Analysis of porcine differential gene expression following challenge with *Salmonella enterica* serovar Choleraesuis using suppression subtractive hybridization

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Abstract

Swine-adapted *Salmonella enterica* subsp. *enterica* serovar Choleraesuis (*S. Choleraesuis*) is the pathogen most frequently isolated from diseased pigs and may affect host gene expression in a species-specific manner. To characterize the porcine transcriptional response to *S. Choleraesuis* infection, the mRNA profiles from the mesenteric lymph nodes of three non-infected and three experimentally infected pigs at 24 h post-inoculation were analyzed by suppression subtractive hybridization (SSH). Forty-four up-regulated and 44 down-regulated genes were revealed by differential cDNA screening of 384 forward and 288 reverse subtracted cDNA clones. The DNA sequence of the cDNA clones identified genes with a role in a variety of cellular functions as well as gene products of unknown function. Seven up-regulated genes (CXCL10, CXCR4, SDCBP, DNAJA1, HSPH1, HSP90 and ANXA5) and two functionally related genes (HSP70 and DNAJA4:pDJA1) were selected for further analysis based on their predicted roles in infection and immunity. Real-time RT-PCR was performed using RNA collected from a time course of infection spanning from the acute phase (8 h) to the chronic phase (21 days) to confirm and quantitate the up-regulation of the SSH-enriched genes. Correlating with the clinical signs of infection (fever, diarrhea and lethargy), the most dramatic induction of gene expression for all nine genes occurred at 48 h post-inoculation. This investigation further defines the...
1. Introduction

With over 2500 serovars, the genus *Salmonella* is widely distributed in the world. Although most serovars have a broad host range (for example, *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*)), several *Salmonella enterica* serovars are adapted to a single host such as Typhi (humans), Dublin (bovine), Abortusovis (small ruminants) and Choleraesuis (swine) (Schwartz, 1999). Unique interactions between host-adapted *Salmonella* and the host are needed to produce a specific systemic infection. Clinical and pathogenic experiments demonstrate that oral inoculation of pigs with different *Salmonella* serovars results in distinct diseases (Schwartz, 1999). Infection of pigs with *S. Choleraesuis* can result in septicemia, enterocolitis, pneumonia and/or hepatitis (as a consequence of bacteremia) whereas infection with *S. Typhimurium* usually only causes enterocolitis (Reed et al., 1986; Schwartz, 1999). Host-specific *Salmonella* serovars also have a greater capacity to survive within their host’s macrophages. For example, *S. Typhi* has an increased ability to survive and proliferate within human macrophages, whereas *S. Typhimurium* survives and replicates better in murine macrophages (Schwan et al., 2000). Additional differences between *Salmonella* serovars were observed in the cellular invasion of microfold (M) cells as a port of entry. In the swine ligated ileal loop model, multiple organisms of *S. Choleraesuis* invaded M cells whereas only one or two organisms of *S. Typhimurium* were found per cell; furthermore, *S. Choleraesuis* caused more extensive apical membrane rearrangements characterized by lamellipodia and filopodia formation or ruffling (Meyerholz and Stabel, 2003).

In response to *Salmonella* infection, the host stimulates nonspecific immune and inflammatory responses as well as specific cellular and humoral immune responses (Jones and Falkow, 1996; Eckmann and Kagnoff, 2001). Phagocytic cells play a central role in innate immunity and are critical in determining the outcome of infection (Jones and Falkow, 1996). Cytokines acting on macrophages, such as interferon γ (IFNG), tumor necrosis factor α (TNF), interleukin 12 (IL12) and IL18, have been extensively studied and determined to be critical for resistance to *Salmonella* (Eckmann and Kagnoff, 2001). Stabel et al. (1995) found that infection of swine with live *S. Choleraesuis* elicits different serum TNF responses than infection with live *S. Typhimurium*.

