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Identification of immunodominant antigens of *Chlamydia trachomatis* using proteome microarrays

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Abstract

Chlamydia trachomatis is the most common bacterial sexually transmitted pathogen in the world. In order to control this infection, there is an urgent need to formulate a vaccine. Identification of protective antigens is required to implement a subunit vaccine. To identify potential antigen vaccine candidates, three strains of mice, BALB/c, C3H/HeN and C57BL/6, were inoculated with live and inactivated *C. trachomatis* mouse pneumonitis (MoPn) by different routes of immunization. Using a protein microarray, serum samples collected after immunization were tested for the presence of antibodies against specific chlamydial antigens. A total of 225 open reading frames (ORF) of the *C. trachomatis* genome were cloned, expressed, and printed in the microarray. Using this protein microarray, a total of seven *C. trachomatis* dominant antigens were identified (TC0052, TC0189, TC0582, TC0660, TC0726, TC0816 and, TC0828) as recognized by IgG antibodies from all three strains of animals after immunization. In addition, the microarray was probed to determine if the antibody response exhibited a Th1 or Th2 bias. Animals immunized with live organisms mounted a predominant Th1 response against most of the chlamydial antigens while mice immunized with inactivated *Chlamydia* mounted a Th2-biased response. In conclusion, using a high throughput protein microarray we have identified a set of novel proteins that can be tested for their ability to protect against a chlamydial infection.

INTRODUCTION

Chlamydia trachomatis is one of the most common bacterial pathogens found in all regions of the world [1–5]. Attempts to produce a vaccine against this pathogen were initiated in the 1960's [2,6]. At that time, *C. trachomatis* was known to cause trachoma, a blinding disease frequent in countries with poor sanitation conditions. Using whole inactivated and viable organisms, trials were performed in humans and in non-human primates. Several

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conclusions were reached from those studies [2,6]. Some vaccine protocols induced protection. In general however, the protection was short-lived, lasting between 1 to 2 years. In addition, the protection appeared to be serovar specific, i.e., of the four ocular isolates, A, B, Ba and C, the protection was effective only against the particular serovar used in the vaccine. Furthermore, some of the immunized individuals developed a hypersensitivity reaction after re-exposure to *Chlamydia*. Although the exact cause of the hypersensitivity reaction was not elucidated, it was attributed to an antigenic component present in *Chlamydia*. Therefore, the need to develop a subunit vaccine was considered.

In the late 1970's, the role of *C. trachomatis* in genital infections was discovered [2,7]. As a result, recent efforts have shifted to engineering a vaccine against *Chlamydia* genital diseases [8,9]. This is an effort worth pursuing since even a vaccine that is not 100% efficacious will have a major impact on the epidemiology of this pathogen [10]. Therefore, the development of an animal model for characterizing the pathogenesis of genital chlamydial infections and for testing vaccine protocols became a research priority. Among the different animal systems that are now available, the mouse model, using the *C. trachomatis* mouse pneumonitis (MoPn) biovar, most closely mimics an infection in humans. Genital inoculation of both female and male mice with this organism induces clinical and pathological changes that parallel those found in humans [11–13]. For example, the production of a severe acute infection, with vaginal shedding, and long-term sequelae in female mice, e.g., infertility, are parameters that can be used to effectively measure the efficacy of a vaccine.

With the goal of establishing a "gold standard" for the vaccine Pal et al. [14,15] immunized intranasally with live *C. trachomatis* MoPn three strains, BALB/c, C3H/HeN and C57BL/6, of female mice and challenged them in the genital tract. Animals immunized with live *Chlamydia* were protected as shown by a significant decrease in the length and severity of vaginal shedding. Subsequently, the mice were mated and the course of the pregnancy was followed. The animals vaccinated intranasally with live *Chlamydia* exhibited protection against infertility. A subunit vaccine using the native major outer membrane protein (MOMP) of *Chlamydia* as the only antigen was subsequently shown to induce a level of protection equivalent to that elicited by intranasal immunization with the live organism [16]. In all likelihood, MOMP is the antigen that elicited the serovar specific protection originally observed in the trachoma vaccine trials [2,6,17]. Therefore, if we want to develop a vaccine that will protect against all, or most, *C. trachomatis* serovars we need to identify antigens that will induce broader protection.

The availability of the complete *C. trachomatis* genome sequence allows the use of proteomic approaches to identify dominant antigens following immunization with live and killed organisms [18,19]. Here, in an effort to identify potential vaccine candidates of *C. trachomatis*, we utilized a new proteome microarray method. Microarray chips coated with *Chlamydia* antigens were prepared using high throughput cloning and protein expression technology [19]. Serum samples collected from mice immunized with *Chlamydia* were used to probe the chips. With this approach, we have identified a new set of antigens that can be tested for their ability to protect against a *C. trachomatis* infection.

MATERIALS AND METHODS

Preparation of stocks of *C. trachomatis*

The mouse *C. trachomatis* biovar MoPn (strain Nigg II; also called *C. muridarum*) was purchased from the American Type Culture Collection (Manassas, VA, USA) [20]. *Chlamydia* was grown in tissue culture flasks using HeLa-229 cells [14]. Eagle’s minimal essential medium supplemented with 5% fetal bovine serum and 1 μg/ml of cycloheximide

were added. To purify elementary bodies (EB) and reticulate bodies (RB) we used the method described by Caldwell et al. [21]. The stocks were stored at −70°C in SPG (0.2 M sucrose, 0.02 M sodium phosphate (pH 7.2) and 5 mM glutamic acid). To determine the number of inclusion forming units (IFU), the stocks of EB were titrated in McCoy cells [14]. EB and RB were inactivated by exposure for 10 min to an UV transilluminator box (Spectroline, Westbury, NY) emitting 302 nm wavelength as previously described [22].

