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Genome-Wide Identification of *Chlamydia trachomatis* Antigens Associated with Tubal Factor Infertility

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Abstract

Objective—To identify *C. trachomatis* antigens that can be used to differentially diagnose tubal factor infertility in comparison to previously reported Heat Shock Protein 60 (HSP60).

Design—*In Vitro* Study

Patients—Infertile women with and without tubal pathology diagnosed laparoscopically.

Setting—Academic medical center.

Main Outcome Measures—Antibody responses to *C. trachomatis* in infertile women with or without tubal pathologies using a *C. trachomatis* genome-wide proteome array.

Results—Comparison of the antibody profiles revealed 30 *C. trachomatis* antigens that were preferentially recognized by tubal factor infertility women with a detection sensitivity and specificity of 80.6% and 56.5%, respectively, 10 of which showed 100% specificity. A combination of CT443 and CT381 antigens yielded the highest detection sensitivity (67.7%) while maintaining 100% specificity.

Conclusion—These findings have demonstrated that antibodies to CT443 and CT381, when used in combination, have higher sensitivity and specificity in predicting tubal factor infertility than other indicators for tubal factor infertility such as HSP60 antibodies (35.5%, 100%) or hysterosalpingogram (65%, 83%). Using a panel of *C. trachomatis* antigens to serologically diagnose tubal factor infertility can save the patients from undertaking expensive and invasive procedures for determining tubal pathology and choosing treatment plans.

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Keywords

Tubal factor infertility; *Chlamydia trachomatis*; Heat Shock Protein 60; Outer Membrane Protein Complex B

Introduction

Twenty-five to 35% of patients presenting for infertility evaluation have tubal disease (1-4). *Chlamydia trachomatis* is the primary sexually transmitted infection responsible for tubal factor infertility (TFI) (5-7) with *C. trachomatis antibodies* in approximately 70% of patients (8). *C. trachomatis* infected cells produce inflammatory cytokines (9, 10) which may contribute to upper genital tract inflammatory damage (11-13). Lunefeld, et al found that among patients undergoing *in vitro* fertilization, those with *C. trachomatis* antibodies had decreased pregnancy rates (14).

C. trachomatis infection is often asymptomatic so patient history cannot dictate the presence of tubal disease (15, 16). Searching for biomarkers to predict chlamydial infection-associated tubal infertility is under intensive investigation. Elevated titers of anti-*C. trachomatis* antibodies are associated with TFI, but detection of overall antibody levels lacks the sensitivity and specificity required for differential diagnosis (17).

Measuring anti-*C. trachomatis* antibodies at the single antigen level may offer increased sensitivity and specificity for predicting TFI. Elevated anti-chlamydial heat shock protein 60 (HSP60, CT110) antibodies are associated with TFI (18-27). Anti-HSP60 antibodies are associated with decreased pregnancy rates in patients with an ectopic pregnancy history (17). When HSP60 antibodies are in follicular fluid, there are decreased implantation rates (28, 29). Some have postulated that chlamydial HSP60 incites a strong inflammatory response that may cross-react with the highly conserved human HSP60 (25, 30, 31). HSP60 may induce T-cell responses that contribute to the tubal damage (32, 33). Regardless of how HSP60 or anti-HSP60 antibodies can mechanistically contribute to tubal disease, the specificity of anti-HSP60 antibody as a predictor for TFI has demonstrated that it may be used to differentially diagnose TFI.

We have developed a *C. trachomatis* whole-genome scale protein array that can profile antigen specificities of anti-*Chlamydia trachomatis* antibodies (34). We hypothesized that *C. trachomatis*-infected women with TFI or controls (IFC) may develop antibodies recognizing unique sets of antigens.

Materials and Methods

Human patients

31 TFI and 23 IFC patients were enrolled at the University of Texas Health Science Center at San Antonio following Institutional Review Board approval. All women were at least 21 years old and underwent diagnostic laparoscopy with chromotubation as part of their infertility evaluation. Diagnosis of tubal infertility was defined as fallopian pathology consistent with hydrosalpinx, fimbrial phimosis, or peri-tubal adhesions. Exclusion criteria included prior tubal ligation, surgical finding of endometriosis, or a history of pelvic infection or inflammation other than pelvic inflammatory disease such as appendicitis. IFC patients had normal pelvic findings and tubal patency at laparoscopy. After the blood draw, serum samples were stored at −20°C until analyzed.

