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Review

Serological diagnosis of *Chlamydia pneumoniae* infection: limitations and perspectives

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Chlamydia pneumoniae is an obligate intracellular human pathogen responsible for a wide range of acute and chronic human diseases, including pneumonia and other respiratory diseases. Serological methods for the diagnosis of *C. pneumoniae* infection vary widely, and several authors have reported significant inter- and intra-laboratory variability in diagnostic methods and criteria. Over the past 10 years, numerous studies have focused on the identification of specific antigens for application in serodiagnosis, including the diagnosis of persistent infections. The use of proteomics may enable the development of serological diagnosis kits that offer reliable sensitivity and specificity and might even differentiate between the various stages of infection with this pathogen.

Introduction

Chlamydia pneumoniae is an intracellular human pathogen that causes acute respiratory diseases (Grayston *et al.*, 1990). It accounts for 7–10 % of community-acquired pneumonia cases in adults and has also been associated with atherosclerosis and cardiovascular disease (Danesh *et al.*, 2000, 2002; Grayston *et al.*, 1995; Gutiérrez *et al.*, 2001; Saikku *et al.*, 1988).

Almost all humans can expect to be infected with *C. pneumoniae* at least once during their lifetime. Reinfections are frequent, and infections may become chronic (Grayston, 2000). After the primary infection, it can persist in the host (Hammerschlag, 2002; Hogan *et al.*, 2004), representing a potential risk factor for chronic inflammatory lung disease (Blasi *et al.*, 1993; Hahn & McDonald, 1998; Laurila *et al.*, 1997) or atherosclerosis (Campbell & Kuo, 2004).

The microimmunofluorescence (MIF) test has been of paramount importance in detecting acute *C. pneumoniae* infections and describing the prevalence of these infections (Persson & Boman, 2000), and it is currently considered the gold standard for the serodiagnosis of *C. pneumoniae* infection (Dowell *et al.*, 2001; Peeling *et al.*, 2000). However, test interpretation problems have resulted in interlaboratory variability in the reporting of MIF titres, and there is a need to develop alternative and standardized serodiagnostic testing methods for routine laboratory diagnoses (Peeling *et al.*, 2000).

Some of the drawbacks of the MIF test may be overcome by using serological tests in an ELISA format (Persson & Boman, 2000). However, although commercially available, they have yet to be approved by the Centers for Disease Control or by the US Food and Drug Administration (Villegas *et al.*, 2008), due to cross-reactivity issues and variations in sensitivity and specificity according to the antigen used.

Various researchers have used a proteomics approach to characterize the *C. pneumoniae* cell surface and identify immunodominant proteins in an attempt to develop a diagnostic kit based on recombinant antigens, as achieved for other species of the *Chlamydiaceae* family (Bas *et al.*, 2001; Longbottom *et al.*, 2001, 2002). This kit would permit reliable diagnosis of *C. pneumoniae* infection and may even allow differentiation among different stages of the disease.

Limitations of serological diagnosis

As noted above, the MIF test is not a wholly satisfactory serological method for diagnosis of *C. pneumoniae* infection. Its results are influenced by the antigen preparation used and by the experience of the professional responsible for reading and interpreting them (Persson & Boman, 2000). Shortcomings of the test include the difficulty of obtaining appropriately paired serum samples, the timing of the serum samples, the high background of IgG antibody prevalence in some adult populations, the lack of standardized testing methods and the shortage of high-quality reagents (Dowell *et al.*, 2001). The MIF test was found to lack specificity during acute infection due to cross-reactive antibodies (Bourke *et al.*, 1989; Gnarpe *et al.*, 2000; Ozanne & Lefebvre, 1992). Furthermore, its ability to discriminate acute infections has been called into question after reports of negative results in cases assessed as positive by other tests (Black *et al.*, 1994; Chirgwin *et al.*, 1991; Emre *et al.*, 1994) and of positive results in unconfirmed or unlikely cases

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(Gaydos *et al.*, 1994a, b; Hyman *et al.*, 1995; Kern *et al.*, 1993). Some studies have suggested that the MIF test is less sensitive and specific than generally thought, and others have claimed that *C. pneumoniae* IgM or IgG reactivity might be caused by heterotypic antibodies (Hyman *et al.*, 1991; Kutlin *et al.*, 1998). The test is evidently unable to discriminate between past and persistent infections.

