Chlamydial Serology: Comparative Diagnostic Value of Immunoblotting, Microimmunofluorescence Test, and Immunoassays Using Different Recombinant Proteins as Antigens

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To improve the reliability of the serodiagnosis of *Chlamydia trachomatis* infections, an immunoblot analysis, a microimmunofluorescence titration, and different immunoassays using synthetic peptides derived from species-specific epitopes in variable domain IV of the major outer membrane protein or recombinant antigens (heat shock protein 70 [hsp70], hsp60, hsp10, polypeptide encoded by open reading frame 3 of the plasmid [pgp3], macrophage infectivity potentiator, and a fragment of the total lipopolysaccharide) were evaluated. Because cross-reactions between chlamydial species have been reported, the microimmunofluorescence tests were also performed with *Chlamydia pneumoniae* and *Chlamydia psittaci* used as antigens, and *C. pneumoniae*-specific antibodies were also determined by immunoassays. Since the presence of antimicrobial antibodies must be interpreted in light of their prevalence in the general population, responses obtained with serum samples from patients with well-defined infection (i.e., with positive urethral or endocervical *C. trachomatis* DNA amplification) were compared to those obtained with samples from healthy blood donors. The best sensitivity (86%) with a specificity of 81% was obtained for immunoblotting results, when the number of individuals with ≥10 immunoglobulin G (IgG) and/or ≥2 IgM responses to the different *C. trachomatis* antigens was considered. A 13-kDa antigen was recognized by most of the samples (86% for IgG) from patients with acute urogenital infection but rarely (3%) by those from healthy blood donors ($P < 0.0001$). The sensitivity and specificity results obtained for serum antibodies to peptides or recombinant antigens were slightly lower than those results obtained for the number of responses to whole *C. trachomatis* antigens, which were 76 and 77%, respectively, when IgG responses to both recombinant hsp60 and pgp3 were considered.

Although serology can never replace methods aiming at the direct detection of *Chlamydia trachomatis*, there are situations in which reliable serological tests can be helpful. Indeed, urogenital infections with these bacteria are frequently inapparent (18, 30, 34, 40). Therefore, determination of antibodies to *C. trachomatis* antigens may be useful in determining whether a patient has had a previous infectious encounter. For example, in chronically infected patients in whom the bacteria are no longer detectable locally, a positive serological test may be the only indication of chlamydial involvement.

Different tests have been used for chlamydial serology. Early studies were performed with a complement fixation test, but this test could not differentiate between chlamydial species, and it lacked sensitivity. The microimmunofluorescent (MIF) test is still considered the serologic "gold standard." Although it is claimed to be species specific, cross-reactions between chlamydial species have been reported (37, 38). Recently, several enzyme-linked immunosorbent assays (ELISAs) have been commercially developed with *Chlamydia* recombinant antigens, some of them known to be *C. trachomatis* specific.

We therefore used different approaches to investigate whether a test or a combination of tests could be sensitive and specific enough to be used for the serodiagnosis of *C. tracho-*

matis infection. We first performed immunoblot assays of *C. trachomatis* antigens, since this technique is widely used in the serodiagnosis of Lyme borreliosis (36) but is not currently used in *Chlamydia* serology. However, because antibodies directed to conformational epitopes can be missed by immunoblot analysis, we also developed an ELISA using, as antigens, five different *Chlamydia* recombinant proteins, most of which were purified in native conditions. The selected proteins were *C. trachomatis* heat shock protein 70 (hsp70), hsp60, hsp10, a polypeptide encoded by open reading frame 3 of the plasmid (pgp3), and a macrophage infectivity potentiator (MIP). hsp70 (5, 13, 28) and MIP (24, 27) have been identified as in vitro targets of neutralizing antibodies. hsp60, which is supposed to play an important role in the host immune response (31), is coexpressed with hsp10 (29), but hsp10 has been reported to be an independent marker (4, 22). pgp3, which is predominantly found in chlamydial outer membrane complex preparations (10), has been found to be a major immunogen in chlamydial infections (11). Antigens were prepared and tested under exactly the same conditions in order to compare the respective sensitivity and specificity of the tests. Two commercially available ELISA tests were also evaluated. One uses synthetic peptides derived from species-specific epitopes in variable domain IV of the major outer membrane protein (MOMP) of *C. trachomatis*, which is not homologous to *C. pneumoniae* MOMP sequence (Labsystems Research Laboratory, Helsinki, Finland). The other ELISA was based on an exclusively *Chlamydia*-specific recombinant fragment of the total lipopolysac-

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TABLE 1. Serum antibody responses to whole *C. trachomatis* antigens determined by immunoblot analysis

Subjects and analysis	Median age in years (range)	% Female	Response (mean no. detectable bands ± SD) to antigen by antibody:		
			IgG	IgM	IgA
Patients with acute urogenital *C. trachomatis* infection	32 (17–65)	27	18.4 ± 9.3	2 ± 2	11.1 ± 8.0
Healthy blood donors	28 (20–39)	29	5.6 ± 5.2	0.5 ± 0.7	3.3 ± 3.7
Statistical analysis (*P*)[a]			<0.0001	0.0004	0.0001

[a] Results (*P* values) from Student's *t* test analysis of indicated antibody responses of both groups to *C. trachomatis* antigens.

charide (LPS) 3-deoxy-D-*manno*-2-octulopyranosonic acid (3-Kdo) (Medac GmbH, Hamburg, Germany). Finally, the MIF tests were performed with *C. trachomatis, C. pneumoniae,* and *C. psittaci* as antigens, and anti-*C. pneumoniae*-specific antibodies were determined with commercially available ELISA tests (Labsystems Research Laboratory) in order to investigate possible cross-reactions.

Since the presence of antimicrobial antibodies must be interpreted in light of their prevalence in the general population, we compared the prevalence of anti-*Chlamydia* antibodies in samples of patients with well-defined disease (i.e., with positive urethral or endocervical *C. trachomatis* DNA amplification) with those in samples from healthy blood donors with a similar age and sex ratio.

MATERIALS AND METHODS

Patients. Serum samples were stored at −70°C until processed. The study subjects were categorized into one of the two following groups: group 1 patients (*n* = 45) had acute *C. trachomatis* urogenital infection with positive findings on urethral or endocervical *C. trachomatis* DNA amplification with the Amplicor test (Roche Diagnostic Systems, Branchburg, N.J.); group 2 subjects (*n* = 31) were healthy blood donors.

The median ages (in years), age range, and percentages of female subjects are given in Table 1.

Recombinant protein preparation. (i) Template DNA. Template DNA for the PCR was obtained from *C. trachomatis* serovar D, strain UW-3/Cx, purchased from the American Type Culture Collection (No. VR-885), or from purified recombinant plasmid clones pUC18 for hsp70 and pCVB2 for hsp60, which were kindly provided by I. Maclean (University of Manitoba, Winnipeg, Canada).

(ii) Primers and DNA amplification. The different *C. trachomatis* sequences amplified (8, 12, 13, 26), with their restriction endonuclease sites and GenBank accession numbers, are indicated in Table 2. Oligonucleotides used as primers were synthesized by Microsynth, Balgach, Switzerland.

