Identification of Immune Responsive Genes in Bovine Airway Using Suppression Subtractive Hybridization

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CHAPTER I

INTRODUCTION

The bovine respiratory disease (BRD) complex is a major cause of the morbidity and economic loss in the cattle industry in North America. The pathogenesis of BRD is a complex process associated with many bacterial and viral agents. *Mannheimia haemolytica* has been identified as the primary bacterial strain that contributes to BRD in the last few decades. The binding of leukotoxin produced by *M. haemolytica* and β2-integrin (CD11/CD18) of macrophages and neutrophils results in the activation and lysis of these inflammatory cells, which in turn leads to severe damages of bovine airway tissues.

In recent years, several differential gene expression techniques, including DNA microarray, suppression subtractive hybridization (SSH), and serial analysis of gene expression (SAGE), have been developed to analyze transcriptional changes and identify immune responsive genes in response to different infections. In this study, we employed SSH to search for the genes that are up-regulated following stimulation of bovine turbinate and tracheal cell lines with *M. haemolytica* as well as the genes that are specifically expressed in bovine lung. As a result, we have identified a total of 116 non-redundant genes that are induced in BT and EBTr cells following infection and 61 non-redundant lung-specific genes. Semi-quantitative and real-time PCR was used to further confirm the expression patterns of six randomly selected genes. Identification and

detailed analysis of these genes will facilitate our understanding of the host defense mechanisms in the bovine airway as well as the development of novel therapeutics against *M. haemolytica*.

CHAPTER II

REVIEW OF LITERATURE

1. Bovine Respiratory Disease (BRD) Complex

1.1. Pathogenesis of BRD

The BRD complex, also known as bovine pneumonic pasteurellosis (BPP) or shipping fever, is one of the major diseases that cause significant economic losses in both the feedlot and dairy cattle industry [1, 2]. Approximately 75% of morbidity and 50% of mortality in the feedlot were caused by BRD [3], with annual of over $3 billion for prevention and treatment in the US [4].

BRD is characterized by interlobular and interalveolar fibrosis and cellular influx of neutrophils into the lung of infected cattle [5, 6]. The inflammatory may prolong to pleural surface in some severe infections [6].

BRD has been found to be associated with a number of bacteria (such as *Mannheimia haemolytica, Pasteurella multocida, Haemophilus somnus,* and *Mycoplasma spp.*) and viruses (such as infectious bovine rhinotracheitis virus, bovine viral diarrhea virus, parainfluenza virus 3, and bovine respiratory syncytial virus) [7]. Among all of the pathogens, *M. haemolytica* is the most important bacterial agent responsible for the symptoms and pathogenesis of BRD [1].

1.2. Virulence factors of M. haemolytica

M. haemolytica is a weakly hemolytic, Gram-negative coccobacillus. There are a total of 12 serotypes of *M. haemolytica* that have been identified. Serotype 1 (A1) does not colonize well in the upper respiratory tract of health cattle. However, the environmental stresses, such as shipping, weaning, overcrowding and infection of other pathogens, can change the situation. Once proliferating in the upper respiratory tract, *M. haemolytica* A1 comes into the lower airway and invades sloughed cells and tissues [1-3].

Several components of *M. haemolytica* have been identified as virulence factors involved in BRD, including leukotoxin (Lkt), lipopolysaccharide (LPS), capsule, outer membrane proteins, adhesins, and neuraminidase. Among them, Lkt and LPS are the primary factors to provoke inflammatory responses and cause lesions of the cells in the bovine airway [2, 8].

Lkt is known to cause severe lesions of bovine lung in BRD. Being a water-soluble and pH-stable glycoprotein of 102-kDa, Lkt is secreted by *M. haemolytica* during the logarithmic phase of growth [2, 8]. It is a pore-forming toxin with six peptide repeats, belonging to the RTX (repeats in toxin) family of multidomain exotoxin [8]. At high concentrations, Lkt can form pores on the membrane of bovine leukocytes and cause the release of the toxic components [5, 9]; while at lower concentrations, Lkt acts as a neutrophil chemoattractant and further stimulates neutrophil degranulation [5]. Cattle infected with Lkt-deficient *M. haemolytica* or inactive Lkt have reduced pathology, which is consistent with the important role of Lkt in BRD pathogenesis [5].

LPS, one of the major components of Gram-negative bacterial cell wall, can recognize and bind to several host receptors, such as membrane CD14, toll-like receptors, and β2-integrins. *M. haemolytica* LPS is known to cause lesions on bovine lung

endothelial cells, while protecting Lkt from degradation by associating with it, which in turn increases the severity of the damage to bovine airway cells [2].

1.3. Host defense factors in the bovine airway

Several innate host defense factors, including mucosal surface, bronchoalveolar macrophages (BAM), and neutrophils, have been well recognized in defense against the BRD pathogens. Mucosal surface, as a physical barrier of the bovine respiratory tract, can filter the air and remove the particles larger than 5 μm before they come into the lung. The mucous on the mucosal surface has antimicrobial functions, containing secretory Ig A, lysozyme as well as a host of other factors. BAM and neutrophils kill pathogens by phagocytosis and release of inflammatory cytokines and host defense molecules [9].

β2-integrins are a group of heterodimeric membrane proteins containing α (CD11a, -b, -c, or d) and β (CD18) subunits and function as adhesion molecules in leukocyte migration [12]. Lkt produced by *M. haemolytica* can bind to macrophages and neutrophils through a type of β2-integrins, namely LFA-1 (CD11a/CD18). Insertion of Lkt into the cell membrane to form a hydrophilic pore leads to lysis of these inflammatory cells through the efflux of K^+ and influx of Ca^{2+}. Subsequently, the release of toxic compounds from these cells causes severe pulmonary lesions.

Several cytokines, such as IL-1β, IL-8 and IFN-γ produced from activated macrophages and neutrophils, function as proinflammatory factors to exacerbate the pathology by enhancing the expression of β2-integrins on bovine leukocytes and susceptibility of these cells to Lkt [1]. Pulmonary expression of IL-1β, IL-8 and TNF-α was also suggested to be responsible for lung lesions in BRD [13]. Platelet-activating

factor (PAF), which was found to mediate the vascular permeability and neutrophil activation in the lung of the BRD cattle in the first few hours of infection, may also be associated with the pulmonary injury [14]. Furthermore, production of nitric oxide (NO) by activated BAMs following *M. haemolytica* infection may also play an important role in mediating the pathogenesis of pulmonary lesions in BRD [16].

It has been revealed recently that bovine herpsvirus-1 (BHV-1) infection leads to the release of inflammatory cytokines, such as IL-1β, TNF-α and IFN-γ, which in turn increases the expression of LFA-1 in bovine peripheral blood mononuclear cells [15]. This may explain why cattle infected with BHV-1 are more susceptible to subsequent *M. haemolytica* infections.

Maternally and naturally acquired antibodies against *M. haemolytia* and *P. multocida* in beef calves were studied. IgM, IgG1and IgG2 that recognize Lkt and entire cells of *M. haemolytia* and outer membrane *P. multocida* proteins (OPMs) have been detected [11]. The data suggested that the calves start to produce antibodies against *M. haemolytia* and *P. multocida* about 60-90 days of age and the best timing for vaccinating beef calves is 4 months of age [11].

1.4. Current therapeutics against BRD

Antimicrobial agents, including tylosin, erythromycin, tetracycline and penicillin, have been used in the therapy of BRD for decades. Recently, several new antimicrobials, such as tilmicosin phosphate, florfenicol and enrofloxacin, were used to treat the cattle with BRD. However, the resistance of bacteria to these antimicrobial has been well documented [17].

A novel antimicrobial, tulathromycin, was developed more recently and displayed a higher efficiency than tilmicosin and florfenicol in the treatment of BRD in stock cattle and cattle at high risk of developing undifferentiated BRD [18-21]. Although tulathromycin-resistant bacteria have not been reported, it's almost certain that the resistance will develop just like other traditional antimicrobial agents after being applied extensively.

To reduce the likelihood of the development of antibiotic resistance, the potential of a new class of membrane-active, peptide antimicrobials in the BRD treatment has been explored. For example, an antibacterial peptide, cecropin B, was over-expressed on bovine nasal mucosa and found to inhibit the colonization of *M. haemolytica* on nasal mucosa *in vivo* [22].

Vaccines are also widely used in the prevention of BRD. Inactivated BRD pathogens or their structural components have been used as antigens to elicit humoral immunity in cattle [23, 24]. An edible vaccine has been developed by using transgenic plants. Expression of a *M. haemolytica* A1 Lkt derivative in white cover was able to elicit the production of anti-Lkt neutralizing antibodies in rabbits [25]. The effectiveness of this vaccine in cattle is yet to be tested [25].

Oral administration of BRD-infected cattle with natural human interferon-α (nHuIFN-α) revealed that nHuIFN-α decreased the occurrence of sick animals and postponed the development of BRD [27]. A study with recombinant interleukin-1β suggested that IL-1β could be used as a vaccine adjuvant (rbIL-1β) at least in BHV-1/*M. haemolytica* co-infected cattle [26].