Various studies have compared the MIF test with an ELISA (Hermann *et al.*, 2002, 2004; Persson & Boman, 2000). Persson & Boman (2000) compared three enzyme immunoassays (EIAs), with the MIF test as gold standard, finding that the sensitivity of the EIAs ranged from 87 to 95 % and the specificity from 91 to 93 %. Hermann *et al.* (2002) studied 11 serodiagnostic kits (ELISAs and EIAs) to detect *C. pneumoniae* in sera from healthy donors and reported a wide variation in their sensitivity, which ranged from 78 to 98 % with reference to MIF test results. The sensitivity of the species-specific tests ranged from 58 to 100 %, with some ELISAs and EIAs failing to detect low IgG titres. However, a later investigation by the same group reported high specificity and sensitivity for ELISA tests, whose results correlated well with MIF test results in sera from children with respiratory tract diseases and from control children (Hermann *et al.*, 2004).

Various studies have described the limitations of serological assays in persistent infections, and improvements are essential to elucidate any possible causal relationship between *C. pneumoniae* and atherosclerosis (Grayston, 2000). Tests that distinguish between past and present *C. pneumoniae* infection may help to resolve the controversy over the role of *C. pneumoniae* in chronic inflammatory diseases (Bunk *et al.*, 2008).

Role of direct diagnosis

Doubts have been raised about the usefulness of serology for the diagnosis of acute infections by *C. pneumoniae.* Some authors found no consistent antibody response in culture-positive or PCR-positive cases of *C. pneumoniae* infection (Black *et al.*, 1994; Emre *et al.*, 1994), whereas others reported reliable antibody responses in cases proven to be positive by these means (Boman *et al.*, 1997; Wang & Grayston, 1998). This discrepancy in findings has yet to be convincingly explained.

It is advisable to confirm serological assays with tests that demonstrate the presence of the organism, such as cell cultures or PCRs, notwithstanding their limitations (Persson & Boman, 2000). Cell culture is a complex process that offers limited sensitivity, and the growth of *C. pneumoniae* is difficult, especially from tissue samples, further hampering the diagnosis of persistent infection. According to some authors, the use of PCR as reference test allows a more accurate calculation of the analytical sensitivity to detect active (acute or chronic) *C. pneumoniae* (Sueur *et al.*, 2006). However, although several in-house PCR assays have shown a high sensitivity and specificity, this type of test has not been adequately validated or standardized, and detection of the pathogen appears to be influenced by the type of sample and treatment applied (Peeling *et al.*, 2000).

A direct detection test cannot be compared with a serological assay for the diagnosis of acute respiratory infections, since the latter requires some time to generate a specific humoral immune response, which may preclude direct detection of the pathogen. Nevertheless, some authors have reported that serological markers for latent or persistent infection can be validated in patients in whom *C. pneumoniae* DNA has been detected in arterial atherosclerotic plaques or blood mononuclear cells (Persson & Boman, 2000).

Future perspectives in serological diagnosis

Most MIF tests and ELISAs are based on the detection of antibodies against whole chlamydial elementary bodies (EBs), explaining their inherent shortcomings with regard to cross-reactivities between *Chlamydia* species and other, even unrelated, micro-organisms (Hermann *et al.*, 2002; Kern *et al.*, 1993; Ozanne & Lefebvre, 1992; Strålin *et al.*, 2001).