Production of *Chlamydia* protein microarray chips

To investigate the chlamydial immune response to immunization in an unbiased manner most of the genes were randomly selected and a few known immunogenic proteins, such as MOMP, the 9-kDa (also known as the 12 kDa lipoprotein) and 60-kDa cysteine rich proteins (crp), and the 10-kDa heat shock protein (hsp) were also included. The protein microarray chips were prepared following a three step process: First, PCR amplification of *Chlamydia* genes (total of 225 open reading frames, Supplemental Table 1); secondly, in vivo recombination cloning into a T7 promoter based plasmid expression vector, and finally, in vitro transcription/translation followed by microarrays chip printing [19]. The *C. trachomatis* MoPn genomic specific PCR primers were designed using 20 bp of the gene-specific sequence and 33 bp of adapter sequences [18, 23]. 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 *Escherichia coli* DH5α cells. At the 5' end of the fusion protein, a polyhistidine (poly-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 following the manufacturer's instructions (RTS 100 kit; Roche; Indianapolis, IN). Microarrays were printed onto nitrocellulose coated glass slides FAST slides (Whatman Inc., Piscataway, NJ) using an OmniGrid Accent microarray printer (DigiLab Inc., Holliston, MA). Protein expression levels were monitored in the microarrays by using anti-poly-His (clone His-1; Sigma-Aldrich; St. Louis, MO) and anti-HA antibodies (clone 3F10, Roche). The microarrays were blocked using 1X-blocking buffer for 30 m while the serum samples were pre-incubating. The blocking buffer was removed and the diluted antibodies were added to the microarrays and hybridized overnight in a humidified box. The following day the arrays were washed three times with Tris buffer-0.05% Tween-20, and the slides were incubated with biotin-conjugated goat anti-mouse, or biotin conjugated goat anti-rat, immunoglobulin diluted 1/1,000 in blocking buffer. Secondary antibodies were added to the slides and incubated for 1 hr at room temperature. Following washing three times with Tris buffer-Tween 20, bound antibodies were detected by incubation with streptavidin-conjugated Sensilight P3 (Columbia Biosciences, Columbia, MD). Following washing as before, additional three washes with Tris buffer saline, and a rinse with ultrapure water (18.2 Ohm), the slides were air dried under centrifugation and examined using a Perkin Elmer ScanArrray Express HT microarray scanner (Waltham, MA). Intensities were quantified using QuantArray software [19]. Measured values at each spot are the intensity at each spot minus the local background average.

Immunization of mice

Groups of 18–20 eight-week-old female BALB/c (H-2^d), C3H/HeN (H-2^k) and C57BL/6 (H-2^b) mice were purchased from Charles River Laboratories (Willmington, MA). Preimmunization sera were collected and used as negative controls. Mice were inoculated using different routes as follows: for intranasal (i.n.) immunization BALB/c and C57BL/6 mice received 10^4 inclusion forming units (IFU) while C3H/HeN mice were infected with 10^1 IFU [14]. For intravaginal (i. vag.) inoculation the mice receive 1.3×10^7 IFU/mouse [24]. For intramuscular plus subcutaneous (i.m.+s.c.) immunization the BALB/c mice were inoculated with 10 μg/mouse of UV-inactivated EB or RB three times at two-week intervals

using complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for the following two [22]. Male BALB/c (H-2^d) and C3H/HeN (H-2^k) mice were inoculated i.n. with 10^4 IFU and 10^1 IFU, respectively.

Probing of *Chlamydia* proteome microarray chips with serum samples

The *Chlamydia*-specific proteome microarray chips were probed with pre-and post-immunization sera. Whole blood was collected and after allowing the sample to clot for 30 m at room temperature, the serum was separated by centrifugation at 5,000×g for 15 min. Serum samples from all the animals in each group were pooled and stored at −70°C until analyses were conducted. Prior to microarray probing, the sera were diluted to 1/100 in Protein Array Blocking Buffer (Whatman) containing *E. coli* lysates at a final concentration of 10% and incubated for 30 min at room temperature while mixing [19]. The microarrays were blocked using 1X-blocking buffer for 30 m while the serum samples were pre-incubating. The blocking buffer was removed and the diluted serum was added to the microarrays and hybridized overnight in a humidified box. Following washing, the slides were incubated with diluted secondary antibodies (Pan IgG or IgG subclasses 1 and 2a or 2c; JacksonImmuno Research Laboratories Inc. West Grove, PA) for 1 hr at room temperature with agitation. Following washing, bound antibodies were detected by incubation with streptavidin-conjugated Sensilight P3 (Columbia Biosciences). Following washing, intensities were quantified using QuantArray software [19]. Microarrays were scanned, quantified, and all signal intensities were corrected for background as described by Sundaresh et al. [25].

Data Analysis Methods

The statistical analysis was performed as described previously [25]. Briefly, the data was calibrated and transformed using the variance stabilizing normalization (vsn) package [26] in the R statistical environment (www.r-project.org). Differential reactivity analysis was then performed using Bayes-regularized t-tests [27]. To control for multiple testing conditions, p-values were adjusted using the Bonferroni procedure for controlling the Family Wise Error Rate (FWER) [28]. All reported p-values are Bonferroni corrected unless otherwise noted. Finally, the data was retransformed into an approximate raw scale for barplot visualizations.

RESULTS

Construction and verification of the *C. trachomatis* proteome microarray

The *Chlamydia* protein microarray included the expression products of 225 unique ORFs (Supplemental Table 1) of *C. trachomatis* MoPn as well as the appropriate positive and negative controls. Each of the 225 expression products and controls was printed once in each microarray. Antibodies that recognize the N-terminal poly-His tag and the C-terminal HA tag were used to assess protein expression. Poly-His and HA staining was done in technical quadruplicates, i.e. 4 microarrays for poly-His and 4 microarrays for HA. The microarrays were scanned with the PerkinElmer ProscanArray HT dual laser microarray scanner, and the intensity for each spot was quantified using the ProscanArray software package. Antigens with mean signal intensities greater than the average control value plus two standard deviations, were positive for the detection of the tag. Tag detection was used as a measure of protein expression. The plots in Figure 1 show the distribution of the signal intensities detected for the His tag (1A) and the HA (1B) tag. Of the 225 ORF arrayed, 225 (100%) stained positive for the N-terminal poly-His and 221 (98.2%) stained positive for the C-terminal HA tag (Table 1).

