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Identification for Different Expression Proteins in Different Virulent *Streptococcus suis* Serotype 7 Strains

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Abstract: *Streptococcus suis* (*S. suis*) secreted numerous proteins are virulence-associated factors that play critical roles in infection. Studies of *S. suis* secreted proteins have been reported in *S. suis* Serotype 2 (SS2) strains and *S. suis* Serotype 9 (SS9) strains. However, data is still scarce. In this study, a comparative proteomics approach was used to distinguish the profiles of the secreted proteins in different virulent SS7 strains. Consequently, 13 individual proteins that were unique to either high pathogenic 07WC11 strain or low pathogenic SJZB408 strain in 2D gels were successfully identified. In addition, researchers attempted to compare the amount of expression 10 identified protein genes (*pft*, *rsp*, *cms*, *esp*, *hpk*, *pk*, *rpp*, *rrf*, *tef*, *tis*) by quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Real-time RT-PCR results showed that pft, rsp, cms, esp, pk, rpp, rrf and tef mRNA expression amount in low pathogenic SS7 strain was lower than in high pathogenic SS7 strain which is accord with the results of 2D. Thus, researchers defined these eight proteins as different expression proteins between low pathogenic and high pathogenic SS7 strain.

Key words: SS7, comparative proteome, secreted proteins, qRT-PCR, PCR

INTRODUCTION

S. suis is an important pathogen associated with a range of diseases in pigs including meningitis, pneumonia, arthritis, septicemia (Gottschalk and Segura, 2000; Staats *et al*., 1997; Juncal *et al*., 1997; Cheng *et al*., 1987) and causes serious human disease occasionally (Arends and Zanen, 1988; Staats *et al*., 1997; Woo, 1986; Fongcom *et al*., 2009; Fittipaldi *et al*., 2009; Feng *et al*., 2009; Haleis *et al*., 2009). There are currently 35 serotypes of *S. suis* recognized based on the immunogenicity of capsular antigens (Gottschalk *et al*., 1991a, b, 1989; Higgins *et al*., 1995; Perch *et al*., 1983). The bacteria secreted proteins which enable bacteria to adapt to and survive in environmental. Some secreted proteins are putative virulence-associated factors that play critical roles in infection process of the host owing to these factors act with host indirect or directly (Trost *et al*., 2005). Studies of *S. suis* secreted proteins have been reported in *S. suis* Serotype 2 (SS2) strain and *S. suis* Serotype 9 (SS9) strain. According to report, about 100 secreted proteins were successfully identified in SS2 strain by Jing *et al*. (2008) and Zhang and Lu (2007). About 13 secreted proteins were identified successfully in SS9 strain GZ0565 by Wu *et al*. (2008). However, data is still rare and comparative analyses of secreted proteins gene expression in pathogenic and nonpathogenic SS7 strains have not been reported. Researchers aim to provide more documents.

In the present study, a comparative proteomics approach was used to compare the two Dimensional electrophoresis (2D) profiles of the secreted proteins of high pathogenic SS7 strain 07WC11 and low pathogenic SS7 strain SJZB408. Some unique secreted proteins to either high pathogenic or low pathogenic SS7 strain in 2D gels were identified successfully by Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS). Moreover, researchers attempted to compare the 10 proteins which were 6-Phosphofructokinase (PFT), 30S Ribosomal Protein S6 (RSP), Chorismate Synthase (CMS), Extracellular Solute-binding Protein family 5 (ESP), HPr

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Kinase/phosphorylase (HPK), Pyruvate Kinase (PK), Ribose-Phosphate Pyrophosphokinase (RPP), Ribosome Recycling Factor (RRF), Translation Elongation Factor Ts (TEF), Triosephosphate Isomerase (TIS) gene expression by qRT-PCR.

MATERIALS AND METHODS

Bacterial strains and culture: Two SS7 strains were used in this study. Strain 07WC11 was isolated from a diseased pig with meningitis in 2007, China. Strain SJZB408 was isolated from the nasal organ of a healthy pig in 2008, China. The strains were plated on a sheep blood agar base and grown in 5% equine serum (HYCLONE) Todd-Hewitt Broth (THB, BD) at 37°C.