Cell culture and chlamydial infection

As previously described, HeLa cells (American Type Culture Collection, Manassas VA 20108) were cultured in DMEM (GIBCO PRL, Rockville, MD) with 10% fetal calf serum (FCS; GIBCO BRL) at 37°C with 5% Carbon dioxide (CO_2) (34-36). *C. trachomatis* serovar D or *Chlamydia pneumoniae* AR39 organisms were grown, purified and titrated as previously described (36-38). For immunofluorescence assay, chlamydial organisms were used to infect HeLa cells grown on glass coverslips in 24-well plates. The sub-confluent HeLa cells were treated with DMEM containing 30 μg/ml of DEAE-Dextran (Sigma, St. Louis, MO) for 10 minutes at 37°C. After removal of DEAE-Dextan 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 2μg/ml of cycloheximide (Sigma, St. Louis, MO).

Immunofluorescence assay (IFA)

Anti-chlamydial organism antibodies in human sera were titrated using an Immunofluorescence assay (IFA) as previously described (34, 36, 39, 40). Briefly, HeLa cells grown on coverslips were infected with *C. trachomatis* or *C. pneumoniae* organisms, fixed 48h post-infection for *C. trachomatis* and 72h for *C. pneumoniae* with 2% paraformaldehyde, and permeabilized with 2% saponin at room temperature for 1 hour. After blocking, human sera were added to the Chlamydia-infected cell samples. The primary Ab binding was visualized with a goat anti-human IgG conjugated with Cy3 (red; Jackson ImmunoResearch Laboratories, West Grove, PA), and DNA was labeled with Hoechst dye (blue; Sigma-Aldrich). The highest dilution of a serum that still gave a positive reactivity was defined as the titer of the given serum sample. Serum samples were serially diluted and the appropriate dilutions were repeated multiple times based on the results obtained from prior dilutions in order to obtain a more accurate titer for each serum. Images were acquired with an Olympus AX70 fluorescence microscope equipped with multiple filter sets (Olympus, Melville, NY) as previously described (36, 40).

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

Glutathione *S*-transferase (GST) fusion protein enzyme-linked immunosorbent assay (ELISA) for detecting human antibody recognition of chlamydial proteins was carried out as previously described (36). Bacterial lysates containing individual chlamydial GST fusion proteins were added to 96 well microplates pre-coated with glutathione (Pierce, Rockford, IL) at a 1:10 dilution in PBS with a total volume of 200 μl/well. Lysates containing GST alone, as negative, and GST-chlamydial protease-like activity factor (CPAF), as positive controls, were also included on each plate. The plates were incubated overnight at 4°C to allow GST fusion proteins to bind to the plate-immobilized glutathione then blocked with 2.5% milk in PBS and washing with PBST (PBS with 0.05% Tween 20; Sigma-Aldrich).

The human sera was pre-absorbed with a bacterial lysates containing GST at 4°C overnight, then incubated with Glutathione beads (bioWorld, Dublin, OH) for 1 hour at room temperature to reduce background caused by non-specific human antibodies. The human antibody reactivity was detected with a goat anti-human-IgG, IgA & IgM conjugated with horse-radish peroxidase (HRP; Jackson ImmunoResearch Laboratories) plus the substrate 2,2′-azino-bi(2-ethylbenzothiazoline-6-sulforic acid) diammonium salt (ABTS; Sigma). The optical density (OD) was measured at 405nm using a microplate reader (Molecular Devices Corporation, Sunnyvale, CA). To confirm the antibody binding specificity, all sera 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 absorption was carried out as following: HeLa cells with or without chlamydial infection were lysed via sonication at 2×10^7 cells per ml of PBS containing a cocktail of protease inhibitors. The

pre-diluted serum samples were incubated with cell lysates overnight at 4°C prior to reacting with the plate-immobilized chlamydial fusion proteins. The antibody binding that remained positive after HeLa-alone lysate absorption but significantly reduced by Chlamydia-HeLa lysate absorption was considered true positive.