The amplification was performed in a reaction volume of 50 µl containing 0.2 mM concentrations of each deoxynucleoside triphosphate, 0.25 µM concentrations of each oligonucleotide primer, and 20 mM Tris-HCl (pH 8.8), 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 0.1 mg of nuclease-free bovine serum albumin (BSA) per ml, and 2.5 U of recombinant *Pfu* DNA polymerase from *Pyrococcus furiosus* (Stratagene, La Jolla, Calif.). Samples were subjected to 33 cycles of amplification in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.) as follows: 1 min (except for the first cycle, which was 3 min) of denaturation at 95°C, 1 min of primer annealing to template at 55°C, and 4 min of primer extension at 72°C. After the last cycle, all samples were incubated for 10 min at 72°C to ensure that the final extension step was complete.

(iii) Cloning of PCR-amplified products in the pQE-60 vector. The pQE-60 vector (Qiagen, Chatsworth, Calif.) was chosen to append a tag of six histidines (6xHis) to the C termini of recombinant proteins for large-scale purification via nickel chelate affinity chromatography. The C-terminal 6xHis tag position allows purification of only full-length proteins. To perform this purification step, the native stop codon was replaced in frame with the *Bam*HI or the *Bgl*II sites (Table 2).

Amplified products were separated on an 0.8% agarose gel, and bands of the expected sizes (Table 2) were excised and purified with the QIAquick spin column purification protocol (Qiagen). Both the amplified insert and the pQE-60 vector were cut with appropriate restriction enzymes (Table 2), ligated with T4 DNA ligase (New England Biolabs) and transformed into M15(pREP4)-competent *Escherichia coli* cells (Qiagen) using standard protocols (CaCl₂ treatment and heat shock).

(iv) Identification of bacterial colonies containing recombinant plasmids. The transformed cells were plated onto 1.5% agar containing kanamycin (Sigma) (25 µg/ml) and ampicillin (Boehringer) (100 µg/ml). Colonies were screened for the presence of an insert of the predicted size (Table 2) by DNA amplification. Oligonucleotides used were the primer-promoter region and the primer-reverse sequencing for pQE vectors (Qiagen).

Bacteria from each tested colony were grown overnight in 1.5 ml of Luria broth (LB) medium containing kanamycin (25 µg/ml) and ampicillin (100 µg/ml). When recombinant clones were identified, glycerol stocks were prepared.

(v) Sequence analysis. The identity of positive clones was confirmed by nucleotide sequencing in both orientations of the entire DNA inserts. Nucleotide sequences were analyzed with the basic local alignment search tool (BLAST) software. They were translated into protein sequences and compared with known sequences in ExPASy.

(vi) In vitro site-directed mutagenesis (for hsp70 and hsp10). In order to change an incorrect base introduced at the cloning step of hsp70 and hsp10, in vitro site-directed mutagenesis was performed with the QuickChange site-directed mutagenesis kit from Stratagene. For the amplification reaction, oligonucleotide primers containing the desired mutations were the hsp70 5′ end, 5′-AGAAATTAACCATGA**A**GCGAAAAAAGAAAGTCTAA-3′; hsp70 3′ end, 5′-TTAGACTTTCTTTTTTCGCTCATGGTTAATTTCT-3′; hsp10 5′ end, 5′-AGA

TABLE 2. Sequences of primers used for DNA amplification and size and localization of the cloned genes

Genes	Location and PCR primer sequences[a]	Restriction enzyme site	Size of DNA fragment (bp)	Localization of DNA fragment (bp)[b]	GenBank accession no.	Reference
hsp70	5′ end, 5′-CATGccatgg[c]GCGAAAAAAGAAAGTCTAACAAAAT-3′	*Nco*I	1,959	151–2109	M27580	13
	3′ end, 5′-CGggatccCTCAGGTTTATCAACAATTTCAACA-3′	*Bam*HI				
hsp60	5′ end, 5′-CATGccatggTCGCTAAAAACATTAAATACAACGA-3′	*Nco*I	1,635	383–2017	M58027	8
	3′ end, 5′-CATGagatctATAGTCCATTCCTGCGCCAGGCATTGC-3′	*Bgl*II				
pgp3	5′ end, 5′-CATGccatggGAAATTCTGGTTTTTATTTGTATAA-3′	*Nco*I	795	4054–4848	J03321	12
	3′ end, 5′-CATGagatctAGCGTTTGTTTGAGGTATTACCTCT-3′	*Bgl*II				
MIP	5′ end, 5′-CATGtcatgaAGAATATATTAAGTTGGATGCTTAT-3′	*Bsp*HI[d]	732	100–831	X66126, S47522, X53481	26
	3′ end, 5′-CGggatccTTCTGTAACAGATACATTATCGTCG-3′	*Bam*HI				
hsp10	5′ end, 5′-CATGccatgg[e]CAGATCAAGCAACGACCCTCAAGAT-3′	*Nco*I	309	37–345	M58027	8
	3′ end, 5′-CATGagatctTTGCAGAACTGCGATAACTTCGCTC-3′	*Bgl*II				

[a] The lowercase characters correspond to restriction enzyme sites.
[b] Nucleotides are numbered according to coordinates of the sequences and refer to their position on the GenBank entries.
[c] G will be replaced by A with the QuickChange site-directed mutagenesis kit of Stratagene, La Jolla, Calif.
[d] The *Bsp*HI restriction site is TCATGA which is able to replace the *Nco*I (CCATGG), since CATG is conserved.
[e] G will be replaced by T with the Quick Change site-directed mutagenesis kit of Stratagene.

AATTAACCATG**T**CAGATCAAGCAACGACCCT-3′; and hsp10 3′ end, 5′-A
GGGTCGTTGCTTGATCTGACATGGTTAATTTCT-3′.

(vii) Small expression cultures and purification of 6xHis tagged proteins. In
order to check the protein molecular weight and the presence of the C-terminal
6xHis tag, 10 ml of culture medium was inoculated with overnight cultures of
recombinant clones. Expression was induced by adding 1 mM of isopropyl-β-D-
thiogalacto-pyranoside (Life Technologies). Purification was performed under
denaturing conditions to isolate any tagged proteins, independently of their
solubility within the cell, according to Qiagen protocol. Nickel-nitrilotriacetic
acid (Ni-NTA) spin columns from Qiagen were used, and the eluted proteins
were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis
(SDS-PAGE). Coomassie blue R-250 was used for staining.

