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Association of tubal factor infertility with elevated antibodies to *Chlamydia trachomatis* caseinolytic protease P

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Abstract

OBJECTIVE—The objective of the study was to assess antibodies against *Chlamydia trachomatis* heat shock proteins (HSP) in patients with tubal factor infertility (TFI), infertility controls (IFC), and fertile controls (FC). HSPs assist organisms in surviving caustic environments such as heat.

STUDY DESIGN—Twenty-one TFI, 15 IFC, and 29 FC patients were enrolled after laparoscopic tubal assessment. The titers of antibodies against *C trachomatis* organisms and 14 chlamydial HSPs were compared among the 3 groups.

RESULTS—TFI patients developed significantly higher levels of antibodies against *C trachomatis* and specifically recognizing chlamydial HSP60 and caseinolytic protease (Clp) P, a subunit of the ATP-dependent Clp protease complex involved in the degradation of abnormal proteins.

CONCLUSION—In addition to confirming high titers of antibodies against *C trachomatis* organisms and HSP60 in TFI patients, we identified a novel link of TFI with anti-ClpP antibodies. These findings may provide useful information for developing a noninvasive screening test for TFI and constructing subunit anti-*C trachomatis* vaccines.

Keywords

antibodies to caseinolytic protease P; *Chlamydia trachomatis*; heat shock protein; tubal factor infertility

Chlamydia trachomatis is the most common reported agent of sexually transmitted infections worldwide.¹ The rate of *C trachomatis* infection in the United States has increased significantly over the last 2 decades.² Infection with *C trachomatis* poses serious health risks, including long-term reproductive tract sequelae such as infertility, chronic pelvic pain, ectopic pregnancy,^{3–6} and development of cervical cancer.^{7,8}

The linkage of tubal factor infertility (TFI) to *C trachomatis* infection has been extensively studied. *C trachomatis* organisms can be isolated from a large portion of women with TFI³ and elevated anti-*C trachomatis* antibodies can be detected in more than 70% of women

with tubal occlusion.⁹ Women with prior *C trachomatis* infection usually maintain high titers of *C trachomatis* antibodies.¹⁰ Although urogenital tract infections with *C trachomatis* is common and has been recognized as a significant cause of tubal infertility, the pathogenic mechanisms of *C trachomatis*-induced tubal damage remain unknown and no effective vaccines are available.

It has been hypothesized that host responses triggered by chlamydial infection contribute to both protective immunity and pathogenesis. Antibodies against the chlamydial major outer membrane protein (MOMP) are associated with protective host immune responses, which is consistent with the recent findings that immunization with a native MOMP-induced protection.^{11,12} In contrast, antibodies to chlamydial heat shock protein (HSP) 60 are associated with pathologies,^{4,13-15} which may provide a partial explanation for the half-century-old observation that whole chlamydial organism-based vaccines designed for preventing trachoma in children actually exacerbated pathologies.¹⁶⁻¹⁸ HSPs assist organisms in surviving stressful environments such as acidity or heat.

Our objective was to test whether human antibodies against other *C trachomatis* HSPs are also associated with chlamydia-induced tubal pathologies by comparing all 14 chlamydial HSPs for their reactivity with antibodies in patients with TFI, infertility controls (IFC), and fertile controls (FC).

Materials and Methods

Human antisera

Following approval by the institutional review board at the University of Texas Health Science Center at San Antonio, 21 TFI, 15 IFC, and 29 FC patients were enrolled. All recruited women underwent diagnostic laparoscopy with chromotubation. Diagnosis of tubal infertility was based on 1 of the following findings: hydrosalpinx, fimbrial phimosis, or peritubal adhesions. Women with prior tubal ligation or a history of pelvic infection or inflammation other than pelvic inflammatory disease such as appendicitis were excluded. IFC patients were women with normal pelvic findings and tubal patency at laparoscopy. FC patients had no history of infertility with at least 1 live birth and normal pelvic findings at time of tubal ligation. All participants underwent a single blood draw. Serum samples were stored at -20°C until analyzed.

Cell culture and chlamydial infection

HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco PRL, Rockville, MD) with 10% fetal calf serum (FCS; Gibco BRL) at 37°C with 5% carbon dioxide (CO_2) as previously described.¹⁹ *C trachomatis* serovar D or *C pneumoniae* AR39 organisms were grown, purified, and titrated as previously described.^{20,21}

After titration, organisms were stored at -80°C . For immunofluorescence assay, chlamydial organisms were used to infect HeLa cells grown on glass coverslips in 24-well plates. The subconfluent HeLa cells were treated with DMEM containing $30\text{ }\mu\text{g/mL}$ of diethylaminoethyl (DEAE)-dextran (Sigma, St Louis, MO) for 10 minutes. After removal of DEAE-dextran solution, chlamydial organisms were added to the wells for 2 hours at 37°C . The infected cells were continuously cultured in DMEM with 10% FCS and $2\text{ }\mu\text{g/mL}$ of cycloheximide (Sigma).