Although many important aspects of the swine response to *Salmonella* infection have been revealed, knowledge about the mechanism underlying *S. Choleraesuis* pathogenesis in swine and *Salmonella* host specificity is scarce. To investigate alterations in the transcriptional profiles of swine infected with *S. Choleraesuis*, suppression subtractive hybridization (SSH) was employed to identify differentially expressed genes while real-time RT-PCR analyzed gene expression over a course of time including the acute and chronic stages of infection.

2. Materials and methods

2.1. Bacterial strains, growth media and antibiotics

*S. Choleraesuis* χ3246 was grown as previously described (Stabel et al., 2002). Briefly, an overnight static culture was grown in Luria Bertani (LB) broth to late log/early stationary phase at 250 rpm, 37 °C. After harvesting by centrifugation, the bacterial pellet was resuspended in phosphate-buffered saline (PBS), pH 7.2. Transformed *E. coli* DH5α were grown on LB agar plates containing 100 μg/ml of ampicillin.
2.2. Animal experiment

Eighteen conventionally raised male and female piglets from *Salmonella* spp.-free sows were weaned at 10 days of age, shipped to the National Animal Disease Center, Ames, IA and raised in isolation facilities. To confirm that all piglets were free of *Salmonella* spp. prior to experiment, bacteriological cultures were performed on rectal swabs. At 7 weeks of age, the pigs were randomly divided in two experimental groups: non-infected \( (n = 3) \) and infected \( (n = 15) \). Three control pigs were necropsied on experimental day -3. On day 0, pigs in the infected group were intranasally challenged with \( \frac{1}{2} \times 10^9 \) CFU of *S. Choleraesuis* x3246. Rectal temperatures and clinical signs (lethargy, loss of appetite and diarrhea) were recorded for each animal daily. At 8 h, 24 h, 48 h, 7 days and 21 days post-inoculation (p.i.), three infected pigs were necropsied.

Tissue samples from the mesenteric lymph nodes were aseptically collected and immediately frozen in liquid nitrogen for future mRNA isolation. Samples of the ileocecal lymph node were used in quantitative bacteriology. All procedures involving animals were lawful and approved by the USDA, ARS, NADC Animal Care and Use Committee.

2.3. RNA isolation

Total RNA was isolated from pig mesenteric lymph node. Briefly, frozen tissues were homogenized in liquid nitrogen using a mortar and pestle. Total RNA was extracted from \( \sim 200 \) mg of tissue using 5 ml of TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s instructions. The resulting total RNA was further purified and treated with DNase I using a RNeasy Midi kit and a RNase-free DNase set (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Messenger RNA was isolated from total RNA using an Oligotex mRNA mini kit (Qiagen) according to the manufacturer’s guidelines. The concentration of total RNA and thereafter mRNA was determined based on absorbance at 260 nm wavelength. RNA integrity, quality and quantity were assessed using the Agilent Bioanalyzer 2100 and RNA Nano 6000 Labchip kit (Agilent technologies, Palo Alto, CA).

2.4. Suppression subtractive hybridization

Suppression subtractive hybridization was performed using PCR Select cDNA Subtraction kit (Clontech, Palo Alto, CA) as described by the manufacturer. Forward and reverse subtractions were carried out using pooled mesenteric lymph node mRNA samples from three infected pigs at 24 h p.i. and three non-infected pigs at day -3.

2.5. Cloning

Secondary (nested) PCR-amplified forward and reverse subtracted cDNA populations were cloned into the pBAD vector (pBAD TOPO TA Expression Kit, Invitrogen, Carlsbad, CA) and transformed into *E. coli* DH5α. Following overnight growth on selective media, random colonies were picked into 96-well plates containing 200 \( \mu \)l of LB media with ampicillin and 20% glycerol. Following overnight incubation at 37 °C, the culture plates were frozen at \(-70 \) °C.

