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Whole genome identification of *C. trachomatis* immunodominant antigens after genital tract infections and effect of antibiotic treatment of pigtailed macaques

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Abstract

The cervix and/or fallopian tubes of pigtailed macaques were experimentally infected with *Chlamydia trachomatis*. Their sera were collected at varying time points and screened for identification of immunodominant antigens using a whole-genome protein microarray. The effect of doxycycline treatment on the antibody response generated in these macaques was also investigated. Twenty-five female macaques were infected with *C. trachomatis* serovars D or E in the cervix and/or fallopian tubes. Bloods were collected at baseline and at various intervals after challenge. Serum samples were tested for antibodies using a *C. trachomatis* serovar D protein microarray. Twenty chlamydial antigens reacted with sera from at least 68% (17/25) of the macaques. In addition to some well-known chlamydial antigens, nine different proteins, not previously recognized as immunodominant, including four hypothetical proteins (CT005, CT066, CT360 and CT578), were identified. Monkeys infected in the fallopian tubes developed a more robust antibody response than animals inoculated in the cervix. Treatment with doxycycline significantly decreased *Chlamydia*-specific antibody levels. In summary, using protein microarray serum samples from experimentally infected pigtailed macaques were screened for immunodominant chlamydial antigens. These antigens can now be tested in animal models for their ability to protect and as markers of disease progression.

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Keywords

Chlamydia trachomatis; pigtailed macaques; *Macaca nemestrina*; antigen; protein microarrays; antibody response; serum; cervix; fallopian tubes; antibiotics

Introduction

In developed countries *C. trachomatis* is the most common bacterial sexually transmitted infection while in regions of the world with poor sanitary conditions this pathogen is the most common cause of preventable blindness [1, 2]. Attempts to control these infections using antibiotics have not been effective. For example, in regions where screening programs, followed by antibiotic treatment, have been implemented, an increase in the number of cases has been reported [3, 4]. Therefore, to eradicate *Chlamydia*, a vaccine is likely the most efficacious approach. A whole organism vaccine against trachoma resulted in a hypersensitivity reaction in some immunized individuals upon re-exposure to this pathogen [2, 5, 6]. The possibility that an antigenic component of *Chlamydia* mediated this hypersensitivity reaction prompted the abandonment of a whole organism vaccine and stimulated the search for a subunit formulation [7–9]. Due to the large number of proteins present in most pathogens it is difficult to identify those that are antigenic. However, recent advances generating whole proteome arrays have led to rapid screening methods to identify proteins that can generate an immune response [10–12].

Non-human primates are the only animal model naturally susceptible to infection with the *C. trachomatis* human serovars and therefore, are the ideal system for testing vaccines [13–15]. The genetic variability in the human population requires *Chlamydia* vaccines that include antigens that can be recognized by individuals with multiple immunogenetic backgrounds. In this study, we screened sera from 25 pigtailed macaques (*Macaca nemestrina*) previously infected in the lower and/or upper genital tract with *C. trachomatis*. We identified 20 immunodominant antigens that can now be tested for their ability to induce protective responses. The effects of antibiotic therapy on the immune response to chlamydial infection were also investigated.

Materials and Methods

Animals

Sexually mature female pigtailed macaques (*Macaca nemestrina*) were enrolled in these studies. All 25 macaques were housed at the Washington National Primate Research Center at the University of Washington, Seattle, WA [15]. Approval was obtained from the Institutional Animal Care and Use Committee at the University of Washington. Animals were handled humanely within the National Institutes of Health's animal use guidelines. To rule out previous or current chlamydial infection all animals were prescreened by cervical culture and serology.

Infection of pigtailed macaques with *C. trachomatis*

Animals underwent different *C. trachomatis* infection regimes using serovars D (P0124) or E (MTW477) (Table 1) [13, 14]. In one study, a single cervical inoculation with serovar E was delivered with a 1 ml tuberculin syringe into the vaginal fornix, thereby exposing the cervix to the organism. In the second study, five weekly cervical challenges were done to establish chronic chlamydial infection. To establish a chronic chlamydial infection of the upper reproductive tract, in the third study, the fallopian tubes were inoculated directly through the fimbrial os at 2-week intervals. Bloods were collected at baseline and at various intervals after challenge. Following infection macaques were treated with antibiotics or placebo as shown in Table 1. At the time the experiments were performed combination therapy with different agents was included.

Production of *C. trachomatis* proteome microarray chips

The *C. trachomatis* protein microarray chips were prepared following a three steps process: 1) PCR amplification of the 894 open reading frames (ORF); 2) in vivo recombination cloning, and 3) in vitro transcription/translation followed by microarrays chip printing (Antigen Discovery, Inc., Irvine, CA). The *C. trachomatis* serovar D (UW-3/Cx; ATCC) genomic specific PCR primers were designed using 20 bp of the gene-specific sequence and 33 bp of adapter sequences [10, 11, 16, 17]. The adapter sequences were designed to be homologous to the cloning site of the linearized T7 expression vector pXT7 and allowed the PCR products to be cloned by homologous recombination in DH5 α *E. coli* cells. At the 5' end of the fusion protein a polyhistidine (His) fragment was incorporated and at the 3' end, a hemagglutinin (HA) sequence and a T7 terminator were included. Plasmids with *Chlamydia* specific sequences were expressed using an in vitro transcription-translation system (RTS 100 kit; Roche). Microarrays were printed onto nitrocellulose coated glass slides (GraceBio) using an OmniGrid Accent microarrays printer (Digilab). Full-length protein expression was monitored in the microarrays by using anti-polyhistidine (clone His-1; Sigma) and anti-hemagglutinin antibodies (clone 3F10; Roche).