For preparing whole-cell lysates, infection was carried out in tissue culture flasks. Infected cultures were processed or harvested 48 hours after infection or as indicated in individual experiments.

Immunofluorescence assay

Antichlamydial organism antibodies in human sera were titrated using an immunofluorescence assay as previously described.^{22,23} Briefly, HeLa cells grown on coverslips were infected with *C trachomatis* or *C pneumoniae* organisms, fixed 48 hours after infection for *C trachomatis* and 72 hours for *C pneumoniae* with 2% paraformaldehyde, and permeabilized with 2% saponin. After blocking, human antisera were added to the chlamydia-infected cell samples. Goat antihuman immunoglobulin (Ig) G conjugated with Cy2 (green; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) was used to visualize human antibody binding and a Hoechst deoxyribonucleic acid (DNA) dye (blue; Sigma) to visualize HeLa and chlamydial DNA. The highest dilution of a serum that still gave a positive reactivity was defined as the titer of the given serum sample.

All human serum samples were serially diluted, and the appropriate dilutions were repeated multiple times based on the results obtained from prior dilutions to obtain a more accurate titer for each serum. For the time-course study, the *C trachomatis*-infected HeLa cells were processed as described above at various time points after infection as indicated in the data figure.

The processed samples were coimmunostained with a mouse anti-HSP60 (unpublished data) or anti-caseinolytic protease (Clp) P (unpublished data) plus rabbit anti-*C trachomatis* serovar D organisms. The primary antibody binding was visualized with a goat antimouse IgG conjugated with Cy3 (red) and a goat antirabbit IgG conjugated with Cy2 (green; both from Jackson ImmunoResearch Laboratories), respectively, and DNA by a Hoechst DNA dye.

Images were acquired with an Olympus AX70 fluorescence microscope equipped with multiple filter sets (Olympus, Melville, NY) as previously described.²³ All microscopic images were processed using an Adobe Photoshop program (Adobe Systems, San Jose, CA).

Chlamydial fusion protein–arrayed microplate enzyme-linked immunosorbent assay (ELISA)

The glutathione *S*-transferase (GST) fusion protein ELISA for detecting human antibody recognition of chlamydial proteins was carried as previously described.²³ The bacterial lysates containing individual chlamydial GST fusion proteins were added to 96-well microplates precoated with glutathione (Pierce, Rockford, IL).

The GST fusion protein lysates included all 14 chlamydial HSP family members: GST-CT110 (GroEL, HSP60); GST-CT111 (GroES, HSP10); GST-CT113 (ClpB, ClpB-related ATP-dependent protease); GST-CT286 (ClpC, Clp protease ATP-binding subunit); GST-CT341 (DnaJ protein); GST-CT395 (GrpE, HSP70 cofactor); GST-CT396 (DnaK, HSP70); GST-CT407 (DksA, probable DnaK suppressor); GST-CT431 (ClpP, ATP-dependent ClpP endopeptidase); GST-CT604 (GroEL, HSP60); GST-CT705 (ClpX, ATP-dependent ClpX-related protease); GST-CT706 (ClpP, ATP-dependent ClpP endopeptidase subunit); GST-CT709 (MreB, Rod shape determining protein MreB/HSP70 sugar kinase); and GST-CT755 (HSP60).

Lysates containing GST alone, as negative, and GST-chlamydial protease-like activity factor, as positive controls, were also included. After blocking, human antisera preabsorbed with a bacterial lysates containing GST alone were reacted with the plate-immobilized fusion proteins. The human antibody reactivity was detected with a goat antihuman-IgG conjugated with horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories) plus the substrate 2,2'-azinobi(2-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS;

Sigma). The optical density (OD) was measured at 405 nm using a microplate reader (Molecular Devices Corp, Sunnyvale, CA).

To confirm the antibody-binding specificity, all antisera were further absorbed with lysates made from either HeLa cells alone or *C trachomatis* serovar D-infected HeLa cells prior to reacting with the fusion protein-coated plates. The antibody binding that remained positive after HeLa-alone lysate absorption but significantly reduced by chlamydia-HeLa lysate absorption was considered true positive.