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Collectively, in spite of numerous attempts in the prevention and treatment of the BRD complex in the cattle, there are still no highly effective agents or strategies available to date. Not surprisingly, BRD remains a major economic threat to the cattle industry. There is an urgent need to develop more effective therapeutic strategies against the BRD complex.

2. Identification of Immune Responsive Genes by Differential mRNA Expression Techniques

A variety of techniques have been developed and applied to the identification of differentially expressed genes following infections of animals or immune cells with different pathogens. Among them, suppression subtractive hybridization (SSH), serial analysis of gene expression (SAGE), and DNA microarray are the most commonly used.

2.1. Suppression subtractive hybridization (SSH)

In SSH, two populations of cDNAs (tester and driver) are compared and the differentially transcript genes are displayed in a subtracted cDNA library [28]. SSH has been widely employed in the identification of differentially expressed genes involved in immune responses. For example, a total of 87 genes were identified in a screening of up-regulated genes in mouse spleen cells following stimulation with immunostimulatory oligodeoxyribo-nucleotides by using SSH [29]. A novel murine CC chemokine gene, namely ABCD-1 was isolated using SSH by challenging the pro/pre B cells with anti-CD40 and IL-4 [31]. By using SSH, a new kinase, IKK-i was identified to be up-

regulated in response to stimulation of mouse macrophage cell line RAW264.7 with LPS [32]. SSH was also employed to search for new antigens in a cancer vaccine study [34].

2.2. Serial analysis of gene expression (SAGE)

SAGE was used in study of the protein function in eukaryotes. 10-14 bp cDNA tags were concatemerized for sequencing, and the result was analyzed in the database. This technique can be used for identification and quantification of gene expression [28]. SAGE has been applied to study the transcriptional profiles of activated Th1 and Th2 cells from human cord blood leukocytes [35]. A number of differentially expressed genes were also identified following the stimulation of human monocytes with LPS [36]. In a study of bovine B lymphocytes in the follicles of Peyer's patches, the gene expression pattern was obtained using SAGE, and the result was further confirmed in functional studies [37].

2.3. DNA microarray

DNA microarray is used to display the global expression profile of a cell. However, it’s relative expensive and not good at searching for novel gene [28]. A limited DNA microarray was employed to define the gene expression profile of the chemokine and chemokine receptor family, cytokines, and signaling pathways in human bronchial epithelial cells in response to different toll-like receptor agonists [38]. The results indicated the TLR signaling may play an important role in the airway defense against inhaled pathogens. DNA microarray was also applied to investigate gene expression changes in human alveolar epithelial cells in response to microbial products and

proinflammatory cytokines including LPS and TNF-α [39]. The results showed that alveolar epithelial cells have distinct immune responses to different stimuli and presumably play an important role in lung defense. To study the innate immune system of the trout, monocytes were isolated and stimulated with LPS or LPS plus cortisol, 1380 genes were analyzed using microarray, and differential gene expression patterns have been revealed between two treatments [40].

SSH and DNA microarray were also combined together to identify immune responsive genes, in which differentially expressed genes were screened first using SSH and further confirmed by DNA microarray. For example, by using a combination of SSH, microarray and Northern blot, a total of 22 genes were identified to be over-expressed in murine macrophages following an infection with *Yersinia pestis* [41]. In another study, five up-regulated and 30 down-regulated genes were identified in rat mononuclear cells after the animals were exposed to wielding fumes by SSH and DNA microarray [42].

In this study, we employed SSH to search for the genes that are differentially expressed following infection of bovine airway cells with live *M. haemolytica*. Furthermore, SSH was also used to identify a number of genes that are preferentially expressed in the bovine lung. Development of a comprehensive DNA microarray featuring lung-specific genes as well as the genes involved in airway immune responses will undoubtedly facilitate a better understanding of innate host defense mechanisms in the bovine respiratory tract and the development of novel therapeutic strategies against the BRD complex.

CHAPTER III

MATERIALS AND METHODS

1. Suppression subtractive hybridization (SSH) procedure

Total RNA was isolated from bovine cells and tissues using Tri Reagent (Sigma, St Louis, MO). SSH was performed by using the PCR-Select cDNA Subtraction Kit following the manufacturer's recommendations (Clontech, Mountain View, CA). Briefly, the first-strand and second-strand cDNA were synthesized, and double-stranded DNA was then digested with *Rsa*I at 37°C for 3 h. Digested tester DNA was ligated with adaptor 1 (5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3') and 2R (5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3'), whereas no ligation is needed for the control driver DNA.

The testers with adaptor 1 and 2R were hybridized separately with driver DNA for 9 h, and then two pools were mixed together with 30-fold more driver DNA for another 16 h. Two rounds of PCR were subsequently performed with diluted DNA mixture to preferentially amplify cDNAs that are differentially expressed. The first round of PCR involved with the primer 1(5'- CTAATACGACTCACTATAGGGC-3') for 27 cycles and the second round of PCR used nested primer 1 (5'-TCGAGCGGCCGCCCGGGCA GGT-3') and primer 2R (5'-AGCGTGGTCGCGGCCGAGGT-3') for 10 cycles. Similarly diluted unsubtracted testers were also performed with the same two rounds of

PCR. To evaluate the efficiency of subtraction, diluted subtracted and unsubtracted nested PCR products were further amplified for 20, 25, 30 and 35 cycles by PCR for a house-keeping gene, GAPDH, and the products were then analyzed by electrophoresis on a 1.2% agarose gel.

To reveal the identities of differentially expressed genes, subtracted PCR products were ligated to pGEM-T-Easy Vector (Promega, Madison, WI) and transformed into competent DH-5α cells by electroporation. White colonies were picked randomly on the LB agar plates containing ampicillin, IPTG and X-gal after overnight incubation at 37°C. Following verification of insertion size by colony PCR, bacterial colonies with different insertions were picked up and sequenced directly. The identities of the genes were revealed by BLAST search of the GenBank databases on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast).

2. Optimization of the infective dose of *M. haemolytica* to bovine airway cells

M. haemolytica (strain 89010807N; a kind gift from Dr. Anthony Confer in the Center for Veterinary Health Sciences at Oklahoma State University) [43] was grown overnight in Brain Heart Infusion (BHI) broth at 37°C. To first obtain the growth curve, bacteria were inoculated 1:1000 in fresh BHI and grown in a shaking incubator for up to 7 h. One ml of aliquots was taken every 1 h and measured for OD_{600}, followed by serial dilution of bacterial culture and spreading onto BHI agar plates for overnight growth. Viable colonies were counted for each hourly bacterial culture in order to obtain the correlation between OD_{600} and bacterial number.

To infect bovine airway cells, overnight bacterial culture was subcultured 1:1000 for 3-4 h in fresh BHI medium until OD_{600} reached 0.4- 0.7 (logarithmic phase). The bacteria were harvested by centrifuging at 1,000 x *g* for 10 min, washed with PBS for three times, and resuspended in 1.0 ml of PBS on ice for further experiments. Embryonic Bovine Tracheal (EBTr) cells and Bovine Turbinate (BT) cells were purchased from ATCC (Manassas, VA) and cultured in DMEM containing 10% horse serum in the present of 5% CO_2 at 37°C. Both cell lines were seeded in 6-well plates and allowed to grow overnight to 80-90% confluence before infected with ***M. haemolytica.*** Different doses of live and heat-inactivated bacteria were used to infect cells for 6 h. For heat inactivation, bacteria were heated to 60°C for 1 h before adding into the cell culture plates.

Following bacterial infection, cell culture medium was aspirated and cells were washed with PBS for three times. Total RNA was isolated from cells using Tri Reagent (Sigma) and the first-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), and semi-quantitative PCR was used to evaluate the expression of interleukin (IL)-6 and IL-8 as described as in former article [44]. The bacterial concentration that gave the strongest induction of IL-6 and/or IL-8 was determined as the optimal dose for large-scale infections as described below.

3. Identification of differentially expressed genes in response to infection with *M. haemolytica*

Once the optimal dose of infection (the ratio of pathogen to host cells as 10:1) was determined, BT and EBTr cells grown in 150 mm tissue culture plates were infected with the optimal dose of live bacteria for 6 h. Total RNA were isolated, and 1 μg of RNA was

used for the first-strand cDNA synthesis by using the Super SMART PCR cDNA Synthesis Kit (Clontech) with primers (5'-AAGCAGTGGTATCAACGCAGAGTAC T $_{(30)}$ VN-3'). The 5'-end of first-strand cDNA was further ligated with 5'-AAGCAGTGG TATCAACGCAGAGTACGCGGG-3'. Amplification of the ligated first-strand cDNA was performed by PCR with the primer (5'-AAGCAGTGGTATCAA CGCAGAGT-3'). To ensure a linear amplification of each sample, different PCR cycles (15, 18, 21 and 24) were performed. The highest cycle number before reaching the saturation of the PCR product was determined to be the optimal cycle. A large volume PCR was performed by using the optimal cycles, and the DNA mixture in the PCR product was further separated by size through CHROMA SPIN 1000 Columns (Clontech). Following collection of DNA of different size ranges, only the high molecular weight DNA was ligated with adaptors and used in SSH as described above with the infected RNA sample as the tester and uninfected control sample as the driver.

