Identification of Early Response Genes in Human Peripheral Leukocytes Infected with *Orientia tsutsugamushi*: The Emergent of a Unique Gene Expression Profile for Diagnosis of *O. tsutsugamush* Infection

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Abstract: Scrub typhus, caused by infection with *Orientia tsutsugamushi*, is one of the most common rickettsial diseases in the Asia-Pacific region. The disease can cause up to 35% mortality if left untreated. In order to get a better understanding of the host responses to *O. tsutsugamushi* infection, freshly isolated peripheral blood mononuclear cells (PBMC) were infected with *O. tsutsugamushi*. The infected cells were collected at 1 h, 4 h, 8 h or 18 h post infection. The gene expression profiles were monitored by cDNA microarray. Among the 7,489 genes, 658 genes were up or down regulated by 2-fold upon infection. ANOVA *t*-test revealed 432 genes with statistically significant fold change ($p < 0.05$). Semi-quantitative PCR using specific primers that flank the mRNA splicing sites of these 658 genes was carried out to verify the microarray results. More than 9000 semi-quantitative PCR reactions were performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene. Of these reactions, 22 genes were confirmed to exhibit up or down regulation. Quantitative PCR using 18S rRNA as the reference further confirmed two genes (NM_001547 and NM_006187) that exhibited significant increase of expression. Analysis of these 22 genes and the specific increase of both NM_001547 and NM_006187 suggest that the 22 genes are unique to *O. tsutsugamushi* infection and have the potential use for differentiating infections caused by virus, bacteria, parasites and other pathogens.

Keywords: Scrub typhus, cDNA microarray, *Orientia tsutsugamushi* infection, semi-quantitative PCR, quantitative PCR, diagnosis, gene expression profiles.

INTRODUCTION

Orientia tsutaugamushi, an obligate intracellular bacterium, is the etiologic agent of scrub typhus, which is transmitted by the bite of larvae of numerous species of trombiculid mites. Although it has long been associated with the genus *Rickettsia* because of its similar obligate intracellular growth in the cytoplasm of infected host cells, in 1995 it was reclassified from *Rickettsia* to the genus *Orientia* due to its clear phenotypic and genotypic differences from *Rickettsia* [1]. These differences include a different cell wall structure which lacks peptidoglycan and lipopolysaccharide. The lack of both peptidoglycan and lipopolysaccharide is also characteristics of the phagosome-enclosed intracellular species in the genera *Anaplasma* and *Ehrlichia* while as the genera *Rickettsia* has both molecules [2]. *Orientia* also has a unique profile of proteins and antigens which distinguish it from all the other Rickettsiales [2, 3].

Scrub typhus accounts for up to 23% of all febrile illnesses in the Asia-Pacific endemic region [4]. Geographic distribution of the disease occurs within an area of about 13 million square kilometers and includes Pakistan, India and Nepal to the west, Japan to the east, southeastern Siberia, China, and Korea to the north and Indonesia, Philippines, northern Australia and the intervening Pacific islands to the south [5-13]. The disease is characterized by fever, rash, eschar, pneumonitis, meningitis, and in some cases, disseminated intravascular coagulation that may lead to circulatory failure [14]. Scrub typhus is one of the most common rickettsial diseases and can cause up to 35% mortality if left untreated [4, 15]. One billion people living in this area are at the risk of scrub typhus. During the World War II, more than 5000 cases were reported among US troops and 30,000 cases for Japanese troops. Scrub typhus ranked only behind malaria as the most important arthropod borne medical problem. During the Vietnam War, scrub typhus was again the number second most important arthropod caused diseases [16].

Orientia can infect a variety of mammalian cells *in vitro*, including macrophages, polymorphonuclear leukocytes, and endothelial cells, and it replicates in the cytoplasm of the infected cells. Prior studies have reported that *O. tsutsugamushi* induces phagocytosis by host cells and then escapes from the phagosome within 30 minutes by lysing the phagosomal membrane [17], but the precise mechanisms of

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14. ABSTRACT

Scrub typhus, caused by infection with Orientia tsutsugamushi, is one of the most common rickettsial diseases in the Asia-Pacific region. The disease can cause up to 35% mortality if left untreated. In order to get a better understanding of the host responses to O. tsutsugamushi infection, freshly isolated peripheral blood mononuclear cells (PBMC) were infected with O. tsutsugamushi. The infected cells were collected at 1 h, 4 h, 8 h or 18 h post infection. The gene expression profiles were monitored by cDNA microarray. Among the 7,489 genes, 658 genes were up or down regulated by 2-fold upon infection. ANOVA t-test revealed 432 genes with statistically significant fold change ($p < 0.05$). Semi-quantitative PCR using specific primers that flank the mRNA splicing sites of these 658 genes was carried out to verify the microarray results. More than 9000 semi-quantitative PCR reactions were performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene. Of these reactions, 22 genes were confirmed to exhibit up or down regulation. Quantitative PCR using 18S rRNA as the reference further confirmed two genes (NM_001547 and NM_006187) that exhibited significant increase of expression. Analysis of these 22 genes and the specific increase of both NM_001547 and NM_006187 suggest that the 22 genes are unique to O. tsutsugamushi infection and have the potential use for differentiating infections caused by virus, bacteria, parasites and other pathogens.

15. SUBJECT TERMS

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entry into host cells and escape from the endocytic pathway have not yet been clearly defined. Other intracellular bacteria have developed different strategies to cope with the harsh environment within phagosome. For example, *Coxiella burnetii* thrives in an acidic compartment [18] and *Mycobacterium avium* [19] and *Legionella pneumophila* [20] attenuate the acidic pH of the compartment in which they reside. *R. prowazekii* has developed mechanisms to lyse the phagosomal membrane and escape into the cytoplasm [21]. All these events must occur soon after the intracellular bacteria enter the host cells to establish their respective growth colonies for propagating within the cytoplasm. It has been shown that *O. tsutsugamushi* may utilize microtubules and dynein to move from the cell periphery to the microtubule organizing center [22]. Therefore, the initial interactions between host and intracellular infectious agents, which lead to the essential responses that are important for the infection and propagation of these agents to successfully grow inside the host cells are crucial events for our understanding of the pathogens.

Microarrays are a promising technique for elucidating and interpreting the mechanistic roles of genes in the pathogenesis of infectious disease. It has been used to analyze the genetic polymorphisms of special loci associated with resistance to antimicrobial agents, to explore the distribution of genes among isolates from the same and similar species, to understand the evolutionary relationship between closely related species, and to integrate the clinical and genomic data. This technique has also been used to study host-pathogen interactions, mainly by identifying genes from pathogens that may be involved in pathogenecity and by surveying the scope of the host response to infection. Experiments investigating host response to infection with *in vitro* models have revealed insights into mechanisms of pathogenesis [23] and have highlighted the potential for the application of microarray technology in studying infection *in vivo*.

As a step toward understanding the host-*Orientia tsutsugamushi* interaction at the molecular level, we used human cDNA microarray technology to examine in detail the host transcriptional profile in a simple laboratory model for the initial encounter between human immune cells and the pathogen, *O. tsutsugamushi*. Human peripheral blood mononuclear cells (PMBCs), encompassing a diverse repertoire of both innate and adaptive immune functions, have well-established roles in surveillance for infectious threats, both directly, through contact with infectious agents, and indirectly, through interactions with infected cells and tissues by means of secreted signaling molecules [24]. By analyzing the host gene expression response, we intend to address the following: i) whether infection by *O. tsutsugamushi* is accompanied by distinct gene expression profiles; ii) which features of the host response to *O. tsutsugamushi* are common to diverse pathogens and which are specific to Orientia; and iii) how virulence mechanisms, which may be unique to *O. tsutsugamushi*, influence and modify these innate response programs.

In this study, we infected freshly isolated human PBMCs with *O. tsutsugamushi* and followed the change of gene expression at 1, 4, 8 and 18 h post infection using a cDNA microarray containing 7,489 different genes. A total of 658 genes were identified with a greater than 2-fold change in expression level. These genes were further confirmed by semi-quantitative PCR using primers flanking the mRNA splice sites for specificity. The expression levels were normalized based on housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Genes confirmed with semi-quantitative PCR were further analyzed with real-time PCR using 18S rRNA as the reference to confirm their altered expression level. Among the genes tested by semi-quantitative PCR, 22 of them showed significant change in expression. Further analysis using real-time PCR confirmed that both NM_001547 and NM_006187 were significantly up-regulated. The profile of 22 responsive genes appeared to be uniquely associated with *O. tsutsugamushi* infection. The exclusivity of this profile is sufficient to differentiate *O. tsutsugamushi* infection from other infections caused by viruses or bacteria including organisms that are closely related to *O. tsutsugamushi* such as *Anaplasma phagocytophilum* and *R. prowazekii*, parasites and other pathogens.