Microarray probing and data collection

A total of 106 serum samples were tested for the presence of antibodies using the *C. trachomatis* serovar D microarray. Briefly, serum samples were diluted 1:100 with 1X protein array blocking buffer (Whatman, Piscataway, NJ) containing 10% *Escherichia coli* lysate (McLab, San Francisco, CA) and incubated at room temperature for 30 minutes with constant agitation. The microarrays were rehydrated in 1X protein array blocking buffer and probed with serum samples [10]. The slides were washed and incubated with biotin-conjugated goat anti-human antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The bound secondary antibodies were detected using streptavidin-conjugated Sensilight P3 (Columbia Biosciences, Columbia, MD). The slides were scanned in a ScanArray Express HT microarray scanner (Perkin Elmer, Waltham, MA), and the fluorescence signal was quantified (QuantArray software; Perkin Elmer, Waltham, MA). Proteins were spotted in triplicate on each array

Bioinformatics analysis

Data processing and normalization—Antigen-specific signal intensities were first corrected for background noise (QuantArray software; Perkin Elmer). Antigens were spotted in triplicate on each array and the average of the three background corrected spots was used as the signal intensity. The signal intensities were then normalized using the variance stabilization and calibration for microarray data (VSN) package implemented in R [18, 19]. The VSN model was built using only the 192 no DNA control spot intensities for each sample, and then the model was applied to the antigen signal intensities.

Reactive antigen selection—To define reactive antigens the following treatment of the data was applied:

1. Transform VSN normalized data back to the raw scale
2. Calculate the mean and standard deviation (SD) of no DNA spots for each of the 106 samples
3. Subtract the no DNA mean plus two standard deviations (no DNA mean + 2*SD) from each antigen intensity
4. Subtract the pre-inoculation sample intensity
5. Remaining intensities greater than 0 are considered reactive

These criteria for defining reactivity were developed in previous projects based on the same technology [10, 12, 20]. This form of the data was not used for statistical analysis, but only for a coarse determination of reactivity with respect to background and the initial pre-inoculation

Prediction of cellular role—Computational prediction of protein cellular role, enzyme class, and gene ontology were downloaded from Comprehensive Microbial Resource website (<http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi>).

Statistical analysis—Only the VSN normalized data was used for statistical analysis. The statistical significance of increases in signal intensity following inoculation was calculated using Cyber-T differential analysis software [21, 22] to perform a Bayes-regularized t-test. The groups being compared were (1) the pre-inoculation samples of the 25 subjects and (2) the latest post-inoculation time point samples before any treatment was given to the 25 subjects. For each protein, the null hypothesis was that the mean of the intensities of the pre-inoculation samples was greater than or equal to the mean of the intensities of the 25 post-inoculation samples. The alternative hypothesis was that the mean of the post inoculation samples was greater. The results of these tests are presented in the Results subsection “Identification of *C. trachomatis* immunodominant antigens” and in Figure 1.

The same approach was used to compare: (1) fallopian tube inoculation versus cervix inoculation – results in Figure 2a; (2) serovar D versus serovar E – results in Figure 2b; (3) pre-doxycycline treatment versus post-doxycycline treatment – results in Figure 3a; and (4) pre-placebo treatment versus post-placebo treatment – results in Figure 3b.

The Chi-square and Fisher's exact tests (<http://www.graphpad.com/quickcalcs/contingency1.cfm>) were used to calculate p-values for the inoculation site, serovar E versus F, and the cell function comparisons.

Results

Identification of *C. trachomatis* immunodominant antigens

The protein microarray included the expression products of 894 unique ORFs of *C. trachomatis* serovar D as well as positive and negative controls. Tag detection was used as a measure of protein expression. Of the 894 ORF arrayed, 864 (96.4%) were positive for both the N-terminal poly-His and the C-terminal HA tags. A summary of the intra- and inter-array coefficients of variation (CVs) is presented in Supplementary Materials.

Microarrays were used to profile the antibody response in 106 serum samples collected from 25 pigtailed macaques that included 25 baseline samples, 40 after infection with *Chlamydia* and 41 after antibiotic or placebo treatment (Table 1). A total of 789 antigens were reactive with at least one serum sample, 184 were reactive with at least 52% (13/25) of the pigtailed macaques, and 54 were reactive with at least 68% (17/25). Considering only sera from macaques infected in the fallopian tubes with serovar D, 674 antigens reacted with at least one sera. Considering only sera from animals inoculated with serovar E, 600 antigens were reactive with at least one sera sample (Tables 1 & 2; on-line). Considering only sera from animals infected in the cervix with serovar E, 684 antigens were reactive. A total of 517 antigens were reactive with at least one serum from the three subsets of subjects described above.

From the 517 common reactive antigens we selected 20 that gave a positive signal with at least one serum sample from 17 (68%) of the 25 macaques and with a signal greater than 2,000 in at least one of the inoculation routes (Table 2). Figure 1 (on line) provides a global view of the increased reactivity levels following inoculation with each dot representing one of the 894 antigens. In the figure the x- and y-axis represent the pre- and post-inoculation mean reactivity values and the 20 selected antigens are indicated by larger dots.

The selected immunodominant antigens included five proteins known to give positive signals with sera from humans infected with *Chlamydia* and with sera from experimental animals, mainly mice and guinea pigs: CT110 (chaperonin GroEL; 60k-Da heat-shock protein; hsp60), CT111 (chaperonin GroES; 10-kDa heat-shock protein; hsp10), CT442 (15-kDa-cysteine-rich protein; 15-kDa crp), CT443 (60-kDa-cysteine-rich protein; 60-kDa crp) and CT681 (major outer membrane protein; MOMP). Two additional proteins identified here, CT841 and CT456, have also being recognized as immunodominant in humans and another four, CT541, CT559, CT664 and CT759, were previously detected using mouse sera. The other nine proteins, including four hypothetical proteins (CT005, CT066, CT360 and CT578), have not previously being identified as immunodominant antigens.

The average signal intensity of pre-inoculation samples and post-inoculation samples and the associated significance of the difference for each of the immunodominant antigens are presented in Fig. 1. The unadjusted p-values of 14 out of the 20 immunodominant antigens

were less than 0.05. Five of these antigens were significant after Benjamini and Hochberg (BH) multiple test correction [23]: CT681, CT443, CT541, CT242, and CT110. Of the remaining 874 antigens 36 had unadjusted p-values less than 0.05 and none were significant after BH correction.