After two rounds of subtractive hybridization and PCR amplification, subtracted PCR products were ligated into T-easy Vector, transformed into DH5α, and grown in LB agar plates containing ampicillin, IPTG and X-gal overnight. A total of 576 white colonies were randomly picked for BT and EBTr cells, and the size of each insertion was verified by colony PCR with the primer (5'-TCGAGCGGCCGCCCGGGCAGGT-3'). 192 colonies with different insertion sizes were picked and sequenced directly. A BLAST search through the NCBI databases was performed to reveal the identities of those differentially expressed genes.

4. Identification of bovine lung-specific transcripts by SSH

To obtain the genes that are specifically expressed in bovine lung, tissue samples including lung, heart, liver, muscle, colon, and rumen were collected from a healthy, 1.5-year-old steer. Total RNA was isolated using Tri Reagent (Sigma), and mRNA was further purified using Oligotex (Qiagen). An equal amount of mRNA from heart, liver, muscle, colon, and rumen was mixed and used as the driver in subsequent SSH, whereas lung mRNA was used as the tester. Double-stranded cDNA were synthesized separately from 2 μg of mRNA from lung and other tissues using the PCR-Select cDNA Subtraction Kit (Clontech) following the manufacturer's recommendation as described above. A total of 288 white colonies was picked and checked for insert sizes by PCR. Ninety-six colonies containing different insert sizes were sequenced and their identities were revealed by BLAST search of the GenBank database.

5. Confirmation of the SSH results by RT-PCR

To confirm the SSH results, BT and EBTr cells grown in 100 mm plates were infected *M. haemolytica* at the ratio of 10:1 (bacteria:host cell). Cells were then harvested at 0, 1, 3, 6 and 12 h post-infection and total RNA was isolated. Sixteen genes that were found to be up-regulated in infected BT and EBTr cells by the SSH analysis were selected and specific exon-spanning primers were designed (Table 2). Semi-quantitative RT-PCR and real-time RT-PCR were used for analysis of gene expression as described [44, 45].

In semi-quantitative RT-PCR, a total of 1.0 μg RNA from sample were reverse transcribed with random hexamers and SuperScript III reverse transcriptase by using a first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions.

The subsequent PCR was carried out with 1/40 of the first-strand cDNA and gene-specific primers for each putative up-regulated gene and β-actin as described [44]. Every pair of primer was designed to locate on different exons to aid in distinguishing PCR products amplified from cDNA vs. genomic DNA. The PCR program used was: 94ºC denaturation for 2 min, followed by different cycles of 94ºC denaturation for 20 sec, 60ºC annealing for 20 sec, and 72ºC extension for 30 sec, followed by a final extension at 72ºC for 5 min. The number of PCR cycle was optimized for each gene to ensure linear amplification. A half of the PCR products were analyzed by electrophoresis on 1.2% agarose gels containing 0.5 μg/ml ethidium bromide.

In quantitative real-time PCR, the first-strand cDNA from 1.0 μg of each RNA sample was synthesized in a reaction volume of 20 μl at 42°C for 60 min by using QuantiTect® Reverse Transcription Kit (Qiagen), followed by real-time PCR amplification by using 0.1 μg of the first-strand cDNA, SYBR® Premix Ex Taq™ (Takara Bio, Japan), and MyiQ® Real-Time PCR Detection System (Bio-Rad) in a total volume of 15 μl. PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. The comparative ΔΔCT method was used to quantify the gene expression levels, where 18s rRNA was used as an internal control for normalization, and IL-6 was used as the positive control. Relative fold changes in gene expression were calculated using the formula $2^{-\Delta\Delta Ct}$. Melting curve analysis (55-95°C) was performed and confirmed the amplification of a single product in each case.

CHAPTER IV

RESULTS

1. Determination of the optimal infective dose of *M. haemolytica*

To be able to quantitate *M. haemolytica* in a liquid culture, we first obtained the bacterial growth curve and the correlation between OD_{600} and viable colony forming units (CFU) of the bacteria (Fig. 1) by using Graphpad prism (Graphpad software, San Diego, CA). A polynomial equation was further derived as follows: $Y = 3.045 \times 10^4 + 3.434 \times 10^6 \times X + 1.329 \times 10^9 \times X^2 - 2.263 \times 10^9 \times X^3 + 1.912 \times 10^9 \times X^4$, where Y is the bacterial number (CFU/ml) and X is OD_{600}. As a result, the number of live *M. haemolytica* in a liquid culture can be calculated directly from OD_{600}.

Next, both live and heat-inactivated bacteria were used to infect two bovine airway cell lines, namely BT and EBTr cells, at different bacteria:host cell rations in order to determine the optimal bacterial dose needed to elicit a robust immune response. IL-6 and IL-8, two commonly used proinflammatory cytokines that are known to be readily inducible under a variety of infectious conditions, were employed as positive indicators. Semi-quantitative RT-PCR was used to determine the expression levels of IL-6 and IL-8. As shown in Fig. 2, IL-6 was dose-dependently induced by 6 h challenge with both live and heat-inactivated bacteria in the two cell types. The same trend was also true with IL-8 (data not shown). Because the strongest up-regulation occurred at the highest

bacteria:host cell ratio of 10:1, this infectious condition was maintained in the following large-scale experiments.

2. Identification of inducible genes in response to infection with *M. haemolytica*

SSH was performed to identify the genes that are induced following 6 h infection of BT and EBTr cells with live *M. haemolytica* on a genome scale. Because of a difficulty in isolating a sufficient amount of mRNA from cells for SSH, 1 μg of total RNA from each sample was used for cDNA synthesis and amplification by using the Super SMART PCR cDNA Synthesis Kit (Clontech). To ensure amplification of cDNA products in the linear range, different cycles (15, 18, 21 and 24) were used for each sample. As shown in Fig. 3A, 18-21 cycles were clearly around the linear range for all four samples. To further identify the optimal amplification cycles more accurately, another round of PCR was performed for different cycles in that range for each sample. The final PCR amplification cycles for control BT cells, infected BT cells, control EBTr cells, and infected EBTr cells were 21, 20, 20 and 19 cycles, respectively (Fig.3B).

Optimally amplified PCR products were further size-separated through the CHROMA SPIN 1000 Columns (Clontech) and 1 ml fractions were collected separately. As indicated in Fig. 4, PCR products of large sizes were primarily concentrated in the fraction B, which therefore was saved for digestion with *Rsa*I and ligation with adaptors.

Uninfected control RNA samples prepared from two cell lines were used as drivers, whereas infected RNA samples were used as testers. Following two rounds of subtractive hybridization and PCR amplification, final PCR products were electrophoresed. A clear difference in the banding pattern was observed between subtracted and unsubtracted

samples (Fig. 5). To further analyze the efficiency of subtraction, a house-keeping gene, GAPDH, was PCR-amplified with subtracted and unsubtracted cDNAs for both cells for 20, 25, 30 and 35 cycles. GAPDH levels were almost completely diminished even after 35 cycles of amplification in subtracted samples as compared with unsubtracted samples (Fig. 6), clearly indicating a high efficiency of subtraction.

Subtracted DNA samples were next ligated to pGEM-T Easy vector (Promega) and transformed into competent bacteria. A total of 288 white colonies were picked randomly for each cell type, and the sizes of all inserts were checked by colony PCR (Fig. 7). To facilitate the identification of inserts with different sizes, all PCR products were further rearranged according to their sizes. As shown in Fig. 8, all inserts varied greatly from <200 bp to >1200 bp, implying a good representation of different types of mRNAs. Ninety-six colonies with inserts of clearly different sizes were selected from each cell type for direct sequencing.

A total of 62 cDNA products from BT cells and 74 inserts from EBTr cells returned readable sequences, which were subsequently searched for their homology with the GenBank database sequence using the BLAST program (Table1). These genes were further categorized based on their biological functions.

As shown in Table 3, among a total of 51 non-redundant genes in the subtracted BT cell cDNA library, 35 were found to have hits in GenBank. Another 16 sequences did not return any good hits in GenBank, indicative of novel genes (data not shown). The genes with known functions can be further categorized into six groups, i.e., innate and adaptive immunity, cell adhesion and migration, general metabolism, transcriptional regulation and processing, signal transduction, and other known functions. For example, 46-kDa

collectin precursor (CL-46) is a C-type lectin involved in innate immunity [46, 47]. Prion protein (PRNP), causing the transmissible spongiform encephalopathies (TSEs) in humans and farm animals, was hypothesized recently as a new type of pattern recognition receptor for viruses, based on the homology in both function and structure [48, 49].