Western blot

Western blot with GST fusion proteins as antigens was carried out as previously described.²⁰ GST fusion proteins (GST-HSP60, GST-HSP10, GST-ClpP) were purified from the corresponding bacterial lysates using glutathione agarose beads as previously described.²⁴ The purified fusion proteins were resolved on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. Membrane-immobilized proteins were reacted with human sera pooled from each patient group and preabsorbed with bacterial lysates containing GST alone. Human antibody binding was detected with a goat antihuman IgG-HRP secondary antibody and visualized with an enhanced chemiluminescence kit (Santa Cruz Biotechnology, Inc, Santa Cruz, CA).

Data analyses

Data were analyzed using SPSS version 15.0 software (IBM, Chicago, IL). As a preliminary step, titer values were log transformed to produce a normal distribution and analyses were performed on transformed values. Analysis of variance was used to assess anti-*C trachomatis* and anti-*C pneumoniae* antibodies to evaluate overall mean differences among the 3 groups of patients.

The Student *t* test was utilized to compare differences between groups. Because the antibody titers had large variations within a given group, the serum titers were evaluated by ranges of less than 1:10 (negative), 1:10 to 1:10,000 (low), and greater than 1:10,000 (high). The χ^2 and Fisher's exact tests were used to compare TFI, IFC, and FC overall antibodies to *C trachomatis* and antibodies to *C pneumoniae*.

Finally, we evaluated pairwise differences between TFI vs IFC, TFI vs FC, and FC vs IFC in *C trachomatis* using logistic regression. ELISA results were analyzed also using χ^2 and Fisher's exact tests as appropriate.

Results

When *C trachomatis*-infected cells were used as antigens to titrate the patient serum antibodies, the TFI group had higher titers than the IFC and FC groups (Table). The titers of anti-*C trachomatis* antibodies were significantly greater in patients with TFI. Because the antibody titers had large variations within a given group, the serum titers were evaluated by ranges of less than 1:10 (negative), 1:10 to 1:10,000 (low), and greater than 1:10,000 (high).

Further logistic regression (Table) analyses revealed significant differences between TFI and IFC in the high compared with both negative and low titers, TFI and FC in the high compared with negative titers, and IFC and FC in the high compared with both negative and low titers. The number of individuals with high anti-*C trachomatis* antibody titers in TFI group is significantly more than those in either the IFC or FC groups.

These results have demonstrated an association of TFI with anti-*C trachomatis* antibodies, which is consistent with various previous observations.²⁵ The anti-*C pneumoniae* antibody

titers among the 3 groups were not significantly different (Table 1). The high titers of anti-*C pneumoniae* antibodies in most of the patients in all 3 groups did not interfere with the measurements of anti-*C trachomatis* antibodies because high titers of anti-*C trachomatis* antibodies were detected only in most TFI patients.

Serum samples from 16 TFI, 7 IFC, and 13 FC patients with high anti-*C. trachomatis* antibodies ($\geq 1:1000$) were further evaluated in a fusion protein ELISA (Figure 1). Antibodies against ClpP were significantly higher in the TFI group as compared with the control groups. Antibodies against the remaining 12 HSPs including HSP10 were not significantly different among the groups.

We further confirmed the specificity of the human antibody binding to ClpP fusion proteins using an absorption approach (Figure 2). In addition to the preabsorption with bacterial lysates containing GST alone, the human sera from the TFI group were further absorbed with either *C trachomatis*-infected or HeLa-alone lysates prior to reacting with the fusion proteins in the ELISA assay.

Absorption with *C trachomatis*-infected HeLa lysate but not the HeLa-alone lysate completely removed ClpP-reactive antibodies from all 4 TFI antisera, demonstrating that the recognition of ClpP by the TFI antisera was specific. Binding of TFI sera to ClpP was confirmed on Western blot (data not shown).

Protein expression of ClpP and HSP60 was assessed over time in cell culture following chlamydial infection (Figure 3). HSP60 was detected as early as 12 hours after infection, whereas ClpP was expressed 24 hours after infection. Both proteins were restricted to the intracellular chlamydia inclusions and persisted throughout the infection cycle.

Comment

Heat shock proteins are stress response proteins that increase expression with stress such as temperature changes and hypoxia. HSPs are evolutionarily highly conserved and found in bacteria and humans.²⁶⁻²⁸ Antibody responses to chlamydial HSP60 and HSP10 have been linked to chlamydia-induced pathologies.²⁹ However, it was unknown whether the antibody responses to any of the remaining 12 chlamydial HSPs are also associated with chlamydial pathogenesis.