Pathogenicity test of SS7 strains: Experiments were performed under Heilongjiang Province Experiment Animal Care and Use Committee-approved Protocols. An 8 weeks old inbred zebrafish line (National Zebrafish Resources of China) was used to test the virulence of the SS7 (07WC11 and SJZB408) strains. Prior to inoculation of the zebrafish, cells were harvested from liquid cultures by centrifugation at 5,000×g for 5 min, resuspended in Phosphate-Buffered Saline (PBS, pH 7.4) and adjusted to the appropriate concentration. Each strain of SS7 was tested at 5 different doses from 5×10^7 to 5×10^3 CFU/fish with ten fish used per dose. The zebrafish were injected by peritoneal cavity and the control fish were injected with PBS. The infected zebrafish were monitored for 1 week. The test yielded reproducible results after being repeated in triplicate. The results were averaged and used to calculate the LD_{50} by the method of Reed and Muench (1938).

Preparation of secretory proteins: Clones of the SS7 strains were picked from sheep blood agar base and grown in 5% equine serum THB at 37°C overnight. Strains 07WC11 and SJZB407 were grown to late log phase (reaching an OD_{600} of 1.0) by adding 1 mL of an overnight bacterial culture to 100 mL of new THB. Bacterial cultures were centrifuged at 10,000×g for 15 min (4°C). The supernatant was again centrifuged (4°C) for 15 min and the residual bacteria were removed by filtering through a 0.22 μm membrane. The filtrate was mixed with prechilled 10% trichloroacetic acid/acetone and incubated overnight (-20°C). After centrifugation at 10,000×g for 10 min (4°C), the pellet was resuspended in 10 mL of prechilled 90% acetone and washed twice. The final pellet was dissolved in hydration buffer (7 M urea, 2% CHAPS, 1% DTT, 0.5% IPG buffer, 0.001% bromophenol blue; GE Healthcare) after the pellet was air-dried.

Two dimensional gel electrophoresis: Secreted proteins were treated using the 2D Clean-Up kit (GE Healthcare). The proteins were then resuspended in a solution compatible with the first dimension (isoelectric focusing) and centrifuged at room temperature at 12,000×g for 15 min to remove nondissolved materials. The protein content was assayed using the 2D Quant kit (GE Healthcare). Sample buffer containing 600 μg of protein in a total volume of 250 μL was absorbed onto a 13 cm Immobiline DryStrip (IPG, immobilized pH gradient, pH range 4-7; GE Healthcare) and IEF was performed in an Ettan IPGphor 3 (GE Healthcare). Altogether, 23 h of IEF was performed at 20°C which was then rehydrated at 30 V for 12 h. Focusing was conducted by a stepwise increase of the voltage as follows: 200 V for 0.5 h, 500 V for 2 h, 1000 V for 2 h, 7000 V for 2 h and 7000 V until 55,000 Vh.

Prior to SDS-PAGE, each IPG strip was washed in 10 mL of equilibration buffer 1 (375 mM Tris-HCl [pH 8.8], 6 M urea, 2% SDS, 20% [v/v] glycerol, 1% DTT; GE Healthcare) for 15 min and in 10 mL of equilibration buffer 2 (375 mM Tris-Cl [pH 8.8], 6 M urea, 2% SDS, 20% [v/v] glycerol, 2.5% iodoacetamide; GE Healthcare) for an additional 15 min. IPG strips were loaded into a homogeneous 12% polyacrylamide gel and sealed with a 1% agarose solution. Electrophoresis was performed in 2 steps at 16°C: 5 mA/gel for 1 h and 20 mA/gel. The gels were stained with PlusOne Coomassie Blue R-350 (GE Healthcare) according to the manufacturer's protocol and destained with 10% acetic acid solution. Three replicates were run for all samples of each strain. Gel evaluation and data analysis were performed using the Image Master 2D Platinum 6.0 (GE Healthcare).

Protein identification by MALDI-TOF-TOF MS and MS/MS analysis: Spots unique to strain 07WC11 or SJZB407 were excised from the 2D gels and sent to Nanjing Medical University for tryptic in-gel digestion coupled with MALDI-TOF-TOF MS and MS/MS. The samples identified by the Peptide Mass Fingerprinting (PMF) were automatically submitted for MS/MS analysis. For MS/MS spectra searching, the spectra were used to search the NCBInr database for matching sequences using MASCOT (Matrix Science). The probability score for the match, Molecular Weight (MW), pI, number of peptide matches and percentage of the total translated ORF sequence covered by the peptides were analyzed for confident spot identification.

qRT-PCR: Specific primers were designed according to the corresponding gene sequences of ten identified proteins using Beacon Designer Software 7.5 (Primer Biosoft International). All the information on the primers

