Pmp21, the largest of the 21 polymorphic *C. pneumoniae* membrane proteins, three fragments were identified with molecular masses of ca. 70, 55, and 45 kDa, respectively, representing an N-terminal fragment (Pmp21-n; spot 36), a middle domain fragment (Pmp21-m; spot 9), and a C-terminal fragment (Pmp21-c; spot 10). With purified RB, no C-terminal Pmp21 fragment was found, while in purified EB only a 47 kDa C-terminal fragment has been previously identified [2, 11]. In contrast, the present combination approach of immunoblotting and high resolution mass spectrometry provided the unequivocal identification of all three Pmp21 fragments (Table 2).

The chlamydial protease/proteasome-like activity factor, Cpaf was identified as a further example where only neo-antigenic fragments were isolated by 2-DE and identified (Table 2). Only a few secreted proteins have been hitherto identified for *C. pneumoniae*. In the present study, we identified both a C- and N-terminal antigenic fragment: a basic 24 kDa fragment (Cpaf-n; pI 7) and an acidic ca. 40 kDa fragment (pI 5; Cpaf-c). The latter fragment had not been previously detected [12], possibly due to its co-migration with the RpoA protein (Spot 14, Supplementary Figure 1). Figure 1 shows the 2D- gel spots of the Cpaf fragments, spot 26 (Cpaf-c) and spot 44 (Cpaf-n), together with the MALDI-FTICR-MS analysis and the LC-MS/MS data for the C-terminal part of Cpaf.

Conclusion

In this study, an affinity-proteomics approach in combination with high performance mass spectrometry, MALDI-FTICR-MS, and LC-MS/MS demonstrated as an efficient tool to identify antigenic structures of *C. pneumoniae* relevant for serodiagnosis. In addition to previously described *C. pneumoniae* proteins, seven new reactive proteins were identified here (Table 1), which may represent specific biomarkers for *C. pneumoniae* infections. High resolution affinity- mass spectrometry proved to be a particularly efficient tool for the identification of neo-antigenic protein fragments of two proteins, the full length precursor proteins of which lack antigenicity, the membrane protein Pmp21, and the chlamydial proteasome like-activity factor Cpaf (Table 2). Thus, the highly antigenic proteins identified here should be useful to (1), enable the development of more standardized serodiagnosis techniques capable to discriminate between past and persistent infections; (2), clarify the role of *C. pneumoniae* in chronic inflammatory diseases; and (3), establish biomarker candidates for future drug development.

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References

1. Kuo, C.C., Jackson, L.A., Campbell, L.A., Grayston, J.T.: *Chlamydia pneumoniae* (TWAR). *Clin. Microbiol. Rev.* **8**, 451–461 (1995)
2. Vandahl, B.B., Birkelund, S., Christiansen, G.: Proteome analysis of Chlamydia pneumoniae. *Meth. Enzymol.* **358**, 277–288 (2002)
3. Ciervo, A., Visca, P., Petrucca, A., Biasucci, L.M., Maseri, A., Cassone, A.: Antibodies to 60-kilodalton heat shock protein and outer membrane protein 2 of *Chlamydia pneumoniae* in patients with coronary heart disease. *Clin. Diagn. Lab. Immunol.* **9**, 66–74 (2002)
4. Kumar, S., Hammerschlag, M.R.: Acute respiratory infection due to Chlamydia pneumoniae: current status of diagnostic methods. *Clin. Infect. Dis.* **44**, 568–576 (2007)
5. Boman, J., Hammerschlag, M.R.: *Chlamydia pneumoniae* and atherosclerosis: critical assessment of diagnostic methods and relevance to treatment studies. *Clin. Microbiol. Rev.* **15**, 1–20 (2002)
6. Maass, M., Gieffers, J., Krause, E., Engel, P.M., Bartels, C., Solbach, W.: Poor correlation between microimmunofluorescence serology and polymerase chain reaction for detection of vascular *Chlamydia pneumoniae* infection in coronary artery disease patients. *Med. Microbiol. Immunol.* **187**, 103–106 (1998)
7. Sanchez-Campillo, M., Bini, L., Comanducci, M., Raggiaschi, R., Marzocchi, B., Pallini, V., Ratti, G.: Identification of immunoreactive proteins of *Chlamydia trachomatis* by western blot analysis of a two-dimensional electrophoresis map with patient Sera. *Electrophoresis* **20**, 2269–2279 (1999)
8. Bunk, S., Susnea, I., Rupp, J., Summersgill, J.T., Maass, M., Stegmann, W., Schrattenholz, A., Wendel, A., Przybylski, M., Hermann, C.: Immunoproteomic identification and serological responses to novel chlamydia pneumoniae antigens that are associated with persistent *C. pneumoniae* infections. *J. Immunol.* **180**, 5490–5498 (2008)
9. Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W.: Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using coomassie brilliant blue G-250 and R-250. *Electrophoresis* **9**, 255–262 (1988)
10. Mortz, E., Vorm, O., Mann, M., Roepstorff, P.: Identification of proteins in polyacrylamide gels by mass spectrometric peptide mapping combined with database search. *Biol. Mass Spectrom.* **23**, 249–261 (1994)
11. Wehrl, W., Brinkmann, V., Jungblut, P.R., Meyer, T.F., Szczepek, A.J.: From the inside out–processing of the chlamydial autotransporter PmpD and its role in bacterial adhesion and activation of human host cells. *Mol. Microbiol.* **51**, 31–334 (2004)
12. Shaw, A.C., Vandahl, B.B., Larsen, M.R., Roepstorff, P., Gevaert, K., Vandekerckhove, J., Christiansen, G., Birkelund, S.: Characterization of a secreted chlamydia protease. *Cell. Microbiol.* **4**, 411–424 (2002)