In the subtracted EBTr cell cDNA library, among a total of 65 non-redundant genes that have been identified, 39 genes have been identified with known functions by BLAST search of the GenBank (Table1 and Table 4). Interestingly, 15 bacterial genes were also identified (data not shown), which are likely to come from *M. haemolytica* adhered to EBTr cells or grown intracellularly. Another 26 sequences did not have any good hits, suggesting that they may be novel genes (data not shown). Among 18 genes with known functions, they can be assigned into three major groups, i.e., innate and adaptive immunity, signal transduction, and other known functions (Table 4).

3. Identification of bovine lung-specific transcripts by SSH

Bovine lung-specific transcripts were also identified similarly by SSH following subtraction of lung RNA samples from a mixture of an equal amount of RNA from heart, liver, muscle, colon, and rumen. A total of 88 inserts returned readable sequences, which were subsequently searched for their identities through the GenBank database using the BLAST program. A total of 61 genes were found to be non-redundant. Among them, 37 genes were found to have hits in GenBank and can be classified in the following five groups, including innate and adaptive immunity, signal transduction, metabolism, DNA repair, and other genes known functions (Table1 and Table 5). The other 24 sequences are novel genes with no good hits (data not shown).

4. Confirmation of the SSH results by RT-PCR

To confirm the SSH results, semi-quantitative and real-time RT-PCR were further performed with 16 randomly selected genes. Among them, five genes were confirmed to be up-regulated following infections of BT and/or EBTr cells with *M. haemolytica* by semi-quantitative RT-PCR (Fig. 9). These five genes include sprouty homolog 4 (Spry4), COBW domain-containing protein 2 (CBWD2), endothelial cell-specific molecule 1 (ESM1), CD164 (Endolyn), and a novel gene, G4. As expected, the positive control, IL-6 gene, showed a robust induction following infections of both cell types (Fig. 9).

Real-time RT-PCR was also independently used to confirm the expression of four selected genes, together with IL-6. As shown in Fig. 10, IL-6 expression was dramatically increased in both cell types after infection. The expression levels of Nck-associated protein 1 (Nap1), CD164, and G4 showed approximately 5-, 6-, and 4- fold changes, respectively, after 6-h infection of BT cells. Similarly, ESM1 also reached the maximum 5.6-fold induction after 12-h infection of BT cells. However, None of these four selected genes was found to be up-regulated in real-time PT-PCR in EBTr cells (Fig. 10).

CHAPTER V

DISCUSSION

The bovine respiratory disease (RBD) complex is the primary cause of economic loss in the beef and dairy cattle industry in North America. It's believed that *M. haemolytica* is a major bacterial agent responsible for the BRD complex. However, the host defense mechanism against *M. haemolytica* remains poorly understood and, not surprisingly, there are no effective therapeutics available. To identify the host factors that are potentially involved in the bovine airway defense and resistance to *M. haemolytica*, SSH was employed to isolate bovine lung-specific genes as well as the genes induced in BT and EBTr cells following infection with *M. haemolytica*.

Among a total of 224 genes with readable sequences identified from three subtracted cDNA libraries (BT cells, EBTr cells and lung), 177 were non-redundant. Among them, 111 genes were found to have known functions and 66 were novel. In subtracted BT and EBTr cell cDNA libraries, a total of 74 sequences have been identified to have homology with known genes in GenBank, and 42 sequences were novel with unknown functions. In subtracted bovine lung cDNA library, 37 sequences were genes with known functions, while 24 genes were novel ones.

Interestingly, sequencing of 96 clones from each of the subtracted BT and EBTr cell cDNA libraries revealed that most of the differentially expressed genes identified are different between the two cell types, expect for T cell receptor gamma and glutamate-

cysteine ligase catalytic subunit (GCLC). Although BT and EBTr cells are both fibroblasts from bovine airway, the differential up-regulation profiles may reflect the fact that the difference in their anatomic locations where they are isolated. BT cells were derived from turbinate mucosa, whereas EBTr cells were from tracheal mucosa. Furthermore, different developmental stages of both cell types may also partially account for the discrepancy in the gene expression profile. BT cells were isolated from young, well-developed calves, while EBTr cells were originated from embryos. In fact, EBTr cells were found to be less responsive to *M. haemolytica* infection than BT cells in their ability to produce IL-6 (Fig. 2) or IL-8 (data not shown). Nevertheless, it is expected that additional differentially expressed genes common in both cell types could be identified following sequencing of a larger number of colonies.

Six were confirmed to be up-regulated in BT and/or EBTr cells following *M. haemolytica* infection as confirmed by semi-quantitative and/or real-time RT-PCR. These genes include endothelial cell-specific molecule 1 (ESM-1), CD164, sprouty homolog 4 (Spry4), COBW domain-containing protein 2 (CBWD2), NCK-associated protein 1 (Nap1), and a novel gene, G4.

ESM-1 was recently found to specifically recognize and bind to integrin CD11a/CD18 on the cell surface of human blood lymphocytes and monocytes, which subsequently inhibits the interaction of integrin with the Intercellular Adhension Molecule-1 (ICAM-1) on the endothelial cells [50]. Interestingly, binding of leukotoxin (Lkt) of *M. haemolytica* to CD11/CD18 of β2-integrins of macrophages and neutrophils is a major cause of severe pulmonary lesions as observed in the BRD complex. An

increase in ESM-1 expression may play a protective role in the bovine airway by competitively inhibiting the binding of Lkt to β2-integrins on leukocytes.

CD164 or endoly is a newly identified sialomucin with 2 extracellular mucin domains with expression on several cell types, including T cells, stromal cells, hematopoietic progenitor cells, and epithelial cells. CD164 functions in cell adhesion and migration and serves as a potent negative regulator of hematopoietic progenitor cell proliferation [51, 52]. It is tempting to speculate that up-regulation of CD164 in response to *M. haemolytica* may limit epithelial cell proliferation and consequently bacterial spreading in the airway.

Spry was firstly identified in *Drosophila* as an inhibitor of fibroblast growth factor (FGF) receptor and receptor tyrosine kinases (RTK) signal transduction. Among four Spry homologous proteins (Spry 1-4) identified in humans, Spry 4 functions as an antagonist of FGF receptor in lung development and inhibits the excessive tracheal branching in embryonic lung [53]. The impact on the up-regulation of Spry 4 in airway on pathogenesis of *M. haemolytica* remains unknown.

Nap1, a GTP-binding protein (Rac)-interacting protein, was found recently to have a regulatory role in cell transformation, activation of c-Jun N-terminal kinases, and membrane NADPH oxidase [54]. The effect of the induction of Nap1 on airway defense and disease resistance is not known. Similarly, the role of CBWD2, a cobalamin (vitamin B12) synthesis protein [55], in the host response to *M. haemolytica* remains unknown.

Within 16 randomly selected genes, only six were found to be truly positive. The high frequency of false positives by SSH may be due to hybridization of the tester cDNA

samples with an insufficient amount of the driver cDNAs prior to PCR amplification of differentially expressed genes in the SSH procedure. Although the manufacturer recommends inclusion of the driver cDNA in 30-fold excess over the tester it seemed that this ratio could be changed to achieve better subtraction efficiency and reduce the frequency of the false positive results.

Taken together, we have identified a diverse array of genes that are potentially induced in the bovine airway following *M. haemolytica* infection as well as the genes that are preferentially expressed in bovine lung. Among 16 randomly selected genes, six have been confirmed to be truly up-regulated in response to infection. Further studies are needed to study their role in disease resistance and/or pathogenesis. The expression patterns and functions of other identified genes, including 66 novel genes, will be investigated in the future as well. Moreover, such a collection of the airway genes can be potentially developed as a microarray tool for comprehensive transcriptional profiling of bovine host responses to infections with the BRD pathogens.

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Table 1. Summary of the sequencing and BLAST results

cDNA Library	Clones screened	Clones sequenced	Readable sequences	Non-redundant genes	Genes with known functions	Novel genes
BT cells (Up-regulated)	288	96	62	51	35	16
EBTr cells (Up-regulated)	288	96	74	65	39*	26
Lung-specific	288	96	88	61	37	24
Total	864	288	224	177	111	66

*Those 39 genes include 24 bovine genes and 15 bacterial genes.