Table 1: Primers for ten genes used in qRT-PCR reactions

Genes	Primer name	Nucleotide sequence	Protein
rsp	Rsp-F	5'-TTGACAATCATGTGACGAAGAATG-3'	30S ribosomal protein
	Rsp-R	5'-GACTCTATCTTGACTGACAACGG-3'	
pft	Pft-F	5'-GAGCACCCATACGAGAAGCG-3'	6-phosphofructokinase
	Pft-R	5'-AGCAGCAGGCGATACAAGTG-3'	
cms	Cms-F	5'-GGTTGACAATGGATACTTCTGACTG-3'	Chorismate synthase
	Cms-R	5'-ACTATGCGTGTGGCTGTTGG-3'	
esp	Esp-F	5'-TCTTTAATCTTCCACGGTCCCATAC-3'	Extracellular solute-binding protein family 5
	Esp-R	5'-CCAGCCTATATCCAACCAGAACAC-3'	
hpk	Hpk-F	5'-ACCACTCCTTCATCCCAATCAAC-3'	HPr kinase/phosphorylase
	Hpk-R	5'-CAAATTACGACGGCAGATATTACCC-3'	
pk	Pk-F	5'-GTTTAGCAATTACACCGTCGTTTTC-3'	Pyruvate kinase
	Pk-R	5'-GATGTTGAAGTTGGTAAGCAAATCC-3'	
rpp	Rpp-F	5'-ACGGTCTCTTTGCTGGAACTG-3'	Ribose-phosphate pyrophosphokinase
	Rpp-R	5'-TTCTTGGATGCGGTGGATGG-3'	
rrf	Rrf-F	5'-TGGCATCACGACGGATATTACG-3'	Ribosome recycling factor
	Rrf-R	5'-CGCAATCTGACGGTACTGTTATC-3'	
tef	Tef-F	5'-TCGTCGCCACCTTCAACAAC-3'	Translation elongation factor Ts
	Tef-R	5'-AAATCTCATTCCGTCGCTTTGC-3'	
tis	Tis-F	5'-CTGCTACTTTGAAGATGCTGGTG-3'	Triosephosphate isomerase
	Tis-R	5'-GACGCTCTGAGTGACCGATAAC-3'	

is listed in Table 1. Gene expression was tested by subjecting the RNA of bacteria grown under standard laboratory conditions to qRT-PCR. RNA was extracted from cultured *S. suis* pellets of 1×10^7 cells using the One Step Trizol reagent (HaiGene) according to the manufacturer's guidelines and the contaminating genomic DNA was eliminated by DNase treatment. The extracted RNA was then diluted to 1 μg μL^{-1} which was quantified by spectrophotometry. Reverse transcription was performed in 0.2 mL PCR tubes in a total volume of 20 μL with 2 μg of total RNA, 5 μM of random primers and 4 μL 5×RT Mix (HaiGene) which contained dNTPs, ribonuclease inhibitor, MMLV reverse transcriptase and the first-strand reaction buffer. This mix was incubated at 30°C for 5 min then 42°C for 30 min and 95°C for another 5 min to inactivate the biomolecules.

The absolute qRT-PCR runs were performed in 96 well plates in triplicate with each containing 1× SYBR Green PCR mix (TaKaRa), 2 μL of cDNA or linear DNA standards as template, 0.2 nM of the specific forward and reverse primers for each gene. qRT-PCR was performed in a Bio-Rad Mini-opticon 2 instrument (Bio-Rad) with conditions as follows: 2 min at 95°C, 40 cycles of 5 sec at 95°C and 30 sec at 60°C followed by a dissolving curve analysis. Standard curves were generated for each gene by serial dilution of PMD-18T cloning vectors containing pft, rsp, cms, esp, hpk, pk, rpp, rrf, tef, tis and GAPDH cDNAs to quantify the amplified products. The CT under the default settings of the qRT-PCR detection software was used to calculate the precise copy numbers of the specific cDNA molecules according to the DNA standards.

Statistical analysis: Rates of ten genes expression in strain 07WC11 and strain SJZB407 were analyzed by a χ^2-test. A t-test was used to compare the results of qRT-PCR. The experiments were carried out in at least three replicates. The results quantifying copy numbers of ten genes mRNA are expressed as the mean of the ratio of ten genes to GAPDH. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Pathogenicity test of SS7: The result of virulence of two SS7 strains was showed in Table 2. The LD_{50} value of strain 07WC11 was 1.25×10^5 CFU/fish. The LD_{50} value for another group of zebrafish injected with strain SJZB408 was 7.90×10^7 CFU/fish. Control fish injected with PBS suffered no mortality.