MATERIALS AND METHODOLOGY

Preparation of Orientia tsutsugamishi for infection. The 1 mL seed inoculum stored at -80^{o}C was thawed at 37^{o}C and mixed well with 19 mL of brain heart infusion (BHI). Confluent L929 cells in T162 were infected with 2 mL of BHI-Orientia suspension and placed on a rocker platform for 60 minutes with rotation at 90 degrees each 15 minutes to ensure the inoculum was dispersed uniformly over all the cells. The flasks were incubated at 35^{o}C with 5% CO_2 in M-199 with Earles salts, 5% fetal bovine serum, 2mM L-glutamine, and 5% tryptose phosphate broth (TPB). The infected L929 cells were harvested 5-8 days post infection (when 30-50% cells deteriorated). The medium was replaced with 10 mL of K36 (16.5 mM KH_2PO_4, 33.3 mM K_2HPO_4, 100 mM KCl and 15.5 mM NaCl, pH 7.2-7.3) buffer solution. Sterile glass beads (5 mm) were added to the flask which was rocked gently to slough the L929 cells from the surface until no cells were attached. The cell suspension was centrifuged at 8,000 rpm in a Sorvall RC-5C centrifuge at 4^{o}C for 30 minutes. The pellet was resuspended in 5 mL filtered SRM (4.9 mM L-glutamine, 3.6 mM KH_2PO_4, 7.1 mM K_2HPO_4, 218 mM sucrose, 1% Renografin 76 and 5 mM $MgCl_2$) and aliquoted into 2 mL cryovials and stored at -80^{o}C until use. A slide smear and control for sterility were done to ensure the quality of preparation.

Preparation of PMBCs. Blood from three volunteers was collected into CPT tubes on three different days. Each blood sample was mixed and centrifuged at 1500 x g in a swinging-bucket rotor at room temp for 45 min. After centrifugation, the top layer of yellowish plasma was aspirated and discarded. The whitish mononuclear cell layer was aspirated and transferred into 50 mL tubes. Mononuclear cells were washed with PBS twice by centrifugation at 180 x g at room temperature for 10 minutes. Cells were resuspended in appropriate volume of PBS (30 mL of PBS for 500 mL blood). The cells were counted using a hemacytometer. An equal number of cells from each individual were used for each time point with or without infection.

Infection of PMBCs with O. tsutsugamushi. PMBCs in PBS were centrifuged at 1,200 rpm for 10 minutes and resuspended with growth medium (GM, RPMI with 7.5% human serum and 1X L-glutamine) to obtain $6x10^6$ cells/mL. A vial of *O. tsutsugamushi* was thawed and added to PMBCs at multiplicity of infection (MOI) of 100 and gently rotated (10 rpm) at 35°C for 45 minutes. For the uninfected control samples, cells were incubated under the same condition without *O. tsutsugamushi*. After incubation, PBS was added to the *O. tsutsugamushi* infected PBMC and centrifuged at 2,000 rpm in a Sorvall RC-5C centrifuge for 5 minutes to wash away any un-internalized *Orientia*. This step was repeated once and the pellet of PMBCs was resuspended in GM. Eight mL of infected and uninfected PMBCs was added into each well in a 4-well plate containing GM. A total of 2 plates (8 wells) were used for each of the uninfected and infected groups. These plates were left in an incubator after infection.

Incubation of infected cells and RNA extraction at indicated times. Cells were incubated at 5% CO_2, 95% humidity and 35°C for additional 1, 4, 8 and 18 hrs post-infection. At each indicated time, both uninfected and infected PMBCs were removed, centrifuged at 2,000 rpm in a Sorvall RC-5C centrifuge for 5 minutes, and the pellet was resuspended in 1.5 mL Trizol (Invitrogen, CA) for RNA extraction which was performed as described by the manufacturer. The purified RNA was subjected to both quantitative and qualitative analyses with Agilent 2100 Bioanalyzer (Agilent Technologies, CA). Only those samples with good quality RNA were used for microarray analysis. High quality RNA with A_{260}/A_{280} ratio greater than 1.9 and enough quantity was obtained for microarray studies. Total DNA was also extracted after RNA extraction by Trizol according to the instruction provided by the manufacturer. Extracted DNA (with A_{260}/A_{280} ratio greater than 1.8) was used as template in PCR with a primer set specific for the GroELS gene [25] of *O. tsutsugamushi* to confirm infection of PBMC by Orientia.

DNA microarray Preparation and Image analysis. The cloned gene library for printing microarray slides was obtained from Research Genetics (Invitrogen, Carlsbad, CA). The slides contained 7,489 genes, including 7,019 known genes, 249 unknown genes,110 expressed sequence tagged genes (ESTs), and 111 positive and negative control genes in replicates. Superamine coated Telechem slides (Telechem Inc., OR) were used for printing the cDNA clones using 12 X 4 pin format, on a Virtek chip writer professional microarrayer at KemTek, Inc, MD. The printed slides underwent UV cross-linking, followed by succinic anhydride treatment. The Micromax™ Tyramide Signal Amplification (TSA)™ Labeling and Detection Kit (PerkinElmer, Inc., MA) was used as recommended by the manufacturer to determine relative gene expression of the collected samples. Human reference RNA was obtained from Stratagene and was used on every slide as the array control to check overall sensitivity of array printing and to monitor reverse transcription, labeling and hybridization efficiencies. Sample hybridization was carried out at 65°C for sixteen hours. A laser detection system was used (GenePix 4000b, Axon Instruments, CA) to scan the finished slides. The intensity of the scanned images was digitalized through Genepix 4.0 software (Axon Inc., CA) [26].

Data Analysis for Microarray. Data filtering and statistical analysis were carried out using Genespring® 7.0 (Agilent Technologies, Santa Clara, CA). Local background was subtracted from individual spot intensity. Genes that failed this 'background check' in any of the given experiments were eliminated from further analysis. Next, each chip was subjected to intra-chip normalization (LOWESS). The genes that varied most between infected and uninfected sample sets were selected *via* ANOVA *t*-test analysis followed by Benjamin correction in order to reduce false discovery rate of less than 5%. A two dimensional hierarchal clustering calculation using Pearson correlation around zero was also performed.

Semi-Quantitative PCR Analyses

a) *cDNA template preparations and the normalization factor determination.* Semi-quantitative PCR analyses were performed to confirm the list of genes of which expression level was affected by *O. tsutsugamushi* infection. The first strand cDNA synthesis reaction was carried out in a 100 µl reaction volume containing 15 µg of the total RNA from each sample, previously denatured at 70°C for 5 min and cooled on ice for 3 min, in the presence of 2 µl oligo dT, dNTPs, DTT, Superscript II RT (Invitrogen) and RT buffer following the manufacturer's instruction. The reaction mixtures were incubated at 42°C for 50 min, then at 70°C for 10 min. PCRs were performed using the gene specific primers for GAPDH (housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase) (Forward: 5'-ACTGGCGTCTTC ACCACCATG-3; Reverse: 5'-ACCACCTGGTGCTCAG TGTAG-3') in the presence of a 1:5 fold serial dilutions of the newly synthesized cDNAs. The PCR mixture was incubated at 94°C for 3 min followed by 30 cycles of a 3-step amplification at 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1.5 min. PCR products (size 551 bp) were separated by electrophoresis on a 1% agarose gel. The unsaturated band from the gel image data was selected from each cDNA sample and semi-quantitation of each sample was performed by using Gel analyzing software, GelPic Analyzer 1.2 (GeneHarbor Inc.Gaithersburg, MD). After background subtraction, the normalization factor of the cDNA template was determined based on the intensity of the band corresponding to GAPDH. The normalization factor was used to semi-quantitate the relative amounts of gene expression based on the amount of GAPDH as the internal standard.

b) *Gene specific primer selection for semi-quantitative PCR.* Specific primers that flank the mRNA splicing sites of these 658 genes were meticulously designed by a highly reliable primer designing algorithm GeneLooper™ 2.0 (GeneHarbor Inc) which provides a uniform annealing temperature and PCR product size (62°C and 300-350 bp, respectively), so that one set of PCR condition can be used for all primer pairs.

c) *Semi-quantitative PCR* PCR was conducted in a 20 µl of reaction volume containing 1 X reaction buffer, 200 uM dNTPs, 250 nM forward and reverse gene specific primers, 1 unit Taq polymerase (GeneCopoeia Inc, Germantown, MD) and the cDNAs obtained from uninfected and infected samples in 96-well plates. Typically 46 genes of interest from uninfected and infected samples were analyzed in

parallel with one negative control and one GAPDH positive control. The PCR mixture was incubated at 94°C for 3 min followed by 32 cycles at 94°C for 30 sec, 62°C for 30sec., and 72°C for 1 min. At the end of PCR amplification, the reaction mixture was held at 72°C for 5 minutes followed by incubation at 4°C. PCR products from uninfected and infected cDNAs were separated by electrophoresis on 1% agarose gel. Semi-quantitation of the PCR gel image data was performed based on the normalization factor for each cDNA template as described previously.