Data Analyses

Data were analyzed using SPSS v. 15.0 software (IBM, Chicago, IL) as previously described (36, 39). Briefly, titer values were log-transformed to produce a normal distribution and analyses were performed on transformed values. Student's *t*-Test was utilized to assess anti-*C. trachomatis* and anti-*C. pneumoniae* antibodies to evaluate overall mean differences between the 2 groups of patients. Because the a priori hypothesis was that the TFI group would have higher titers than the IFC group, a one-tailed analysis was used for the *C. trachomatis* data, but a two-tailed analysis was performed on the *C. pneumonia* because there was no a priori hypothesis. Because the antibody titers had large variations within a given group, the serum titers were evaluated by ranges of <1:10 (Negative), 1:10 to 1:10,000 (Low), and >1:10,000 (High). Chi-Squared and Fisher's Exact Test were employed to compare overall antibodies to *C. trachomatis* and antibodies to *C. pneumoniae*.

ELISA results were analyzed using Student's *t*-Test and Fisher's Exact Test as appropriate. For the genome-wide ELISA, both Student's *t*-Test (for comparing quantitative OD value data) and Fisher's Exact Test (for comparing the number of sera positively reacted with a given antigen) were preformed. Using both methods allows us to identify C. trachomatis antigens that are both clinically and statistically significant. When Student's *t*-Test was utilized, the OD values after subtracting background from the same plate were used. When Fisher's Exact Test was utilized, a response was determined positive when the OD value was equal to or greater than 2 standard deviations above the mean calculated from the same 96 well plate as described previously (39, 41).

Results

I. Infertile women with laparoscopy-identified tubal pathologies developed significantly higher titers of anti-*C. trachomatis* antibodies

Sera from TFI or IFC were titrated using HeLa cells infected with either *C. trachomatis* or *C. pneumoniae* organisms as antigens in an IFA. TFI Patients developed high titers of antibodies to *C. trachomatis* ($p<0.001$) but not *C. pneumoniae* ($p=0.269$) (Table 1). When the patients were categorized based on levels of anti-chlamydial antibodies, most TFI patients developed high titers of anti-*C. trachomatis* antibodies (61.3%) while most IFC patients displayed lower titers (82.6%; $p<0.001$).

II. Identification of *C. trachomatis* antigens preferentially recognized by infertile women with or without tubal pathology

It is difficult to use the quantitative difference in overall anti-*C. trachomatis* antibodies to diagnose TFI. To identify antigens that are recognized by TFI patients, Anti-*C. trachomatis* antibodies in 24 TFI and 11 IFC patients were mapped at the genome-wide scale since these patients displayed an overall anti-*C. trachomatis* antibody titer above 1:1000. As shown in Fig.1, these 35 sera recognized *C. trachomatis* antigens distributed across the genome (panel A) with 265 antigens recognized by at least one antiserum and 643 antigens not detected by any sera (B). Many *C. trachomatis* antigens are recognized by both groups of patients, but there are antigens preferentially recognized by either group as highlighted with color-coded brackets (C). Thirty antigens were significantly recognized by TFI based on either mean OD values (Student's *t*-test) or recognition frequency (Fisher's exact test). Reactivity was

confirmed using absorption against either HeLa alone or *C. trachomatis*-infected HeLa lysates as described previously (36).

III. Identification of *C. trachomatis* antigens uniquely recognized by TFI patients

To identify antigens that can be used to predict TFI in infertility clinics, we identified antigens that were uniquely recognized by TFI patients. The 30 antigens preferentially recognized by TFI patients (as described above) were reacted with sera from all 54 patients (including 31 TFI and 23 IFC) regardless of their overall anti-*C. trachomatis* antibody titers. As listed in Figure 2, the HSP60 (CT110) reacted with 22 of the 31 TFI and 4 of 23 IFC sera with a specificity and sensitivity of 82.6% and 71.0% respectively in predicting TFI.

To further increase specificity, a 5-fold dilution (final dilution of 1:4000) was used to reduce the false-positive rate (Figure 2). At this dilution, 10 of the 30 antigens, including CT110, CT322, CT376, CT381, CT414, CT443, CT681, CT795, CT798 & CT813, failed to react with any sera from the IFC group. Thus, these 10 antigens were uniquely recognized by TFI patients with a detection specificity of 100% (panel A). Dilution of the samples decreased detection sensitivity. HSP60 (CT110) only reacted with 11 out of 31 TFI sera, dropping the detection sensitivity to 35.5% along with three immunodominant antigens (CT795, CT798, CT813). CT443 reacted with 18 of the 31 TFI sera, maintaining a sensitivity of 58.1%. When the reactivity of the 10 antigens was analyzed at each individual antiserum level (Panel B), we found that the 10 antigens all together reacted with 21 independent sera of 31 total TFI sera, with a sensitivity of 67.7%. More importantly, this sensitivity can be maintained using fewer antigens. Combining HSP60 with CT376, CT381 & CT798 (total 4 antigens) or CT443 with CT381 (only 2 antigens) maintains sensitivity of 67.7%. As for other immunodominant antigens with a detection specificity of <100%, their detection sensitivity can be 80% even after serum dilution. Due to their ability to generating false positive results by reacting with IFC samples, it is clinically undesirable to use these antigens for screening for TFI.