Initial research on the identification of *C. pneumoniae* antigens used various immunological assays (Western blot, dot blot, MIF, immunoprecipitation and immunoelectron microscopy). Several outer-membrane complex proteins of *C. pneumoniae* were identified as immunogenic, including MOMP (Iijima *et al.*, 1994; Jantos *et al.*, 1997; Wolf *et al.*, 2001), PorB (Kubo & Stephens, 2000), CrpA (Klein *et al.*, 2003; Melgosa *et al.*, 1993), OmcB (Mygind *et al.*, 1998; Stephens *et al.*, 2001), a 76 kDa protein (Perez Melgosa *et al.*, 1994) and some members of the polymorphic membrane protein family (Christiansen *et al.*, 2000; Knudsen *et al.*, 1999) (Table 1).

However, these antigens have yielded variable results with respect to the frequency and pattern of recognition of *Chlamydia* species, showing either high specificity but low immunogenicity (MOMP) or high immunogenicity but low specificity (OmcB, also known as Omp2; CrpA). Hence, further research is required to seek new epitopes that are specific and highly immunodominant and might be useful for the development of new serodiagnostic assays.

The sequencing of *C. pneumoniae* genomes (Kalman *et al.*, 1999; Read *et al.*, 2000) has provided an opportunity for comparisons with closely related organisms. However, genome sequences do not identify the proteins that are expressed or the time when they are present, and the developmental cycle of *Chlamydia* is insufficiently understood (Vandahl *et al.*, 2001). The intrinsic difficulty of working with *C. pneumoniae* and the lack of adequate methods for its genetic manipulation limit the molecular definition of the chlamydial cell surface (Montigiani *et al.*, 2002).

After sequencing the genome, Vandahl *et al.* (2001) published a two-dimensional reference map of the *C.*

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Table 1. Summary of studies that describe *C. pneumoniae* antigen/immunogen candidates with possible application in serological diagnosis

Type of study	Reference
Characterization of MOMP as an immunogen	Iijima *et al.* (1994); Jantos *et al.* (1997); Wolf *et al.* (2001)
Characterization of PorB as an immunogen	Kubo & Stephens (2000)
Characterization of CrpA as an immunogen	Melgosa *et al.* (1993); Klein *et al.* (2003)
Characterization of OmcB as an immunogen	Mygind *et al.* (1998); Stephens *et al.* (2001)
Characterization of a 76 kDa protein as an immunogen	Perez Melgosa *et al.* (1994)
Characterization of members of the PMP family as immunogens	Knudsen *et al.* (1999); Christiansen *et al.* (2000)
Identification of 263 *C. pneumoniae* proteins	Vandahl *et al.* (2001)
Identification of 53 *C. pneumoniae* antigens, 41 of which were immunogens	Montigiani *et al.* (2002)
Evaluation of six proteins (enolase, OmpH, HtrA, ArtJ, Pmp2, Pmp10) as vaccine candidates	Finco *et al.* (2005)
Evaluation of 54 kDa protein (CPn0980) as an immunogen	Campbell *et al.* (2001); Sueur *et al.* (2006)
Description of CrpA, OmcB, MOMP-VD2 and MOMP-VD3 as immunogens	Klein *et al.* (2003)
Identification of Omp11, PmpG and type III secretion system ATPase as immunogens	Park *et al.* (2009)
Studies in persistent infections	
Identification of upregulated proteins	Molestina *et al.* (2002)
Identification of altered protein expression pattern	Mukhopadhyay *et al.* (2004)
Identification of upregulated proteins	Mukhopadhyay *et al.* (2006)
Identification of eight immunogens (RpoA, MOMP, YscC, Pmp10, PorB, Pmp21-m, GroEL and Cpaf-c)	Bunk *et al.* (2008)

pneumoniae proteome, which contained 263 proteins identified in EBs by the matrix-assisted laser desorption/ionization-MS technique and classified according to the *Chlamydia* Genome Project (Stephens *et al.*, 1998). Most authors have reported that EBs are metabolically inactive, but this study identified a large number of EB proteins involved in various pathways. It was long believed that *Chlamydia* species are energy parasites that are strictly auxotrophic for ATP, but this study revealed the presence of several proteins related to the production of energy by a type III secretion system in EBs.