Quantitative PCR

a) *Quantitative PCR using SYBR Green.* The quantitative real time-PCR assays were carried out using the same cDNA templates for semi-quantitative PCR as described previously. SYBR green qPCR experiments were performed in the iCycler (BioRad, Hercules, CA) using the light cycler DNA master SYBR green I kit (Roche Diagnostics, Indianapolis, Indiana). The 18S rRNA was used as control (house keeping gene, HKG) to normalize the raw real-time PCR data of the genes of interest. Sequence information for each primer set is listed in Supplementary Table **1**.

Sensitivity experiments were performed using pBAC-2cp as a template and plasmid-specific primers designed to produce a 311 bp amplicon. The PCR was initiated with a 2-minute denaturation at 95°C followed by 40 cycles at 95 °C for 15 seconds, 20 seconds annealing at 60 °C, and 30 seconds extension at 72 °C. After the completion of 40 cycles, the reaction mixture was held at 72°C for 5 min followed by incubation at 4 °C. The standard curve had a serial 1:10 dilution of DNA template starting from 1 ng down to 100 fg. One additional ten-fold dilution was made after the standard was undetectable. The analyses of data were accomplished using the iCycler Software.

Serial dilutions were used to determine the efficiency (E) of each primer set according to the following equation: $E = (10^{-1}/m)^{-1}$ where m represents the slope of the best fitted straight line of the graph of Ct (threshold cycle) *vs* the corresponding range of dilution factors of cDNA.

The Ct values for all the genes were converted to a fold change using the formula $[(1 + E)\Delta Ct]_{GOI}/ [(1 + E)\Delta Ct]_{HKG}$, where ΔCt denotes the difference between the Ct' values of uninfected and infected samples of a given gene. GOI and HKG symbolizes genes of interest and house keeping genes (18S rRNA) respectively.

b). *Quantitative TaqMan real time PCR. Fluorogenic probe and oligonucletide primers selection and synthesis.* The complete ORF regions of the two genes (NM-001547 and NM_006187) were analyzed to develop probes for TaqMan quantitative PCR. The assay was formulated to employ a uniquely designed internal fluorescence-labeled probe complementary to a target sequence of each amplicon using a pair of flanking primers. The primer pairs and probes of NM_001547 and NM_006187 genes for real time PCR were designed using Primer Express software (PE Applied Biosystem Inc., Foster City, CA) and are shown in Supplementary Table **2**. The fluorescence labeled oligonucleotide probe was labeled with 5'-FAM (reporter dye) and 3'-TAMRA dyes (quencher dye). After hybridizing to the target amplicon, fluorescent signal was generated by separating the reporter dye from the quencher dye through 5'-nuclease activity of DNA Taq polymerase [27, 28]. Labeled probes were synthesized by Eurogentec (San Diego, CA) and unlabeled primer pairs were synthesized by MWG Biotech Inc. (High Point, NC).

PCR was conducted in 50 µl reaction volumes containing 2µl cDNA template, 5 µl 10X Taqman buffer (PE ABI), 4 µl 1.25 mM dNTPs, 8 µl 25 mM $MgCl_2$, 200 mM forward and reverse primers, 20 nM fluoregenic probe, and 1.25 units AmpliTaq Gold DNA polymerase (PE ABI). A gene detection system from MJ research (DNA Engine Opticon 2 Real-Time Cycler) was employed for PCR cycling amplification, real time data collection and analysis. PCR mixtures were pre-incubated at 50°C for 2 min, then 95°C for 10 min followed by 40 cycles of two-step amplification at 95°C for 15 second and 60°C for 1 min.

RESULTS

Using cDNA arrays and various bioinformatics tools, gene expression profiles induced by intracellular *O. tsutsugamushi* in human PBMC were measured at early stages of infection. Expression ratios of genes in the cDNA arrays were determined by comparing the levels of mRNA in *Orientia* infected cells *vs* uninfected cells at each time point. The results were represented as the average of blood samples analyzed from three separate donors.

Confirmation of Infection of PBMCs by O. tsutsugamushi: The infection of PBMCs with *O. tsutsugamushi* was confirmed by Giemsa staining (data not shown). Furthermore, the presence of *Orientia* DNA in infected samples was demonstrated by PCR using primers for the groELS. The results in Fig. (**1A**) show clearly that the *O. tsutsugamushi* DNA was only detectable in the infected samples (amplicon of 548 bp long as indicated) at all time points but not in the uninfected samples confirming that PBMCs were infected by *O. tsutsugamushi.*

Quality evaluation of extracted RNA: The quality of the extracted RNA was evaluated by the integrity of the 18S and 28S rRNA [29]. As shown in Fig. (**1B**), it was clear that the majority of the RNA preparations was of high quality and could be used in the downstream microarray application. The apparent differences in mobility were inspected by using analysis software provided by the manufacturer (Agilent Technologies).

Gene expression profiles regulated in PBMC by Orientia infection and Identification of early responsive genes. A total of 658 genes or 9% of the genes on the microarray showed more than 2-fold change in expression during *Orientia* infection. These genes belong to many different functional categories such as cytokines, transcription factors, kinases and phosphatases, genes involved in hemostasis, coagulation and apoptosis (Fig. **2**, Panels **A** and **B**). Up and down regulations of a variety of different classes of gene families were observed in a time dependent manner (Fig. **2**, Panel **C**). The 2-fold change of expression was selected arbitrarily as the cutoff for further studies due to the excessive number of genes. There are 432 genes with statistically significant changes based on ANOVA *t*-test ($p < 0.05$).