2.6. Differential screening

Subtracted clones were subjected to differential screening to confirm their unique gene expression. The cDNA inserts were amplified by PCR using the following conditions: 95 °C for 1 min followed by 30 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 1 min and a final cycle at 72 °C for 5 min. PCR products were analyzed on 2% agarose gels to identify insert-containing clones. To prepare for Southern hybridization, two identical nylon membranes were made using the Bio-Dot apparatus (BioRad, Hercules, CA) by blotting 5 ml of denatured, PCR amplified cDNA inserts. For probe preparation, the forward and reverse subtracted secondary PCR products were digested with RsaI to remove the adaptors then purified using QIAquick PCR purification kit (Qiagen). Probes (100 ng of denatured cDNA) were generated by alkaline phosphatase labeling with Gene Images AlkPhos Direct Labeling and Detection System (Amersham Biosciences Corp, Piscataway, NJ) as instructed by the manufacturer. The two identical membranes were hybridized overnight at 55 °C, one with the forward and the other with the reverse subtracted probes. The hybridization signals were visualized using X-ray film or a Multi-Imager Light...
Cabinet (Alpha Inotech Corporation, San Leandro, CA) and quantified using a Chemi-Imager 4000 Low Light Imaging System densitometer (Alpha Inotech Corporation, San Leandro, CA) and a GS-800 calibrated densitometer with Quantity One Software (BioRad). cDNA clones were considered differentially expressed when blots probed with the subtracted tester repeatedly demonstrated a signal intensity >1.5-fold different than blots probed with the subtracted driver. Plasmids containing differentially expressed cDNA were extracted and sequenced by dideoxy chain termination using an ABI 3700 DNA Analyzer (Applied Biosystems Inc., Foster City, CA) at the Iowa State University DNA sequencing and synthesis facility. DNA similarity searches were conducted using the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) (Altschul et al., 1997). All selected plasmids were re-blotted in triplicate on nylon membranes and re-hybridized to forward and reverse subtracted secondary PCR products using the above described technique.

2.7. Real-time RT-PCR

Differential expression of the clones of interest was verified using quantitative one-step RT-PCR. Total RNA, isolated from 3 individual swine mesenteric lymph node samples at day -3 (non-infected), 8 h, 24 h, 48 h, 7 days and 21 days p.i. served as a template for amplification using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). RT-PCR transcripts from each pig were amplified in triplicate and detected using the QuantiTect SYBR Green RT-PCR kit (Qiagen). Thermal cycling parameters were as follows: 50 °C for 30 min, 95 °C for 15 min, 35 cycles at 94 °C for 15 s, 55 °C to 60 °C for 30 s (the annealing temperature varied depending on the gene specific primers), 72 °C for 5 s. At the 72 °C step, fluorescent data acquisition was performed. Following PCR cycling, disassociation curve analysis was performed at 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s; data acquisition was performed at the final 60–95 °C ramp and the final 95 °C step. Analysis of the disassociation curves (as well as agarose gel electrophoresis) confirmed that fluorescent signal was generated only from specific cDNA transcripts. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as the endogenous assay control (primer sequence: forward 5’-CAG-CAATGCCTCTGTACCA-3’ and reverse 5’-GATGC CGAAGTTGTCATGGA-3’, GenBank accession no. AY008846 (Vallee et al., 2003)). Gene expression data were normalized to the amount of total RNA amplified (Bustin, 2002) based on the accurate quantitation of RNA using the Agilent Bioanalyser 2100. Relative quantification of gene amplification by real-time RT-PCR was performed using the cycle threshold (Ct) values (Dawson et al., 2004). Fold change in expression of the target gene is presented as log2 of the difference between averaged Ct values for the control and infected pigs.

2.8. Statistical analysis

Data from the cDNA Southern hybridization and real-time RT-PCR (Ct values) were analyzed using the unpaired t test from GraphPad InStat software (GraphPad Software Inc., San Diego, CA). Results were considered to be significant when P < 0.05.