Table 2. Primers for semi-quantitative and real-time RT-PCR

Target cDNA	Primer Name	Primer sequence (5'-3')	PCR product size (bp)
Osteonectin	bSPACRC-SN	CCTGTGACCTGGACAACGAC	140
	bSPACRC-AS	GTCAAAGAGAGAGAATCTGGT	
B5 (Unknown gene)	B5-EBTr-SN	CCGTCTATGGGGTTGCACAG	142
	B5-EBTr-AS	TGAGTAAGGAGAACTCTGGTG	
NID67 (Unknown gene)	bNID67-SN	AGACCTGGCACGATCCAGCA	143
	bNID67-AS	ATGACCCAGATGTCCAGGATG	
Tumor protein, translationally-controlled 1 (TPT1)	bTPT1-SN	GGACTACCGTGAGGATGGTG	129
	bTPT1-AS	CAGTAGCCAGCCAGTTATGAC	
Decorin (DCN)	bDecorin-SN	GACTGAGTTTCAACAGCATCT	175
	bDecorin-AS	GAGCCGATTGCAGAGATATTG	
Myeloid cell leukemia protein 1 (MCL1)	bMCL1-SN	CTTGAGCCTCTGAATGTGACC	117
	bMCL1-AS	AAGGCTTAGAGATGGGCAGG	
CD47	bCD47-SN	GACTTGGTTTAATTGTACTTCC	182
	bCD47-AS	AGGACCATGCACTGGGATAC	
Programmed cell death 4	bPDCD4-SN	CTGGATGTCCCACATTCATAC	156
	bPDCD4-AS	GCTCTCTGGTTTAAGACGACC	
NCK-associated protein 1 (NCKAP1)	bNCKAP1-SN	CTTGCATCCTCCAGTCTACT	163
	bNCKAP1-AS	CATTTCTCAGCAAGACATAGG	
COBW domain-containing protein 2 (CBWD2)	bCBWD2-SN	GGAAGACGACAGTGAGAGAAC	145
	bCBWD2-AS	TGGACAAACTTGATCTTCTTGG	
G4 (Unknown gene)	G4-EBTr-SN	GTCGTTGCTCTCATTACTATC	130
	G4-EBTr-AS	GTCATAAGAAGCTGTGGCGAC	
Prion Protein (PRNP)	bPRNP-SN	AAGTTCTTGGTGCAAATGTGTC	108
	bPRNP-AS	GCTAAGGACAACACGGAAGAG	
Sialomucin (CD164)	bCD164-SN	ACCACAACTCTGCCTTCCACT	106
	bCD164-AS	AGGTAGACTTCCGCGTAGGT	
Ferritin heavy polypeptide 1 (FTH1)	bFTH1-SN	AAGTGTGAATCAGTCACTACTG	115
	bFTH1-AS	TTTGATGGCTTCCACCTGCTC	
Sprouty homolog 4	bSPRY4-SN	TGCACTGGCCTTCTGGTTTATG	110
	bSPRY4-AS	CACCGACTTACCCACAGCAAAG	
Endothelial cell-specific molecule 1 (ESM1)	bESM1-SN	CAACAGGGTGACCGGCAAATG	114
	bESM1-AS	CCGTCTCCAGATGCCACGTC	

Table 3. *M. haemolytica*-induced genes in BT cells identified by SSH

Best BLAST hit	Identity (%)	Score (bits)	E value
Innate and adaptive immunity			
46-kDa collectin precursor (CL-46)	92 (305/331)	450	7e-124
Propreprion protein (PRNP) and prion-like protein doppel(PRND)	93 (627/668)	985	0
prion protein (PRNP)	96 (626/650)	1088	0
T cell receptor gamma	98 (253/258)	466	2e-128
Cell adhesion and migration			
Connective tissue growth factor	99 (369/372)	718	0
Sialomucin (CD164 antigen)	99 (296/297)	581	8e-163
Fibronectin 1	94 (79/84)	137	6e-30
Decorin	99 (399/403)	720	0
Thymosin beta 4	100 (345/345)	630	1e-177
Endothelial cell-specific molecule 1 (ESM1)	86 (87/101)	90	7e-15
Prostaglandin E receptor 2	100 (64/64)	127	2e-26
Prostaglandin E receptor 2 (subtype EP2), 53kDa (PTGER2)	100 (64/64)	127	2e-26
General metabolism			
Glutamate-cysteine ligase catalytic subunit (GCLC)	89 (157/176)	190	4e-45
Low density lipoprotein receptor, transcript variant 6	99 (174/175)	339	2e-90
Ferritin heavy polypeptide 1 (FTH1)	99 (387/390)	708	0
Phosphatidylinositol glycan class V	100 (35/35)	70	5e-09
Transcriptional regulation and processing			
Splicing factor, proline-and glutamine-rich	90 (260/287)	315	7e-83
Elongation of very long chain fatty acids -like 1	100 (416/416)	825	0
Chromodomain helicase DNA binding protein 9	100 (81/81)	161	3e-36
Signal transduction			
Sequestosome 1, transcript variant 3	99 (189/190)	369	3e-99
Mus musculus sprouty homolog 4	93 (120/128)	198	1e-47
Ras suppressor protein 1 (RSU1)	99 (519/520)	1023	0
Moesin	99 (754/759)	1471	0
90-kDa heat shock protein beta	92 (183/197)	299	2e-78
Other genes with known functions			
Eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	100 (526/526)	1043	0
Solute carrier family 35 member 3 (slc35a3)	93 (212/227)	331	2e-87
Myostatin	96 (213/221)	377	3e-101
X-inactivation center region, Jpx and Xist	95 (228/238)	377	1e-101

Mus musculus sprouty homolog 4 (Spry4)	93(120/128)	198	1e-47
Genes with unknown functions			
CG10979-PA, transcript variant 1	100 (129/129)	256	9e-65
Hypothetical LOC511583	95 (237/247)	410	9e-112

Table 4. *M. haemolytica*-induced genes in EBTr cells identified by SSH

Best BLAST hit	Identity (%)	Score (bits)	E value
Innate and adaptive immunity			
T cell receptor gamma (TCRG)	96 (494/511)	882	0
Apoptosis			
Programmed cell death 4	100 (336/336)	666	0
General metabolism			
ATP synthase	99 (461/464)	896	0
Glutamate-cysteine ligase catalytic subunit (GCLC)	89 (157/176)	190	4e-45
Decorin	100 (377/377)	706	0
Signal transduction			
Sus scrofa myeloid cell leukemia protein 1 (MCL1)	86 (347/401)	285	1e-73
Platelet-activating factor acetylhydrolase	99 (593/596)	1118	0
NCK-associated protein 1	90 (38/42)	60	1e-5
14-3-3 protein zeta/delta (protein kinase C inhibitor protein 1	90 (188/208)	278	1e-71
Zinc finger and BTB domain containing protein	99 (424/425)	835	0
Progesterone membrane binding protein	96 (503/519)	934	0
Other genes with known functions			
Osteonectin	99 (290/291)	571	9e-160
Tumor protein, translationally-controlled 1	89 (364/406)	537	7e-150
Sus scrofa topoisomersae II (TOPOII)	87 (341/389)	440	2e-120
Actin, cytoplasmic 2	98 (713/727)	1352	0
COBW domain-containing protein 2	99 (411/412)	809	0
Tnni3 gene for cardiac troponin I	89 (161/179)	208	2e-50
Homo sapiens v-myb myeloblastosis viral oncogene homolog (avian)-like 1	89 (169/189)	210	2e-51
Genes with unknown functions			
Homo sapiens cDNA clone IMAGE:3897094	91 (52/57)	70	7e-09
Synthetic construct arsenic-like protein gene	97 (48/49)	92	2e-15
Ornithine decarboxylase (ODC)	89 (94/105)	121	2e-24
Bos taurus cDNA clone MGC:128424 IMAGE:7954811	98 (333/338)	634	5e-179
Homo sapiens similar to hypothetical protein MGC17347	87 (343/391)	379	4e-102
Homo sapiens chromosome 2 open reading frame 12, transcript variant 1 (C2orf12)	97 (271/278)	488	2e-134

Table 5. Lung-specific genes identified by SSH

Best BLAST hit	Identity (%)	Score (bits)	E value
Innate and adaptive immunity			
Amyloid β(A4)	98(296/299)	529	1e-149
T cell receptor α	89(134/149)	176	4e-43
Cell adhesion and migration			
Integrin α-3 precusor	99(538/542)	1043	0.0
Chemokine ligand 16	100(160/160)	317	8e-86
Cadherin 11	99(158/159)	307	5e-83
Fibronectin 1	100(176/176)	349	2e-95
Apoptosis			
RhoB	99(206/208)	317	9e-86
Calpain	95(211/220)	383	2e-105
Surfactant protein A (SP-A)	100 (23/23)	46	4e-4
Surfactant, pulmonary-associated protein C (SFTPC)	93 (154/164)	262	4e-69
ATP-binding cassette sub-family E member 1	96 (764/789)	1372	0
Signal transduction			
Caveolin 1	94(239/254)	406	1e-112
Serum/glucocorticoid regulated kinase	98 (209/213)	398	2e-110
Class I cytokine receptor	98(227/231)	432	3e-120
AHNAK nucleoprotein isoform 1	98 (119/121)	228	6e-59
Metabolism			
UDP-glucose dehydrogenase	98 (343/350)	607	8e-173
Stearoyl-coenzyme A desaturase (SCD)	95 (369/387)	658	0
Beta-1,2-N-acetylglucosaminyltransferase II	98 (306/312)	579	2e-164
Adenosine deaminase (ADA)	99 (354/355)	698	0
11-beta hydroxysteroid dehydrogenase type 2	100 (22/22)	44	0.006
DNA repair			
APEX nuclease (multifunctional DNA repair enzyme) 1	97 (415/425)	779	0
Double-strand-break repair protein	99 (345/347)	674	0
Transcriptional regulation			
Methyl-CpG binding domain protein	99 (251/252)	494	6e-139
Other genes with known functions			
Futb and rtlf	83 (125/150)	88	2e-16
Alpha-2-macroglobulin precursor	100 (486/486)	963	0
Gap junction protein, alpha 5	99 (112/113)	216	7e-55
Eukaryotic translation factor 5	95 (353/371)	620	7e-177
Hypoxia-inducible factor 1 alpha inhibitor	99 (155/156)	303	4e-81
Transmembrane anchor protein 1	96 (192/196)	357	1e-97