The selection of the 20 immunodominant antigens was performed as described above (i.e. clear increases following inoculation in at least 68% of the macaques) because in order to be of interest as a potential vaccine candidate, or to be useful as a marker for disease progression or treatment, an antigen must generate a strong immune response in a significant majority of the macaques. Alternative approaches for selecting antigens (e.g. statistical significance, mean increase) do not guarantee that these criteria would be met; however, the average intensity increases following inoculation were significant for 5 of 894 proteins after BH correction and all 5 were among the set of 20 immunodominant antigens.

The immunodominant antigens were also utilized for the additional comparisons in this study (i.e. inoculation site, serovar, and treatment). When all 894 proteins are tested for each of the additional comparisons none of the results are significant after BH correction; thus, no significant result is excluded by presenting results only for the immunodominant antigens.

Distribution of positive immunodominant antigens based on site of infection and *C. trachomatis* serovar used to infect

To determine if we could identify antigens preferentially associated with upper or lower genital tract infections the results were evaluated based on inoculation site (Table 2). More macaques inoculated in the fallopian tubes gave positive signals with the immunodominant antigens than animals infected in the cervix. Four of the 20 antigens CT111, CT443, CT461 and CT791, were positive at clearly higher frequency in animals infected in the fallopian tubes when compared with those infected in the cervix (Table 3). These four proteins were positive in 87 to 93% of the monkeys after tubal challenge but were only positive in 40 to 50% of the monkeys after cervical challenge. For the remaining 16 proteins, 15 showed similar percentage of positive monkeys in fallopian tubes and cervix infection groups. One protein, CT759, had a higher percentage of positive monkeys in the cervix inoculation group.

The signal intensity for the 20-immunodominant antigens was also stronger in sera from macaques inoculated in the fallopian tubes than in those infected in the cervix (Fig. 2a). Of the 20 antigens, 19 (95%) gave stronger average signals with sera from macaques infected in the fallopian tubes when compared with those inoculated in the cervix. Out of these 19 antigens with higher average signal for fallopian tubes CT110 and CT461 resulted in unadjusted p-values < 0.05; however, neither result is significant after BH correction [23]. Only CT456 had a stronger signal in macaques inoculated in the cervix when compared with those infected in the fallopian tubes.

When comparing the percentage of pigtailed macaques infected with *C. trachomatis* serovars D or E in the fallopian tubes that gave a positive signal, no significant differences were observed for any of the 20-immunodominant antigens (Table 2). When comparing the signal intensities between macaques infected with serovar D versus serovar E in the

fallopian tubes, CT242 was the only immunodominant antigen that resulted in an unadjusted p-value < 0.05; however, the result is not significant after BH correction (Fig. 2b). In addition, none of the other 894 antigens result in a significant difference after BH correction.

Effect of antibiotic treatment on antibody responses

To determine if antibiotic treatment affects antibody responses elicited by *C. trachomatis* infection we evaluated sera from monkeys infected with serovar D in the fallopian tubes. As shown in Table 3, most macaques treated with doxycycline showed a decrease in signal intensity for 16 (80%) of the 20-immunodominant antigens. For three (15%) proteins, CT066, CT443, CT841, there was an increase in signal intensity and for (CT759), equal number of monkeys had an increase or a decrease in signal following antibiotic treatment. In animals treated with placebo the opposite trend occurred. In more macaques there was an increase in signal intensity for 13 (65%) of the antigens, while for the other seven proteins no change was observed.

The average signal intensity for the immunodominant antigens before and after doxycycline treatment of monkeys infected in the fallopian tubes with serovar D is shown in Fig. 3a. For 15 of the 20 antigens the average signals were higher before than after doxycycline treatment. For the other five antigens CT066, CT443, CT456, CT759 and CT841, the average signal intensities increased following treatment. In contrast, for the placebo treated monkeys the signal intensity increased for all, but one (CT442), of the antigens. Using an odds ratio confidence interval test, the observed decreased in signal in monkeys treated with antibiotic has an odds ratio of 10.1729, which falls within the 95% confidence interval (between 4.1288 to 25.0652). None of the doxycycline or placebo comparisons using the 20-immunodominant antigens resulted in an unadjusted p-value < 0.05 and none of the other 894 antigens was significant after BH correction.

Assignment of cell function

Using the Comprehensive Microbial Resource (CMR) from the J. Craig Venter Institute (JCVI; <http://www.jcvi.org>), we assigned the predicted cellular roles to the 894 *Chlamydia* ORFs, the 789 proteins that gave a positive signal with at least one serum and the 20-immunodominant antigens selected using the protein microarray. Each protein is assigned to one main cellular role category (Fig. 4). The greatest number (56.78%) of the total positive antigens is categorized as unknown function (hypothetical proteins). Most of the categorized cellular roles of all the identified positive antigens are in close proportion to the percentage represented in the *C. trachomatis* genome. In contrast, proteins from the cell envelope, cellular processes, protein fate and unknown function categories have a higher representation in the immunodominant antigen selection when compared with the positive antigens, or the entire genome, and for the protein fate group this difference is significant ($P < 0.05$).

Discussion

Archived serum samples from pigtailed macaques, infected with *C. trachomatis* in the cervix or in the fallopian tubes, were used to identify chlamydial antigens that elicited a

humoral immune response. A protein microarray corresponding to 96.4% of the ORF of the *C. trachomatis* serovar D was used to screen the samples. We selected 20 chlamydial proteins that were recognized by sera from 68% (17/25) of the macaques. In addition to well-known chlamydial antigens such as MOMP, the 15- and 60-kDa crp and the 10-kDa and 60-kDa hsp, we identified nine antigens not previously recognized as immunodominant. Macaques infected in the fallopian tubes mounted a more robust immune response and reacted with antigens that may be used as markers of upper genital pathology. Furthermore, we showed that antibiotic treatment following *C. trachomatis* infection decreases the antibody response. The immunodominant antigens we have identified can now be tested in animal models to determine their ability to elicit protection and evaluate their potential role as markers of disease progression.