3. Results

3.1. Suppression subtractive hybridization (SSH) to identify differentially expressed porcine genes

To identify specific aspects of the porcine response to S. Choleraesuis at the molecular level, SSH was employed to characterize differential gene expression following infection. To obtain the porcine tissues for SSH, an experimental swine infection with S. Choleraesuis was performed over a 3-week period. The pigs developed clinical signs of disease manifested by a loss of appetite, lethargy and diarrhea at day 2 p.i. that continued for about a week. The rectal temperature of the infected animals peaked at infection day 2 (41.6 ± 0.4 °C) and gradually declined to the temperature of the non-infected controls (~39.7 °C). Salmonella was detected in the ileocecal lymph nodes throughout the 21 day experiment (data not shown).

Using the SSH technique, the mRNA populations of the mesenteric lymph nodes from non-infected and infected pigs at 24 h p.i. were compared to identify genes expressed more highly in one population than in the other. Following SSH, the forward and reverse
subtracted cDNA populations were cloned and a subtracted library representing differentially expressed genes was created. Differential cDNA screening by Southern hybridization was performed on 384 forward subtracted cDNA clones and 288 reverse subtracted cDNA clones, revealing 44 up-regulated and 44 down-regulated cDNA clones. DNA sequence was determined for the 88 differentially expressed clones, within which we found 12 duplicates and thus 76 different cDNAs. Based on their sequence identity with the GenBank database, the identified genes were grouped into various categories (Fig. 1). Twenty-six of the forward subtracted sequences (up-regulated swine genes during S. Choleraesuis infection) showed homology with known genes, 12 sequences aligned with genes of unknown function and 6 sequences were novel genes that had no homology with the GenBank database. The cellular roles of the genes identified by forward subtraction involve the following functions: heat shock response, translation, transcription, immunity, cell metabolism, vesicular transport, signal transduction, cell cycle regulation, apoptosis and membrane proteins. Only one forward subtracted cDNA sequence was duplicated. As for the reverse subtracted clones (down-regulated swine genes during S. Choleraesuis infection), 37 sequences showed identity with known genes, 4 sequences aligned with genes of unknown function and 3 sequences had no homology in the database. The cellular functions of the genes identified by reverse subtraction include cell metabolism, cell cycle regulation, immunity, signal transduction, transcription and translation as well as genes encoding for mitochondrial, cell membrane, ribosomal and cytoskeleton proteins. Eleven reverse subtracted cDNA sequences were repeated. Based on their potential role in the stimulation of host’s immune and inflammatory response to S. Choleraesuis infection, seven up-regulated genes from the SSH study were selected for confirmation by real-time RT-PCR (Table 1).

3.2. Real-time RT-PCR of differentially expressed genes

To analyze the expression profiles of the SSH identified genes over the 3-week period of infection...
### Table 1
SSH-identified, up-regulated porcine genes during *S. Choleraesuis* infection