Microarray hybridization reproducibility

To verify the consistency of data, microarray chips were probed using three specified serum samples. The same sera and protocol was repeated on three sequential days, using fresh batches of reagents each time. Each serum sample tested was probed on three microarrays in each of the three days, and the probing process was repeated by a different operator each time. The microarrays were scanned and the signal intensity of each antigen was normalized. The average signal was calculated from the technical triplicate data points, one from each array, for each antigen using the data obtained from the quantification of the microarrays that were probed on the same day. Scatter plots and distribution correlations were used to compare the data sets obtained from probing performed on three different days. Figure 2 is a representative scatter plot and indicative of the data obtained for three parallel experiments. The three different comparisons, day 1 vs. day 2, day 1 vs. day 3, day 2 vs. day 3 (Figure 2A, 2B, and 2C respectively) showed R^2 value greater then 0.9, indicating very high correlation between the data sets collected from the different probing events. The distribution of the correlations was analyzed to validate the reproducibility of the data by making correlation matrices for all the data. The intra-day correlations for each sample were calculated. The mean value for the intra-day correlations was 0.9703557 for the three samples. The mean value for inter-day correlation was 0.8899541 for the three samples. It is interesting to note that all the inter-day correlations were above 0.95, except for the correlation involving one sample on day 2.

Microarray analysis reveals a targeted and specific response following immunization of mice with C. *trachomatis*

The protein microarray chips were used to profile the antibody response in mice following immunization. Sera from the different strains of female and male mice were used to probe the microarray chip. Each serum sample was probed nine times using nine arrays.

We defined immunologically reactive antigens using three criteria. The antigen must have a mean signal intensity that is greater than two standard deviations above the average control value, the antigen has a mean signal intensity that is greater than two standard deviation above the mean of the pre-immunization sera, and the antigen must have a CyberT Bonferroni corrected p-value (P) that is less than 0.05 between pre-immunization and post-immunization. A summary of the P for all of the reactive antigens can be found in Table 2, and will be described in more detail in the following text.

a) Antibody responses in BALB/c mice—Seven antigens were recognized by BALB/c serum after live *Chlamydia* immunization irrespective of gender and immunization route (Figures 3A–C and Table 2). Antibodies against the following *Chlamydia* antigens were detected: TC0052 (MOMP), TC0189 (hypothetical protein), TC0387 (co-chaperonin GroES or HSP10), TC0582 (V-type ATP synthase subunit A, AtpA), TC0660 (arginine binding protein, ArtJ), TC0726 (15-kDa, sulfur rich protein) and TC0828 (peptidylprolyl cis-trans isomerase, Mip). Two antigens were recognized by two of the three groups of BALB/c mice. TC0166 (hypothetical protein) was recognized by i. vag. immunized female mice and i.n. immunized male mice (Figures 3B and 3C). TC0210 (serine protease, HtrA) was recognized by male and female mice serum after i.n. immunization (Figures 3A and 3C). Additionally, four hypothetical proteins (TC0328, TC449, TC0500, TC816 and TC0909), TC0181 (glycogen synthase, GlgA), and TC0338 (iron binding protein YtgA) were recognized by at least one group of immunized BALB/c mice.

In a parallel experiment, female BALB/c mice were immunized with 10 ug of UV treated *C. trachomatis* MoPn EB or RB, using Freund's adjuvant, via the i.m. and s.c. routes. UV treated MoPn EB elicited responses to five antigens that are recognized after live *Chlamydia*

immunization. Detectable levels of antibodies against TC0052 (MOMP), TC0210 (HtrA), TC0387 (hsp-10), TC0660 (ArtJ), and TC0828 (Mip) were detected. Two additional proteins were recognized after immunization with killed MoPn EB, TC0584 (V-type ATP synthase subunit E, AtpE) and TC0846 (dihydrolipoamide dehydrogenase) (Figure 3D and Table 2). Immunization with UV inactivated MoPn RB produced a much less robust immunological response, eliciting detectable antibodies to only TC0052 (MOMP) (Figure 3E and Table 2).

b) Antibody responses in C3H/HeN mice—To determine which antibodies are elicited in response to infection in the C3H/HeN mouse strain, female and male mice were inoculated with live *Chlamydia*. Serum samples were collected from the mice and subsequently used to profile the immune response.

Ten antigens were recognized after live *Chlamydia* immunization in C3H/HeN mice irrespective of gender and immunization route (Figures 4A–C and Table 2). Detectable levels of antibodies were observed for TC0052 (MOMP), TC210 (HtrA), TC0387 (hsp-10), TC0453 (hypothetical protein), TC0660 (ArtJ), TC0726 (Srp), TC0727 (60-kDa crp), TC0816 (hypothetical protein), TC0828 (Mip), and TC0865 (type III secretion chaperone SycD) after immunization with live *Chlamydia*. Two additional antigens were recognized by two out three mouse groups. TC0338 (YtgA) was reactive in the i. vag. immunized female mice and the i.n. immunized male mice, while TC0850 (type III secretion system protein SctL) was reactive after i.n. immunization of male and female mice. Three hypothetical proteins (TC0189, TC0867 and TC0911), TC0137 (UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminoligase, MurF), TC0181 (GlgA), and TC0582 (AtpA) were recognized by at least one group of immunized C3H/HeN mice.

c) Antibody responses in C57BL/6 mice—Eight antigens were recognized after C57BL/6 mice were immunized with live *Chlamydia* via i.n. and i. vag. routes (Figures 4D, E and Table 2). Detectable levels of antibodies were seen in both i.n. and i. vag. immunized mice for TC0052 (MOMP), TC0582 (AtpA), TC0660 (ArtJ), TC0726 (Srp), TC0727 (60-kDa crp), TC0828 (Mip), and two hypothetical proteins (TC0328 and TC0816).