Implementation of high throughput screening platforms, such as the one utilized here facilitates the characterization of immune responses to multiple antigens. Some of these approaches have been applied to the identification of *C. trachomatis* vaccine candidates. For example, Wang et al. [24] using recombinant proteins coated in microtiter plates, tested sera from 99 females with acute *C. trachomatis* urogenital infection. From the 908 *C. trachomatis* proteins screened, they selected 27 that were recognized by more than 50% of the antisera. Six of the antigens, that Wang et al. [24] identified as dominant are also included in our list of 20 immunodominant proteins (CT110, CT442, CT443, CT456, CT681 and CT841). Similarly, to identify antibody-producing proteins, Finco et al. [25] utilized a panel of 120 serovar D recombinant proteins to screen sera from *C. trachomatis* infected patients. The 79 proteins that gave positive results were used to stimulate splenocytes from *C. trachomatis*-infected mice. Five of the proteins (CT119, CT372, CT443, CT681 and CT823) induced CD4+/IFN- γ . Two of the proteins (CT443 and CT681) identified by Finco et al. [25] as having T- and B-cell epitopes are included in the list of 20 immunodominant antigens selected in this study. Also, to select B and T-cell epitopes, Follmann et al. [26] used a panel of 116 recombinant *C. trachomatis* serovar D proteins to screen serum from 46 and peripheral blood mononuclear cells, from 10 *C. trachomatis* infected patients. CT082, CT089, CT322, CT396 and CT681 proteins bound antibodies, CT004, CT043, CT184, CT509 and CT611, were recognized exclusively by T cells and CT110 and CT443 were identified by both, antibodies and T cells. The last two proteins, CT110 and CT443, in addition to CT681, are included in our list of immunodominant antigens. From the immunodominant antigens identified in these studies, MOMP (CT681) is the protein that has been shown to induce the most robust protection in various animal models, including non-human primates [7, 27–30]. In a mouse model, Olsen et al. [31] have shown limited protection with a recombinant fusion protein of CT443 and CT521.

Trying to identify markers of disease progression Rodgers et al. [32] used an array of 30 serovar D recombinant proteins to screen sera from 31 patients with tubal factor infertility (TFI) and 23 sera from infertile patients. Based on the ability of sera to react with the 30 chlamydial antigens, they concluded that using a combination of CT381 and CT443, as reactive markers, they had 67% sensitivity and 100% specificity to identify patients with TFI. In our study, CT111, CT443, CT461 and CT791 were detected by the majority (~90%) of samples from monkeys infected in the fallopian tube while less than 50% of sera from

animals infected in the cervix gave positive signals. Therefore, our analysis confirms the finding by Rodgers et al. [32] that reactivity with the CT443 (60kDa crp) is preferentially associated with upper genital pathology. Interestingly, it has previously been shown that CT443, through antigenic mimicry on the signal peptide, may be involved in the pathogenesis of *Chlamydia*-related heart diseases [33]. It is possible that a similar immune mechanism may account for the association between antibodies to this protein and upper genital pathology. Neither Rodgers et al. [32], nor this study, found an association between upper genital infection and the 60kDa hsp (CT110). It has been proposed that this protein is the antigen responsible for the hypersensitivity reaction observed during the trachoma vaccine trials and some studies, have also found an association of reactivity to the 60kDa hsp and upper genital tract pathology [34–36].

We also analyzed the effect of antibiotic treatment on the antibody response in pigtailed macaques infected in the fallopian tubes with *C. trachomatis* serovar D. Countries that have implemented screening programs for *C. trachomatis*, followed by antibiotic treatment, have noticed an increase in the prevalence of the infection [3, 4]. Treatment with antibiotics was proposed to result in weakening of the immune response to *Chlamydia* and therefore, possibly increased susceptibility to reinfection and/or recurrences [4, 37]. There is evidence in humans and animal models to support this hypothesis [38, 39]. Our results confirm that there is decline in antibody levels following antibiotic treatment therefore, supporting this hypothesis. Antibodies are known to play a critical role in protection against *Chlamydia* and therefore, reduction in antibody levels may render an individual more susceptible to infection [40, 41].

Our study has several limitations. The microarray used here is a bacterial-based expression system that does not control for post-translational modifications, such as phosphorylation, glycosylation or lipidation. Therefore, antibodies that recognized those structures may not be identified. However, using vaccinia virus arrays produced by this method, all the known glycosylated proteins were recognized by sera from immunized humans and animals [20]. Since proteins on these arrays were not glycosylated, these findings suggest that at least some antibody responses are directed against epitopes not post-translationally modified.

Similar limitations may occur with conformational epitopes and those formed by disulfide bonds. The *Chlamydia* MOMP (CT681) has disulfide bonds and forms trimers in the outer membrane [42, 43]. Monoclonal antibodies that only bind to the MOMP trimer do not recognize the MOMP printed on this array, but monoclonals to linear epitopes do react (unpublished data). However, as shown here, anti-MOMP antibodies produced in monkeys following infection with live *Chlamydia* were detected with this array.

The limitations of all the screening assays may explain, in addition to the different types of samples analyzed, the apparent discrepancies between some studies. In this respect, most investigators have chosen to use proteins from the *C. trachomatis* serovar D in their platforms since its genome sequence is available [44]. Although, there is more than 98% DNA homology between the 15 *C. trachomatis* serovars, single nucleotide polymorphism and mutations may result in antigenic variation that may be reflected in the immune response to certain proteins. Therefore, screening human samples with proteins from a

single *C. trachomatis* serovar may bias the results since multiple serovars cause infections. For example, our results will suggest that serovar D elicits a more robust humoral immune response than serovar E. However, we cannot be certain of this conclusion since our microarray utilized serovar D proteins. However, certain *Chlamydia* proteins are consistently identified by different methodologies using various types of samples and therefore, these antigens may be the best candidates for further testing.