Discussion

C. trachomatis organisms cause pathologies in the fallopian tubes, leading to complications such as ectopic pregnancy and infertility. Since infertility can be caused by many different factors, distinguishing tubal infertility from other causes is required for guiding treatment plans. There is an ongoing debate on whether *C. trachomatis*-specific serology can aid in differential diagnosis of TFI. The goal of the current study is to test whether we can identify *C. trachomatis* antigens that can improve specificity and sensitivity in detecting TFI.

The finding that anti-*C. trachomatis* but not anti-*C. pneumoniae* antibodies are highly associated with TFI is consistent with a well-established concept in the literature (42-44). Efforts have been made to develop individual *C. trachomatis* antigen-based detection methods. Previous reports demonstrate that anti-HSP60 antibodies are detected in 70-80% of TFI patients (21, 22, 45, 46). Our genome-wide search for additional markers of TFI not only confirmed these findings but also revealed new information for further increasing specificity and sensitivity in detecting TFI.

30 antigens were preferentially recognized by TFI patients. At 1:800 dilution, HSP60 reacted with 22 of the 31 TFI sera (71% sensitivity) and 4 of the 23 IFC sera (82.6% specificity), which is consistent with previous findings and suggests our ELISA is comparable to other assays. When the sera were diluted to 1:4000 in order to further increase specificity, 10 of the 30 antigens achieved 100% specificity. Although the sensitivity decreased, careful examination revealed that a combination of 2 antigens [CT381 and CT443 (Outer membrane complex B, OmcB)], or 4 antigens [CT110 (HSP60), CT376,

CT381 & CT798] detected TFI with a specificity and sensitivity of 100% and 67.7% respectively (Fig. 2). We conclude that these combinations of antigens improved the *C. trachomatis* serology approach for diagnosing tubal infertility over using HSP60 alone (35.5% sensitivity), which represents a clinically significant improvement.

Hysterosalpingogram (HSG) has a detection specificity and sensitivity of 83% and 65% respectively for detecting tubal pathology (47). *C. trachomatis* antigen-based serology diagnosis has numerous advantages over HSG besides improved detection, including sparing patients from the discomfort, radiation, and potential for infectious sequellae. This conclusion is consistent with previous reports that elevated chlamydial antibody levels are comparable to HSG (48) in diagnosing TFI and that HSG does not add to the medical knowledge on whether *C. trachomatis* infection contribute to tubal pathology (49).

It is unknown whether these antigens themselves or antigen-specific immune responses contribute to the inflammatory pathologies in the fallopian tubes. The protein CT443, or OmcB, displayed the highest rate of reactivity with TFI patient sera. OmcB is a highly conserved immunodominant antigen, but the precise location of OmcB in the organisms and its role during infection is poorly understood (34, 50-52).

Despite the overwhelming evidence of *C. trachomatis* infection association with TFI, not every patient is infected with *C. trachomatis* or developed immune responses to *C. trachomatis*. Interventions such as early antibiotic therapy may cause a negative or low titers in patients, but it is unlikely that tubal pathology would be attributed to the C. trachomatis infection in these patients. Tubal pathology in TFI patients without positive *C. trachomatis* titers might be caused by other sources of infection such as *Neisseria gonorrhoeae* (53, 54) and *Mycoplasma genitalium* (55, 56). Thus, to further increase the sensitivity in diagnosing TFI, other infection causes should also be taken into account.

The current study has presented a promising approach for globally identifying *C. trachomatis*-specific serological markers for diagnosis of TFI, Although statistically, the identified antigens or their combinations seem to provide definitive diagnosis of TFI, large sample sizes of infertile women from more diverse sources must be analyzed with a genome-wide scale proteome assay to maximize the potential of the *C. trachomatis* antigen-specific serology-based diagnosis of TFI. Efforts are underway to expand our studies to China where we hope to enroll several thousand patients.