<table>
<thead>
<tr>
<th>Clone</th>
<th>Fold difference (Southern hybridization)</th>
<th>BLAST submission (bp)</th>
<th>Identified gene</th>
<th>Gene symbol</th>
<th>Similarity and GenBank accession number</th>
<th>Accession number of submitted ESTs</th>
<th>Primer sequences for real-time RT-PCR (5′–3′)</th>
<th>RT-PCR amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2/B2 D2</td>
<td>1.986, <em>P</em> = 0.0015&lt;sup&gt;+&lt;/sup&gt;</td>
<td>510</td>
<td>Annexin A5</td>
<td>ANXA5</td>
<td>Human 93%, NM_001154.2</td>
<td>CX309655</td>
<td>f GAAGCACAGGT TGAACAAGATG, r CTCTAATGTGAC ACACCTCGTG</td>
<td>116</td>
</tr>
<tr>
<td>T2/B2 G12</td>
<td>7.584, <em>P</em> &lt; 0.0001&lt;sup&gt;+&lt;/sup&gt;</td>
<td>781</td>
<td>Chemokine ligand 10</td>
<td>CXCL10</td>
<td>Pig 99%, NM_00108691</td>
<td>CX309657</td>
<td>f CCCACATGTTG AGATATTGC, r CATCCTTAATCA GTAGTGCCG</td>
<td>168</td>
</tr>
<tr>
<td>T2/B5 E7</td>
<td>1.59, <em>P</em> = 0.0032&lt;sup&gt;+&lt;/sup&gt;</td>
<td>360</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
<td>CXCR4</td>
<td>Pig 100%, AB116560.1</td>
<td>CX309659</td>
<td>f GTCGTCTATG TCACCGTCTG, r CACCCTAACCC ACACCTCACTG</td>
<td>132</td>
</tr>
<tr>
<td>T2/B3 A11</td>
<td>2.075, <em>P</em> = 0.0002&lt;sup&gt;+&lt;/sup&gt;</td>
<td>368</td>
<td>DNAJ (Hsp40) homolog, subfamily A, member 1</td>
<td>DNAJA1</td>
<td>Human 95%, NM_001539.2</td>
<td>CX309658</td>
<td>f CTTCAATTTGG ATTCTTATCAOG, r GAGCCAAAC CACCACCTCC</td>
<td>146</td>
</tr>
<tr>
<td>T2/B5 A10</td>
<td>1.74, <em>P</em> = 0.0209&lt;sup&gt;+&lt;/sup&gt;</td>
<td>192</td>
<td>90 kDa heat shock protein</td>
<td>HSP90</td>
<td>Pig 100%, NM_213973.1</td>
<td>CX309661</td>
<td>f GATGTTGTCTTCT GATGAAGAG, r GTATGTCACCT GTGGTTCTTG</td>
<td>136</td>
</tr>
<tr>
<td>T2/B5 F8</td>
<td>3.6, <em>P</em> &lt; 0.0001&lt;sup&gt;+&lt;/sup&gt;</td>
<td>407</td>
<td>Heat shock 105 kDa/110 kDa protein 1</td>
<td>HSPH1</td>
<td>Human 92%, BC018124.2</td>
<td>CX309660</td>
<td>f CTGAACCTCCT CACCAGAATG, r GTCTCAGTATGT TATGTGAATACCA</td>
<td>150</td>
</tr>
<tr>
<td>T2/B2 A8</td>
<td>1.79, <em>P</em> = 0.0002&lt;sup&gt;+&lt;/sup&gt;</td>
<td>273</td>
<td>Syndecan binding protein (Syntenin)</td>
<td>SDCBP</td>
<td>Rat 89%, NM_031986.1</td>
<td>CX309656</td>
<td>f GCATCTGCGAG GTGATAAGTG, r GTGACATTCA CACCTGTTGA</td>
<td>137</td>
</tr>
</tbody>
</table>

* Statistically significant (*P* < 0.05).
with S. Choleraesuis, real-time RT-PCR analysis was performed. The expression of CXCL10, CXCR4, SDCBP, HSPH1, HSP90, DNAJA1 and ANXA5 was quantified based on the $C_t$ values obtained for the total RNA transcripts of the non-infected and infected porcine mesenteric lymph nodes (Table 2). Only significant results ($P < 0.05$) are described. The expression of CXCL10 was increased at 24 h p.i., exhibited the greatest induction at 48 h p.i. and remained up-regulated to 7 days p.i. (Fig. 2a). The expression of CXCR4 and SDCBP peaked at 48 h p.i. (Fig. 2b and c). Up-regulation of HSPH1 and DNAJA1 was detected at 24 h p.i. with the highest expression level at 48 h p.i. (Fig. 2d and e). Induction of HSP90 and ANXA5 was detected at 24 h p.i. and continued to 21 days p.i. with the highest up-regulation at 48 h p.i. (Fig. 2f and g).