**(viii) Large-scale expression and purification under native conditions of
6xHis-tagged chlamydial recombinant proteins.** A small inoculate of glycerol
stock was added to 25 ml of LB containing kanamycin (25 µg/ml) and ampicillin
(100 µg/ml) and was incubated overnight at 37°C. This seeding culture was added
to 500 ml of LB that also contained kanamycin and ampicillin, incubated at 37°C
for about 1 h, and induced with 1 mM isopropyl-β-D-thiogalactopyranoside
(except for MIP, for which a concentration of 0.4 mM was used) for about 3.5 h
(2.5 for MIP) with vigorous shaking. Cultures of *E. coli* were pelleted by using an
RC 5 B Sorvall centrifuge with a GS-3 rotor at $4,000 \times g$ for 20 min at 4°C. Pellets
were washed in phosphate-buffered saline (PBS) and frozen at −20°C until used.
Pellets were thawed and resuspended in lysis buffer (50 mM Na_2HPO_4/NaH_2PO_4
buffer [pH 8.0], 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl
fluoride, 20 mM 2-mercaptoethanol, and 1 mg of lysozyme per ml [Sigma]). They
were sonicated for 6×10 s on ice to ensure complete resuspension. Lysate was
then centrifuged at $10,000 \times g$ for 30 min at 4°C to pellet cell debris.

Fast protein liquid chromatography (FPLC) equipment (Pharmacia) was used
to purify proteins under native conditions by nickel chelate affinity chromatog-
raphy (Qiagen Ni-NTA Superflow resin).

For MIP purification a different experimental procedure was used. The re-
combinant protein was extracted by Triton X-114. A cushion of 6% (wt/vol)
sucrose–10 mM Tris-HCl (pH 7.4)–150 mM NaCl–0.06% Triton X-114 was
placed at the bottom of a centrifuge tube. The lysis supernatant was overlaid on
this sucrose cushion, and the tube was incubated for 10 min at 30°C. Clouding of
the solution occurred. The tube was centrifuged for 10 min at $300 \times g$ at room
temperature with a swinging bucket rotor. The upper aqueous phase was re-
moved from the tube and received 0.5% fresh Triton X-114. After dissolution of
the surfactant at 0°C, the mixture was again overlaid on the sucrose cushion used
previously, incubated 10 min at 30°C, and centrifuged (6). The Triton X-114
phase was then diluted in lysis buffer and passed through the column. After
extensive washing with wash buffer containing 0.5% 3[(3-cholamidopropyl)-dim-
ethylammonio]-1-propane-sulfonate (CHAPS), MIP was eluted by a 20 to 250
mM imidazole gradient containing 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 8.0),
300 mM NaCl, and 0.5% CHAPS. Fractions containing MIP were pooled and
dialyzed against PBS with 0.5% CHAPS (25).

The protein concentration was determined with the Bradford assay (Bio-Rad
Laboratories, Richmond, Calif.). Purified proteins were subsequently character-
ized by SDS-PAGE and immunoblotting, separated into aliquots, and frozen at
−80°C.

**(ix) Characterization of expressed recombinant proteins: control of apparent
molecular weight, in-frame translation of the 6xHis tag, and binding by IgG
from *C. trachomatis*-infected patients.** Precast Tris-glycine polyacrylamide gels (4
to 20% with a 4% stacking gel; 10 wells) were purchased from Novex (San Diego,
Calif.). Protein preparations were reduced by heating at 95°C for 5 min in the
presence of 0.0625 M Tris (pH 6.8)–10% (vol/vol) glycerol–3% (wt/vol) SDS–
0.003% (wt/vol) bromophenol blue–3% (vol/vol) 2-mercaptoethanol. Ten micro-
liters of protein preparation or 3 to 5 µl of protein standard (Mark12 wide range
protein standard from Novex for the Coomassie blue staining gel, 6xHis protein
ladder from Qiagen for the immunoblot assay with anti-His antibody, and
prestained, multicolored protein standard from Novex for the immunoblot assay
with a pool of sera from *C. trachomatis*-infected patients) was loaded in each
well. Electrophoresis was performed for 1 h and 50 min at a constant voltage of
125 V in an Xcell II vertical slab gel system (Novex). The running buffer con-
sisted of 0.025 M Tris, 0.192 M glycine, and 0.1% (wt/vol) SDS (pH 8.3).

Three identical gels were prepared, one of which was stained for protein
visualization with Coomassie blue and the other two of which were transferred to
a Hybond C-extra nitrocellulose membrane purchased from Amersham, Little
Chalfont, England. The transfer was performed for 1 h and 30 min at a constant
voltage of 40 V by semidry blotting in an Xcell II blot module (Novex) at
4°C. The transfer buffer consisted of 0.012 M Tris, 0.096 M glycine, and 20%
(vol/vol) methanol (pH 8.3). The blotted nitrocellulose membranes were incu-
bated in blocking buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 3% [wt/vol] BSA,

in order to block unspecific binding sites. The membrane was then washed twice
for 10 min with Tris-buffered saline (TBS)-Tween-Triton buffer (20 mM Tris [pH
7.5], 500 mM NaCl, 0.05% [vol/vol] Tween 20, and 0.2% [vol/vol] Triton X-100)
and 10 min with TBS buffer (10 mM Tris [pH 7.5], 150 mM NaCl).

For the 6xHis tag detection, a mouse monoclonal penta His antibody (Qiagen)
was diluted 1/1,000 in blocking buffer and incubated with the membrane for 1 h
at room temperature. The membrane was then washed twice for 10 min with
TBS-Tween-Triton buffer and 10 min with TBS buffer and incubated for 1 h, at
room temperature, with the second antibody (alkaline phosphatase-conjugated
$F(ab')_2$ fragment of polyclonal sheep anti-mouse IgG) (Cappel, Embrach, Swit-
zerland) at a dilution of 1:1,000 in blocking buffer.

For the binding by IgG-specific antibodies, a pool of 22 sera from patients with
positive urethral or endocervical *C. trachomatis* DNA amplification was diluted
1:100 and incubated for 2 h at 37°C. The second antibody, an alkaline phos-
phatase-conjugated $F(ab')_2$ fragment of polyclonal goat IgG anti-human IgG (Fc
specific) (Cappel) was diluted 1:1,600 and incubated for 2 h at 37°C.

The membranes were then washed four times with TBS-Tween-Triton buffer.
The color was developed by adding a buffer containing 100 mM Tris (pH 9.5),
100 mM NaCl, 5 mM $MgCl_2$, 0.33 mg of nitroblue tetrazolium per ml, and 0.165
mg of 5-bromo-4-chloro-3-indoyl phosphate per ml. Color development was
stopped by washing the membrane with water after 15 to 20 min.

**Measurement of IgG and IgA antibodies against the 6xHis-tagged native
recombinant proteins by ELISA.** The solid phase was Ni-NTA HisSorb strips
(Qiagen), which were used to allow the oriented binding of the 6xHis-tagged
native proteins. These flat-well strips were coated with 100 µl of each of the five
recombinant protein solutions, prepared at the same concentration of 100 pmol
per ml of PBS with 0.25% (wt/vol) BSA and incubated overnight at 4°C. The
strips were washed four times in PBS-Tween 20. Serum samples were tested in
quadruplicate; they were diluted 1:100 for IgG and 1:50 for IgA determination in
PBS with 0.25% BSA and incubated overnight at 4°C. After four washes in
PBS-Tween, 100 µl of an alkaline phosphatase-conjugated $F(ab')_2$ fragment of
polyclonal goat IgG, anti-human IgG (Fc specific), or IgA (α chain specific)
(Cappel), diluted with PBS-Tween 20 containing 0.25% BSA, was added to each
well and incubated for 2 h at 37°C. After four washes in PBS-Tween, 100 µl of
substrate (1 mg of *p*-nitrophenyl phosphate per ml in 10% diethanolamine) was
added for 1 h at 37°C. Color development was stopped by the addition of 3 M
NaOH (25 µl/well). Optical density (OD) at 410 nm was measured by a Dynatech
MR 5000 Microplate Reader (Dynatech, Alexandria, Va.). The reactivity of each
serum was also tested in four noncoated wells. The net OD values are presented
(mean OD value obtained for the four coated wells − nonspecific OD [mean OD
value obtained for the four noncoated wells]).