Translocation protein 1	93 (260/279)	436	1e-121
Polyribonucleotide nucleotidyltransferase 1	95 (104/109)	186	5e-46
Sorting nexin 17 (SNX17)	100 (180/180)	357	1e-97
Secretoglobin precursor	98 (240/244)	458	6e-128
Chromodomain helicase DNA binding protein 8	99 (450/452)	884	0
Beta-2-microglobulin (B2M)	94 (172/182)	301	4e-81
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a2	100 (205/205)	406	1e-112
Apolipoprotein L, 3	98 (104/106)	194	6e-49
RNA-dependent helicase p68	88 (165/187)	238	4e-62
Zinc finger, matrin type 2	99 (232/233)	454	1e-126
RAN, member RAS oncogene family	100 (113/113)	224	1e-57
Genes with unknown functions			
Hypothetical protein LOC613306	100 (246/246)	446	2e-124
Cell surface retention sequence binding protein 1	96 (583/604)	1067	0
Chromosome 17 open reading frame 27	93 (607/651)	969	0
C10 protein	100 (32/32)	64	6e-9
NudC domain containing 1	83 (180/216)	198	1e-49
Ring finger protein 130	99 (285/287)	553	1e-156

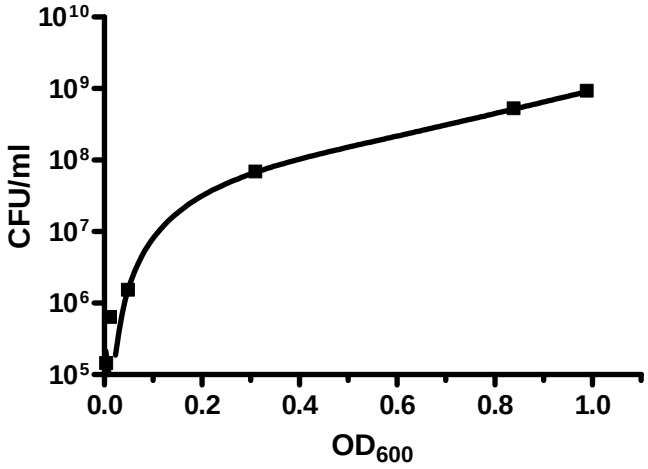

Fig. 1. Growth curve of *M. haemolytica*. Overnight bacterial culture was subcultured 1:1000 and measured at OD_{600} for turbidity on an hourly basis. Each hourly bacterial culture was serially diluted and spread into medium agar plates for bacterial colony counting.

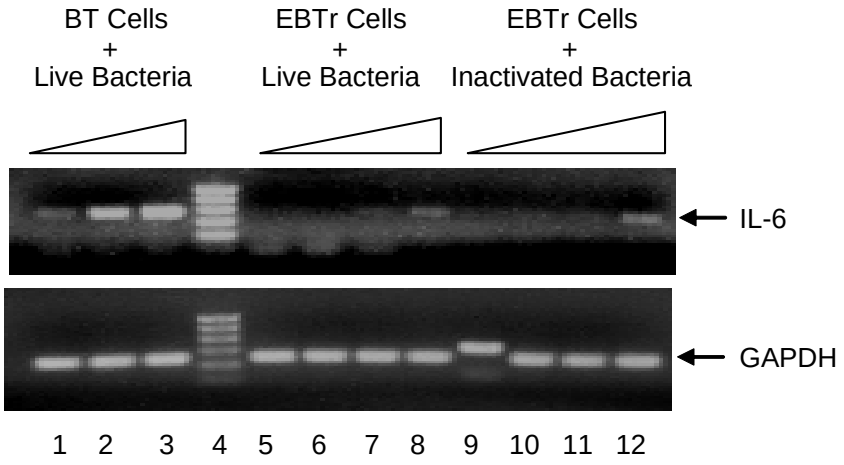

Fig. 2. Semi-quantitative RT-PCR Analysis of IL-6 mRNA expression in BT and EBTr cells following a 6-h infection with different doses of *M. haemolytica.* BT cells were left uninfected (lane 1) or infected with live bacteria at bacteria:host cell ratios of 1:1 (lane 2) and 10:1 (lane 3), whereas EBTr cells were left uninfected (lane 5) or infected with live bacteria at bacteria:host cell ratios of 0.1:1 (lane 6), 1:1 (lane 7), and 10:1 (lane 8) or heated-inactivated bacteria at bacteria:host cell ratios of 0.1:1 (lane 9), 1:1 (lane 10), 10:1 (lane 11), and 100:1 (lane 12). Total RNA was then isolated from each sample and subjected to semi-quantitative RT-PCR. PCR amplification cycles for GAPDH and IL-6 are 25 and 32 cycles, respectively. To facilitate visualization of PCR products, 100 bp DNA ladder was loaded in lane 4.

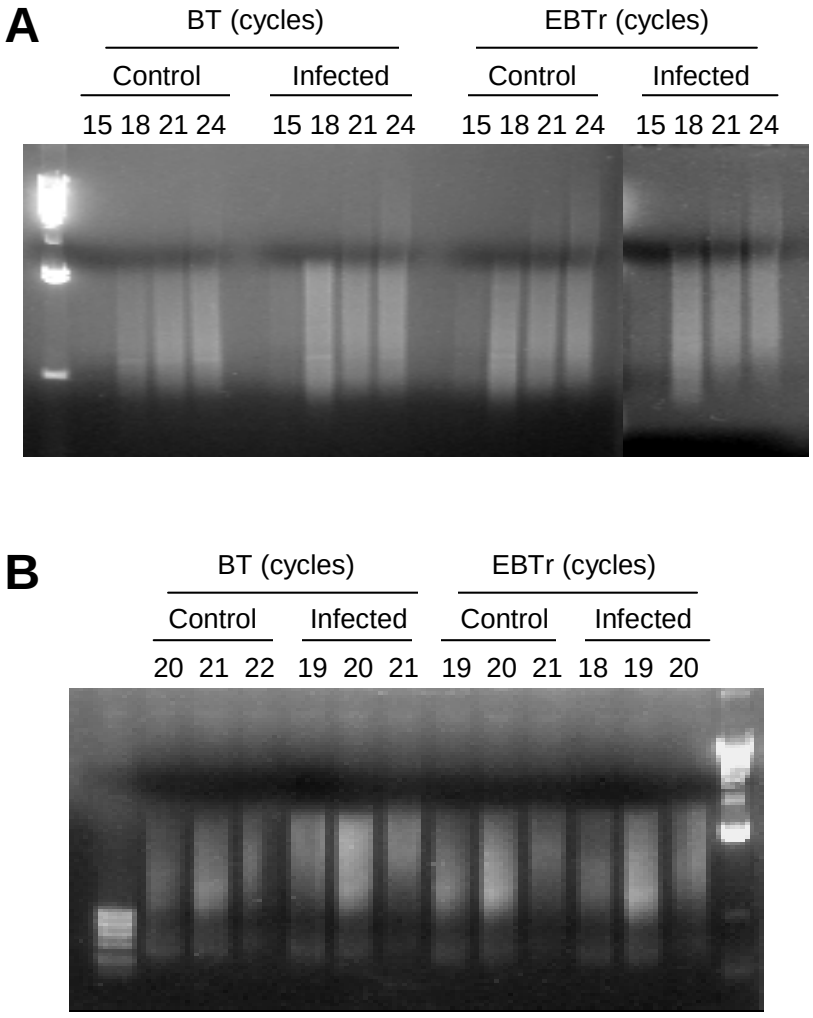

Fig. 3. Determination of the optimal number of cycles for linear cDNA amplification. In panel A, the first-stranded cDNA from control and infected cells was subjected to 15, 18, 21 and 24 cycles of amplification. In panel B, the optimal cycle number was further refined based on the results from panel A. The final optimal number of amplification cycle was determined as 21, 20, 20 and 19 for control BT cells, infected BT cells, control EBTr cells, and infected EBTr cells, respectively.