In addition, there are 13 antigens for which we detected antibodies in the sera of i.n. immunized C57BL/6 mice (Figure 4D). These antigens are TC0079 (ATP dependant Clp protease, ClpP), TC0137 (MurF), TC0257 (glycogen branching enzyme, GlgB), TC0289 (signal peptidase), TC0715 (ATP dependant Clp protease, ClpP-2), TC0752 (sensor histidine kinase), TC0852 (SctS), TC0892 (anti-oxidant AhpCTSA family protein) as well as five hypothetical proteins encoded by TC0189, TC0449, TC0671, TC0908 and TC0909.

Characterization of the Th1/Th2 response to *Chlamydia* antigens

The pan IgG results pointed to a targeted response with a relatively small number of antigens eliciting antibodies. To gain further insight, the proteome microarrays were probed with the same sera described above, and visualized to determine the effect of the immunization protocol on the production of the IgG1 and IgG2 antibody subtypes. The signal intensity values were used to calculate subtype fractions for IgG1 and IgG2 and to determine the average ratios for each antigen.

Immunizing BALB/c mice with live *Chlamydia* elicits mostly a IgG2a antibody response (Figure 5A). Eight out of ten proteins recognized by female mice immunized i.n. are recognized primarily by IgG2a antibodies. There were higher levels of IgG1 antibodies against only one protein, TC0387 (hsp-10). After i. vag. immunization of female mice the recognition of all 12 antigens is mostly by IgG2a antibodies. There was a very clear bias towards IgG2a antibodies, high IgG2a/IgG1 ratio, in all but one instance (TC0660; amino

acid ABC transporter, periplasmic amino acid-binding protein) for which the ratio was only slightly above 1 (Figure 5B). Immunizing male mice i.n. yields much the same IgG2a bias as the female mice showed in Figure 5A. Nine out of ten proteins were recognized by IgG2a antibodies almost exclusively (Figure 5C). Like in female mice only the TC0387 (hsp-10) elicited a Th2 response.

While immunization of BALB/c with live *Chlamydia* elicits primarily IgG2a antibodies, BALB/c mice show higher levels of IgG1 antibodies in response to immunization with UV killed EB or RB using Freund's adjuvant. After immunization with killed MoPn EB, antigens that were recognized primarily by IgG2a antibodies after immunization with live *Chlamydia*, including TC0052 (MOMP), TC0660 (amino acid ABC transporter, periplasmic amino acid-binding protein), and TC0828 (Mip), are being recognized primarily by IgG1 antibodies (Figure 5D). TC0052 (MOMP) was the only protein detected by antibodies from mice immunized with UV-killed MoPn RB, and there is a clear bias towards a IgG1 response (Figure 5E).

A very distinct IgG2a bias against chlamydial proteins after inoculation with live organism is observed with C3H/HeN mice (Figure 6A–C). Ten of eleven proteins elicited strong IgG2a responses, while only one antigen, TC0865 (SctD), elicited a strong IgG1 response after i.n. immunization of female C3H/HeN mice (Figure 6A). Figure 6B shows that eleven out of thirteen of the antigens, showed a very clear recognition bias with IgG2a antibodies after i. vag. immunization of female C3H/HeN mice. TC0210 (htrA) and TC0453 (hypothetical protein) elicited equivalent levels of IgG1 and IgG2a antibodies after immunization. I.n. immunization of male C3H/HeN mice elicited a very clear IgG2a response with little to no detectable IgG1 levels for all of the proteins (Figure 6C).

Live *Chlamydia* immunization of C57BL/6 female elicits primarily IgG2c antibodies (Figures 6D, 6E). All the antigens were strongly recognized by IgG2c antibodies and there was very little detectable IgG1. Only TC0892 (hypothetical protein) showed detectable levels of IgG1 that were close to the level of IgG2c antibodies detected in i.vag. immunized female mice (Figure 6D).

DISCUSSION

The recent implementation of vaccines using well-defined recombinant antigens, for instance the hepatitis B virus (HBV) and human papillomaviruses (HPV), has created a new impetus for engineering recombinant vaccines for other pathogens such as *C. trachomatis* [29–32]. However, formulation of subunits vaccines requires the identification of protective antigens. This is a major challenge since pathogenic organisms have complex genomes with hundreds of potential vaccine candidates. Here, using a partial proteome microarray of the *C. trachomatis* MoPn ORFome, we have identified a group of seven immunodominant antigens that were recognized by sera from the three strains of mice inoculated with this bacterium. In addition to known immunogens, such as MOMP (TC0052) and the 60-kDa crp (TC0727), we have detected novel antigens that can be tested for their ability to induce a protective immune response.

The development of high throughput systems, including the proteome microarray used in this study, allows the rapid analysis of immune response to thousand of immunogens. Some of these techniques have been applied for the identification of potential chlamydial vaccine candidates [33,34]. For example, using recombinant proteins coated in microtiter plates, Sharma et al. [33] tested 15 serum samples from females with acute *C. trachomatis* urogenital infections. Utilizing 156 chlamydial fusion proteins, they identified seven reactive chlamydial antigens. Of these, five (CT089, CT147, CT226, CT694, CT795;

corresponding to the following *C. trachomatis* MoPn ORF: TC0364, TC0424, TC0497, TC0066 and TC0177, respectively), were encoded by hypothetical proteins. In addition, two proteins previously known to be antigenic, MOMP (CT681; TC0052) and CPAF (CT858; TC0248), were recognized by the serum samples from the acutely infected patients.