The determination of absorbance cutoff values was of 14 sera from patients
with acute urogenital *C. trachomatis* infection and 31 from healthy blood donors
was performed by using the prediction limit calculated from child serum values,
used as negative controls, and a 99% confidence level. For IgG and IgA anti-
bodies to all of the proteins, the absorbance cutoff values were found to be 0.1
(14). To evaluate the reproducibility of the immunoassay results, one positive
serum was tested 11 times. The reproducibilities of the ODs from run to run were
±10%.

Immunoblot analysis of sera. Immunoblot analysis of 14 sera from patients
with acute urogenital *C. trachomatis* infection and 31 from healthy blood donors
was performed as previously described (2). Briefly, elementary body antigens of
C. trachomatis LGV2 strain 434 were purchased from Biodesign International
(Kennebunk, Maine). The antigens were suspended in sample buffer, heated to
100°C for 5 min, and centrifuged at $10,000 \times g$ for 5 min. Two hundred micro-
liters (145 µg of protein) was loaded in a two-dimensional well of precast
Tris-glycine polyacrylamide gels (10 to 27% with a 4% stacking gel; two-dimen-
sional well) purchased from Novex. After electrophoresis, *C. trachomatis* anti-
gens were transferred to a Hybond C-extra nitrocellulose membrane purchased
from Amersham. The blotted nitrocellulose membrane was cut into strips 4 mm
in width. These strips were incubated overnight at 4°C in blocking buffer, incu-
bated for 2 h at 37°C in sera diluted 1:100 in PBS-Tween, and incubated for 2 h
at 37°C with alkaline phosphatase-conjugated $F(ab')_2$ fragment of polyclonal
goat IgG anti-human IgG (Fc-specific), IgM (µ chain specific) or IgA (α chain
specific) (Cappel) diluted with PBS-Tween containing 5% (wt/vol) BSA. After
color development, the numbers of bands and their intensities on nitrocellulose
paper were evaluated both visually (blots were assessed blindly, always by the
same person) and with a GS-700 imaging densitometer and with Molecular
Analyst software version 2.1 (Bio-Rad Laboratories, Hercules, Calif.) from weak
(+) to very strong (++++) intensity. In order to avoid biases due to limitations
of reproducibility, at least two immunoblots were done using each serum sample
and were evaluated independently. The percentage of patients with serum anti-
bodies to each antigen was calculated. The total response corresponded to all

bands visually detectable, and the high-intensity responses corresponded only to bands with +++ and ++++ intensities.

Commercially available ELISA. The ELISA kits used to detect the IgG and IgA anti-*C. trachomatis* MOMP antibodies and those used to detect IgG, IgM, and IgA anti-*C. pneumoniae* were purchased from Labsystems Research Laboratory (Helsinki, Finland). The ELISA kits used to detect the IgG, IgM, and IgA anti-*Chlamydia* LPS antibodies were purchased from Medac GmbH (Hamburg, Germany). The assays and calculations were performed according to the manufacturers' instructions. However, equivocal results for the anti-MOMP and anti-*C. pneumoniae* assays or gray-zone results for anti-LPS assays were considered negative. Results were considered positive when the signal/cutoff value was ≥1.4 for the anti-MOMP antibodies or >1.1 for the IgM anti-*C. pneumoniae* antibodies, when enzyme immunounits were >45 for IgG or >12 for IgA anti-*C. pneumoniae* antibodies and when OD values were greater than or equal to cutoff plus 10% (+15% for IgM) for the anti-LPS antibodies.

MIF tests. Specific IgG, IgM, and IgA antibodies to *C. trachomatis, C. pneumoniae*, and *C. psittaci* antigens were determined with slides purchased from Labsystems. The assays were performed according to the manufacturer's instructions. Results were considered positive when titers were ≥1:16 for IgG and IgA and ≥1:10 for IgM.

Calculations. Sensitivity, specificity, positive predictive value, negative predictive value, and false-positive and false-negative rates were calculated as described previously (1).

Statistical analysis. Where appropriate, results were analyzed by the Student's *t* test and chi-square test.

RESULTS

Analysis of cloned genes. The nucleotide sequences of cloned genes were analyzed with BLAST, and those giving the best alignment showed a high similarity to the previously published *C. trachomatis* sequences. When the protein sequences were compared, the hsp70 sequence was found to have 100% identity to the protein sequence obtained from the AE001312 (GenBank accession number) nucleotide sequence, hsp60 to have 99.4% identity to M31739, pgp3 to have 100% identity to M19487, MIP to have 100% identity to AE001324, and hsp10 to have 100% identity to AE001285.

Analysis of purified recombinant proteins. Protein solubility was determined according to Qiagen protocol; hsp70, hsp60, pgp3, and hsp10 were found to be present mainly in soluble fractions. MIP was found to be present mainly with insoluble matter. The five recombinant proteins were, however, prepared in native conditions. Mild nonionic (Triton X-114) and amphoteric (CHAPS) detergents were used to purify MIP.

After migration on SDS-PAGE and Coomassie blue staining (Fig. 1A), immunodetection with the penta-His antibody (Fig. 1B), or immunodetection with a pool of sera from *C. trachomatis*-infected patients (Fig. 1C), purified proteins showed a band representing the expected apparent molecular weight and confirming correct insertion of the coding fragments and in-frame translation of the 6xHis tag, and these showed a recognition by IgG from infected patients.

Antibody responses to whole *C. trachomatis* antigens determined by immunoblot analysis of serum samples from healthy blood donors and *C. trachomatis*-infected patients. Immunoblot analysis showed that the IgM response seemed to be limited to a small number of antigens (maximum, 6), whereas IgA, and particularly IgG, recognized a high number of antigens (maximum, 23 and 31, respectively).