Among studies on immunogenic proteins, we highlight the identification by Montigiani *et al.* (2002) of immunogenic EB surface proteins. These authors adopted a combined genomic–proteomic approach based on *in silico* prediction, followed by the heterologous expression and purification of selected proteins and the production of mouse immune sera against recombinant proteins to be used in Western blot and fluorescence-activated cell sorting (FACS). They identified 53 antigens, including 28 that were shown to be surface-exposed by FACS. Their study represented the first successful attempt to systematically analyse proteins located on the outer surface of *C. pneumoniae*. FACS analysis can be used to follow antibody interaction with *C. pneumoniae* EBs, only revealing interactions with surface-exposed proteins but with the disadvantage of possible false-positive results.

The same research group carried out another study (Finco *et al.*, 2005) to identify novel candidate vaccines against *C. pneumoniae*. They reported that three (enolase, HtrA and ArtJ) of the six proteins studied, which would be expected to have a cytoplasmic or periplasmic location in *Chlamydiae*, were immunoaccessible in infectious EBs. Homologues of enolase and HtrA have been described as surface-exposed but only in Gram-positive bacteria (Bergmann *et al.*, 2001; Pancholi & Fischetti, 1998; Poquet *et al.*, 2000). A structural explanation of the interference with *C. pneumoniae* infectivity by antibodies to these proteins must await a better molecular characterization of the chlamydial surface.

Researchers have studied the immunogenic capacity of recombinant *C. pneumoniae* surface proteins with the aim of improving serological diagnosis kits. Sueur *et al.* (2006) assessed a 54 kDa recombinant protein encoded by the CPn0980 gene for use in a *C. pneumoniae*-specific ELISA. Recombinant P54 is a *C. pneumoniae*-specific immunodominant protein, and its recombinant antigen was evaluated by using a panel of 105 serum samples from 62 patients with community-acquired pneumonia. When an outer-membrane complex ELISA was taken as reference, anti-P54 IgG antibodies showed a sensitivity of 45 % and specificity of 100 %, and when a direct PCR test was the standard, anti-P54 IgG antibodies showed a sensitivity of 55.5 % and specificity of 84.9 % and anti-IgM antibodies a sensitivity of 22.2 % and specificity of 94.3 %.

A direct detection test is not the optimal reference standard to assess a serological assay, not only due to the time required to induce the specific humoral immune response, as already mentioned, but also because if samples are positive for the antibodies and negative for the pathogen it is not known whether this is attributable to a lack of test sensitivity or the elimination of the pathogen.

Campbell *et al.* (2001) previously evaluated the diagnostic usefulness of a recombinant antigen of 54 kDa by Western

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blotting. Recognition of the 54 kDa (CPn0980) protein showed a sensitivity of 79 % and a specificity of 95.8 % with reference to the MIF test. Klein *et al.* (2003) carried out a comparative serological analysis of several recombinant surface antigens of *C. pneumoniae* and *Chlamydia trachomatis* in immunoblot assays, finding that neither PorB nor the 9 kDa outer-membrane protein (OmcA) of *C. pneumoniae* were recognized by IgG or IgA antibodies in any of the 41 human sera tested. They also confirmed that denatured chlamydial OmcB fusion proteins, which have been reported to be major immunogens in chlamydial infections (Mygind *et al.*, 1998; Watson *et al.*, 1994), share immunodominant epitope(s). They demonstrated that the frequently reported cross-reactivities of antibodies can be avoided by dissecting MOMP into fragments according to its extracellular variable domains (VDs), proposing that identification of the VD2 and VD3 regions of *C. pneumoniae* as species-specific domains within the MOMP may prove valuable in improving species-specific assay systems for *C. pneumoniae*.