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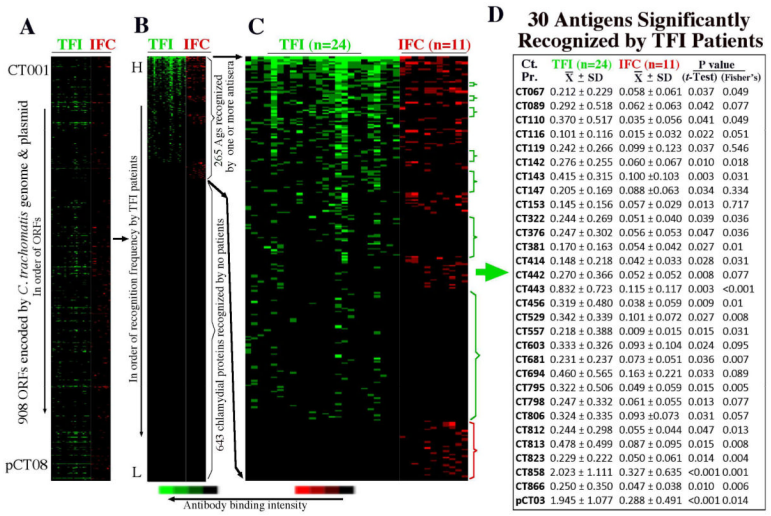
Capsule

Chlamydia trachomatis proteome array identified 30 antigens which are preferentially recognized by tubal factor infertility patients compared to controls. Combining CT443 and CT381 has 100% specificity and 67.7% sensitivity.



Ct. Pr.	TFI (n=24) $\bar{X}$ ± SD	IFC (n=11) $\bar{X}$ ± SD	P value (t-Test)	P value (Fisher's)
CT067	0.212 ± 0.229	0.058 ± 0.061	0.037	0.049
CT089	0.292 ± 0.518	0.062 ± 0.063	0.042	0.077
CT110	0.370 ± 0.517	0.035 ± 0.056	0.041	0.049
CT116	0.101 ± 0.116	0.015 ± 0.032	0.022	0.051
CT119	0.242 ± 0.266	0.099 ± 0.123	0.037	0.546
CT142	0.276 ± 0.255	0.060 ± 0.067	0.010	0.018
CT143	0.415 ± 0.315	0.100 ±0.103	0.003	0.031
CT147	0.205 ± 0.169	0.088 ±0.063	0.034	0.334
CT153	0.145 ± 0.156	0.057 ± 0.029	0.013	0.717
CT322	0.244 ± 0.269	0.051 ± 0.040	0.039	0.036
CT376	0.247 ± 0.302	0.056 ± 0.053	0.047	0.036
CT381	0.170 ± 0.163	0.054 ± 0.042	0.027	0.01
CT414	0.148 ± 0.218	0.042 ± 0.033	0.028	0.031
CT442	0.270 ± 0.366	0.052 ± 0.052	0.008	0.077
CT443	0.832 ± 0.723	0.115 ± 0.117	0.003	<0.001
CT456	0.319 ± 0.480	0.038 ± 0.059	0.009	0.01
CT529	0.342 ± 0.339	0.101 ± 0.072	0.027	0.008
CT557	0.218 ± 0.388	0.009 ± 0.015	0.015	0.031
CT603	0.333 ± 0.326	0.093 ± 0.104	0.024	0.095
CT681	0.231 ± 0.237	0.073 ± 0.051	0.036	0.007
CT694	0.460 ± 0.565	0.163 ± 0.221	0.033	0.089
CT795	0.322 ± 0.506	0.049 ± 0.059	0.015	0.005
CT798	0.247 ± 0.332	0.061 ± 0.055	0.013	0.077
CT806	0.324 ± 0.335	0.093 ±0.073	0.031	0.057
CT812	0.244 ± 0.298	0.055 ± 0.044	0.047	0.013
CT813	0.478 ± 0.499	0.087 ± 0.095	0.015	0.008
CT823	0.229 ± 0.222	0.050 ± 0.061	0.014	0.004
CT858	2.023 ± 1.111	0.327 ± 0.635	<0.001	0.001
CT866	0.250 ± 0.350	0.047 ± 0.038	0.010	0.006
pCT03	1.945 ± 1.077	0.288 ± 0.491	<0.001	0.014

Figure 1. Reactivity of 54 infertility sera with 933 GST-chlamydial fusion proteins representing 908 unique ORFs of *C. trachomatis*