We demonstrated that TFI patients displayed significantly higher levels of anti-*C trachomatis* antibodies, whereas there was no significant difference in the anti-*C pneumoniae* antibody titers between TFI and control patients, which is consistent with what has been previously reported.¹⁰ *C pneumoniae* is a ubiquitous human respiratory pathogen. Although infection with *C pneumoniae* has been associated with both airway allergic diseases and cardiovascular pathologies, *C pneumoniae* infection has not been linked to tubal factor infertility. Indeed, we found that there were no significant differences in anti-*C pneumoniae* antibodies among the 3 groups.

This observation has not only confirmed the lack of association of *C pneumoniae* infection with TFI but also suggested that coinfection with *C pneumoniae* did not significantly affect the detection specificity when measuring anti-*C trachomatis* antibodies despite the fact that *C pneumoniae* and *C trachomatis* share a very similar genome.

Using fusion protein ELISA, we both confirmed the association of the antichlamydial HSP60 antibodies with TFI and found a new link of TFI to human antibodies against *C trachomatis* ClpP. ClpP is a proteolytic subunit of the ATP-dependent Clp protease complex. The endopeptidase Clp is also called Ti endopeptidase or ATP dependent

endopeptidase Ti, which is found in prokaryotes, chloroplasts, and mitochondria and plays an important role in the degradation of abnormal proteins. The remaining 12 HSPs were not associated with TFI in our patients.

The mechanisms on how the HSPs and their antibodies contribute to the tubal pathologies are still unknown. Some have proposed that the large amounts of bacterial HSPs secreted during infection can lead to an autoimmune response, resulting in tubal pathologies.^{30,31} Although immune dominant B cell epitopes of chlamydia HSP60 has been mapped,^{32,33} potential autoreactive epitopes have been identified³⁰ and the association between chlamydial HSP60-induced circulating autoantibodies and tubal pathologies has been established,³⁴⁻³⁶ there is still a lack of direct demonstration for a role of the HSP60 autoreactive epitopes in chlamydial pathogenesis.

Antibody responses might just indicate the presence of chlamydial antigens in the host, and it is the chlamydial antigen-induced inflammatory^{37,38} and cellular immune responses³⁹ that may be mainly responsible for causing the pathologies. Chlamydia HSP60 is a powerful inflammatory stimulus that can activate both macrophages and epithelial cells to secrete inflammatory cytokines.³⁸ HSP60 can also induce T cell responses,³⁹ which can be pathogenic, depending on the phenotype, time, and extent of the responses.⁴⁰⁻⁴²

ClpP is a proteolytic subunit of the ATP-dependent Clp protease complex. The Clp proteases represent a distinctive family of energy-dependent serine proteases that are highly conserved throughout bacteria and eukaryotes.⁴³ Chlamydial ClpP share 45% amino acid sequence identity with its homolog in humans (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence alignment analysis led to the identification of 5 distinct regions each with more than 5 identical amino acids between chlamydial and human ClpPs. These 5 stretches of sequences may serve as potential cross-reactive linear epitopes. It is possible that some of the antichlamydial ClpP human antibodies may recognize the cross-reactive epitopes and attack human ClpP in the tubal tissues.

Although our sample size is limited, the significantly elevated anti-ClpP antibodies in TFI patients may serve as a potential marker for aiding in diagnosis of chlamydia-induced tubal damage. Diagnostic laparoscopy with chromotubation is the gold standard for evaluating tubal patency in an infertility evaluation.

Hysterosalpingogram (HSG) is less invasive in evaluating tubal patency, but HSG does have inherent risks of peritonitis or endometritis. Furthermore, a metaanalysis suggests that HSG has a sensitivity of only 65% and specificity of 83% in diagnosing tubal occlusion.⁴⁴ Thus, there is an urgent need for developing a more reliable and noninvasive marker for diagnosing tubal infertility. The observation that detection of anti-*C trachomatis* antibody titers can be as good as HSG in diagnosing tubal occlusion⁴⁵ suggests that chlamydial proteinspecific antibodies can be explored for predicting TFI.

Efforts have been made to use antibodies against chlamydial HSPs for screening for TFI.⁴⁵⁻⁴⁷ In the population recruited into the current pilot study, the anti-HSP60 antibodies can be used to detect TFI with 56% sensitivity and 95% specificity (Figure 1). Inclusion of anti-ClpP antibody detection would increase the sensitivity of this screening test to 69%. The negative predictive value of using ClpP plus HSP60 is 79% and the positive predictive value is 92%. Thus, anti-ClpP antibody may prove to be a valuable marker for improving both detection sensitivity and specificity for the antibody-based diagnosis of tubal occlusion.