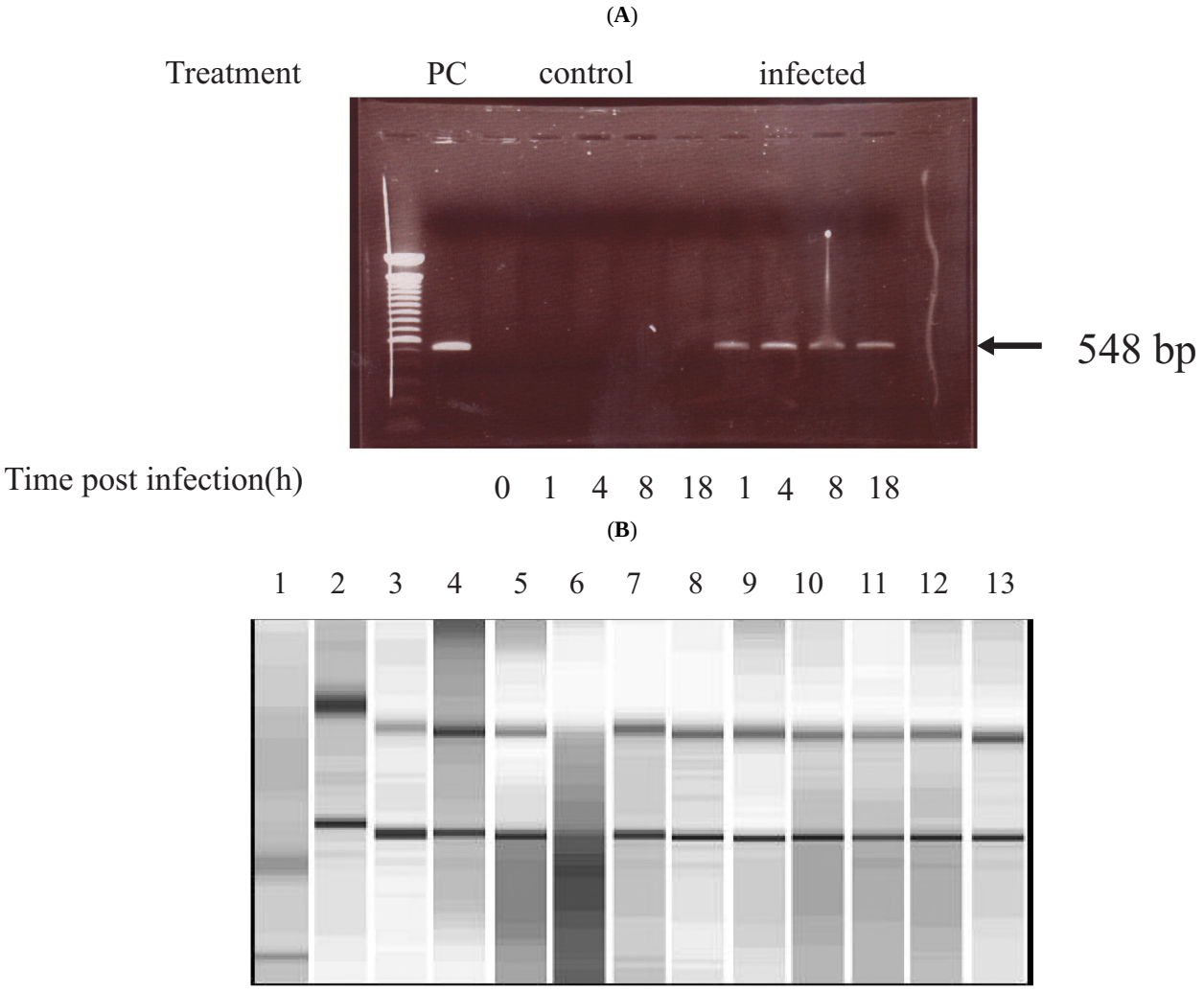
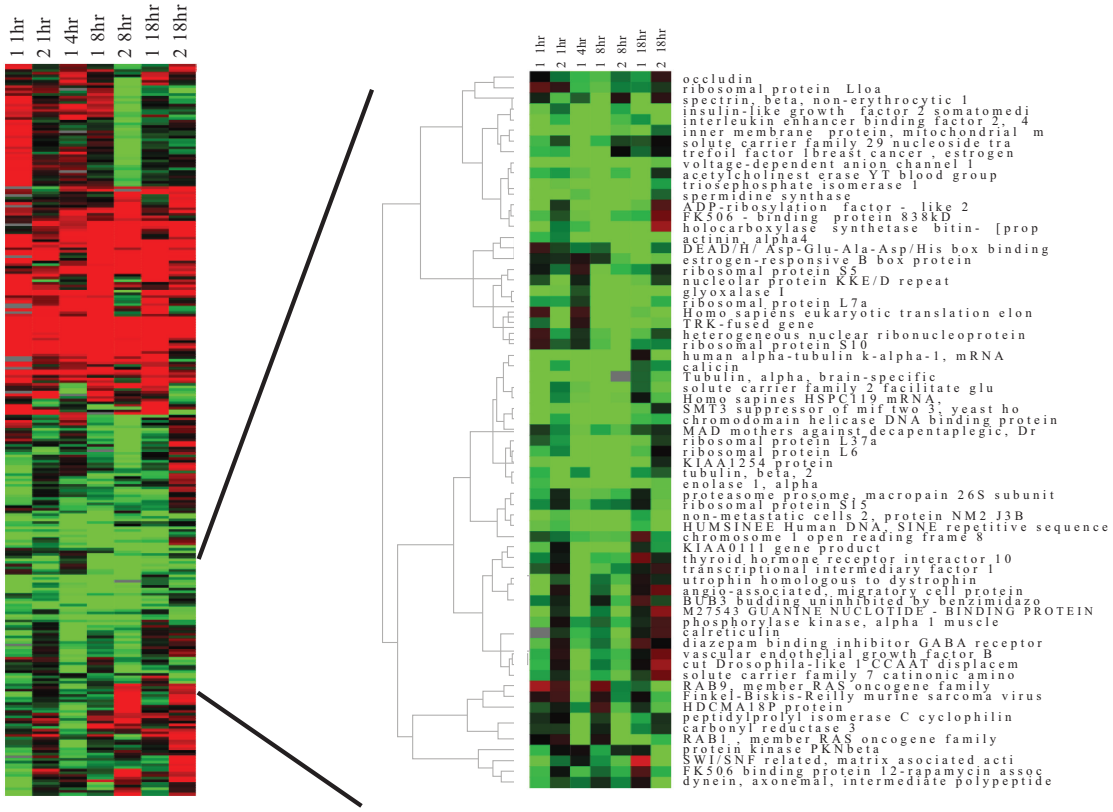


Fig. (1). Verification of PBMC infection by *O. tsutsugamushi* and quality analysis of RNA extracted from uninfected and infected PBMC. Panel **A** shows the PCR amplicon of GroEL gene from the *O. tsutsugamushi* genome. DNA from each sample was used as template in PCR to generate 548 bp amplicon representing a segment of the conserved GroELS gene in *O. tsutsugamushi.* Pure genomic DNA extracted from *O. tsutsugamushi* Karp strain was used as positive control (lane 2). Uninfected samples (lane 3-7) and infected samples (lanes 8-11) obtained 1, 4, 8 and 18 hrs post infection from one of the donors were used for the experiment. Lane 1 is 1 kb DNA ladder standard. Panel **B** shows the quality of RNA examined using Agilent Bioanalyzer 2100 as described in Materials and Methods. The 28S and 18S rRNA were observed as two distinct bands in most of the samples. Lanes 1-13 are duplicated samples from different time points from one donor.

Semi-quantitative analysis of regulated genes. To confirm the expression difference of these 658 genes, we undertook a high-throughput PCR approach using specific primer sets designed to cover the mRNA splicing sites of the 658 genes of interest. This was performed in order to eliminate the possibility of PCR amplification due to contaminated DNA in the RNA preparations and to allow for the detection of specific amplicons from matured mRNA. The densitometry analysis of the amplicons of GAPDH was used to normalize the amount of input cDNA. The ratio of area of blue bar/area of red bar (Supplementary Fig. **1A**) represents the correcting factor used to normalize the variation of amounts of cDNA in each corresponding uninfected and infected sample pair. An example of data from one 96-well plate is shown in Supplementary Fig. (**1B**). The green circle indicated the set of gene that was up-regulated due to *O. tsutsugamushi* infection and the red circle indicated the gene that was down-regulated. Semi-quantitative analysis of the intensity of amplicons using GAPDH as an internal control has indicated that 22 genes exhibited different levels of expression in infected *vs* uninfected samples. The accession number, names of the genes and their biological processes are listed in Table **1**. Examples of semi-quantitative analysis of two 8 h post-infection samples are shown in Supplementary Fig. (**2**). Not all of these genes showed up-or down-regulation relative to the expression in uninfected sample at all time points. These 22 genes represented 3.3% of the total number of genes identified as 2-fold up-or down-regulated by microarray assays. The protein products of these 22 genes are involved in the following biological processes: signal transduction (9 genes), nucleotide and nucleic acid metabolism (5 genes), immune response (3 genes), and one gene each in cell growth and/or maintenance, metabolism and energy