Each of the human Antibodies (displayed along the x-axis on top of each panel) after 1/2000 dilution was reacted with each of the 933 GST fusion proteins (listed along the y-axis) immobilized onto the 96-well microplates. Each colored bar represents a positive reactivity between a given fusion protein and a patient serum with the tubal factor infertility (TFI) patient sera in green and infertile control (IFC) in red. The results are expressed as optical density (OD) readings obtained at the wavelength of 405 nm. Any reaction with an OD ≥ mean + 2 Standard deviations calculated from the same plate is defined positive. The positive OD values are expressed as binding intensity in increasing brightness of fluorescent color. The negative OD values are black. The 933 fusion proteins representing the *C. trachomatis* genome are first listed in order of the ORFs from CT001 to pCT08 (panel A). Both letter and number extensions were used to distinguish multiple fusion proteins and protein fragments that share the same ORF names. (B) The 933 fusion proteins were then reordered based on binding frequency by the TFI sera from high (top) to low (bottom). As indicated on the right of the panel, a total of 265 ORFs were recognized by one or more sera. (C) By visual inspection of the expanded view of the positive antigens, the fusion proteins preferentially recognized by either TFI (green) or IFC (red) patients were marked with brackets on the right of the panel. (D) A total of 30 antigens were significantly recognized by TFI patients.

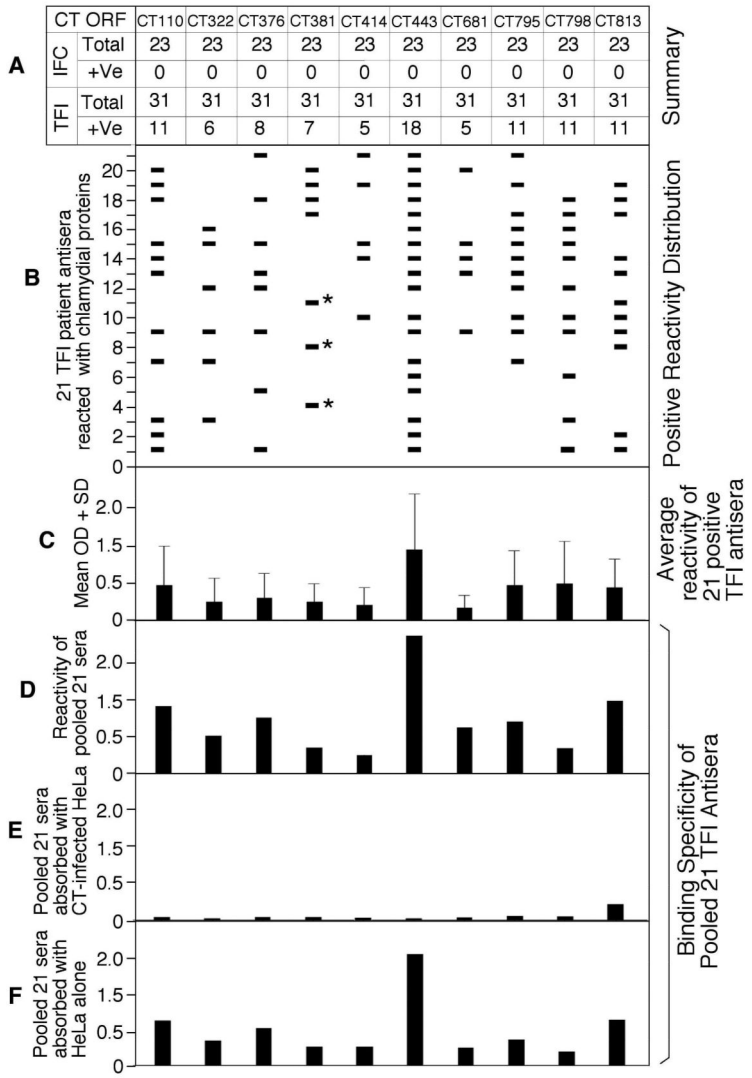


Figure 2. Reactivity of 30 *C. trachomatis* antigens with 54 patient sera at 1:4000 dilution