The discovery of a unique marker for detecting TFI using the limited number of chlamydial fusion proteins has encouraged us to expand the scope of our assay to include the entire genome. We obtained fusion protein clones covering all open reading frames encoded by *C*

trachomatis genome and plasmid and are in the process of preparing a whole-genome scale proteome ELISA for screening the TFI patient sera as more patient sera are obtained. We hope to use the whole genome scale approach to identify additional unique markers for TFI so that we can further improve the detection specificity and sensitivity of the antibody-based diagnostic approach.

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REFERENCES

- Centers for Disease Control and Prevention. Sexually transmitted disease surveillance, 2007. U.S. Department of Health and Human Services; Atlanta, GA: December. 2008
- Centers for Disease Control and Prevention. Sexually transmitted disease surveillance 2007 supplement, Chlamydia Prevalence Monitoring Project Annual Report 2007. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention; Atlanta, GA: January. 2009
- Keilani A, Bouliou D, Raudrant D, Carraz M, Quenin P. [Role of Chlamydia trachomatis in tubal pathology (acute salpingitis and tubal sterility). Microbiological study of 175 samples of peritoneal fluid]. J Gynecol Obstet Biol Reprod (Paris). 1989; 18:167–72. [PubMed: 2723346]
- Arno JN, Yuan Y, Cleary RE, Morrison RP. Serologic responses of infertile women to the 60-kd chlamydial heat shock protein (hsp60). Fertil Steril. 1995; 64:730–5. [PubMed: 7672143]
- Cates W Jr, Wasserheit JN. Genital chlamydial infections: epidemiology and reproductive sequelae. Am J Obstet Gynecol. 1991; 164:1771–81. [PubMed: 2039031]
- Wolner-Hanssen P. Silent pelvic inflammatory disease: is it overstated? Obstet Gynecol. 1995; 86:321–5. [PubMed: 7651634]
- Anttila T, Saikku P, Koskela P, et al. Serotypes of Chlamydia trachomatis and risk for development of cervical squamous cell carcinoma. JAMA. 2001; 285:47–51. [PubMed: 11150108]
- Paavonen J, Karunakaran KP, Noguchi Y, et al. Serum antibody response to the heat shock protein 60 of Chlamydia trachomatis in women with developing cervical cancer. Am J Obstet Gynecol. 2003; 189:1287–92. [PubMed: 14634555]
- Moore DE, Spadoni LR, Foy HM, et al. Increased frequency of serum antibodies to Chlamydia trachomatis in infertility due to distal tubal disease. Lancet. 1982; 2:574–7. [PubMed: 6125729]
- Persson K, Osseer S, Birkelund S, Christiansen G, Brade H. Antibodies to Chlamydia trachomatis heat shock proteins in women with tubal factor infertility are associated with prior infection by C. trachomatis but not by C. pneumoniae. Hum Reprod. 1999; 14:1969–73. [PubMed: 10438411]
- Kari L, Whitmire WM, Crane DD, et al. Chlamydia trachomatis native major outer membrane protein induces partial protection in nonhuman primates: implication for a trachoma transmission-blocking vaccine. J Immunol. 2009; 182:8063–70. [PubMed: 19494332]
- Pal S, Peterson EM, de la Maza LM. Vaccination with the Chlamydia trachomatis major outer membrane protein can elicit an immune response as protective as that resulting from inoculation with live bacteria. Infect Immun. 2005; 73:8153–60. [PubMed: 16299310]
- Zhang YX, Stewart SJ, Caldwell HD. Protective monoclonal antibodies to Chlamydia trachomatis serovar- and serogroup-specific major outer membrane protein determinants. Infect Immun. 1989; 57:636–8. [PubMed: 2463971]
- Brunham RC, Peeling RW. Chlamydia trachomatis antigens: role in immunity and pathogenesis. Infect Agents Dis. 1994; 3:218–33. [PubMed: 7866655]
- Zhong G, Berry J, Brunham RC. Antibody recognition of a neutralization epitope on the major outer membrane protein of Chlamydia trachomatis. Infect Immun. 1994; 62:1576–83. [PubMed: 7513303]