The objective of a recent study by Park *et al.* (2009) was also to discover new immunogenic proteins for the diagnosis of *C. pneumoniae* infection by using immunoproteomics. They selected three proteins of *C. pneumoniae* as candidate diagnostic immunogens (Omp11, PmpG and a type III secretion system ATPase), which were detected in all of 20 *C. pneumoniae* sera using two-dimensional Western blotting.

The identification and characterization of recombinant immunogenic antigens should lead to an alternative serological test that allows *C. pneumoniae* to be diagnosed in a reliable, precise and invariable manner that can also be validated.

Despite years of efforts by research groups worldwide, a vaccine against human chlamydial infection remains unavailable. After the unsatisfactory results obtained with single antigens, it is now widely accepted that effective anti-*Chlamydia* immunization will only be achieved by using balanced combinations of several antigens (Finco *et al.*, 2005). Results of the search for antigens with diagnostic application have also been disappointing. Although most of them are highly antigenic for individual sera, they have low overall reactivity and none has been proven sufficiently immunodominant and specific to allow a more sensitive and specific serological assay to be developed. Hence, the combination of several antigens in a single test is the current objective.

Serological tests based on different selective combinations of specific recombinant antigens would greatly improve the serodiagnosis of *C. pneumoniae* infection (Bunk *et al.*, 2008).

Proteomic analysis in persistent infections

The hypothesis that *C. pneumoniae* is involved in atherogenesis at some stages (Campbell & Kuo, 2004) is supported by direct detection of the pathogen in atherosclerotic plaques and by data from animal and *in vitro* experiments.

Saikku *et al.* (1988) first reported a link between coronary artery disease and serological evidence of past infection with *C. pneumoniae*. Since then, numerous studies have reported a positive association but an equal number have found no association. This discrepancy can be attributed to the poor validity of current *C. pneumoniae* serodiagnosis (Apfalter, 2006; Boman & Hammerschlag, 2002; Hermann *et al.*, 2002; Ieven & Hoymans, 2005).

Various studies (Byrne *et al.*, 2001; Mathews *et al.*, 2001; Molestina *et al.*, 2002; Mukhopadhyay *et al.*, 2004, 2006) have shown that *C. pneumoniae* expresses genetic and proteomic transcription patterns that are altered during persistent *in vitro* infection induced by interferon-γ (IFN-γ), since subinhibitory IFN-γ levels allow the development of a persistent state of *C. pneumoniae in vitro*, a phenomenon that is characterized by the formation of reticulate bodies. The stimulation of host cells by IFN-γ inhibits the growth of *C. pneumoniae* by inducing indoleamine 2,3-dioxygenase activity, which removes tryptophan from the organism (Mehta *et al.*, 1998; Pantoja *et al.*, 2000; Summersgill *et al.*, 1995).

Mathews *et al.* (2001) identified five genes [*ompA*, *ompB* (*porB*), *pyk*, *nlpD* and Cpn0585] that were clearly upregulated under persistent conditions in comparison to normal cultures. Byrne *et al.* (2001) showed that the expression of genes involved in cell division (*ftsK*, *ftsW*) was downregulated, consistent with the very slow cell division but complex metabolic activity in the persistent state.

With regard to the pattern of differential protein expression, Molestina *et al.* (2002) carried out a proteomic analysis of the expression of proteins from *C. pneumoniae* intracellularly labelled with [^{35}S]methionine/cysteine under normal conditions and IFN-γ-mediated persistence. Comparison of electrophoretic maps of *C. pneumoniae*-infected HEp-2 cells grown with or without IFN-γ treatment showed significant upregulation of various proteins with different metabolic functions, including MOMP, GroEL, PgK, GlgP, GyrA, RpoA, PnP, Rrf and SctN.

Mukhopadhyay *et al.* (2004) analysed five *C. pneumoniae* proteins (Adk, AhpC, CrpA, Map and Cpn0710) under normal versus IFN-γ-induced persistent growth conditions at different time intervals, and observed alterations in the protein expression pattern. Subsequently, the same group (Mukhopadhyay *et al.*, 2006) used two models of chlamydial persistence, IFN-γ treatment and iron limitation, observing (in both models) an upregulation of the proteins involved in protein folding, assembly and modification (GroEL, GroES, DnaK, DksA, GrpE, HtrA, ClpP1, ClpP2, ClpC, ClpX and ClpB), which indicates a strong stress component.