The 30 antigens were reacted with the 54 human sera as described in table Table 2 note except that each serum was diluted 1:4000 (data not shown). (A) Ten of the 30 antigens, failing to react with any of the 23 IFC sera, were thus presented in the figure. Note that HSP60 (CT110) and OmcB (CT443) maintained a detection sensitivity of 35.5% and 58% respectively. (B) The reactivity of each of the 10 antigens was analyzed at individual antiserum level. Note that the combinations of CT443 with CT381 or HSP60 with CT376, CT381 & CT798 can have the highest sensitivity of 67.7% while maintaining 100% specificity. (C) The reactivity intensity between each antigen and the 21 positive sera (measured at individual antiserum level) was expressed as mean OD plus standard deviation. (D) Each of the 10 antigen were reacted with an antiserum sample pooled from the 21 sera at equal ratio without (D) or with absorption with *C. trachomatis* (CT)-infected HeLa lysate (E) or HeLa alone lysate (F). Note that absorption with CT-HeLa but not HeLa alone lysates removed the reactivity of each antigen with the pooled antiserum.

Table 1

Titers of antibodies against *C. trachomatis* and *C. pneumoniae* in infertile women with or without tubal pathology

Serum samples from women with TFI and IFC were 2 fold serially diluted starting with 1:10 and reacted with HeLa cells infected with either *C. trachomatis* or *C. pneumoniae*. The antibody reactivity was detected using an immunofluorescence assay as described in the materials and method section. The highest dilution that still gave a positive reactivity was defined as the serum titer. Each serum sample was titrated in triplicate and the average was used as the geometric titer of a given serum sample. Student's *t*-Test was used to quantitatively analyze the differences between the two groups of patients. There is a statistically significant difference in titers of antibodies against *C. trachomatis* ($p<0.001$) but not *C. pneumoniae* ($p=0.269$) organisms. When the serum samples were divided into 3 categories (Negative, Low & High) based on antibody titers, the qualitative analysis with Chi-squared test still revealed a significant difference in the number of sera in different categories between the two groups of patients for antibodies against *C. trachomatis* ($p<0.001$) but not C. *pneumoniae* ($p=0.634$) organisms. Further pairwise Chi-squared analyses of the anti-C. *trachomatis* antibodies revealed significant differences between the high vs. low and high vs. negative groups. The number of individuals with high titers of anti-C. *trachomatis* antibodies in TFI group is significantly higher than that in the IFC group.

		Antibodies to *C. trachomatis*		**Antibodies to C. *pneumoniae***	
		TFI (n=31)	**IFC (n=23)**	**TFI (n=31)**	**IFC (n=23)**
Quantitative analyses	Mean	69928	3814	41503	25861
	Standard Deviation	106709	8270	65848	35847
	Student's *t* Test	p=0.0009		p=0.269	
Categorization of serum samples into Negative, Low and High titer groups					
Qualitative analyses	Negative titers (<1:10)	1 (3.2%)	3 (13.0%)	3 (9.7%)	4 (17.4%)
	Low titers (1:10-1:10000)	11 (35.5%)	19 (82.6%)	11 (34.5%)	6 (26.1%)
	High titers (>1:10000)	19 (61.3%)	1 (4.4%)	17 (54.8%)	13 (56.5%)
	χ^2 Test	$p < 0.001$		$p = 0.634$	
	Pairwise χ^2 Tests	High vs. Negative	$p = 0.008$	N/A	
		High vs. Low	$p < 0.001$		
		Low vs. Negative	$p = 0.556$		

Table 2

Reactivity of 30 significant *C. trachomatis* proteins with 31 TFI & 23 IFC patient antisera (@1:800)

The 30 *C. trachomatis* antigens significantly recognized by 24 TFI patients as identified in Fig.1 were reacted with 54 patient sera (33 TFI and 21 IFC). All sera were diluted at 1:800 regardless of their over anti-*C. trachomatis* antibody titers as determined with the immunofluorescence assay. The mean ODs of each antigen were compared between TFI and IFC groups were compared using Student *t*-Test and the corresponding p values were listed in the last column of the left panel, confirming that all 30 antigens were significantly recognized by TFI patients. The number of positive recognition (determined as described Fig. 1 legend) by either TFI or IFC was used to calculate recognition specificity and sensitivity as well as positive or negative predicting values (PPV or NPV; right panel). Note that HSP60 (CT110) displayed a detection specificity of 82.6% and sensitivity of 71% and many other immundominant antigens such as pCT03 (Pgp3, a plasmid-encoded secreted protein), CT858 (CPAF, a chlamydial protease/proteasome-like activity factor that is secreted into host cell cytosol), CT823 (cHtrA, a secreted stress response serine protease), CT813 (an inclusion membrane protein), CT443 (OmcB, outer membrane complex protein B) & CT143 (a hypothetical protein) behaved similarly. Only the hypothetical protein CT557 had a 100% specificity but its sensitivity was only 29%. Thus, under this assay condition, no single antigen or combinations of antigens can achieve 100% specificity with a sensitivity of >50%.