Here, we immunized BALB/c (H-2^d), C3H/HeN (H-2^k) and C57BL/6 (H-2^b) mice with live EB of *C. trachomatis* MoPn in order to identify new potential vaccine candidates that will induce antibody responses in animals with a diverse immunogenetic background. In addition, BALB/c mice were immunized with UV-treated EB and RB. We decided to immunize the mice with live and UV-treated EB and RB because there are antigens that will only be expressed at certain stages of the developmental cycle of *Chlamydia* and will not be present in inactivated EB [33,34]. It is also possible that certain antigens may induce an antibody response only if the animal is immunized using a particular route. Therefore, for these experiments we decided to use four different routes of immunization: intranasal, intravaginal, intramuscular and subcutaneous. From the 225 *Chlamydia* antigens tested seven of them induced an antibody response in all three strains of mice. Of the seven immunodominant antigens, MOMP (TC0052), ArtJ(TC0660), Srp (TC0726) and Mip (TC0828), gave positive results with all the serum samples tested from mice immunized with live organism and all but Srp gave positive results with inactivated EB. MOMP and Mip$_{[D1]}$ have previously been reported to be immunodominant while the ABC transport arginine binding protein ArtJ (TC0660) and Srp (TC0726) have not [35–37]. Although ArtJ is a novel immunodominant antigen, this is not the first report of a *Chlamydia* ABC binding protein being immunodominant$_{[D2]}$. *C. trachomatis*-infected patients generate antibodies against the ABC transporter iron binding protein$_{[D3]}$ YtgA, for which we were able to detect antibodies in BALB/c and C3H/HeN mice [38]. Srp (TC0726) was identified as a crp by Newhall [39] and Clark et al. [40] and de la Maza et al. [41] described the ORF of this protein downstream from the 60 kDa crp. Unlike the 60- and 9-kDa crp we are not aware of a publication describing the antigenicity of this protein [42].

In addition to ArtJ (TC0660) and Srp (TC0726) we identified three novel immunodominant proteins. TC0189 (hypothetical protein), TC0582 (AtpA), and TC816 (hypothetical protein) gave a positive signal with at least one serum sample from each of the three strains of mice. TC0189 encodes a 450 amino acid protein with multiple transmembrane domains. AtpA (TC0582) is part of the catalytic domain of ATP synthase, which is a hexamer of three AtpA and three AtpB subunits located in the cytosol. While we are not aware of *Chlamydia* AtpA as an immunodomiant antigen, there are reports of AtpB from different bacteria being an immunodominat antigen [43,44]. $_{[D4]}$TC0816 is a hypothetical protein that corresponds to CT529, an inclusion membrane associated protein known as Cap1. Cap1 is recognized by CD8+ T cells and vaccination of mice with this protein reduces the level of infection in the spleen after an intravenous challenge$_{[D5]}$ [45].

In addition to testing the microarrays using an anti pan-Ig secondary antibody we also assayed the same chips using specific anti-IgG1, anti-IgG2a, for BALB/c and C3H/HeN mice, and anti-IgG1 and anti-IgG2c, for C57BL/6 mice [46]. Our goal was to compare the levels of IgG subclasses as a measurement of the Th1 versus Th2 response [47]. The main component of a non-viable vaccine that drives the immune response towards a Th1 or a Th2 response is the adjuvant [48–50]. In addition, some antigens may have adjuvant-like effects and therefore, can also influence the host towards a Th1 or a Th2 immune response [50,51]. For live vaccines, the intrinsic adjuvanticity of the pathogen can drive the immune response towards a Th1 or a Th2 response. Moreover, the genetic make-up of the host can favor a Th1 or a Th2 response. Overall, C57BL/6 mice are considered to have a strong Th1-biased immune response while the C3H/HeN mice lean more towards Th2 and the BALB/c have a balanced Th1/Th2 response [51,52]. This bias correlates with the susceptibility to a *C.*

trachomatis genital infection and infetility [24]. Furthermore, IgG subclass bias seen using vaccinated mice serum samples to interrogate protein microarray was previously shown to be linked to protection [53]. Here, our data clearly shows that infection of the three strains of mice with live *Chlamydia* results in a predominant Th1 response against most of the antigens independent of the host genetic background or the route of immunization. Interestingly, only TC0387, the 10-kDa hsp, an antigen associated with tubal infertility factor in humans, elicited a Th2-biased response in the BALB/c and C3H/HeN mice immunized with live *Chlamydia*, while C57BL/6 mice, the most resistant strain, did not respond to this antigen [54]. We also showed that in BALB/c mice immunized with UV-treated EB or RB using Freund's adjuvant, an adjuvant that induces a Th2 response, all the chlamydial antigens elicit a Th2-biased response (48, 49). As far as we know, this is the first time that, Th1 or Th2 humoral immune responses to specific *C. trachomatis* antigens has been reported. This is an important finding because it suggests that it may be possible to bias the immune response towards Th1 or Th2 for most of the chlamydial antigens independent of the particular genetic background of the host or the route of immunization. Humans have very heterogeneous immunogenetic backgrounds and therefore, it will be important to formulate a vaccine with broad efficacy with the appropriate adjuvants.

There are some limitations of the proteome microarray system utilized in this study. Based on our results, and previous data collected with other organisms, it appears that the antibody response mounted against a pathogen is limited to a reduced number of antigens [53,55–57]. For example, in the case of *Francisella tularensis* only 48 antigens, out of a total of 1,741 expressed ORF, reacted with sera from mice immunized with killed organisms [53]. Furthermore, sera from mice immunized subcutaneously with viable bacteria reacted only with nine antigens. Similarly, only 15% of the ORF products of *Borrelia burgdorferi* were recognized by sera from humans infected with this pathogen [57]. It could be argued that the sensitivity of this proteomic microarray is limited and therefore, it is possible that a number of reactive immunogens will not be detected.[D6] For example, we expected to see antibodies to the 60-kDa crp (TC0727) in the sera from BALB/c immunized mice (22). An alternative explanation is that the immune response to an infection is specific and targeted to a few antigens. To confirm if this is a limitation of this system, comparison of microarray results with data collected using an alternative serological detection system should be considered.