A highly significant ($P \leq 0.0004$) increase (three- to fourfold) in the number of IgG, IgM, and IgA responses to whole *Chlamydia* antigens obtained after immunoblotting was observed for serum from *C. trachomatis*-infected patients compared to serum from healthy blood donors (Table 1).
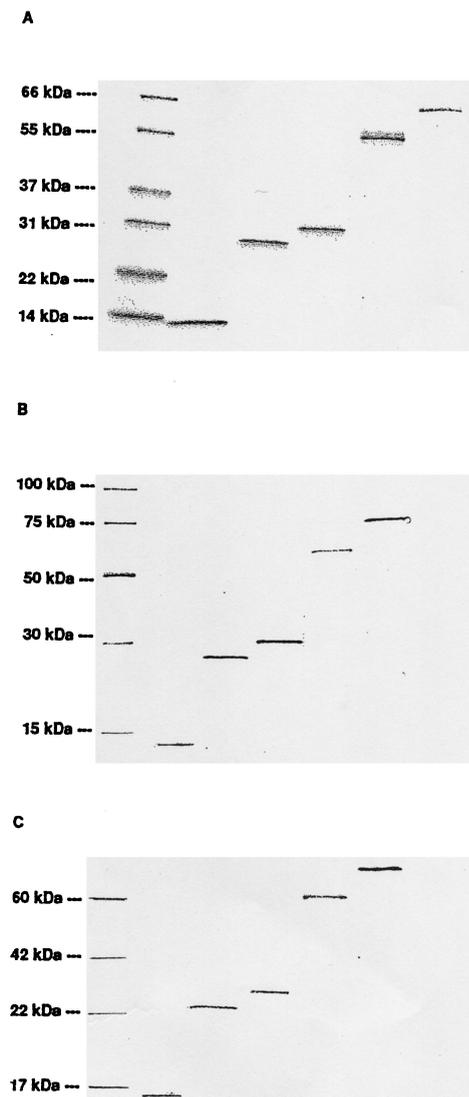

FIG. 1. Characterization of expressed recombinant proteins. The separation of proteins by SDS-PAGE was performed in three identical gels, one of which was stained for protein visualization with Coomassie blue (A). The two other gels were transferred to a Hybond C-extra nitrocellulose membrane and immunodetected with either anti-His antibody (penta-His antibody) according to Qiagen instructions for 6xHis-tagged protein detection (B) or a pool of 22 sera from patients with positive urethral or endocervical *C. trachomatis* DNA amplification for IgG-specific antibody binding (C). Lanes (left to right) MW standard, hsp10, MIP, pgp3, hsp60, and hsp70.

Antibody responses to recombinant or synthetic *Chlamydia* antigens determined by ELISA. When the percentage of individuals with serum antibodies to synthetic peptides or recombinant antigens was examined, *C. trachomatis*-infected patients had significantly more IgG to LPS, MOMP, hsp60, and pgp3 and more IgA to LPS and MOMP than healthy blood donors. The highest significant difference was observed for IgG responses to hsp60 (Table 3).

The patterns of the IgG and IgA responses to the different peptides or recombinant antigens obtained for *C. trachomatis*-infected patients were different for each individual. Detailed IgG responses to different individual antigens in the seven

TABLE 3. Percentages of individuals with serum antibodies to recombinant or synthetic *Chlamydia* antigens as determined by ELISA

Antigen (source)	Serum antibody response	% of patients with acute *C. trachomatis* urogenital infection ($n = 45$)	% of healthy blood donors ($n = 31$)[a]	Statistical analysis (chi-square test) between both groups[b]
LPS (Medac)	IgG	84	52	9.62; $P = 0.0019$
	IgM	9	3	NS
	IgA	62	23	11.61; $P = 0.0007$
MOMP (Labsystems)	IgG	58	32	4.80; $P = 0.029$
	IgA	47	16	7.61; $P = 0.0058$
Recombinant proteins prepared in our laboratory				
hsp70	IgG	29	17	NS
	IgA	2	0	NS
hsp60	IgG	62	20	13.64; $P = 0.0002$
	IgA	24	13	NS
pgp3	IgG	53	20	8.87; $P = 0.0029$
	IgA	9	3	NS
MIP	IgG	20	7	NS
	IgA	0	0	NS
hsp10	IgG	2	3	NS
	IgA	2	0	NS

[a] Only 30 healthy blood donors were tested for serum antibodies to recombinant proteins.
[b] NS, not significant.

immunoassays as well as the number of IgG responses to whole *C. trachomatis* antigens determined after immunoblotting are presented in Fig. 2. Except for one donor (number 11), an IgG response to pgp3 was observed only for samples in which >10 antigens of *C. trachomatis* were recognized after immunoblotting.

The number of IgG responses to *Chlamydia* antigens determined after immunoblotting and the total number of positive IgG responses in the seven immunoassays were highly correlated (0.87; 95% confidence interval, 0.78 to 0.93; $P < 0.0001$ [Z test]).

Antibody responses to *C. trachomatis, C. pneumoniae*, or *C. psittaci* antigens determined by MIF test. When the percentage of individuals with serum antibodies to the three different species of *Chlamydia* was examined, *C. trachomatis*-infected patients had significantly more IgG to *C. trachomatis* and *C. pneumoniae* than did healthy blood donors. However, the difference observed for *C. pneumoniae* was at the limit of a nonsignificant result ($P = 0.046$) (Table 4).

Antibody responses to *C. pneumoniae* antigens determined by ELISA. No significant difference was observed between the percentages of *C. trachomatis*-infected patients with IgG, IgM, or IgA antibodies and those percentages obtained for healthy blood donors (Table 5).

Comparison of the results obtained for *C. pneumoniae* antibodies by MIF test and ELISA. The ELISA appeared to have significantly higher sensitivity than the MIF test, particularly for the IgA determination. For the IgG measurement in healthy blood donor sera, 73% of donors were found to be positive with ELISA, and 42% were found to be positive with the MIF test ($P = 0.036$). For IgA, 25% of *C. trachomatis*-infected patients were found to be positive with ELISA, but none were found with the MIF test ($P = 0.007$), and 54% of healthy blood donors were found to be positive with ELISA, but none were found with the MIF test ($P = 0.0001$).

Comparison of *C. trachomatis*- and *C. pneumoniae*-specific assays with anti-*Chlamydia* LPS ELISA. The presence of IgG anti-*Chlamydia* LPS is associated with the presence of IgG anti-*C. pneumoniae* alone (donors 13 and 18), with IgG anti-

C. trachomatis alone (patients 31, 37, and 38), or with both in a high number of cases (Fig. 3).

Diagnostic value of the different antibody determinations. From immunoblot results, when the numbers of individuals with ≥10 IgG, ≥2 IgM, or ≥6 IgA responses to *C. trachomatis* antigens were considered, the best results were obtained for IgG, with a sensitivity of 79% and a specificity of 84%. When the numbers of individuals with ≥10 IgG and/or ≥2 IgM responses were considered, the best sensitivity (86%) was obtained with a slight decrease of specificity (81%). With these criteria, a negative predictive value (likelihood that a negative result means the patient does not have disease) of 93% was obtained.

When the numbers of individuals with serum antibodies to peptides or recombinant antigens were considered, the diagnostic values of the tests were always lower. The highest sensitivity was obtained for IgG anti-LPS (84%) but with a low specificity (48%). The best results were obtained for IgG anti-hsp60 (sensitivity, 62%; specificity, 80%). When the numbers of individuals with IgG responses to hsp60 and/or pgp3 were considered, a higher sensitivity was obtained (76%) for a slightly lower specificity (77%).

The MIF test presented good specificity (89%) but poor sensitivity (44%) (Table 6).