16. Grayston JT, Wang SP, Woolridge RL, Alexander ER. Prevention of trachoma with vaccine. *Arch Environ Health*. 1964; 8:518–26. [PubMed: 14107660]
17. Grayston JT, Woolridge RL, Wang S. Trachoma vaccine studies on Taiwan. *Ann N Y Acad Sci*. 1962; 98:352–67. [PubMed: 13901337]
18. Rockey DD, Wang J, Lei L, Zhong G. Chlamydia vaccine candidates and tools for chlamydial antigen discovery. *Expert Rev Vaccines*. 2009; 8:1365–77. [PubMed: 19803759]
19. Zhong G, Fan P, Ji H, Dong F, Huang Y. Identification of a chlamydial protease-like activity factor responsible for the degradation of host transcription factors. *J Exp Med*. 2001; 193:935–42. [PubMed: 11304554]
20. Li Z, Chen D, Zhong Y, Wang S, Zhong G. The chlamydial plasmid-encoded protein pgp3 is secreted into the cytosol of Chlamydia-infected cells. *Infect Immun*. 2008; 76:3415–28. [PubMed: 18474640]
21. Luo J, Liu G, Zhong Y, et al. Characterization of hypothetical proteins Cpn0146, 0147, 0284 and 0285 that are predicted to be in the Chlamydia pneumoniae inclusion membrane. *BMC Microbiol*. 2007; 7:38. [PubMed: 17504539]
22. Wang J, Chen L, Chen F, et al. A chlamydial type III-secreted effector protein (Tarp) is predominantly recognized by antibodies from humans infected with Chlamydia trachomatis and induces protective immunity in mice against inflammatory pathologies in the upper genital tract. *Vaccine*. 2009; 27:2967–80. [PubMed: 19428908]
23. Sharma J, Zhong Y, Dong F, Piper JM, Wang G, Zhong G. Profiling of human antibody responses to Chlamydia trachomatis urogenital tract infection using microplates arrayed with 156 chlamydial fusion proteins. *Infect Immun*. 2006; 74:1490–9. [PubMed: 16495519]
24. Sharma J, Dong F, Pirbhai M, Zhong G. Inhibition of proteolytic activity of a chlamydial proteasome/protease-like activity factor by antibodies from humans infected with Chlamydia trachomatis. *Infect Immun*. 2005; 73:4414–9. [PubMed: 15972540]
25. Toye B, Laferriere C, Claman P, Jessamine P, Peeling R. Association between antibody to the chlamydial heat-shock protein and tubal infertility. *J Infect Dis*. 1993; 168:1236–40. [PubMed: 7901289]
26. Singh SK, Grimaud R, Hoskins JR, Wickner S, Maurizi MR. Unfolding and internalization of proteins by the ATP-dependent proteases ClpXP and ClpAP. *Proc Natl Acad Sci USA*. 2000; 97:8898–903. [PubMed: 10922052]
27. Ellis RJ. Molecular chaperones: assisting assembly in addition to folding. *Trends Biochem Sci*. 2006; 31:395–401. [PubMed: 16716593]
28. Neuer A, Spandorfer SD, Giraldo P, Dieterle S, Rosenwaks Z, Witkin SS. The role of heat shock proteins in reproduction. *Hum Reprod Update*. 2000; 6:149–59. [PubMed: 10782573]
29. Peeling RW, Kimani J, Plummer F, et al. Antibody to chlamydial hsp60 predicts an increased risk for chlamydial pelvic inflammatory disease. *J Infect Dis*. 1997; 175:1153–8. [PubMed: 9129079]
30. Campanella C, Gammazza A, Marino A, Mularoni L, Cappello F, Zummo G, Di Felice V. A comparative analysis of the products of GROEL-1 gene from Chlamydia trachomatis serovar D and the HSP60 var1 transcript from Homo sapiens suggests a possible autoimmune response. *Int J Immunogenet*. 2009; 36:73–8. [PubMed: 19207939]
31. Cappello F, de Macario E, Conway, Di Felice V, Zummo G, Macario AJ. Chlamydia trachomatis infection and anti-Hsp60 immunity: the two sides of the coin. *PLoS Pathog*. 2009; 5:e1000552. [PubMed: 19714222]
32. Yi Y, Zhong G, Brunham RC. Continuous B-cell epitopes in Chlamydia trachomatis heat shock protein 60. *Infect Immun*. 1993; 61:1117–20. [PubMed: 7679373]
33. Zhong G, Brunham RC. Antibody responses to the chlamydial heat shock proteins hsp60 and hsp70 are H-2 linked. *Infect Immun*. 1992; 60:3143–9. [PubMed: 1639484]
34. Witkin SS, Askienazy-Elbhar M, Henry-Suchet J, Belaisch-Allart J, Tort-Grumbach J, Sarjdine K. Circulating antibodies to a conserved epitope of the Chlamydia trachomatis 60 kDa heat shock protein (hsp60) in infertile couples and its relationship to antibodies to C. trachomatis surface antigens and the Escherichia coli and human HS P60. *Hum Reprod*. 1998; 13:1175–9. [PubMed: 9647542]