The type of microarray used in the current study has been developed as a high-throughput screening tool to profile the immune response to infection or diseases of self [19]. Currently, we can not correlate the amount of antigen present in each spot with the antibody reactivity towards the poly-His or the HA tags. These values cannot be used to quantitate the amount of antigen present in each individual spot. The binding of the antibodies to the poly-His and to the HA tags appears to be affected by many factors that may include the availability of the tags for binding due to folding and, in the case of the C-terminal tag, the fact that not the entire protein population will be full length. We cannot therefore compare quantitatively the antibody response to different antigens, as the observed difference in antibody reactivity could be due to variation in levels of protein expression rather than actual serum antibody titers. We can however, quantitatively follow the antibody response to a particular antigen since the amount of protein per spot for each antigen is the same from array to array.

Because of the limitations of a bacterial-based expression system, antibodies that recognize proteins based on post-translational modification, such as phosphorylation, glycosylation or lipidation, will not be identified with this method. However, it has been shown, using vaccinia virus arrays, that all the known glycosylated proteins are recognized by serum from immunized humans and animals [55]. Since none of the proteins on the array are glycosylated, this finding implies that at least a portion of the natural polyclonal response to

these proteins is directed against epitopes, or domains, that have not been post-translationally modified.

Similar limitations may occur with conformational epitopes and those formed by disulfide bonds. The MOMP (TC0052) from *Chlamydia* has disulfide bonds and naturally forms a trimer in the outer membrane of the organism [58–60]. Monoclonal antibodies that only recognize the native trimer do not recognize the MOMP printed on the array, but monoclonals antibodies to linear epitopes do react (data not shown). Epitopes requiring disulfide bonds for antibody recognition can either be seen or not depending on whether they are expressed in vitro under oxidizing conditions and the protein is correctly folded. As shown here, anti-MOMP antibodies produced following immunization of mice with live or UV-treated *Chlamydia* were detectable using this system.

Some of the limitations of the microarray system utilized in this study could, on the other hand, be considered as an advantage. If the long-term goal is to produce a vaccine with a recombinant protein, using an immunogen that induces protection as a result of post-translationally modification, or conformational epitopes, may represent a shortcoming for large-scale manufacturing.

In conclusion, in a mouse model, we have validated the use of a high throughput microarray analysis for the characterization of the antibody response to chlamydial antigens. We are currently in the process of expanding our characterization to include the entire ORFeome of *C. trachomatis* MoPn. Correlation of the findings obtained using human samples and those from animal models will be very helpful for identifying chlamydial antigens that can be tested in vaccine formulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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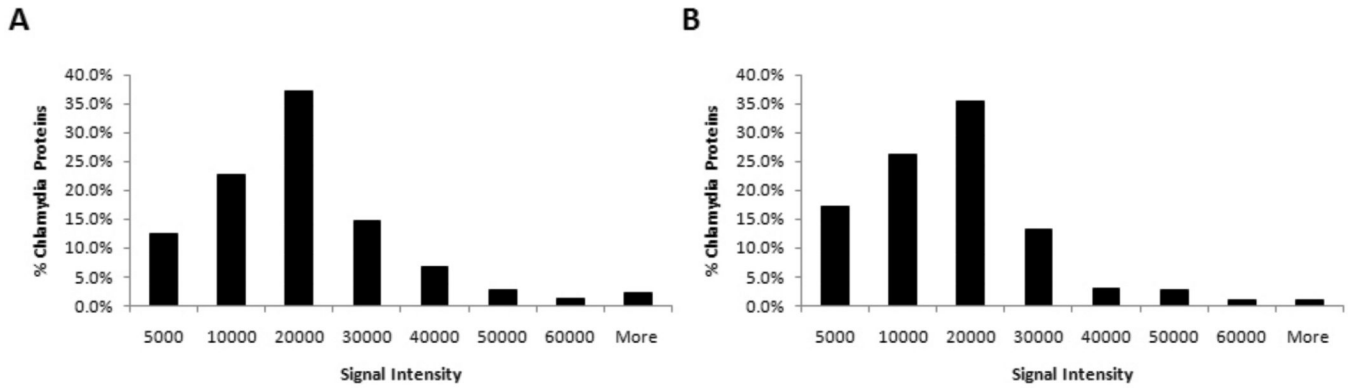

Figure 1. Distribution plot of the raw data indicating the range and percentages of the signal intensities for the poly-His and HA tags

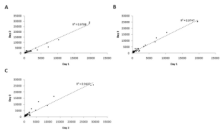

Figure 2. Scatter plots of the data obtained for three parallel experiments performed on three separate days

A) Day 1 versus 2; B) Day 1 versus 3; C) Day 2 versus 3.

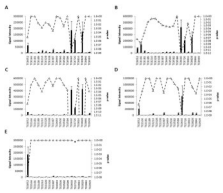

Figure 3. Identification of immuno reactive *C. trachomatis* MoPn proteins using serum samples collected from BALB/c mice

Sera from female BALB/c mice immunized A) i.n. and B) i.vag. with live *Chlamydia*. C) Sera from male mice inoculated i.n. with live *Chlamydia*. Sera from female mice immunized i.m.+s.c. with UV-treated: D) EB and E) RB. Open triangles: P values; Black and White bars: signal intensities for post-immunization and pre-immunization respectively.

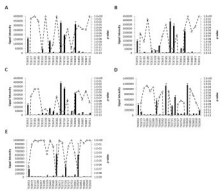

Figure 4. Identification of reactive proteins using serum samples collected from C3H/HeN and C57BL/6 mice inoculated with live *C. trachomatis* MoPn

Female C3H/HeN mice sera from animals immunized A) i.n. and B) i.vag. C) Sera from C3H/HeN male mice inoculated i.n. Sera from female C57BL/6 mice immunized D) i.n. and E) i.vag. Open triangles: P values; Black and White bars: signal intensities for post-immunization and pre-immunization respectively