Our group (Gutiérrez *et al.*, 2005) analysed the relationship between infection by *C. pneumoniae* and peripheral artery

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disease, since the pathogenesis of coronary and peripheral arterial disease appears to differ. In another study (Gutiérrez *et al.*, 2006), we used indirect (Western blot, MIF, ELISA) and direct (PCR) detection in 68 cases and 50 controls. With Western blotting, a significant presence of anti-39 kDa and anti-54 kDa IgG was found in some cases and was related to MIF results and associated with the presence of *C. pneumoniae* DNA. Anti-LPS, anti-92 kDa and anti-Hsp60 kDa IgA were also detected and related to the presence of the DNA. Among all of the bands significantly detected in the Western blot assay, only a few antibodies were associated with the presence of DNA.

Other relevant studies were recently published on persistent *C. pneumoniae* infections. Bunk *et al.* (2008) combined a proteomic approach with two-dimensional gel immunoblotting in order to analyse the antibody response pattern of different sera from subjects with and without PCR evidence of persistent *C. pneumoniae* infection. Out of the 31 *C. pneumoniae* antigens identified, 8 (RpoA, $MOMP_{4.9}$, YscC, Pmp10, PorB, Pmp21-m, GroEL and Cpaf-c) showed higher reactivity with sera from PCR-positive donors. They also observed that the antibody response pattern of PCR-positive donors correlated with the altered protein expression observed in persistent *C. pneumoniae* infection *in vitro*. These data offer the first evidence of differences in serological response to *C. pneumoniae* as a function of the infection status of donors. The antibody response of *C. pneumoniae* DNA-positive donors showed specific reactivity toward antigens selectively upregulated during *C. pneumoniae* persistence (Bunk *et al.*, 2008).

The use of antigens that are differentially expressed during acute versus persistent infection might allow the diagnosis of persistently infected individuals, an important prerequisite for clinical studies investigating the role of *C. pneumoniae* in chronic infections. In addition, the proteomic analysis of *C. pneumoniae* in persistent infections may be useful in identifying potential virulence factors and therapeutic targets (Molestina *et al.*, 2002) and may represent a tool for identifying patients with persistent infection.

Many of the antigens found to be upregulated during persistent infection are not located on the outer surface of *C. pneumoniae* but may be previously inaccessible bacterial proteins released from disintegrated cell walls and exposed to B-cell antigen receptors. Furthermore, previously inaccessible linear motifs can be exposed to serve as B-cell epitopes upon protein unfolding after limited proteolysis.

Importantly, the ability of chlamydiae to persist in host tissue for extended time periods is attributed to activation of the host cell-mediated immune response, which plays an important role in the protection and pathogenesis of *C. pneumoniae* infections (Halme *et al.*, 2000; Hammerschlag *et al.*, 1992; Penttilä *et al.*, 1998).

Current data from studies on the potential value of antigens as components of a future human vaccine indicate that antibodies alone are not sufficient to prevent or clear chlamydial dissemination and infection and that cell-mediated mechanisms are at least equally important (Finco *et al.*, 2005).

Conclusions

Numerous studies have been carried out to identify *C. pneumoniae*-specific antigens that can be recognized during human infection in order to improve serodiagnosis of this infection, but no clear pattern of reactivity indicating *C. pneumoniae* infection has emerged.

Given the major problems with inter- and intra-laboratory reproducibility, further research is required to identify new candidate antigens for the development of diagnostic techniques and vaccines against this human pathogen.

Serological assays that differentiate between past and persistent infections may offer the key to understanding the role of this infection in coronary artery disease. Proteomics may make a major contribution to improvements in the serodiagnosis of these patients by identifying selective antigens or combinations of antigens associated with persistent infection.

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