35. Yi Y, Yang X, Brunham RC. Autoimmunity to heat shock protein 60 and antigen-specific production of interleukin-10. *Infect Immun*. 1997; 65:1669–74. [PubMed: 9125545]
36. Domeika M, Domeika K, Paavonen J, Mardh PA, Witkin SS. Humoral immune response to conserved epitopes of *Chlamydia trachomatis* and human 60-kDa heat-shock protein in women with pelvic inflammatory disease. *J Infect Dis*. 1998; 177:714–9. [PubMed: 9498452]
37. den Hartog JE, Morre SA, Land JA. *Chlamydia trachomatis*-associated tubal factor subfertility: immunogenetic aspects and serological screening. *Hum Reprod Update*. 2006; 12:719–30. [PubMed: 16832042]
38. Bulut Y, Faure E, Thomas L, et al. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J Immunol*. 2002; 168:1435–40. [PubMed: 11801686]
39. Ramage JM, Young JL, Goodall JC, Gaston JS. T cell responses to heat-shock protein 60: differential responses by CD4+ T cell subsets according to their expression of CD45 isotypes. *J Immunol*. 1999; 162:704–10. [PubMed: 9916689]
40. Chen L, Lei L, Chang X, et al. Mice deficient in MyD88 develop a Th2-dominant response and severe pathology in the upper genital tract following *Chlamydia muridarum* infection. *J Immunol*. 2010; 184:2602–10. [PubMed: 20124098]
41. Igietseme JU, He Q, Joseph K, et al. Role of T lymphocytes in the pathogenesis of *Chlamydia* disease. *J Infect Dis*. 2009; 200:926–34. [PubMed: 19656067]
42. Kinnunen AH, Surcel HM, Lehtinen M, et al. HLA DQ alleles and interleukin-10 polymorphism associated with *Chlamydia trachomatis*-related tubal factor infertility: a case-control study. *Hum Reprod*. 2002; 17:2073–8. [PubMed: 12151439]
43. Yu AY, Houry WA. ClpP: a distinctive family of cylindrical energy-dependent serine proteases. *FEBS Lett*. 2007; 581:3749–57. [PubMed: 17499722]
44. Swart P, Mol BW, van der Veen F, van Beurden M, Redekop WK, Bossuyt PM. The accuracy of hysterosalpingography in the diagnosis of tubal pathology: a meta-analysis. *Fertil Steril*. 1995; 64:486–91. [PubMed: 7641899]
45. Mol BW, Dijkman B, Wertheim P, Lijmer J, van der Veen F, Bossuyt PM. The accuracy of serum chlamydial antibodies in the diagnosis of tubal pathology: a meta-analysis. *Fertil Steril*. 1997; 67:1031–7. [PubMed: 9176440]
46. Dadamessi I, Eb F, Betsou F. Combined detection of *Chlamydia trachomatis*-specific antibodies against the 10 and 60-kDa heat shock proteins as a diagnostic tool for tubal factor infertility: results from a case-control study in Cameroon. *FEMS Immunol Med Microbiol*. 2005; 45:31–5. [PubMed: 15985220]
47. Claman P, Honey L, Peeling RW, Jessamine P, Toye B. The presence of serum antibody to the chlamydial heat shock protein (CHSP60) as a diagnostic test for tubal factor infertility. *Fertil Steril*. 1997; 67:501–4. [PubMed: 9091338]

FIGURE 1. Reactivity of human antibodies with chlamydial fusion proteins arrayed to microplate wells
The bacterial lysates containing individual chlamydial GST fusion proteins or GST alone (listed along the X-axis) were directly added to glutathione-coated microplates. Human antisera from 3 groups of patients (listed along the Y-axis) were first preabsorbed with bacterial lysates containing GST alone and then reacted with the plate-immobilized chlamydial fusion proteins. The human antibody binding was detected with a goat antihuman IgG conjugated with HRP plus the soluble substrate ABTS (Sigma, St Louis, MO) and measured in OD values at 405 nm. A reaction with an OD value of 2 SD about the mean was considered positive as indicated with *horizontal bars*. The number of positive individuals from different groups of patients was compared with Pearson's χ^2 test. The number of sera that positively recognized HSP60 ($P = .001$) or ClpP ($P = .03$) was significantly higher in the TFI group when compared with either the IFC or the FC groups. *ClpP*, caseinolytic protease P; *FC*, fertile controls; *GST*, glutathione *S*-transferase; *HSP*, heat shock proteins; *IFC*, infertility controls; *OD*, optical density; *SD*, standard deviation; *TFI*, tubal factor infertility.