Three heat shock proteins (DNAJA1, HSPH1 and HSP90) were identified in the SSH study and confirmed by real-time RT-PCR. These heat shock proteins are known to be functionally associated with HSP70 (Yamagishi et al., 2000; Gotoh et al., 2004). Although not identified in the SSH experiment, the expression of HSP70 was specifically analyzed by real-time RT-PCR from the mesenteric lymph-node of S. Choleraesuis infected swine because of its intricate involvement with the three HSPs identified by SSH. Real-time RT-PCR results indicated that HSP70 was significantly up-regulated at 8 h, 24 h, 48 h and 21 days p.i. (Table 2 and Fig. 3). Similar to the other heat shock proteins, HSP70 demonstrated its greatest induction at 48 h p.i.

In our study, a member of the large HSP40 (DNAJ) protein family was identified by SSH (DNAJA1). As stated above, the DNAJ proteins interact with HSP70 proteins to form a chaperone complex, and the specific function of HSP70 in the different cellular compartments is determined by the DNAJA1 protein (Rassow et al., 1995). To extend the understanding about the possible role of the DNAJ homologs in the S. Choleraesuis infection model, the expression profile of a chaperone recently described in swine, DNAJA4:pDJA1 (Depre et al., 2003), was analyzed by real-time RT-PCR. The expression of DNAJA4:pDJA1 was up-regulated at 24 and 48 h p.i. (Table 2 and Fig. 4), consistent with the highest induction of its partner chaperones, HSP70 and HSPH1.

### 4. Discussion

To identify differentially expressed porcine genes during a Salmonella infection, SSH analysis was performed on swine experimentally infected with S. Choleraesuis. The pigs developed a typical S. Choleraesuis infection, exhibiting clinical symptoms at 48 h p.i. with high levels of Salmonella in the ileocecal lymph node detected from 24 h p.i. To

<table>
<thead>
<tr>
<th>Group</th>
<th>Non-infected</th>
<th>8 h p.i.</th>
<th>24 h p.i.</th>
<th>48 h p.i.</th>
<th>7 days p.i.</th>
<th>21 days p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVG</td>
<td>S.D.</td>
<td>AVG</td>
<td>S.D.</td>
<td>AVG</td>
<td>S.D.</td>
</tr>
<tr>
<td>SSH identified genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANXA5</td>
<td>32.1</td>
<td>0.3</td>
<td>31.8</td>
<td>0.1</td>
<td>31.0</td>
<td>0.2</td>
</tr>
<tr>
<td>CXCL10</td>
<td>28.9</td>
<td>1.4</td>
<td>28.7</td>
<td>0.2</td>
<td>26.2</td>
<td>1.0</td>
</tr>
<tr>
<td>CXCR4</td>
<td>29.0</td>
<td>0.4</td>
<td>29.0</td>
<td>0.4</td>
<td>28.1</td>
<td>0.5</td>
</tr>
<tr>
<td>DNAJA1</td>
<td>34.2</td>
<td>0.8</td>
<td>33.7</td>
<td>0.1</td>
<td>32.6</td>
<td>0.4</td>
</tr>
<tr>
<td>HSP90</td>
<td>25.5</td>
<td>0.3</td>
<td>25.1</td>
<td>0.2</td>
<td>24.2</td>
<td>0.5</td>
</tr>
<tr>
<td>HSPH1</td>
<td>34.5</td>
<td>0.6</td>
<td>33.8</td>
<td>0.7</td>
<td>32.1</td>
<td>1.2</td>
</tr>
<tr>
<td>SDCBP</td>
<td>28.4</td>
<td>0.4</td>
<td>28.5</td>
<td>0.0</td>
<td>27.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Additional genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNAJA4:pDJA1</td>
<td>37.0</td>
<td>0.3</td>
<td>36.9</td>
<td>0.5</td>
<td>35.8</td>
<td>0.7</td>
</tr>
<tr>
<td>HSP70</td>
<td>37.2</td>
<td>0.9</td>
<td>34.8</td>
<td>0.3</td>
<td>33.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Control gene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3PDH</td>
<td>30.8</td>
<td>0.8</td>
<td>30.6</td>
<td>0.7</td>
<td>30.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$^a$ Data was averaged (AVG ± S.D.) for each day post-inoculation (p.i.).
investigate the onset/development of the host’s response, RNA isolated from the mesenteric lymph nodes at 24 h p.i. of infected and non-infected pigs was selected for SSH analysis. Seven of the up-regulated genes (CXCL10, CXCR4, SDCBP, HSPH1, DNAJA1, HSP90 and ANXA5) identified by SSH were selected for further analysis due to their potential role in the host’s response to *S. Choleraesuis* infection. Real-time RT-PCR confirmed the up-regulation of the SSH identified genes at 24 h p.i., although not significantly for two of the genes. Interestingly, the most dramatic changes in gene expression were observed at the 48 h time point, correlating with the symptoms of the *S. Choleraesuis* infection. Thus, by day 2 p.i., *S.
Choleraesuis is actively colonizing the host and mediators of inflammation and the immune response are responding to the infection.