From immunoblot results, when the percentages of individuals with serum antibodies to different *C. trachomatis* antigens were analyzed, the nine bands giving the most significant differences between patients with acute urogenital infection and healthy blood donors were of about 77, 71, 64, 61, 52, 36, 21, 18, and 13 kDa. The last band was particularly discriminant since $P \leq 0.0001$ for IgG, IgM, and IgA when all of the responses were considered, and $P \leq 0.0001$ for IgG and IgA when only the responses of high intensity were considered (Tables 7 and 8).

DISCUSSION

Reliable serological tests could be of assistance in the diagnosis of *C. trachomatis* infection when a test for direct detec-
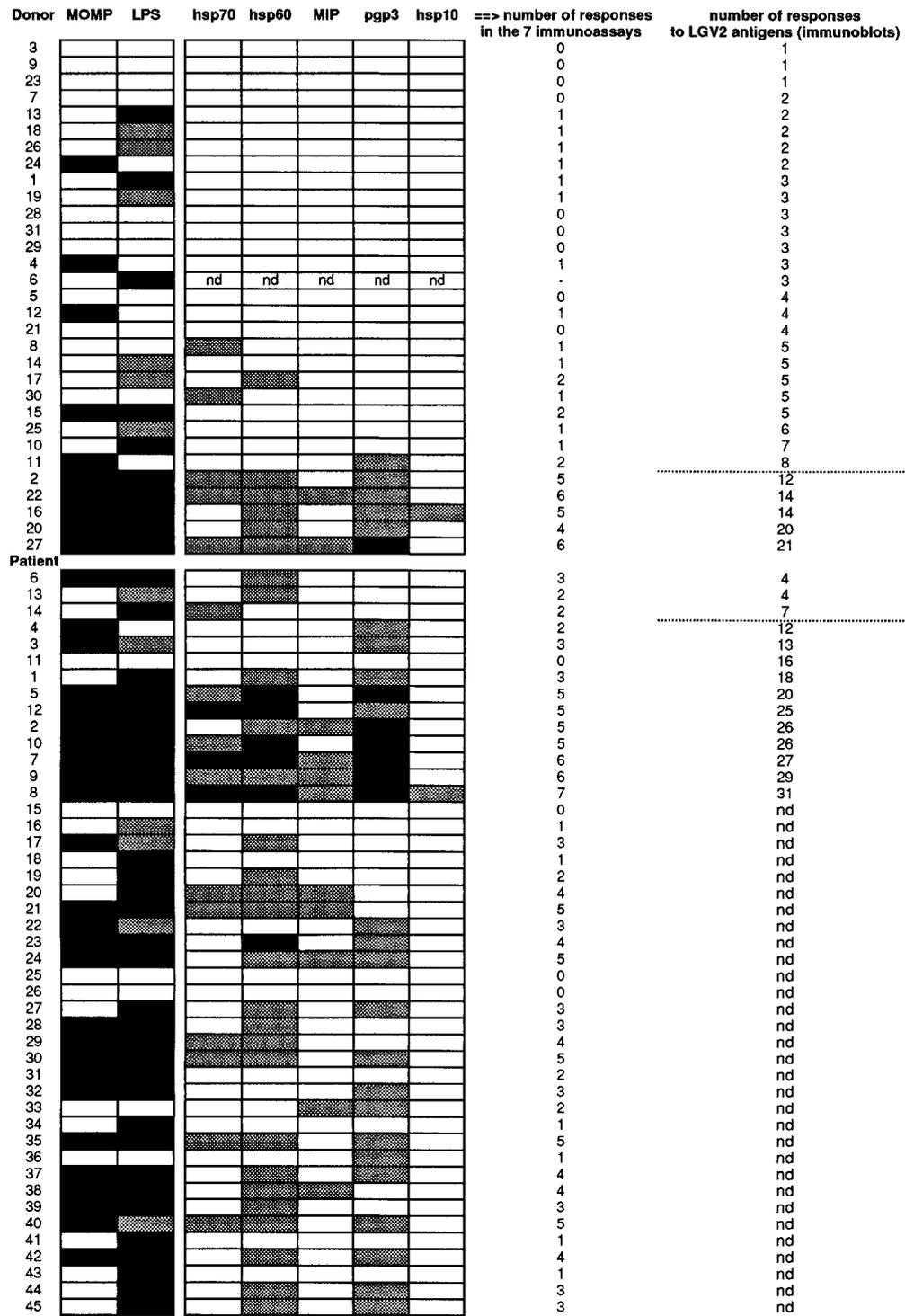
FIG. 2. Sera from healthy blood donors or *C. trachomatis*-infected patients tested in immunoassays have different patterns of reactivity with synthetic peptides or recombinant antigens. MOMP antigens are synthetic peptides derived from species-specific epitopes in the variable domain IV of the MOMP of *C. trachomatis* and are not homologous to *C. pneumoniae* MOMP (Labsystems Research Laboratory). LPS is an exclusively *Chlamydia*-specific recombinant fragment of the total LPS (3-deoxy-D-*manno*-2-octulopyranosonic acid) (Medac GmbH). hsp70, hsp60, MIP, pgp3, and hsp10 are native purified recombinant antigens prepared in our laboratory. Two mild detergents were used to purify MIP, which otherwise was prepared under the same conditions as the other recombinant proteins (cf., Materials and Methods). Black boxes, high reactivity (≥cutoff value with OD 1.0); gray boxes, lower reactivity (≥cutoff value with OD < 1.0); white boxes, reactivity below the cutoff values. The number of positive IgG responses obtained for each sample tested in the seven immunoassays is compared with the number of IgG responses to LGV2 antigens observed in immunoblots.

TABLE 4. Percentages of individuals with serum antibodies to
C. trachomatis, pneumoniae, or *psittaci* antigens as
determined by microimmunofluorescence test

Bacteria	Serum antibody	% of patients with acute *C. trachomatis* urogenital infection (n = 25)	% of healthy blood donors (n = 19)	Statistical analysis (chi-square test) between both groups[a]
C. trachomatis	IgG	44	11	5.81; $P = 0.016$
	IgM	4	0	NS
	IgA	0	0	NS
C. pneumoniae	IgG	72	42	3.99; $P = 0.046$
	IgM	0	0	NS
	IgA	0	0	NS
C. psittaci	IgG	0	0	NS
	IgM	0	0	NS
	IgA	0	0	NS

[a] NS, not significant.

tion of bacteria is negative or difficult to perform, as in cases of upper genital tract infections. In this study the diagnostic value of two complementary approaches, a global and a detailed one, were compared. A global view of the humoral response was obtained by immunoblot analysis and was quantified by counting the number of immunoreactive bands obtained. The molecular specificity of the response was studied in two different ways: its ability to recognize 27 different *Chlamydia* antigens after immunoblotting and its reactivity to seven *Chlamydia* peptides or recombinant antigens in immunoassays. To retain an antigenic structure capable of detecting antibodies directed to conformational epitopes, which could be missed by immunoblot analysis, five of these tests were performed with recombinant proteins prepared in their native conditions. These five antigens were tested by ELISA in exactly the same way in order to determine the key immunodominant antigens.