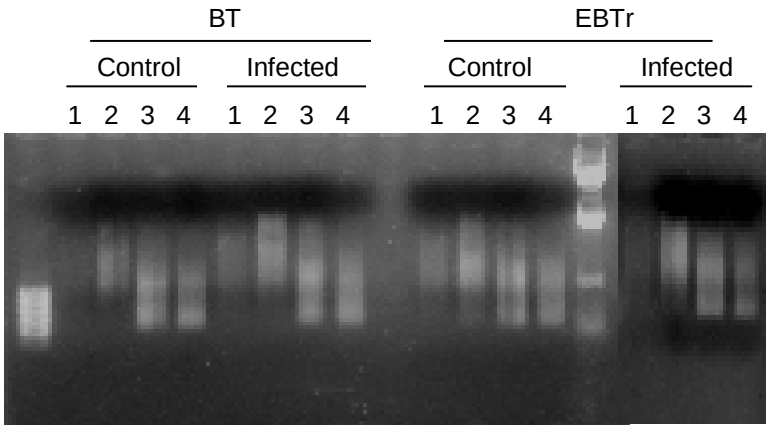

Fig. 4. Analysis of cDNA recovered from using BD CHROMA SPIN 1000 columns. Following linear amplification, cDNA samples were further fractionated by size-exclusion chromatography. Lanes: 1, cDNA sample before chromatography; 2, eluted fraction B; 3, eluted fraction C; 4, eluted fraction D. To facilitate visualization of cDNA sizes, 100 bp ladder DNA marker and Lamda DNA/*Eco*R I + *Hin*d III marker were loaded as well.

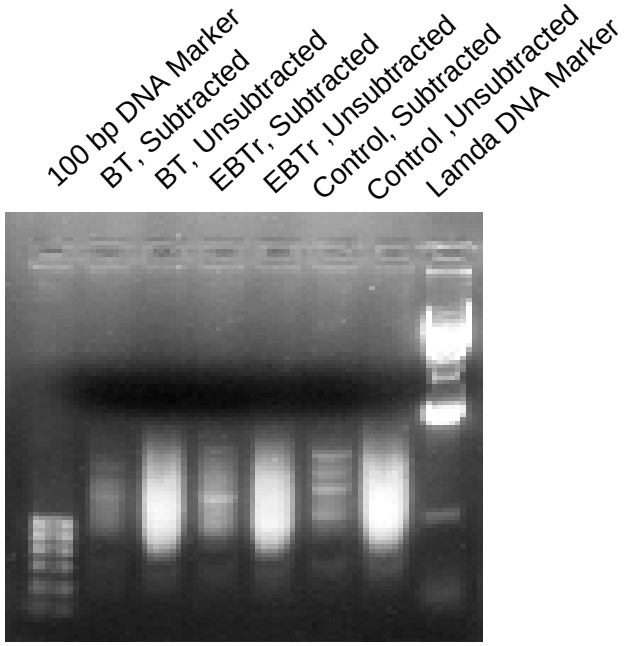

Fig. 5. Analysis of subtracted cDNA samples by gel electrophoresis. Nested PCR products of subtracted and unsubtracted testers of BT cells, EBTr cells, and positive control samples (provided in the SSH kit) are compared side by side to show the different banding patterns.

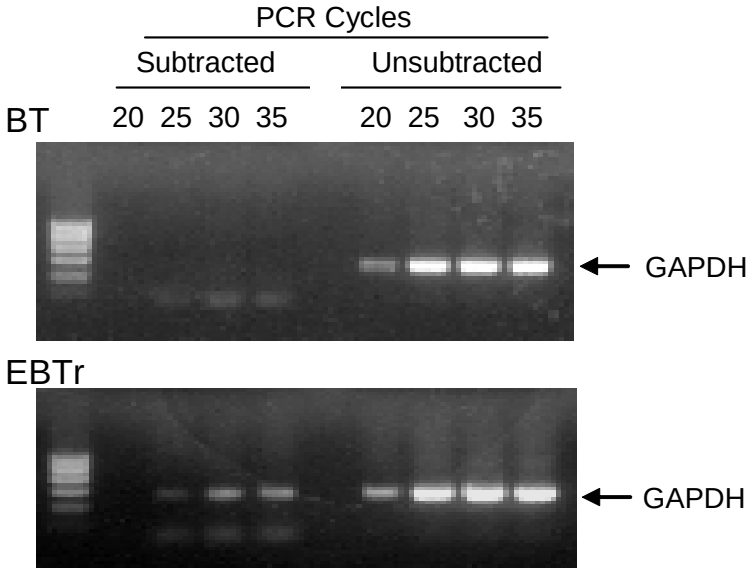

Fig. 6. Analysis of the subtraction efficiency. PCR was performed for 20, 25, 30, and 35 cycles for both subtracted and unsubtracted cDNA samples from BT and EBTr cells. Note a significant reduction in GAPDH abundance in subtracted samples.

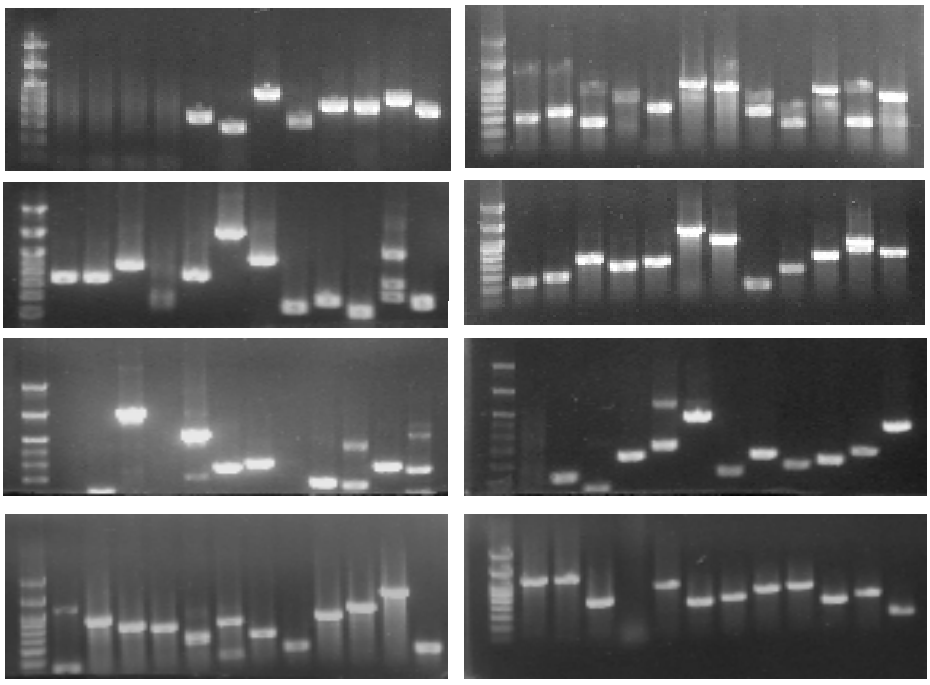

Fig. 7. Screening of the inserts by colony PCR. The inserts were PCR-amplified from 96 random plasmids in the subtracted BT cell cDNA library by Nested Primer 1 and 2R. 100 bp DNA ladder was loaded in the leftmost lane in each gel.

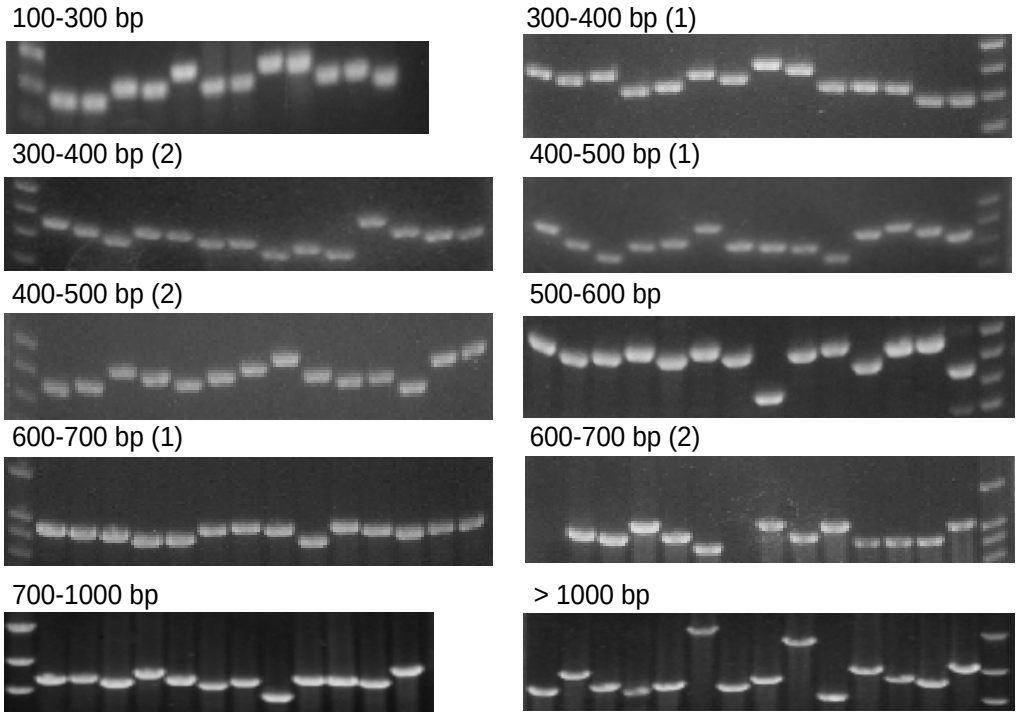

Fig. 8. Rearrangement of the inserts based on their sizes. The inserts with similar sizes were compared in the same agarose gel to facilitate the selection of 96 non-redundant inserts with different sizes for direct sequencing.