In conclusion, using sera from *C. trachomatis*-infected pigtailed macaques we have identified immunodominant antigens that can be tested in animal models for their ability to protect against this pathogen. Amongst the immunodominant antigens some were predominantly recognized by sera from macaques inoculated in fallopian tubes rather than in the cervix and therefore, may be markers for upper genital tract pathology. In addition, we have shown that antibiotic treatment following infection weakens antibody responses and thus, may make the individual more susceptible to reinfection. Screening patients infected with *C. trachomatis* with whole proteome arrays may help stage the infection and the response to therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

This is the first time that *Chlamydia trachomatis* immunodominant antigens have been identified in pigtailed macaques following a uterine cervix or a fallopian tubes infection. These immunodominant antigens can now be used to vaccinate non-human primates and determine their ability to protect against a *C. trachomatis* genital infection. Proteins that are protective can subsequently be tested in humans. Amongst the immunodominant antigens some were predominantly recognized by sera from macaques inoculated in the fallopian tubes rather than in the cervix and therefore, may be markers for upper genital tract pathology. In addition, treatment with doxycycline following infection significantly decreased *Chlamydia*-specific antibody levels. This information can be used to evaluate the efficacy of antibiotic treatment and potentially susceptibility to reinfection.

Highlights

- Pigtailed macaques were infected with *Chlamydia trachomatis* in the cervix and fallopian tubes.
- A protein microarray with all the open reading frames of *C. trachomatis* was probed with sera.
- Twenty chlamydial antigens reacted with sera from at least 68% (17/25) of the macaques.
- Treatment with doxycycline significantly decreased *Chlamydia*-specific antibody levels.
- Immudominant antigens can be tested as vaccine antigens and to evaluate antibiotic therapy.

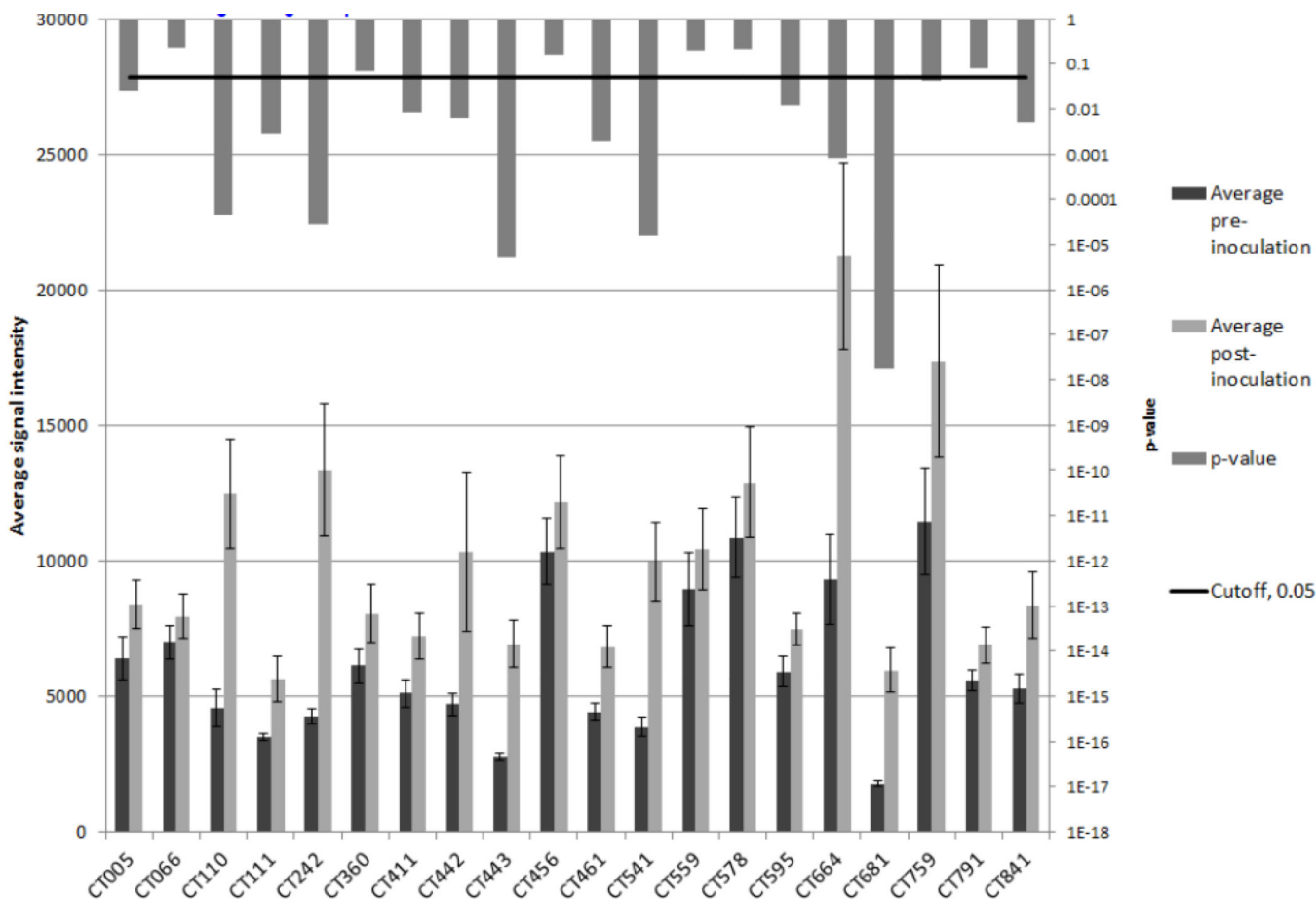


Figure 1. Comparison of pre-inoculation and post-inoculation average signal intensities for the immunodominant antigens

The pre-inoculation samples (n=25) were compared to the set of the latest post-inoculation samples for the subjects (n=25). For each of the 20-immunodominant antigens the barplots compare the average signal intensities (left vertical axis) of the groups and the error bars show standard error. The vertical bars descending from the top of the figure indicate the p-values resulting from t-tests (right vertical axis).