The immunoblot analysis was performed with a lymphogranuloma venereum (LGV) serovar, although it is an infrequent cause of sexually transmitted infections. However, human strains of *C. trachomatis* are closely related, and the LGV2 preparation seems to have sufficient cross- or group-reactive antigens to detect antibodies against most infecting strains. Antibodies directed against antigens common to all serovars of *C. trachomatis* have been detected but not antibodies directed against a particular serovar. In any case, it was not possible to test all trachoma serovars by this method. When immunoblots were analyzed, some *C. trachomatis* antigens appeared to have high reactivities with healthy blood donor samples. These bindings could correspond to cross-binding reactions with antigenic proteins from other microorganisms. Chlamydial proteins having homologues in other bacteria have been reported (33). However, the total number of immunoreactive bands appeared

TABLE 5. Percentages of individuals with serum antibodies to
C. pneumoniae antigens determined by ELISA

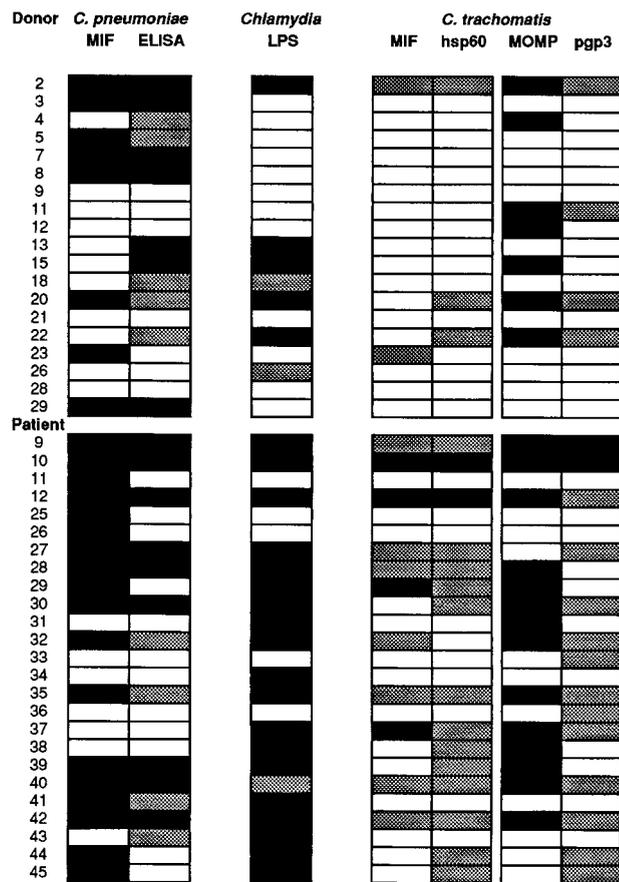| Serum antibody	% of patients with acute *C. trachomatis* urogenital infection (n = 32)	% of healthy blood donors (n = 26)	Statistical analysis (chi-square test) between both groups[a]
IgG	50	73	NS
IgM	0	0	NS
IgA	25	54	NS

[a] NS, not significant.

FIG. 3. Sera from healthy blood donors or *C. trachomatis*-infected patients tested for IgG anti-*C. pneumoniae* antibodies (MIF test and ELISA from Labsystems), anti-*Chlamydia* LPS antibodies (ELISA kit from Medac) and anti-*C. trachomatis* antibodies (MIF test and ELISA kit for anti-MOMP antibodies from Labsystems; anti-hsp60 and pgp3 antibody tests developed in our laboratory). For anti-*C. pneumoniae* MIF results, no titration was performed and positive results (≥1:16) are depicted as black boxes; for anti-*C. trachomatis* MIF results, high reactivity (≥1/128) is depicted as black boxes, and lower reactivity (1:16 to 1:64) is depicted as gray boxes. For IgG anti-*C. pneumoniae* antibodies determined with ELISA, high reactivity (>100) is depicted as black boxes, and lower reactivity (45 to 100) is depicted as gray boxes. For anti-LPS, MOMP, hsp60, and pgp3 antibodies, high reactivity (≥cutoff value with OD of >1.0) is depicted as black boxes; lower reactivity (≥cutoff value with OD of <1.0) is depicted as gray boxes. For all tests, reactivity below the cutoff values is depicted by white boxes.

to be useful for diagnosis. Indeed, the best sensitivity, specificity, and negative predictive values for the serodiagnosis of *C. trachomatis* infection were obtained when considering serum specimens with at least 10 IgG bands and/or 2 IgM bands in immunoblot results. Thus, this study shows that immunoblotting is significantly more sensitive and specific for diagnosing *C. trachomatis* infection than the ELISAs. It is also more sensitive than the MIF test. A similar conclusion has already been obtained for serodiagnosis of early Lyme disease (15, 16) in which an immunoblot test has been recommended by the Centers for Disease Control and Prevention (7).

A *C. trachomatis* antigen of about 13 kDa was recognized by most of the samples (86% for IgG) from patients with acute urogenital infection and rarely (3%) by those from healthy

TABLE 6. Best results obtained for the different anti-*Chlamydia* antibody assays

Assay	Sensitivity (%)	Specificity (%)	Predictive value (+) (%)	Predictive value (−) (%)	False-positive rate (%)	False-negative rate (%)
IgG anti-*C. trachomatis* (blot) (≥10 bands)[a]	79	84	69	90	16	21
IgM anti-*C. trachomatis* (blot) (≥2 bands)[a]	50	94	78	81	6	50
IgA anti-*C. trachomatis* (blot) (≥6 bands)[a]	71	87	71	87	13	29
IgG anti-*C. trachomatis* (blot) (≥10 bands) + IgM anti-*C. trachomatis* (blot) (≥2 bands)[a]	86	81	67	93	19	14
IgA anti-*C. trachomatis* (blot) (≥6 bands) + IgM anti-*C. trachomatis* (blot) (≥2 bands)[a]	79	81	65	89	19	21
IgG anti-LPS[b]	84	48	70	68	52	16
IgG anti-MOMP[b]	58	68	72	53	32	42
IgG anti-hsp60[c]	62	80	82	59	20	38
IgG anti-pgp3[c]	53	80	80	53	20	47
IgG anti-MOMP + pgp3[c]	71	67	76	61	33	29
IgG anti-hsp60 + pgp3[c]	76	77	83	68	23	24
IgG anti-*C. trachomatis* (MIF test)[d]	44	89	85	55	11	56

[a] Positive patients were 14 patients with *C. trachomatis* urogenital infection, and negative individuals were 31 healthy blood donors.
[b] Positive patients were 45 patients with *C. trachomatis* urogenital infection, and negative individuals were 31 healthy blood donors.
[c] Positive patients were 45 patients with *C. trachomatis* urogenital infection, and negative individuals were 30 healthy blood donors.
[d] Positive patients were 25 patients with *C. trachomatis* urogenital infection, and negative individuals were 19 healthy blood donors.

blood donors ($P < 0.0001$) and could therefore be of great diagnostic value, but its identity was not determined. It is unlikely that this antigen is an LPS fraction or hsp10, since no similar results were obtained with immunoassays using these recombinant antigens. Another possible antigen is the ribosomal protein L7/12 (33).