CT	TFI (n=31)	IFC (n=23)	*t*-Test	Specificity	Sensitivity	PPV	NPV
ORF	X +/− SD	X +/− SD					
CT067	0.407 ± 0.483	0.045 ± 0.115	<0.001	87.0%	51.6%	84.2%	57.1%
CT089	0.645 ± 0.936	0.189 ± 0.407	0.020	78.3%	45.2%	73.7%	51.4%
CT110	0.679 ± 0.756	0.069 ± 0.112	<0.001	82.6%	71.0%	84.6%	67.9%
CT116	0.176 ± 0.252	0.012 ± 0.059	0.001	95.7%	32.3%	90.9%	51.2%
CT119	0.375 ± 0.501	0.082 ± 0.196	0.005	87.0%	48.4%	83.3%	55.6%
CT142	0.468 ± 0.522	0.098 ± 0.138	0.001	78.3%	54.8%	77.3%	56.3%
CT143	1.012 ± 0.818	0.166 ± 0.220	<0.001	73.9%	71.0%	78.6%	65.4%
CT147	0.789 ± 0.678	0.303 ± 0.185	0.001	34.8%	80.6%	62.5%	57.1%
CT153	0.404 ± 0.561	0.071 ± 0.110	0.003	91.3%	45.2%	87.5%	55.3%
CT322	0.366 ± 0.586	0.055 ± 0.112	0.007	95.7%	41.9%	92.9%	55.0%
CT376	0.453 ± 0.616	0.072 ± 0.097	0.002	95.7%	41.9%	92.9%	55.0%
CT381	0.330 ± 0.346	0.059 ± 0.074	<0.001	95.7%	51.6%	94.1%	59.5%
CT414	0.327 ± 0.469	0.061 ± 0.082	0.004	95.7%	51.6%	94.1%	59.5%
CT442	0.486 ± 0.622	0.055 ± 0.070	0.001	91.3%	48.4%	88.2%	56.8%
CT443	1.145 ± 1.020	0.110 ± 0.173	<0.001	87.0%	71.0%	88.0%	69.0%
CT456	0.803 ± 0.879	0.241 ± 0.558	0.006	73.9%	64.5%	76.9%	60.7%
CT529	0.854 ± 0.644	0.444 ± 0.310	0.003	13.0%	87.1%	57.4%	42.9%
CT557	0.358 ± 0.638	0.028 ± 0.057	0.007	100%	29.0%	100%	51.1%

CT	TFI (n=31)	IFC (n=23)	*t*-Test	Specificity	Sensitivity	PPV	NPV
ORF	X +/− SD	X +/− SD					
CT603	0.579 ± 0.654	0.141 ± 0.161	0.001	78.3%	61.3%	79.2%	60.0%
CT681	0.363 ± 0.386	0.060 ± 0.078	<0.001	87.0%	51.6%	84.2%	57.1%
CT694	0.698 ± 0.848	0.150 ± 0.293	0.002	78.3%	54.8%	77.3%	56.3%
CT795	0.647 ± 0.771	0.034 ± 0.101	<0.001	95.7%	61.3%	95.0%	64.7%
CT798	0.622 ± 0.827	0.038 ± 0.089	<0.001	91.3%	51.6%	88.9%	58.3%
CT806	0.673 ± 0.772	0.104 ± 0.212	<0.001	82.6%	54.8%	81.0%	57.6%
CT812	0.555 ± 0.667	0.061 ± 0.087	<0.001	91.3%	54.8%	89.5%	60.0%
CT813	0.673 ± 0.689	0.095 ± 0.165	<0.001	78.3%	67.7%	80.8%	64.3%
CT823	0.649 ± 0.709	0.071 ± 0.074	<0.001	91.3%	71.0%	91.7%	70.0%
CT858	1.947 ± 1.276	0.338 ± 0.666	<0.001	78.3%	74.2%	82.1%	69.2%
CT866	0.574 ± 0.738	0.062 ± 0.079	0.001	91.3%	48.4%	88.2%	56.8%
pCT03	1.761 ±1.366	0.166 ± 0.573	<0.001	82.6%	74.2%	85.2%	70.4%