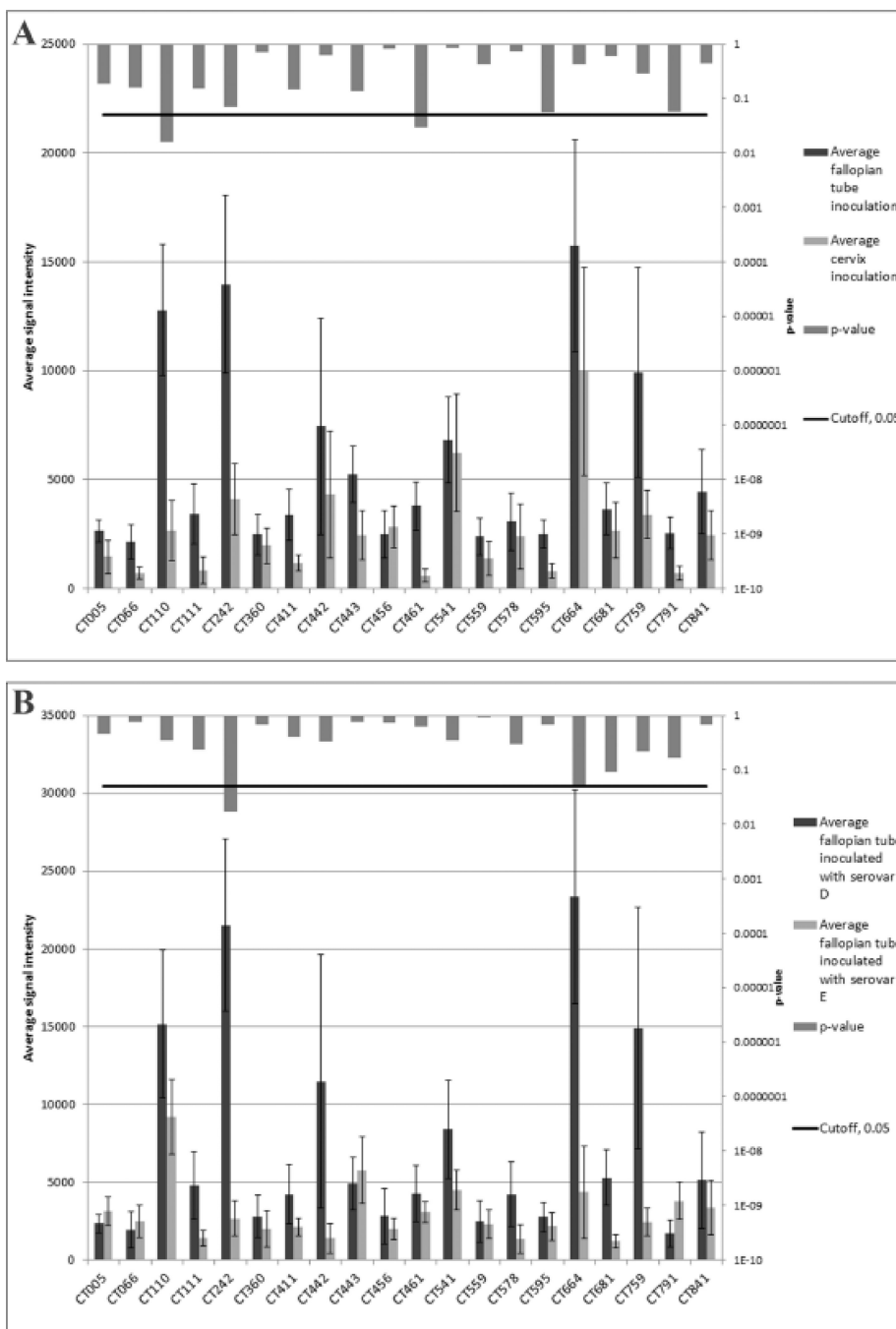


Figure 2. Comparison of inoculation site (a) and serovar type (b) average signal intensities to immunodominant antigens

Panel (a) compares serum samples from pigtailed macaques inoculated in the cervix to those inoculated in the fallopian tubes. Panel (b) compares serum samples from pigtailed macaques inoculated with serovar D to those inoculated with serovar E. For each of the 20-immunodominant antigens the barplots compare the average signal intensities (left vertical axis) of the groups and the error bars show standard error. The vertical bars descending from the top of the figure indicate the p-values resulting from t-tests (right vertical axis).