(A)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ribosomal protein S5
nucleolar protein KKE/D repeat
glyoxalase I
ribosomal protein L7a
Homo sapiens eukaryotic translation elon
TRK-fused gene
heterogeneous nuclear ribonucleoprotein
ribosomal protein S10
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calicin
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SMT3 suppressor of mif two 3, yeast ho
chromodomain helicase DNA binding protein
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ribosomal protein L37a
ribosomal protein L6
KIAA1254 protein
tubulin, beta, 2
enolase 1, alpha
proteasome prosome, macropain 26S subunit
ribosomal protein S15
non-metastatic cells 2, protein NM2 J3B
HUMSINEE Human DNA, SINE repetitive sequence
chromosome 1 open reading frame 8
KIAA0111 gene product
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transcriptional intermediary factor 1
utrophin homologous to dystrophin
angio-associated, migratory cell protein
BUB3 budding uninhibited by benzimidazo
M27543 GUANINE NUCLOTIDE - BINDING PROTEIN
phosphorylase kinase, alpha 1 muscle
calreticulin
diazepam binding inhibitor GABA receptor
vascular endothelial growth factor B
cut Drosophila-like 1 CCAAT displacem
solute carrier family 7 catinonic amino
RAB9, member RAS oncogene family
Finkel-Biskis-Reilly murine sarcoma virus
HDCMA18P protein
peptidylprolyl isomerase C cyclophilin
carbonyl reductase 3
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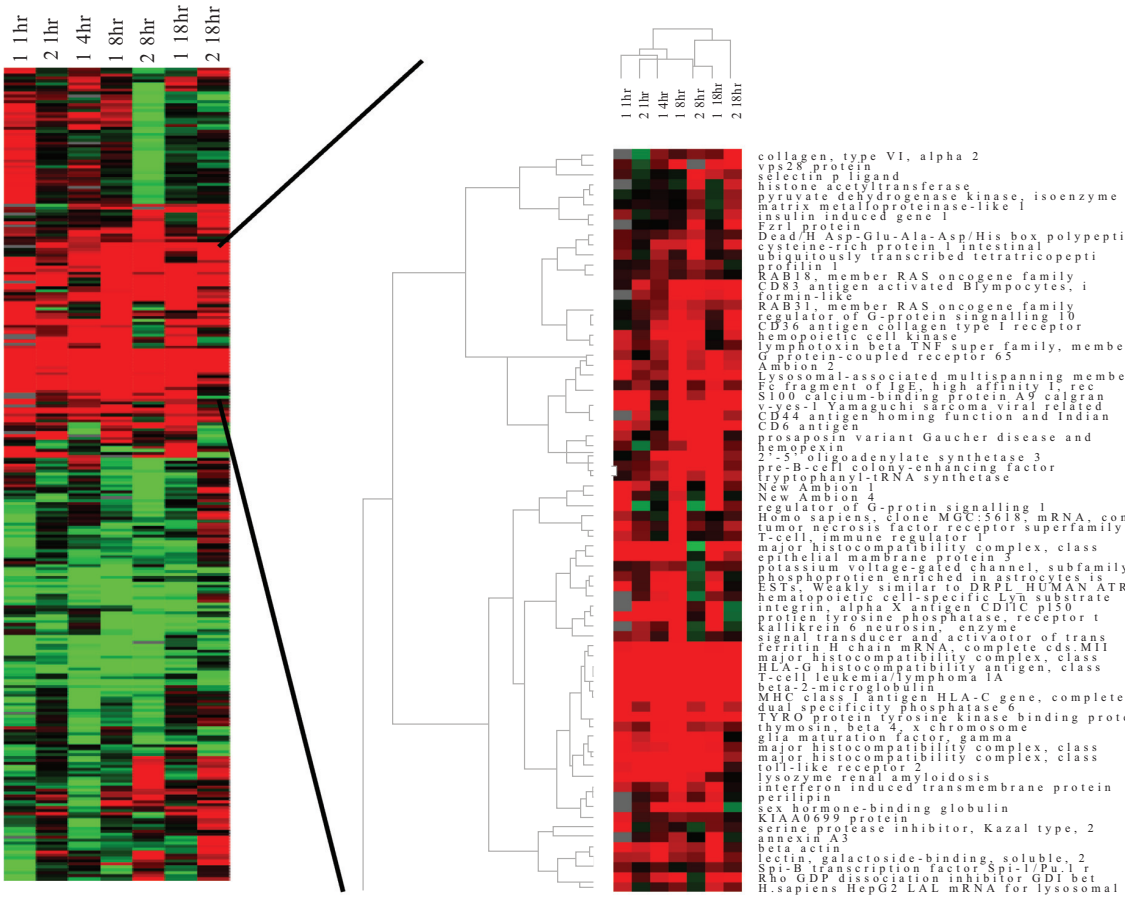
FK506 binding protein 12-rapamycin assoc
dynein, axonemal, intermediate polypeptide

(B)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histone acetyltransferase
pyruvate dehydrogenase kinase, isoenzyme
matrix metalloproteinase-like 1
insulin induced gene 1
Fzr1 protein
Dead/H Asp-Glu-Ala-Asp/His box polypeptide
cysteine-rich protein 1 intestinal
ubiquitously transcribed tetratricopepti
profilin 1
RAB18, member RAS oncogene family
CD83 antigen activated Blympocytes, i
formin-like
RAB31, member RAS oncogene family
regulator of G-protein singnalling 10
CD36 antigen collagen type I receptor
hemopoietic cell kinase
lymphotoxin beta TNF super family, member
G protein-coupled receptor 65
Ambion 2
Lysosomal-associated multispanning member
Fc fragment of IgE, high affinity I, rec
S100 calcium-binding protein A9 calgran
v-yes-1 Yamaguchi sarcoma viral related
CD44 antigen homing function and Indian
CD6 antigen
prosaposin variant Gaucher disease and
hemopexin
2'-5'- oligoadenylate synthetase 3
pre-B-cell colony-enhancing factor
tryptophanyl-tRNA synthetase
New Ambion 1
New Ambion 4
regulator of G-protin signalling 1
Homo sapiens, clone MGC:5618, mRNA, comp
tumor necrosis factor receptor superfamily
T-cell, immune regulator 1
major histocompatibility complex, class
epithelial mambrane protein 3
potassium voltage-gated channel, subfamily
phosphoprotein enriched in astrocytes, is
ESTs, Weakly similar to DRPL_HUMAN ATROP
hematopoietic cell-specific Lyn substrate
integrin, alpha X antigen CD11C p150
protien tyrosine phosphatase, receptor t
kallikrein 6 neurosin, enzyme
signal transducer and activaotor of trans
ferritin H chain mRNA, complete cds.MII
major histocompatibility complex, class
HLA-G histocompatibility antigen, class
T-cell leukemia/lymphoma 1A
beta-2-microglobulin
MHC class I antigen HLA-C gene, complete
dual specificity phosphatase 6
TYRO protein tyrosine kinase binding protein
thymosin, beta 4, x chromosome
glia maturation factor, gamma
major histocompatibility complex, class
major histocompatibility complex, class
toll-like receptor 2
lysozyme renal amyloidosis
interferon induced transmembrane protein
perilipin
sex hormone-binding globulin
KIAA0699 protein
serine protease inhibitor, Kazal type, 2
annexin A3
beta actin
lectin, galactoside-binding, soluble, 2
Spi-B transcription factor Spi-1/Pu.1 r
Rho GDP dissociation inhibitor GDI bet
H.sapiens HepG2 LAL mRNA for lysosomal

(Fig. 2) contd…..