FIGURE 2. Absorption of human sera with endogenous *C trachomatis* antigens blocks the binding of human antibodies to chlamydial fusion proteins

The bacterial lysates containing individual chlamydial GST fusion proteins or GST alone (as listed along the *left side* of the figure) were allowed to bind to microplates, and the ELISA was carried out as described in the legend for Figure 1. The 4 human antisera from the TFI group as listed on *top* of the figure were preabsorbed with bacterial lysates containing GST alone and then further absorbed with either HeLa alone or chlamydia-infected HeLa lysates prior to reacting with the chlamydial fusion proteins on the microplate. Please note that none of the 4 sera bound to the other subunit of the ClpP complex (GST-CT706) and the binding of the 4 sera to both GST-CT431 and GST-CT858 was completely blocked by absorption with the chlamydia-infected but not HeLa alone lysates.

ClpP, caseinolytic protease P; *ELISA*, enzyme-linked immunosorbent assay; *GST*, glutathione *S*-transferase; *TFI*, tubal factor infertility.

FIGURE 3. Expression of CT110 (HSP60) and CT431 (ClpP) during *C. trachomatis* infection
 HeLa cells grown on coverslips were infected with *C. trachomatis* serovar D organisms, and at various times after infection as listed on the top of the figure, the infected cultures were processed for immunofluorescence labeling with mouse antibodies against HSP60 or ClpP (red). The samples were colabeled with an anti-MOMP antibody (green) and a DNA dye (blue). Please note that HSP60 was detected as early as 12 hours (yellow = red overlapping with green), whereas ClpP was detected only by 24 hours (white arrows).
ClpP, caseinolytic protease P; *HSP*, heat shock proteins; *MOMP*, major outer membrane protein.

TABLE 1

Titers of human antibodies against *C trachomatis* and *C pneumoniae*

Variable	Antibodies to <i>C trachomatis</i>			Antibodies to <i>C pneumoniae</i>		
	TFI (n = 21)	IFC (n = 15)	FC (n = 29)	TFI (n = 21)	IFC (n = 15)	FC (n = 29)
Mean	90,199	4488	36,994	56,010	32,027	56,429
SD	123,700	10,110	83,570	75,580	41,930	65,140
ANOVA	<i>P</i> = .018			<i>P</i> = .45		
Student <i>t</i> test	TFI vs IFC, <i>P</i> = .012					
	TFI vs FC, <i>P</i> = .075					
	IFC vs FC, <i>P</i> = .142					
Categorization of serum samples into negative, low, and high titer groups						
Negative titers (<1:10)	1 (5%)	2 (13%)	1 (3%)	3 (14%)	4 (27%)	0 (0%)
Low titers (1:10-1:10,000)	6 (29%)	12 (80%)	15 (52%)	3 (14%)	1 (7%)	5 (17%)
High titers (>1:10,000)	14 (67%)	1 (7%)	13 (45%)	15 (71%)	10 (67%)	24 (83%)
χ^2 test	<i>P</i> = .009			<i>P</i> = .09		
Logistic regression	TFI vs IFC	High vs negative		<i>P</i> = .04		
	TFI vs IFC	High vs low		<i>P</i> = .004		
	TFI vs FC	High vs low		<i>P</i> = .03		
	FC vs IFC	High vs negative		<i>P</i> = .04		
	FC vs IFC	High vs low		<i>P</i> = .03		

Serum samples from women with TFI, IFC, or FC were 2-fold serially diluted starting with 1:10 and reacted with HeLa cells infected with either *C trachomatis* or *C pneumoniae*. The highest dilution that still gave a positive reactivity was defined as the serum titer. Each serum sample was titrated 3 times, and the average from the 3 independent titrations was used as the geometric titer of a given serum sample. ANOVA was used to analyze the overall differences among the 3 groups. There is a statistically significant difference in titers of antibodies against *C trachomatis* ($P = .018$) but not *C pneumoniae* ($P = .45$) organisms. The significant difference was determined between the TFI and IFC groups by Student *t* test ($P = .012$). When the serum samples were divided into 3 categories (negative, low, and high) based on antibody titers, the χ^2 test still revealed a significant difference in the number of sera in different categories among the 3 groups of patients for antibodies against *C trachomatis* ($P = .009$) but not *C pneumoniae* ($P = .09$) organisms. Further logistic regression analyses of the anti-*C trachomatis* antibodies revealed significant differences between the TFI and IFC, TFI and FC, and the IFC and FC groups. The number of individuals with high anti-*C trachomatis* antibody titers in the TFI group is significantly higher than those in either the IFC or FC groups, although there are also differences between the IFC and FC groups.

ANOVA, analysis of variance; FC, fertile controls; IFC, infertility controls; TFI, tubal factor infertility.