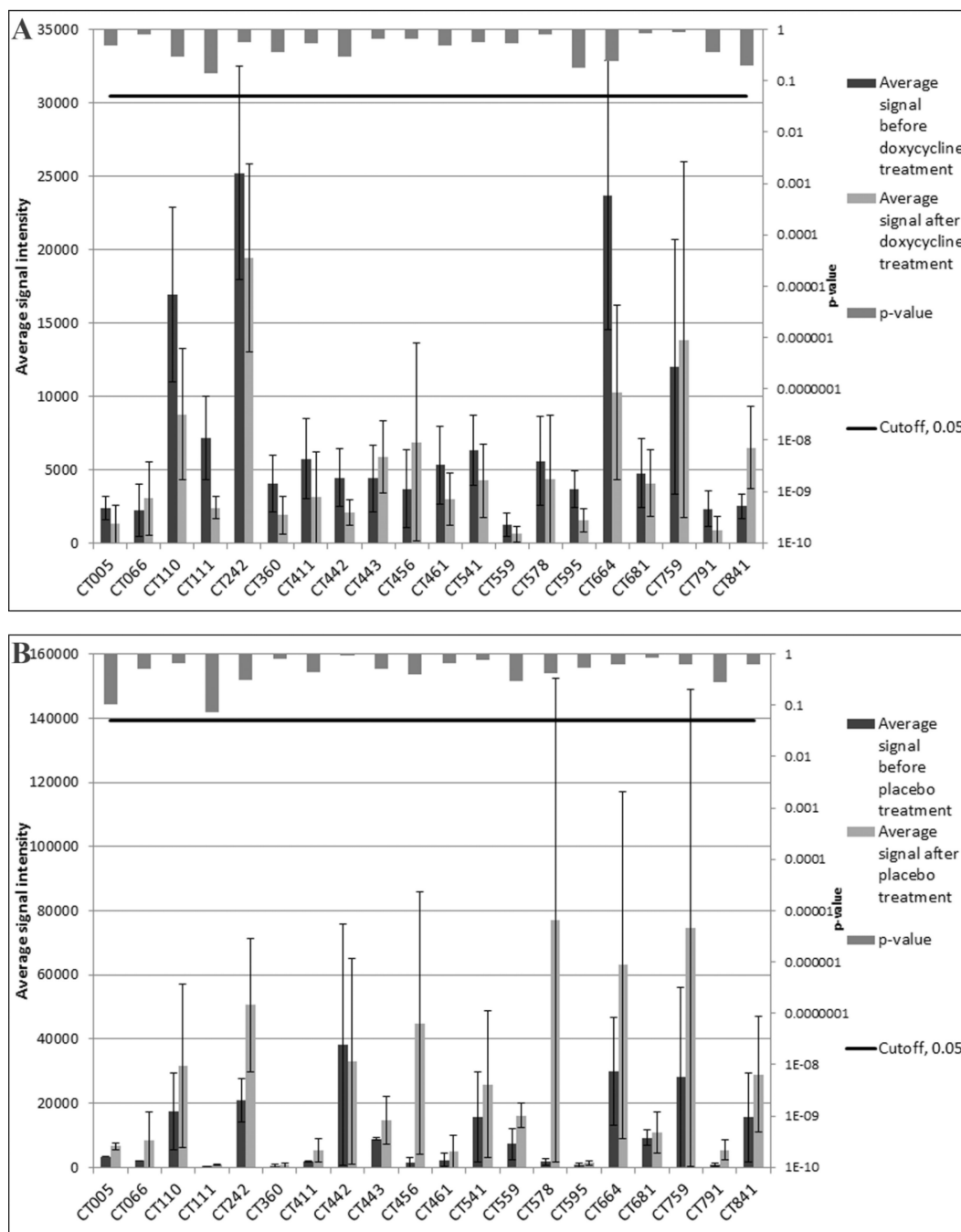


Figure 3. Comparison of pretreatment and post-treatment with doxycycline (a) and placebo (b) average signal intensities to immunodominant antigens
 Panel (a) compares serum samples from pigtailed macaques treated with doxycycline before and after treatment. Panel (b) compares serum samples from pigtailed macaques treated with placebo before and after treatment. For each of the 20 immunodominant antigens the barplots compare the average signal intensities (left vertical axis) of the groups and the error bars show standard error. The vertical bars descending from the top of the figure indicate the p-values resulting from t-tests (right vertical axis).

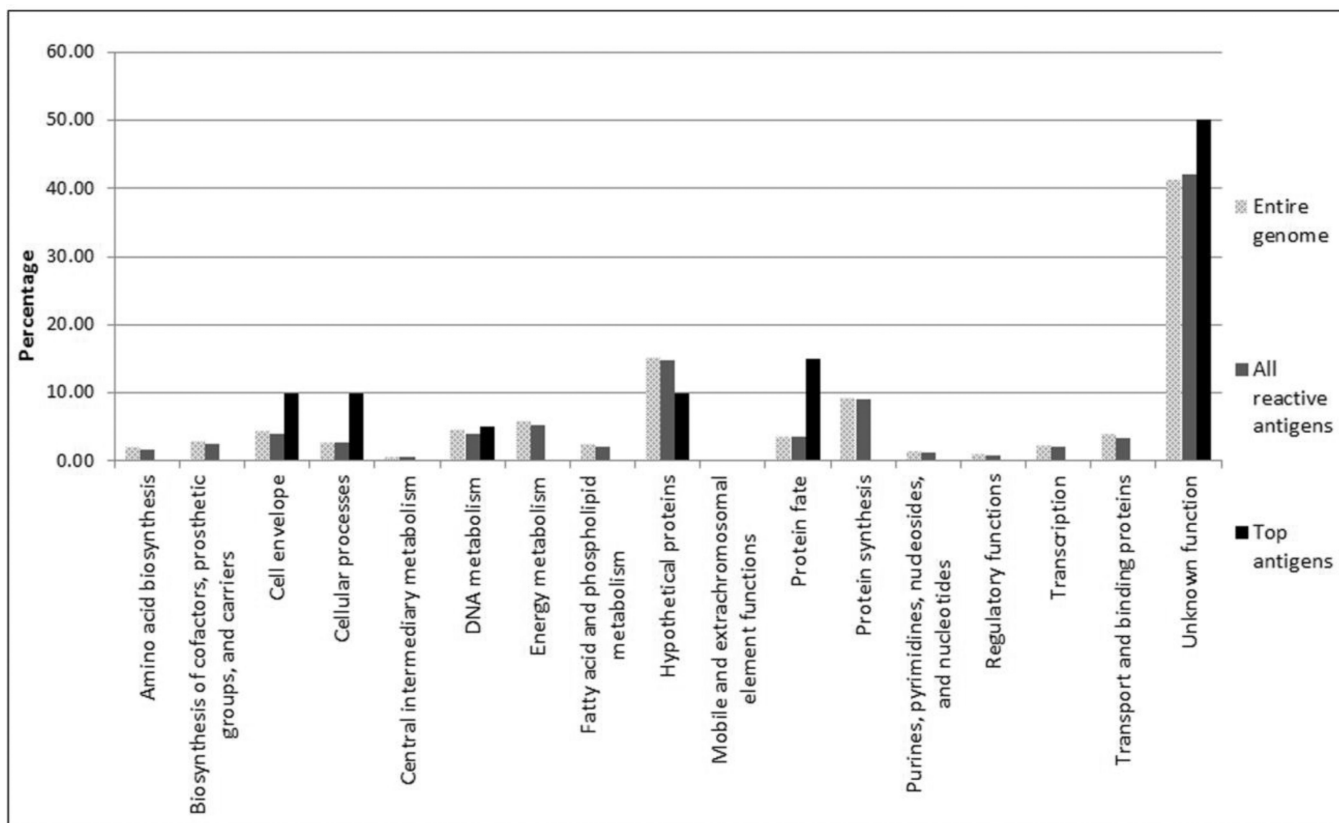


Figure 4. Functional roles of the ORFs of the whole *C. trachomatis* serovar D genome, all the positive antigens identified following infection of the pigtailed macaques and the 20 selected immunodominant antigens

The distribution of the predicted roles of the 20-immunodominant antigens is compared to the whole *C. trachomatis* serovar D and to all the reactive chlamydial antigens using the JCVI cellular role categories.