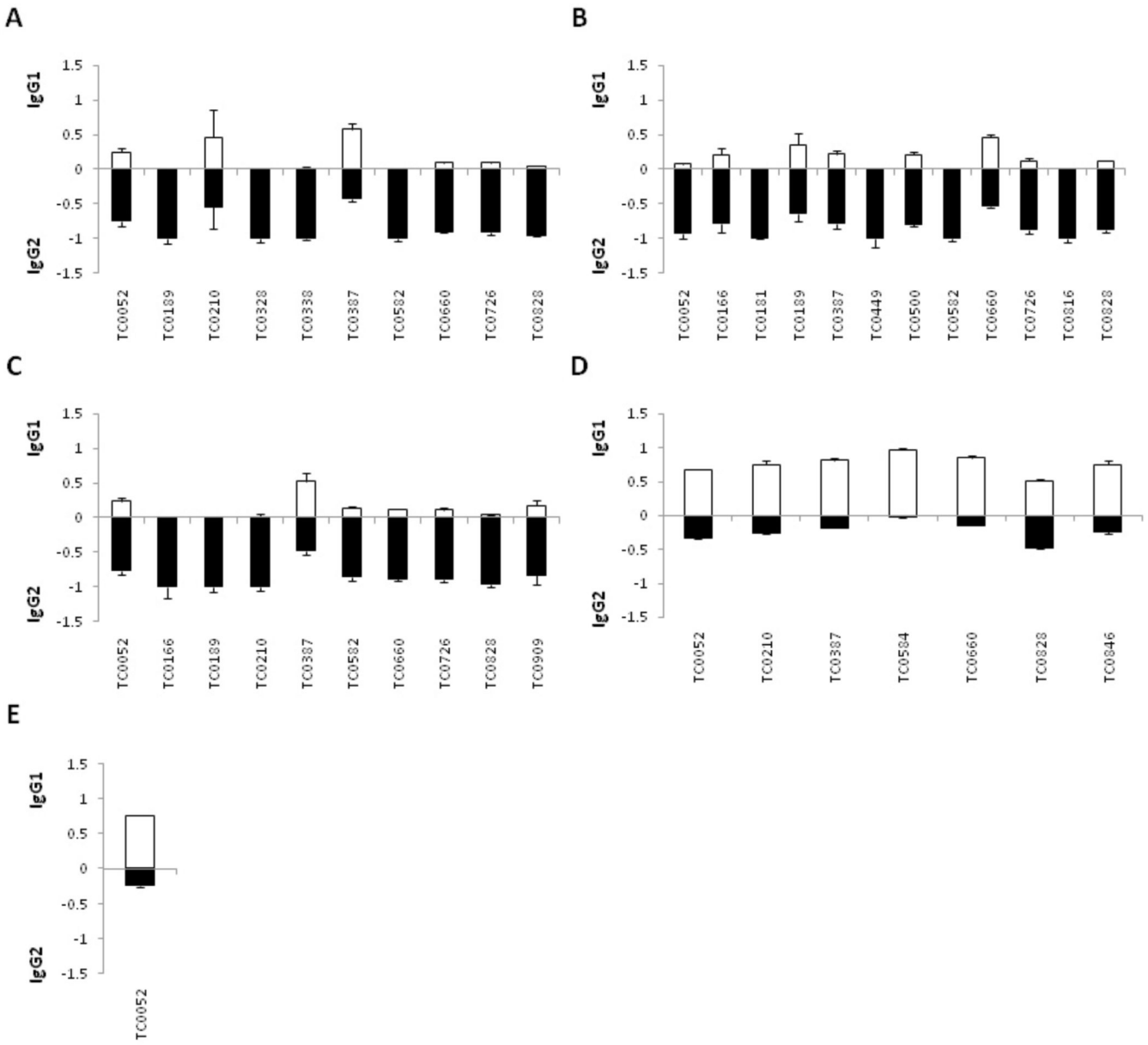

Figure 5. Detection of IgG2a and IgG1 antibodies from *Chlamydia* immunized BALB/c mice

Sera from female animals immunized A) i.n. and B) i.vag. with live *C. trachomatis* MoPn. C) Sera from male mice immunized i.n. with live *Chlamydia*. Sera from female mice immunized with UV-treated D) EB and E) RB.