Significant induction of chemokine CXCL10 was detected as early as 24 h p.i., indicating an early activation of the naïve immune response by the host. CXCL10, produced by many different cell types in the body, is a potent chemoattractant, targeting receptor CXCR3 and predominantly attracting activated CD4+ T lymphocytes and natural killer (NK) cells expressing this receptor on their surface (Taub et al., 1993; Loetscher et al., 1996). Salmonella and Salmonella-derived LPS can induce cultured mouse osteoblasts to produce high levels of CXCL10 (Gasper et al., 2002), and neutrophils stimulated by IFNG and bacterial LPS produce CXCL10 which attracts CXCR3 expressing immune cells to the site of infection (Gasperini et al., 1999). In our study, swine infected with S. Choleraesuis exhibited an increase in expression of CXCL10 for at least a week, suggesting that CXCL10 is important in facilitating the clearance of Salmonella by attracting immune cells, T lymphocytes and NK cells to the site of inflammation during the acute infection. Furthermore, because CXCL10 is a mediator of the Th1 type immune response, up-regulation of CXCL10 in the infected pigs supports the recent suggestion by Chiu et al. (2004) that swine initiate a Th1 mediated response to S. Choleraesuis.

The innate immune system recognizes the LPS of Salmonella, an essential component of the bacterial outer membrane and a major determinant of Salmonella virulence (Chiu et al., 2004). HSP70, HSP90, CXCR4 and growth differentiation factor 5 (GDF5) have been identified as LPS receptor molecules or LPS associated proteins (LAPs) that play an essential role in LPS ligation and delivery of an activation signal into the host cell, thereby triggering multiple signaling pathways within the cell including the pro-inflammatory responses (Triantafilou and Triantafilou, 2002). Two LAPs (CXCR4 and HSP90) were identified in the SSH study and confirmed by real-time RT-PCR. A third LAP, HSP70 was selected for gene expression analysis by real-time RT-PCR. The peak up-regulation of all three LAPs was detected at 48 h p.i., indicating that S. Choleraesuis is actively triggering the host innate immune response and the expression of multiple immune mediators.
In addition to its implicated role as one of the LPS receptors, HSP70 is also a molecular chaperone. Studies indicate that under normal conditions, HSP70, with the help of its co-chaperones (DNAJ proteins), is involved in protein folding and re-folding of misfolded proteins as well as intracellular protein transport (Kiang and Tsokos, 1998). Under stress conditions, HSP70 can bind to damaged proteins and facilitate their refolding or target them for degradation (Samali and Orrenius, 1998). HSP70 is functionally connected with the HSP40 (DNAJ) family of proteins and HSPH1. Both DNAJA1 and HSPH1 were found by SSH to be induced in S. Choleraesuis infected pigs, and real-time RT-PCR determined that both genes were up-regulated during the initial 48 h of infection. As a member of the HSP70/DNAJ chaperone complex in the cell, DNAJ proteins stimulate intrinsic HSP70 ATPase activity, facilitating the efficient ATP-dependent binding of HSP70 to the polypeptide substrate (Minami et al., 1996; Yamagishi et al., 2000). HSPH1 not only functions as a chaperone preventing thermal aggregation of proteins but also is a regulator of the HSP70/DNAJ complex (Yamagishi et al., 2000). Furthermore, HSPH1 suppresses the aggregation of heat denatured proteins in the presence of ADP rather than ATP. Thus, under conditions whereby cellular ATP levels may be diminished (such as stress or infection), HSPH1 may substitute for the HSP70 family of proteins since they require ATP to suppress denatured protein aggregation (Yamagishi et al., 2003).