Pigtailed macaques entered in the experiment *C. trachomatis* serovar used to infect, site of inoculation, antibiotic treatment and number of samples collected

Table 1

# Pigtailed macaques	Inoculation site	# serum samples before inoculation	Chlamydia serovar	# of inoculations	# serum samples after inoculation	# Pigtailed macaques	Treatment	# serum samples after tx
15	Fallopian tubes	9	6×10^6 Serovar D (PO124)	3X	13	6	Doxycycline e: 2.2mg/kg/d ay orally, 10 days Doxycycline e: 2.2mg/kg/d ay orally, 10 days + Triamcinolone: 0.2mg/kg each, 3 days	16
						2	Placebo x 10 days Doxycycline e: 2.2mg/kg/d ay orally, 10 days Doxycycline e: 2.2mg/kg/d ay orally, 10 days Doxycycline e: 2.2mg/kg/d ay orally, 10 days Doxycycline e: 2.2mg/kg/d ay orally,	9
		2	5×10^6 Serovar E (MTW477)	2X	3	2		3
		4	6×10^6 Serovar E	2X	6	1		1

# Pigtailed macaques	Inoculation site	# serum samples before inoculation	Chlamydia serovar	# of inoculations	# serum samples after inoculation	# Pigtailed macaques	Treatment	# serum samples after tx
			(MTW477)			2	10 days + Triamcinolone; n: 0.2mg/kg each, 3 days	4
						1	Placebo x 10 days	0
			5 × 10 ⁴ Serovar			4	Placebo x 14 days	7
10	Cervix	7	E (MTW477)	5X	10	3	Placebo x 14 days	0
			5 × 10 ⁴ Serovar E (MTW477)	1X	8	3	Azithromycin; n: 14mg/kg each, 5 days	0

Table 2

Number of monkeys (% +) that gave positive signals with the 20 immunodominant antigens following infection with *C. trachomatis*

<i>C. trachomatis</i> geneID no.	No. (%) of positive monkeys w/ fallopian tube inoc	No. (%) of positive monkeys w/ cervix inoc	No. (%) of positive monkeys w/ serovar D fallopian tube inoc	No. (%) of positive monkeys w/ serovar E fallopian tube inoc	Total number of positive monkeys (%)	Common name for protein	Nucleic acid length
CT005	13 (87)	7 (70)	7 (78)	6 (100)	20 (80)	Hypothetical protein CT005	1092
CT066	13 (87)	6 (60)	8 (89)	5 (83)	19 (76)	Hypothetical protein CT066	477
CT110	14 (93)	6 (60)	8 (89)	6 (100)	20 (80)	Chaperonin GroEL; 60-kDa hsp	1635
CT111	13 (87)	4 (40)	8 (89)	5 (83)	17 (68)	Co-chaperonin GroES; 10-kDa hsp	309
CT242	13 (87)	7 (70)	9 (100)	4 (67)	20 (80)	OmpH-like outer membrane protein	522
CT360	11 (73)	9 (90)	7 (78)	4 (67)	20 (80)	Hypothetical protein CT360	627
CT411	14 (93)	6 (60)	8 (89)	6 (100)	20 (80)	Lipid-A-disaccharide synthase	1824
CT442	11 (73)	9 (90)	7 (78)	4 (67)	20 (80)	15 kDa cysteine-rich protein	453
CT443	14 (93)	5 (50)	8 (89)	6 (100)	19 (76)	60kD cysteine-rich outer membrane protein	1662
CT456	11 (73)	9 (90)	6 (67)	5 (83)	20 (80)	Hypothetical protein CT456	3018
CT461	14 (93)	4 (40)	9 (100)	5 (83)	18 (72)	Phosphohydrolase	990
CT541	14 (93)	7 (70)	8 (89)	6 (100)	21 (84)	Peptidyl-prolyl cis-trans isomerase	732
CT559	12 (80)	7 (70)	7 (78)	5 (83)	19 (76)	Yop proteins translocation lipoprotein J	981
CT578	12 (80)	7 (70)	9 (100)	3 (50)	19 (76)	Hypothetical protein CT578	1464
CT595	13 (87)	6 (60)	8 (89)	5 (83)	19 (76)	Thio:disulfide interchange protein	2079
CT664	15 (100)	7 (70)	9 (100)	6 (100)	22 (88)	FHA domain-containing protein	2490
CT681	13 (87)	6 (60)	8 (89)	5 (83)	19 (76)	Major outer membrane protein	1182
CT759	10 (68)	9 (90)	6 (67)	4 (67)	19 (76)	Muramidase	738
CT791	13 (87)	4 (40)	8 (89)	5 (83)	17 (68)	Excinuclease ABC subunit C	1797
CT841	13 (87)	7 (70)	8 (89)	5 (83)	20 (80)	ATP-dependent zinc protease	2742

Table 3

Antibody responses in monkeys to the 20 immunodominant *C. trachomatis* antigens following antibiotic/placebo treatment

ID	Fallopian tube Serovar D, Doxy tx		Fallopian tube Serovar D, Placebo tx		p-value
	Increase	Decrease	Increase	Decrease	
CT005	1	4	2	0	0.1429
CT066	5	1	1	1	0.4643
CT110	1	5	2	0	0.1071
CT111	0	6	2	0	0.0357
CT242	2	4	2	0	0.4286
CT360	1	5	1	0	0.2857
CT411	0	6	2	0	0.0357
CT442	2	3	1	1	1.0000
CT443	4	1	1	1	1.0000
CT456	1	4	2	0	0.1429
CT461	1	5	1	1	0.4643
CT541	2	4	2	0	0.4286
CT559	1	4	2	0	0.1429
CT578	1	5	2	0	0.1071
CT595	0	6	1	1	0.2500
CT664	0	6	1	1	0.2500
CT681	1	4	1	1	1.0000
CT759	2	2	2	0	0.4667
CT791	0	6	2	0	0.0357
CT841	5	1	2	0	1.0000