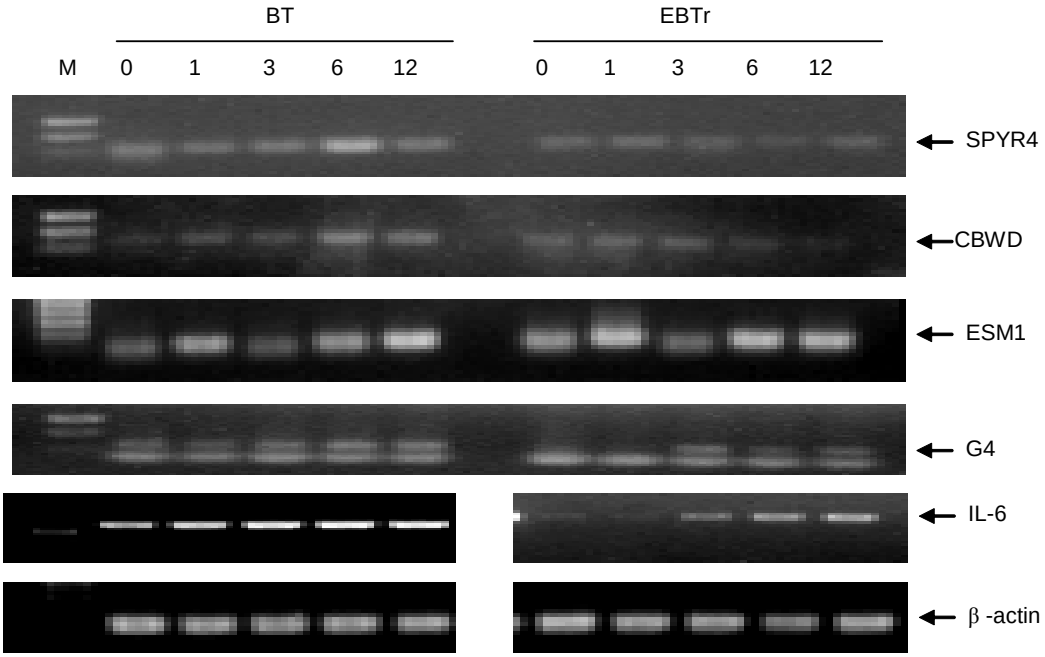

Fig. 9. Semi-quantitative RT-PCR confirmation of five *M. haemolytica*-induced genes identified by SSH. BT and EBTr cells were infected with *M. haemolytica* for 0, 1, 3, 6 and 12 h, followed by RNA isolation and RT-PCR for five different genes normalized against the β-actin expression. IL-6 was used as a positive control.

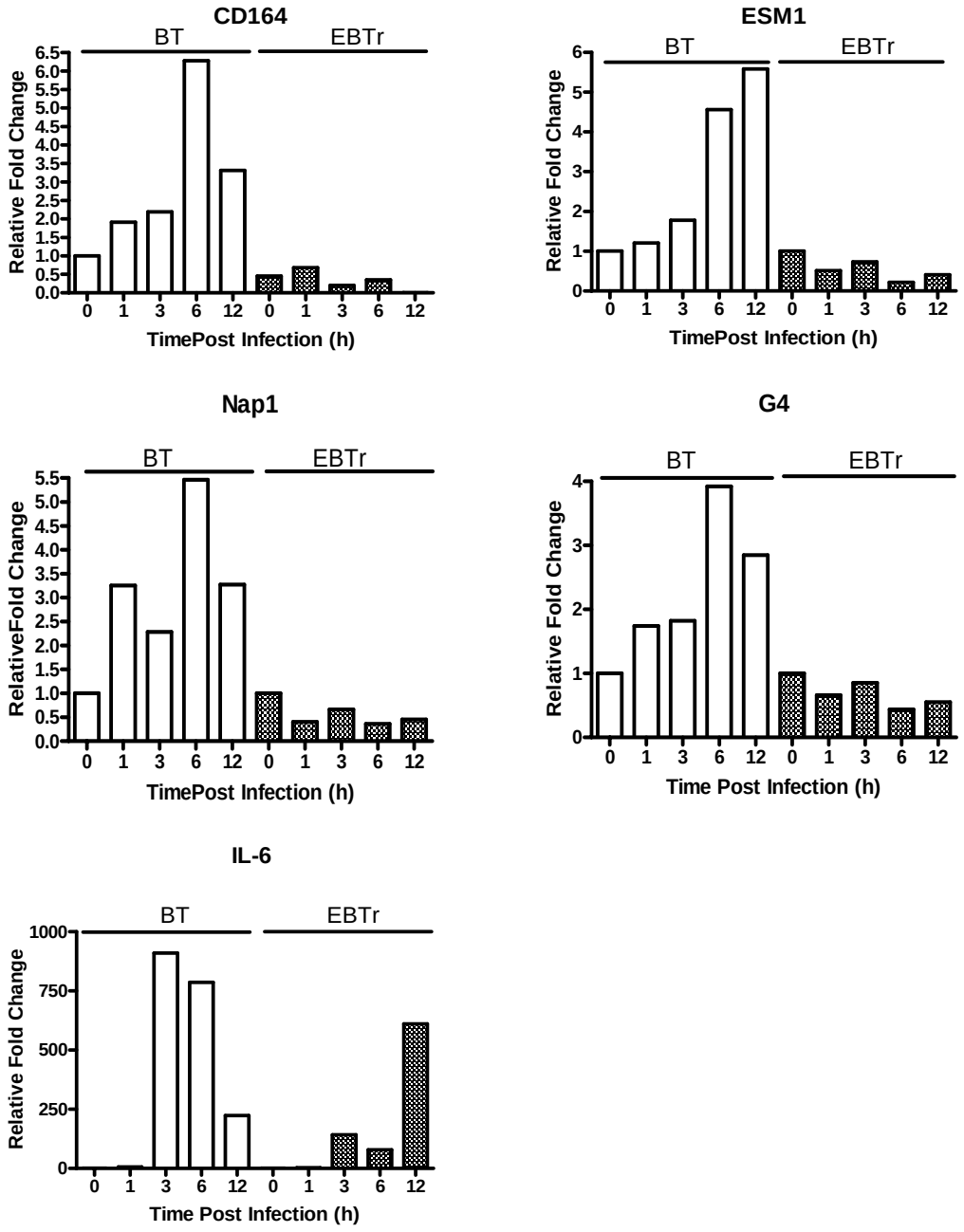

Fig. 10. Real-time RT-PCR confirmation of five *M. haemolytica*-induced genes identified by SSH. BT and EBTr cells were infected with *M. haemolytica* for 0, 1, 3, 6 and 12 h, followed by RNA isolation and real-time RT-PCR for four different genes normalized against 18S rRNA expression. IL-6 was used as a positive control.

VITA

Yibin Cai

Candidate for the Degree of

Master of Science or Arts

Thesis: IDENTIFICATION OF IMMUNE RESPONSIVE GENES IN BOVINE AIRWAY USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION

Major Field: Animal Science

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Name: Yibin Cai Date of Degree: December, 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: IDENTIFICATION OF IMMUNE RESPONSIVE GENES IN BOVINE AIRWAY USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION

Pages in Study: 50 Candidate for the Degree of Master of Science/Arts
Major Field: Animal Science

Scope and Method of Study: To identify the host factors that are potentially involved in the bovine airway defense and resistance to M. haemolytica, we employed suppression subtractive hybridization (SSH) to isolate bovine lung-specific genes as well as the genes that are induced in two bovine airway cell lines, namely Embryonic Bovine Tracheal (EBTr) and Bovine Turbinate (BT) cells, following infection with *M. haemolytica*.

Findings and Conclusions: Among a total of 224 genes sequenced from three subtracted cDNA libraries, 177 are non-redundant, including 66 novel genes. The expression levels of 16 genes have been further selected for confirmation by semi-quantitative and quantitative real-time RT-PCR. Six have been confirmed to be truly up-regulated in response to *M. haemolytica* infection. The expression patterns and functions of additional genes will be investigated in the future. Importantly, such a collection of differentially expressed airway genes can be potentially developed as a microarray tool for comprehensive transcriptional profiling of bovine host responses to respiratory infections to facilitate the understanding of airway defense mechanisms as well as the development of more effective therapeutic strategies against the BRD pathogens.

ADVISER'S APPROVAL: Guolong Zhang