As well as the roles of LPS receptor and molecular chaperone, studies indicate that HSP70 in conjunction with the DNAJ chaperone family can prevent cellular apoptosis by preventing the activation of apoptotic signaling pathways, such as stress-activated protein kinase SAPK/JNK (c-Jun N-terminal kinase) and caspase-3 (Mosser et al., 1997; Gotoh et al., 2004). It has been reported that HSP70 plays an important role in protecting macrophages against TNF mediated cell death as a consequence of Salmonella infection (Nishimura et al., 1997). Treatment of cultured monocyte/macrophage cells with a HSP70 anti-sense oligonucleotide dramatically increased cell death in response to S. Choleraesuis infection. In addition, Depre et al. (2003) reported that apoptosis was significantly decreased in cardiac myocyte cells transduced with DNAJA4:pDJAI, another DNAJ family member (specifically identified in swine) with elevated gene expression at 48 h p.i. in our S. Choleraesuis infection study. Furthermore, heart muscle cells over-expressing HSP70 and DNAJA4 were more resistant to severe heat shock (Abdul et al., 2002). Therefore, with their implicated functions in bacterial LPS ligation, protein chaperoning, apoptosis prevention and cytoskeleton formation/protection (Liang and MacRae, 1997), the up-regulation of DNAJA4:pDJAI as well as DNAJA1 in conjunction with HSP70 and HSPH1 in the S. Choleraesuis swine infection model indicates an important role for the HSP complexes in the host’s cellular response to Salmonella infection and survival under infectious stress conditions.

SDCBP was identified in the SSH study and confirmed by real-time RT-PCR to be up-regulated at 48 h p.i. Through its PDZ domain, SDCBP binds syndecans, proteins that co-ordinate actin cytoskeleton rearrangements in the cell and regulate signaling through the Rho family of GTPases (Bass and Humphries, 2002). It is known that Salmonella uses virulence genes of the Type III secretion apparatus that act on GTPases of the Rho subfamily to induce cell membrane and cytoskeleton rearrangements that facilitate its entry into the cell (Galan, 2001). Thus, S. Choleraesuis may cause the induction of SDCBP to initiate the cytoskeleton rearrangement events that support cellular invasion.

DNA sequences encoding ANXA5 were enriched by SSH, and real-time RT-PCR analysis revealed the up-regulation of the gene from 48 h to 21 days p.i. The annexin family of proteins possesses the common feature of binding phospholipids in a calcium dependent manner and has been implicated in multiple cellular processes including membrane trafficking, ion-channel formation, anticoagulation, signal transduction, inflammation and apoptosis (Raynal and Pollard, 1994; Blankenberg et al., 2001). Errasfa and Russo-Marie (1989) observed a decrease in leukocyte migration and neutrophil accumulation in inflammatory sites of mice upon i.v. injection of ANXA5. In the S. Choleraesuis infection model, ANXA5 may play a protective role for host cells and tissues as an anti-inflammatory agent as well as be involved in vesicular trafficking within phagocytic cells.

In summary, this study was performed to investigate the porcine transcriptional response to S. Choleraesuis over a time course of infection ranging.
from the acute stage (8 h) to the carrier stage (21 days). SSH identified and real-time RT-PCR confirmed the up-regulation of porcine genes involved in innate immunity and the Th1 type immune response following experimental inoculation with S. Choleraesuis. Furthermore, swine genes involved in cytoskeleton regulation and rearrangement, a host structure manipulated by Salmonella during cellular invasion, were also induced in response to S. Choleraesuis infection.

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