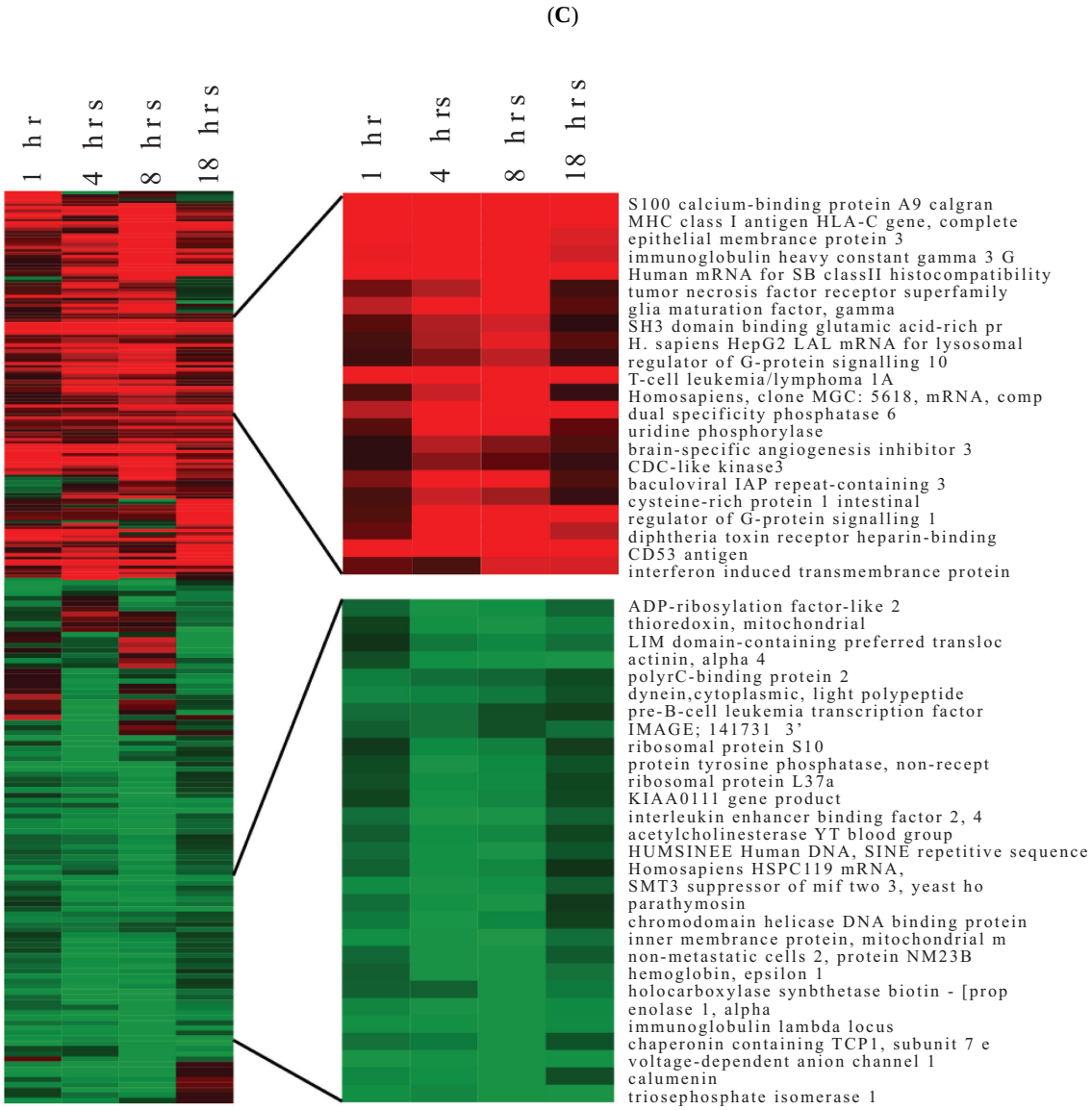

Fig. (2). Gene expression profiles of PBMCs infected by *O. tsutsugamushi*. The RNA was extracted at 1, 4, 8 and 18 hrs post infection. The DNA microarray was performed as described in the Materials and Methods. The up-regulated (in red) and down-regulated (in green) genes are shown with a blown-up view of certain regions on the right. Samples are labeled as 1 and 2 for uninfected and infected samples, respectively. Panel **A** shows an example of down-regulated genes from PBMCs isolated from a single donor. Panel **B** shows an example of up-regulated genes from PBMC isolated from the same donor. Panel **C** shows the result of average gene expression profile of *O. tsutsugamushi* infected PBMCs isolated from three individual donors.

pathways, regulation of cell cycle, cell adhesion, and apoptosis according to human protein reference database (www.hprd.org) [30].

Quantitative real-time PCR analysis of genes expression using SYBR green. The 22 genes confirmed by semi-quantitative analysis were further examined by SYBR green quantitative real-time PCR. Previous results indicated that most genes showed the greatest difference at 8 h post infection, thus we decided to focus our analysis on the 8 h post infection samples. All 22 genes showed differences between the control and infected samples (data not shown) but the differences were most prominent for NM_001547 (IFIT2) and NM_006187 (OAD3), known to be involved in cell growth and/or maintenance and immune response, respectively. Similar results for these two genes were observed for samples from different time points although the magnitude of differences was lower than those observed from 8 h post infection samples (data not shown).

Fluorgenic probes for quantitative real time PCR. Both NM_001547 and NM_006187 were further evaluated using quantitative real time PCR with primers and probes designed specifically for the two genes. The 4, 8, and 18 hours post infection infected and uninfected samples from one of the three donors were analyzed. The results are shown in Fig. (**3**) (similar results were observed with the other donors, data not shown). In panels **A** through **D**, the results of NM_001547

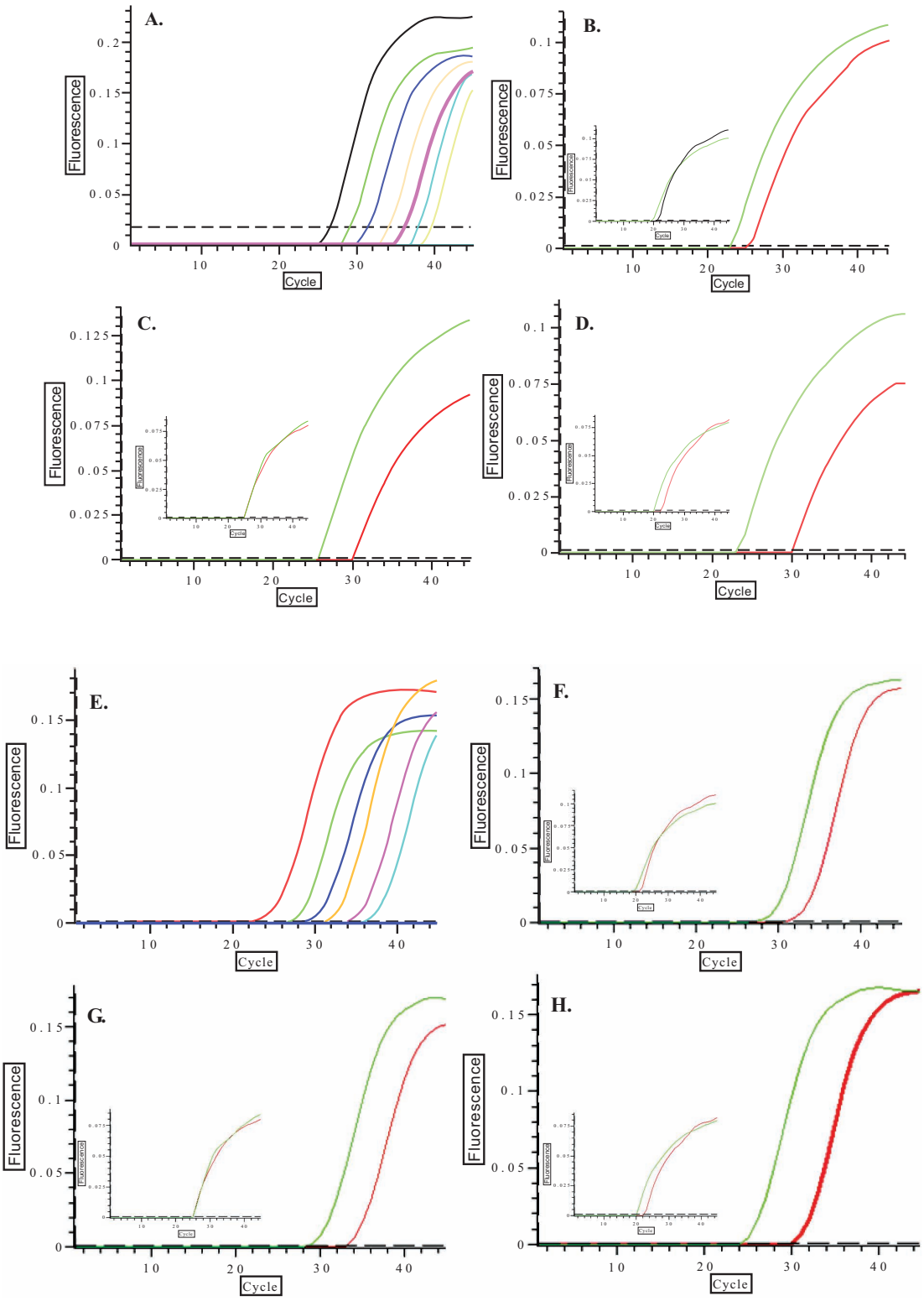

Fig. (3). Flourogenic real time PCR determination of NM_001547 and NM_006187. Uninfected and infected samples from donor 2 were used for analysis as described in the Materials and Methods. Panels **A** through **D** are the results of NM_001547 and panels **E** through **H** are the results of NM_006187. Panels **A** and **E** show two independently run standard curves using different amounts of 18S rRNA. Panel **B** and **F** show the difference of each gene between uninfected and infected samples 4 hrs post infection. Panel **C** and **G** show the difference of each gene between control and infected samples 8 hrs post infection. Panel **D** and **H** show the difference of each gene between uninfected and infected samples 18 hrs post infection. Insets in panels **B-D** and **F-H** are the signals of 18S rRNA from uninfected and infected samples to indicate equal amounts of RNA were loaded in the reaction. Green curves in panels **B-D** and **F-H** represent the signals of infected samples and red curves in the same panels represent the signals of uninfected samples.