Concerning the antigens tested by ELISA, the highest sensitivity (84%) was observed for the IgG reactivity to LPS but this determination had a low specificity (45%), confirming our previous observations (3) which attributed these results to the high prevalence of anti-*C. pneumoniae* antibodies in the population (19, 21), since LPS is a genus-specific antigen. This high prevalence was also observed in the present study, in which as high as 73% of healthy blood donors were found to have IgG anti-*C. pneumoniae*. The highest specificity (80%) was obtained with the IgG response to both hsp60, another conserved antigen, and pgp3, a *C. trachomatis*-specific antigen. When results obtained for IgG anti-hsp60 were associated with those obtained for pgp3, the sensitivity was increased (76%) with only a slight decrease of specificity (77%). This highest specificity observed with hsp60 was not expected, since it is one of the most conserved proteins in evolution and *C. trachomatis* and *C. pneumoniae* hsp60 are immunologically similar (20, 39).

However, in this study, we found no evidence that anti-hsp60 antibodies might be elicited by other microorganisms, as was suggested by Sziller et al. (35), since the anti-hsp60 antibodies detected in our blood donor samples were always present with other *Chlamydia*-specific antibodies. Indeed, one blood donor sample also had IgG anti-LPS, and five samples had IgG targeting *C. trachomatis*-specific antigens (MOMP and pgp3). Moreover, none of the six blood donors with evidence of IgG anti-*C. pneumoniae* but without evidence of IgG anti-*C. trachomatis* (donors 3, 5, 7, 8, 13, and 18) had IgG anti-hsp60. Our results agree rather with those of Persson et al. (32), which suggest that anti-hsp60 antibodies are related to *C. trachomatis* but not to *C. pneumoniae*, and with those of Chernesky et al. (9), who obtained a high specificity with an ELISA using this protein. However, it could be useful to evaluate the specificity of anti-*C. trachomatis* hsp60 antibody determination in serum samples of patients with various bacterial infections.

Concerning the MIF test for *C. trachomatis* antibody detection, a high specificity was obtained for IgG, but this test had poor sensitivity. No IgA was detected when 47% of *C. trachomatis*-infected patients had IgA anti-MOMP antibodies and 71% recognized more than six antigens of *C. trachomatis* in immunoblots. We did not observe cross-reactions between chla-

TABLE 7. *C. trachomatis* antigens of diagnostic value

Antigens (kDa)	% of individuals with serum antibodies to *C. trachomatis* antigens (total responses)								
	IgG			IgM			IgA		
	Patients with urogenital infection (n = 42)	Healthy blood donors (n = 31)	P	Patients with urogenital infection (n = 42)	Healthy blood donors (n = 31)	P	Patients with urogenital infection (n = 42)	Healthy blood donors (n = 31)	P
77	79	6	<0.0001	5	0	NS[a]	42	3	<0.0001
71	65	0	<0.0001	28	0	0.0011	56	6	<0.0001
64	74	13	<0.0001	33	0	0.0003	56	10	<0.0001
61	84	26	<0.0001	35	10	0.011	88	35	<0.0001
52	81	26	<0.0001	35	0	0.0002	70	26	0.0003
36	79	3	<0.0001	12	0	0.047	65	10	<0.0001
21	81	26	<0.0001	28	3	0.0051	63	0	<0.0001
18	79	48	0.0072	63	10	<0.0001	74	10	<0.0001
13	86	3	<0.0001	51	0	<0.0001	77	3	<0.0001

[a] NS, not significant.

TABLE 8. *Chlamydia trachomatis* antigens of diagnostic value

Antigen size (kDa)	% of individuals with serum antibodies to *Chlamydia trachomatis* antigens (high-intensity responses only)[a]					
	IgG			IgA		
	Patients with urogenital infection (n = 42)	Healthy blood donors (n = 31)	P	Patients with urogenital infection (n = 42)	Healthy blood donors (n = 31)	P
77	19	0	0.01	2	0	NS
71	30	0	0.0006	12	0	0.047
64	44	6	0.0006	16	0	0.017
61	47	10	0.0006	12	3	NS
52	35	0	0.0002	21	3	0.025
36	33	3	0.0017	21	0	0.0059
21	14	3	NS	9	0	NS
18	28	13	NS	37	0	0.0001
13	40	0	<0.0001	37	0	0.0001

[a] NS, not significant.

mydial species, since no antibody was found to recognize *C. psittaci*.

As already reported (17, 33), a considerable heterogeneity was observed in the antigens recognized by sera from *C. trachomatis*-infected patients after immunoblotting as well as by ELISA. When the patterns were compared between the 45 infected patients, no diagnostic profile of antibodies to *C. trachomatis* antigens was found but a great diversity was observed. These individual variations in humoral response could be due to differences in the host ability to develop an immune response to a given antigen; it may be related to HLA class II alleles, since Zhong and Brunham (41) have reported that the antibody responses to *C. trachomatis* hsp60 and hsp70 are major histocompatibility complex-linked in mice. This finding also underlines the usefulness of testing different *Chlamydia* antigens in order to avoid false-negative results. Thus, four *C. trachomatis*-infected patients (9%) were found to have no IgG, IgA, or IgM response to the seven different *Chlamydia* antigens tested by immunoassays. However, in one of them a high number of *Chlamydia* antigens was recognized after immunoblotting. Therefore, in terms of diagnostic value, increasing the number of tested antigens decreases the number of patients negative results. The time course of the antibody appearance could also differ between the different antigens or between LPS and protein antigens. Immunoaccessibility is also certainly an important factor, and the different reactivities observed for the five recombinant proteins are probably a function of their surface exposure, since bacterial infection usually induces serum antibody against surface-exposed components. Thus, the poor reactivity to MIP can be explained by its limited immunoaccessibility (28). The different reactivities observed in the present study between hsp70, hsp60, and hsp10, and already reported for hsp60 and hsp10 (4, 22) could be explained either by a greater surface exposure of hsp60, different genetic regulation of antibody responses to the different hsp types, or lack of epitopes due to the absence of posttranslational modification, as suggested by LaVerda and Byrne for hsp10 (23).

In conclusion, although the MIF test is still considered as the serologic "gold standard," we did not find it to be the best test. No cross-reactions between chlamydial species were observed, but a lower sensitivity (44%) was obtained than with other tests

(86%). Because this test has also been criticized for its requirement of considerable expertise, its subjective interpretation, and its unsuitability for testing large numbers of specimens, we think that immunoblotting or ELISA with appropriate antigen(s) is more useful. Thus, by comparing the humoral immune responses of patients with proven *C. trachomatis* infection with those of healthy blood donors of similar ages and sex ratios, the best balance between sensitivity and specificity was found with the total number of IgG and IgM reactive bands obtained by immunoblotting. As a complementary method, the association of two recombinant proteins (hsp60 and pgp3), prepared under native conditions and used as antigens in ELISA, improved both specificity and sensitivity compared to individual recombinant antigens.

The anti-LPS antibody test from Medac appears to be of low usefulness due to its nonspecificity. Concerning anti-*C. pneumoniae* assays, we observed no good agreement between results obtained from MIF and ELISA tests.

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