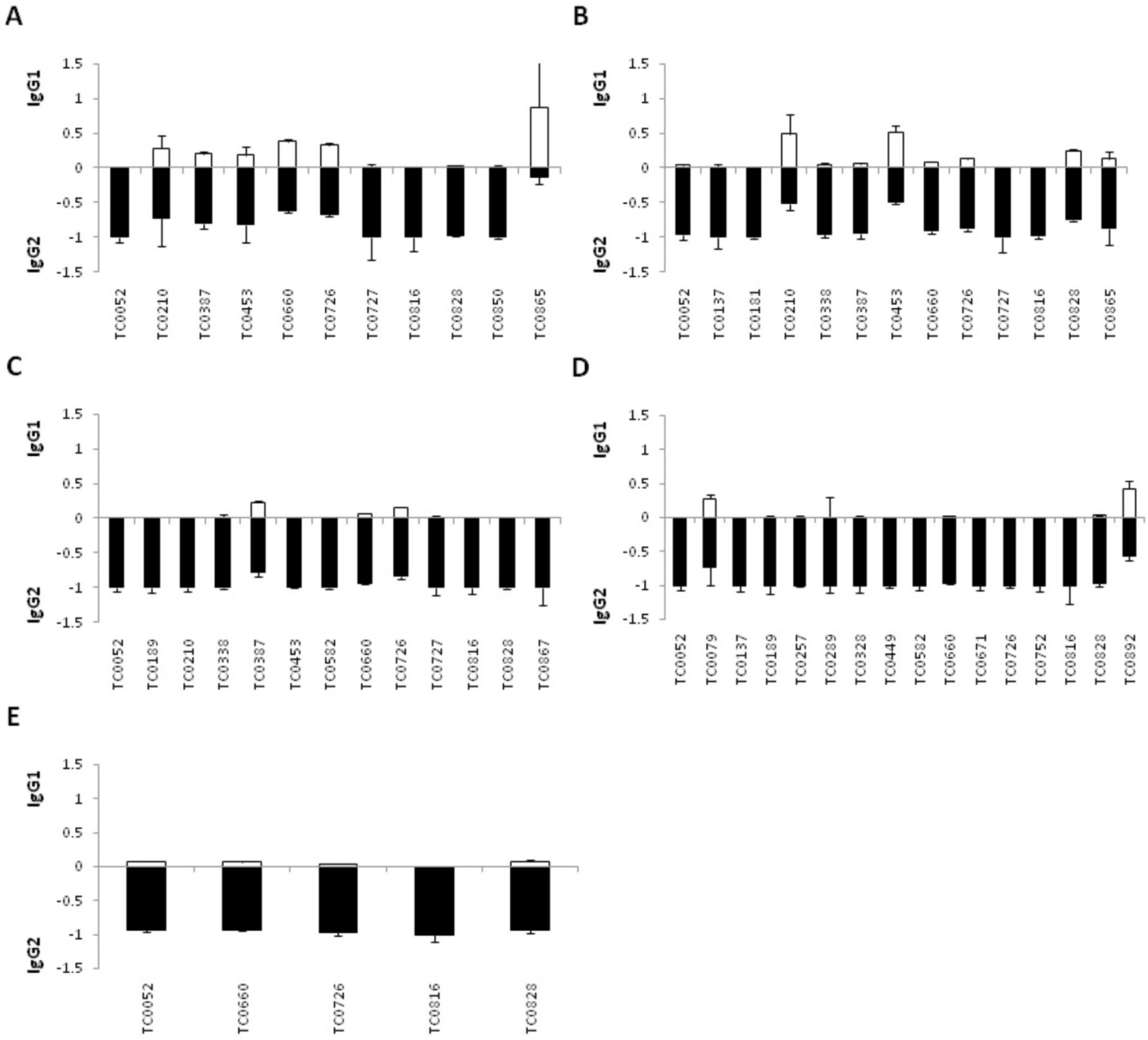

Figure 6. Relative quantities of IgG2a and IgG1 antibodies from C3H/HeN mice and IgG2c and IgG1 from C57BL/6 mice immunized with live *C. trachomatis* MoPn

Sera from female C3H/HeN mice inoculated by the A) i.n. and B) i.vag. routes. C) Samples from male C3H/HeN mice immunized i.n. Sera from female C57BL/6 mice inoculated by the D) i.n. and E) i.vag. routes.

Table 1

Tag detection as a measure of expression summary.

positive	225	221	221	0
total	225	225	225	225
% **positive**	100%	98.2%	98.2%	0%

Table 2

Bonferroni corrected p-values for antigens with detectable antibody titers after immunization

		BALB/c	BALB/c	BALB/c	BALB/c	BALB/c	C3H/HeN	C3H/HeN	C3H/HeN	C57BL/6	C57BL/6	
		F	F	M	F	F	F	F	M	F	F	
		i.n.	i.v.	i.n.	i.m.+s.c.	i.m.+s.c.	i.n.	i.v.	i.n.	i.n.	i.v.	
		MoPn	MoPn	MoPn	EB (UV)	RB (UV)	MoPn	MoPn	MoPn	MoPn	MoPn	frequency of reactivity
Locus	**Common Name**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	
TC0052	major outer membrane Pro., porin	■	■	■	■	■	■	■	■	■	■	10
TC0660	amino acid ABC transporter, periplasmic amino acid-binding Pro.	■	■	■	■		■	■	■	■	■	9
TC0828	peptidyl-prolyl cis-trans isomerase Mip	■	■	■	■		■	■	■	■	■	9
TC0726	sulfur rich Pro.	■	■	■			■	■	■	■	■	8
TC0387	10 kDa chaperonin	■	■	■	■		■	■	■			7
TC0210	serine protease, HtrA/DegQ/DegS family	■		■	■		■	■	■			6
TC0582	ATP synthase, subunit A	■	■	■					■	■	■	6
TC0816	conserved Hypo. Pro.		■				■	■	■	■	■	6
TC0189	conserved Hypo. Pro.	■	■	■					■	■		5
TC0727	60 kDa outer membrane Pro.						■	■	■	■	■	5
TC0328	conserved Hypo. Pro.	■								■	■	3
TC0338	ABC transporter, periplasmic substrate-binding Pro., putative	■						■	■			3
TC0453	conserved Hypo. Pro.						■	■	■			3
TC0865	type III secretion chaperone SycD						■	■	■			3
TC0137	UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanyl ligase MurF, putative							■		■		2
TC0166	conserved Hypo. Pro.		■	■								2
TC0181	glycogen synthase		■					■				2
TC0449	conserved Hypo. Pro.		■							■		2
TC0850	type III secretion translocase sctL						■		■			2
TC0909	conserved Hypo. Pro.			■						■		2

		BALB/c	BALB/c	BALB/c	BALB/c	BALB/c	C3H/HeN	C3H/HeN	C3H/HeN	C57BL/6	C57BL/6	
		F	F	M	F	F	F	F	M	F	F	
		i.n.	i.v.	i.n.	i.m.+s.c.	i.m.+s.c.	i.n.	i.v.	i.n.	i.n.	i.v.	
		MoPn	MoPn	MoPn	EB (UV)	RB (UV)	MoPn	MoPn	MoPn	MoPn	MoPn	frequency of reactivity
Locus	**Common Name**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	**p-val**	
TC0079	ATP-dependent Clp protease, proteolytic subunit											1
TC0257	1,4-alpha-glucan branching enzyme											1
TC0289	signal peptidase, putative											1
TC0500	Hypo. Pro.											1
TC0584	ATP synthase, subunit E, putative											1
TC0671	conserved Hypo. Pro.											1
TC0715	ATP-dependent Clp protease, proteolytic subunit											1
TC0752	sensor histidine kinase											1
TC0846	2-oxo acid dehydrogenase, E3 component, lipoamide dehydrogenase, putative											1
TC0852	type III secretion inner membrane Pro. SctS											1
TC0867	conserved Hypo. Pro.											1
TC0892	antioxidant, AhpC/Tsa family											1
TC0908	conserved Hypo. Pro.											1
TC0911	conserved Hypo. Pro.											1
	# of reactive antigens	10	12	10	7	1	11	13	16	21	8	