Table 1. List of Genes Confirmed by Semi-Quantitative PCR*

Accession Number	Name of Genes	Biological Processes
NM_000530	myelin protein zero (Charcot-Marie-Tooth neuropathy 1B) (MPZ),	Signal transduction
NM_000595	lymphotoxin alpha (TNF superfamily, member 1) (LTA),	Signal transduction
NM_000801	FK506 binding protein 1A, 12kDa (FKBP1A), transcript variant 12B	Signal transduction
NM_001547	interferon-induced protein with tetratricopeptide repeats 2 (IFIT2),	Cell growth and/or maintenance
NM_001838	chemokine (C-C motif) receptor 7 (CCR7),	Signal transduction
NM_002498	NIMA (never in mitosis gene a)-related kinase 3 (NEK3), transcript variant 1,	Signal transduction
NM_002922	regulator of G-protein signalling 1 (RGS1),	Signal transduction
NM_002946	replication protein A2, 32kDa (RPA2),	Nucleotide and nucleic acid metabolism
NM_002983	chemokine (C-C motif) ligand 3 (CCL3),	Immune response
NM_003205	transcription factor 12 (HTF4, helix-loop-helix transcription factors 4) (TCF12), transcript variant 3	Nucleotide and nucleic acid metabolism
NM_003404	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (YWHAB), transcript variant 1	Signal transduction
NM_003906	MCM3 minichromosome maintenance deficient 3 (S. cerevisiae) associated protein (MCM3AP)	Signal transduction
NM_004551	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase) (NDUFS3),	Metabolism, Energy pathways
NM_005082	tripartite motif-containing 25 (TRIM25),	Nucleotide and nucleic acid metabolism
NM_005623	chemokine (C-C motif) ligand 8 (CCL8),	Signal transduction
NM_006187	2'-5'-oligoadenylate synthetase 3, 100kDa (OAS3),	Immune response
NM_007215	polymerase (DNA directed), gamma 2, accessory subunit (POLG2),	Nucleotide and nucleic acid metabolism
NM_015369	TP53TG3 protein (TP53TG3), transcript variant 1	Regulation of cell cycle
NM_021991	junction plakoglobin (JUP), transcript variant 2	Cell adhesion
NM_033340	caspase 7, apoptosis-related cysteine peptidase (CASP7), transcript variant beta	Apoptosis
NM_080657	radical S-adenosyl methionine domain containing 2 (RSAD2),	Immune response
NM_152998	enhancer of zeste homolog 2 (Drosophila) (EZH2), transcript variant 2	Nucleotide and nucleic acid metabolism

*Gene names are all found in Homo sapiens and the biological processes were assigned based on human protein reference database (HPRD, www.hprd.org). Gene names in bold are those confirmed as down-regulated genes.

are shown and panels **E** through **H** show the results of NM_006187. The insets in panels **B** to **D** and **F** to **H** showed that the amounts of cDNA for the 18S rRNA were equivalents for both control and infected samples, indicating that the differences in the cycle number for both target genes in each sample are due to differences in the number of their transcripts (cDNA copies). Thus both NM_001547 and NM_006187 are up-regulated in infected samples and the up-regulation is persistent for at least 18 hours post infection.

DISCUSSION AND CONCLUSION

The gene expression profile induced by *O. tsutsugamushi* infection was investigated in this study. The initial DNA microarray data revealed 658 genes with 2-fold up-or down-regulation at different time post infection. All these genes were subjected to a semi-quantitative analysis using GAPDH as the internal reference. Among the 658 genes, only 22 genes appeared to have robust differences in expression, particularly at 8 h post infection. The list of these genes was used to search against the Gene Expression Omnibus (GEO) database in NCBI regardless of the platform of microarray used, the up-or down-regulation of the gene of interest, and the time post infection (Table **2**). The purpose of this investigation was to determine whether the regulation of these genes upon *O. tsutsugamushi* infection was unique and specific. Each of the 22 genes had been previously identified as regulated by one or several different infectious agents, including virus and bacteria. Some of the infectious agents appeared to simultaneously regulate several of the 22 genes but none of the infectious agents showed regulation of all 22 genes identified in this study. This suggested that the gene expression profile composed of all these 22 genes is *O. tsutsugamushi* infection specific. Among all the infectious agents searched, *A. phagocytophilum* induced the regulation of 18 out of 22 genes in promyelocytic cells (NB4). Interestingly, based on the 16S rRNA gene sequence, it is known that both *Anaplasma* and *Orientia* along with *Rickettsia*, *Ehrlichia*, *Neorickettsia* and *Wolbachia* belong to the order Rickettsiales [31]. Taken together, the results suggest that even infection by *A. phagocytophilum*, one of the most closely-related infectious agents to *O. tsutsugamushi*, can be differentiated from infection by *O. tsutsugamushi* using the expression profile of these 22 genes.

Table 2. Genes Previously Shown to be Involved in Various Bacterial Infection

Gene[#]	GDS Record[@]	Infectious Agent Used in Experiment
000530	GDS 1022	*Pseudomanas aeruginosa*
	GDS 1774	Hepatitis C
000595	GDS 625	*Helicobacter pylori*
	GDS 940 and 1676	Herpesvirus
	GDS 1022	*Pseudomanas aeruginosa*
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1449, 2168, 2649	HIV
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 1667	Human papillomavirus
	GDS 1774	Hepatitus C
	GDS 2023	Syncytial virus
	GDS 2180	bacillus Calmette-Guerin
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 2362	Malaria
	GDS 2749	*Aspergillus fumigatus*
	GDS 2833	Polyomavirus JCV
000801	GDS 940	Herpesvirus
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1449, 2168, 2649	HIV
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 1667	Human papillomavirus
	GDS 1774	Hepatitus C
	GDS 2023	Syncytial virus
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 2362	Malaria
	GDS 2749	*Aspergillus fumigatus*
	GDS 2833	Polyomavirus JCV
001547	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 1774	Hepatitis C
001838	GDS 625	*Helicobacter pylori*
	GDS 940 and 1676	Herpesvirus
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1449, 2168, 2649	HIV
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 1667	Human papillomavirus
	GDS 1774	Hepatitus C
	GDS 2023	Syncytial virus
	GDS 2180	bacillus Calmette-Guerin
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 2362	Malaria
	GDS 2749	*Aspergillus fumigatus*
	GDS 2756	Measles
	GDS 2833	Polyomavirus JCV
002498	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 1774	Hepatitis C

(Table 2) contd…..

Gene[#]	GDS Record[@]	Infectious Agent Used in Experiment
002922	GDS 940	Herpesvirus
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 1022	*Pseudomanas aeruginosa*
	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 1667	Human papillomavirus
	GDS 2023	Syncytial virus
	GDS 2168, 2649	HIV
	GDS 2180	bacillus Calmette-Guerin
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 2362, 2822	Malaria
	GDS 2749	*Aspergillus fumigatus*
	GDS 2756	Measles
	GDS 2833	Polyomavirus JCV
002946	GDS 940	Herpesvirus
	GDS 1022	*Pseudomanas aeruginosa*
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1449, 2168, 2649	HIV
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 1667	Human papillomavirus
	GDS 1774	Hepatitis C
	GDS 2023	Syncytial virus
	GDS 2180	bacillus Calmette-Guerin
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 2362	Malaria
	GDS 2749	*Aspergillus fumigatus*
	GDS 2833	Polyomavirus JCV
002983	GDS 260	*Leishmania major, Leishmania donovani, Toxoplasma gondii, Mycobacterium tuberculosis, Burgia malyi*
	GDS 625	*Helicobacter pylori*
	GDS 940	Herpesvirus
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1449, 2168, 2649	HIV
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 1667	Human papillomavirus
	GDS 2023	Syncytial virus
	GDS 2180	bacillus Calmette-Guerin
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 2333	*Neisseria meningitidis*
	GDS 2362	Malaria
	GDS 2749	*Aspergillus fumigatus*
	GDS 2756	Measles
	GDS 2833	Polyomavirus JCV
003205	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1774	Hepatitis C
	GDS 2180	*bacillus Calmette-Guerin*
003404	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 2180	bacillus Calmette-Guerin

(Table 2) contd…..

Gene[#]	GDS Record[@]	Infectious Agent Used in Experiment
003906	GDS 940	Herpesvirus
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 1237	**Anaplasama phagocytophilum**
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 1667	Human papillomavirus
	GDS 1774	Hepatitis C
	GDS 2023	Syncytial virus
	GDS 2168, 2649	bacillus Calmette-Guerin
	GDS 2180	HIV
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 2362	Malaria
	GDS 2749	*Aspergillus fumigatus*
	GDS 2833	Polyomavirus JCV
004551	GDS 940	Herpesvirus
	GDS 2833	Polyomavirus JCV
	GDS 1667	Human papillomavirus
	GDs 2749	*Aspergillus fumigatus*
	GDS 1237	**Anaplasma phagocytophilum**
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 1774	Hepatitis C
	GDS 2023	Syncytial virus
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 2168, 2649, 1449	HIV
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection bacillus Calmette-Guerin
	GDS 2180	Malaria
	GDS 2362	
005082	GDS 1237	**Anaplasma phagocytophilum**
	GDS 940	Herpesvirus
	GDS 1667	Human papillomavirus
	GDS 2833	Polyomavirus JCV
	GDS 2749	*Aspergillus fumigatus*
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 1774	Hepatitis C
	GDS 2023	Syncytial virus
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 2168, 2649, 1449	HIV
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection bacillus Calmette-Guerin
	GDS 2180	Malaria
	GDS 2632	
005623	GDS 1774	Hepatitis C
	GDS 1676	Herpesvirus
	GDS 2180	bacillus Calmette-Guerin
	GDS 625	*Helicobacter pylori*
006187	GDS 1667	Human papillomavirus
	GDS 2833	Polyomavirus JCV
	GDS 940	Herpesvirus
	GDS 2749	*Aspergillus fumigatus*
	GDS 1237	**Anaplasma phagocytophilum**
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 1774	Hepatitis C
	GDS 2023	Syncytial virus
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 2168, 2649, 1449	HIV
	GDS 2362	Malaria
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 2180	bacillus Calmette-Guerin

(Table 2) contd…..

Gene[#]	GDS Record[@]	Infectious Agent Used in Experiment
007215	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 2180	bacillus Calmette-Guerin
	GDS 940	Herpesvirus
	GDS 1667	Human papillomavirus
	GDS 2833	Polyomavirus JCV
	GDS 2749	*Aspergillus fumigatus*
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 1774	Hepatitis C
	GDS 2023	Syncytial virus
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 2168, 2649, 1449	HIV
	GDS 2362	Malaria
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
015369	GDS 1667	Human papillomavirus
	GDS 2833	Polyomavirus JV
	GDS 940	Herpesvirus
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 2749	*Aspergillus fumigatus*
	GDS 1774	Hepatitis C
	GDS 2023	Syncytial virus
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 2168, 2649	HIV
	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
	GDS 2362	Malaria
021991	GDS 1237	**Anaplasma phagocytophilum**
	GDS 1667	Human papillomavirus
	GDS 2833	Polyomavirus JCV
	GDS 940	Herpesvirus
	GDS 2749	*Aspergillus fumigatus*
	GDS 2332, 2333	*Neisseria meningitidis*
	GDS 2023	Syncytial virus
	GDS 2287	*Pseudomanas aeruginosa* rsmA mutant
	GDS 1063	Primary effusion lymphomas and associated viral infection
	GDS 2168, 2649, 1449	HIV
	GDS 2362	Malaria
	GDS 1646	HIV and Neisseria gonorrhoeae co-infection
033340	GDS 1022	*Pseudomanas aeruginosa*
080657	GDS 1774	Hepatitis C
	GDS 1237	**Anaplasma phagocytophilum**
152998	GDS 1022	*Pseudomanas aeruginosa*

*Search results were performed using GEO profile available in NCBI website (www.ncbi.nlm.nih.gov).

[#]Genes listed are by accession number as NM_xxxxxx.

[@]GDS record represents the results when a query was performed using both gene number and infection (i.e. NM_152998 and infection) to search the whole GEO profiles. The DNA array platform used in each experiment was not necessarily the same and was not considered in the analysis. Gene numbers that are bolded (**001547** and **006187**) are those confirmed over expressed in infected samples at 8 h post infection by TaqMan real-time PCR.

Real time PCR analysis of samples from 8 h post infection showed that both NM_001547 and NM_006187 were significantly up-regulated. The up-regulation of both genes were not observed in the infection of NB4 by *A. phagocytophilum*, indicating that one may be able to use just these two genes to differentiate *O. tsutsugamushi* infection from *A. phagocytophilum*. Although these two genes were not up-regulated by *A. phagocytophilum* infection, infections by other agents did result in up-regulation of both genes. Table **3** shows the results of the expression of both NM_001547 and NM_006187 in PBMCs infected by different infectious agents 8 h post infection. Only virus infection did not result in the up-regulation of both genes whereas bacteria infection or exposure to toxin led to the up-

regulation of both genes. Therefore, the change of expression of these two genes alone is not sufficient to differentiate various bacterial infections. The up-regulation of these 2 genes was also observed at 18 h post infection, suggesting that up-regulation was a sustained effect for at least 18 h post infection. Some of the 22 genes were similarly regulated at 18 h post infection as they were at 8 h post infection. Therefore, the 8 h post infection *in vitro* is probably the optimal time to obtain samples for differentiating *O. tsutaugamushi* infection from other infections. It is thus plausible to construct a diagnostic platform based on these 22 genes along with necessary control genes to monitor the expression of these genes. Recent advancement in microarray technology makes it possible to perform expression profiling experiment with complete data analysis within 1 day, making DNA microarray analysis an attractive method to effectively diagnose early Orientia infection (8-18 hours post infection).

Table 3. Fold Change of Gene Expression from PBMC Infected by Various Agents*

Pathogen or Toxin Used[b]	Fold Change[a]	
	NM_001547	NM_006187
Anthrax infection	5.3	5.4
VEE	0.48	0.85
SEB	5.5	2.7
BOT	23.5	18.4
Dengue	0.86	2.7
Rickettsia	9.4	8.8

*RNA was extracted from PBMC at 8 h post infection or exposure to toxin.

[a]Experiment was performed using 18S rRNA as the reference for quantitation as described in the Materials and Methods. The primer set was designed specifically for individual pathogen or toxin as described elsewhere (26).

[b]VEE: Venezuelan Equine encephalitis virus, SEB: *Staphylococcal enterotoxin* B, BOT: C. botulinum toxin.

Data analysis using a simple 2-fold change filter showed that many genes are apparently up-and down-regulated after infection by *O. tsutsugamushi*. This 2-fold change is a rather arbitrary choice and does not imply biological significance. Although various methods have been developed to relate the biological significance with fold changes of expression, it has been very difficult to correlate these two different parameters. The significance analysis of microarrays (SAM) [32] has been the most popular method employed for microarray data analysis. The method assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than the adjustable threshold, SAM estimates the percentage of genes identified by chance i.e. the false discovery rate (FDR). The impact of selected thresholds on the output from SAM may critically alter the conclusion of a study. Larsson *et al.* recently demonstrated that variation on the "fold change" setting when applying SAM can have a significant effect on the final number of "significant" genes [33], suggesting caution is needed when selecting threshold for data analysis. Mariani *et al.* [34] also developed a new program to generate a variable fold-change threshold to eliminate the intensity-specific variability. In spite of all these efforts, the correlation of fold changes and biological significance has not been well established. Alternatively, the microarray data can be used as a screening tool to investigate responsive gene from a large set of gene library, and the selected genes can be further confirmed using quantitative real-time PCR analysis. This approach was used in this study. After semi-quantitative high throughput PCR confirmation, only a few (3.4% or 22 genes) of the 658 genes with 2-fold change in expression appeared significantly up-or down-regulated. These results are consistent with the notion that the gene list identified using fold changes needs further analyses to verify its significance.

The majority of genes found to be differentially regulated are not surprising but they do not seem to respond to *O. tsutsugamushi* infection through common pathways. Although these genes did not provide clear pathways on how the host counteracts *O. tsutsugamushi* infection, these genes do provide a unique profile to differentiate *O. tsutsugamushi* infection from other infectious agents. A recent study of THP cells infected by *R. prowazekii* has revealed a very different set of genes that are regulated during infection [35], although *R. prowazekii* is closely related to *O. tsutusgamushi*, more so than *A. phagocytophilum*, these 22 genes can still uniquely differentiate infection caused by this organism. The difference in responsive genes upon infection by *R. prowazekii* and *O. tsutsugamushi* could be attributed to not only the difference in organism but also the difference in infected cells. THP is a cell line generated from mouse monocytes while the fresh purification of human PBMC are a mixture of cell populations, including 15-30 % of monocytes. In addition, Whitney *et al.* has shown that different types of cells within PBMC responded differently to the same infection [36]. The overall response was observed in this study.

In conclusion, we have shown that infection of human PBMC by *O. tsutsugamushi* resulted in the regulation of 22 specific genes as early as 8 hours after infection. Initial DNA microarray screening revealed that the expression of 658 genes was affected by Orientia infection. Subsequent semi-quantitative PCR analysis of all 658 genes confirmed the regulation of 22 genes by *O. tsutsugamushi* infection. The real-time RT-PCR conclusively showed that two genes were highly up-regulated. Both genes are shown regulated by viral and *A. phagocytophilum* infection, consistent with the intracellular nature of virus and *O. tsutsugamushi* as well as taxonomy closeness between *O. tsutsugamushi* and *A. phagocytophilum*. The 22 genes are uniquely regulated by *O. tsutsugamushi* infection as evidenced by the available database in GEO which indicates no other infection that results in differential expression of the same 22 genes. Therefore, we propose that these 22 genes can be used to differentiate infections by *O. tsutsugamushi* from infection by virus, bacteria and other closely related intracellular organisms.

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Leukopheresis units were obtained from volunteer donors using the procedures outlined in our approved human use protocol, reviewed by the established Institutional Review Board at WRAIR. This study used only purchased cell covered under the non-human subject research protocol

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