Protein identification: The profiles of the secreted proteins of the two SS7 strains were resolved by 2D. Protein spots observed at similar locations in both gels were marked with the same numbers (Fig. 1, labeled with white arrowheads). Twenty three protein spots in the two gels that were unique to 07WC11 or SJZB408 were marked (Fig. 1, labeled by black arrowheads; the gels were stained with CBB. The different identified protein spots in the 2 gels are marked with black arrowheads and numbers and were subjected to mass spectrometric analyses. Similar protein spots in the 2 gels are marked with white arrowheads and numbers) and analyzed by MALDI-TOF-TOF MS and MS/MS. PMF and MS/MS spectral data were searched against the NCBInr database using MASCOT. As shown in Table 3, among 23 protein spots, only 18 protein spots were successfully identified. The probability nominal mass (MW), score for the match, number of peptide matches, the percentage of the total translated open reading frame sequences covered by the peptides and calculated pI value were all used as confidence factors in protein identifications. Because two proteins were identified in multiple spots at different positions on the 2D gels, 13 individual proteins were successfully identified from 18 protein spots. Most of the

identified proteins are key enzymes involved in glycolysis/glycometabolism, protein synthesis and gene regulation.

Comparative analysis of ten secreted proteins gene expression: About 10 secreted proteins genes (*pft*, *rsp*, *cms*, *esp*, *hpk*, *pk*, *rpp*, *rrf*, *tef*, *tis*) were selected for analysis by using a qRT-PCR assay. Amplification curve and dissolution curve consistent with quantitation request. In 0.5 μg total RNA, the *tis*, *pft*, *rsp*, *cms*, *esp*, *pk*, *rpp*, *rrf*, *tef*, *hpk* gene expression quantitation were 3.28×10^6 copies, 5.56×10^4 copies, 2.88×10^7 copies, 1.32×10^5 copies, 0.13×10^4 copies, 5.73×10^4 copies, 1.53×10^6 copies, 7.4×10^6 copies, 1.44×10^6 copies, 4.98×10^4 copies in strain SJZB408, respectively and 2.20×10^6 copies, 10.44×10^4 copies, 3.93×10^7 copies, 13.83×10^5 copies, 12.76×10^5 copies, 8.82×10^4 copies, 3.37×10^6 copies, 12.9×10^6 copies, 2.04×10^6 copies, 4.58×10^4 copies in strain 07WC11, respectively. As shown in Fig. 2, the amount of *esp*, *cms* and *rpp* gene expression in avirulent strain SJZB408 were significantly lower than in virulent strain 07WC11 ($p<0.01$). The amount of *pft*, *rrf*, *pk* and *tis* gene expression from strain SJZB408 were less than that from strain 07WC11 ($p< 0.05$). The mRNA level of HPK, TEF and RSP were not obviously different between strain SJZB408 and strain 07WC11 ($p>0.05$).

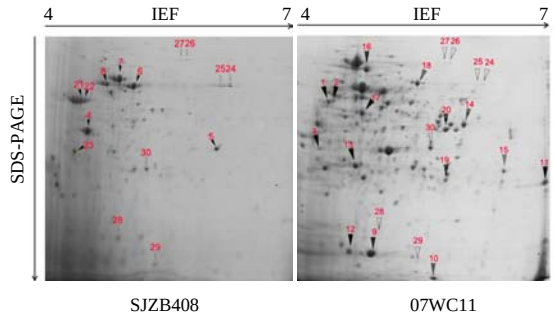

Fig. 1: 2D maps of the secreted proteins of strains SJZB408 and 07WC11

Table 2: LD_{50} of the two strains of SS7

Strains	Infectious dose (CFU)	Total death rate	LD_{50} (CFU)
07WC11	5×10^3 to 5×10^7	23/50	1.25×10^5
SJZB408	5×10^3 to 5×10^7	3/50	7.90×10^7
Control		0/10	∞

Researchers established a zebrafish SS7 infection model where researchers detected the virulence of SS7. When researchers compared the virulence of the 07WC11 and SJZB408 strains we determined that 07WC11 was more virulent in the Zebrafish Infection Model with LD_{50} of 1.25×10^5 CFU mL^{-1} compared to SJZB408-infected zebrafish that presented with clinical symptoms of branchia and hypogastric region bleeding.

In this research, gene primers were designed and synthesized based on the ten genes sequence used in the Taqman RT-qPCR assay described. Data showed the existence of ten genes mRNA, not only in 07WC11 but also SJZB408. Also, researchers showed new differences in mRNA expression quantities of ten genes between 07WC11 and SJZB408. The result of 8 genes less expressed at the mRNA level in SJZB407 compared to 07WC11 is coincidence with the result for 2D. Thus,

Table 3: Protein spots identified successfully by MALDI-TOF-TOF MS and MS/MS

Spot number	Identified protein: Mascot results[a]	Annotation/Species	Nominal mass (MW) (Da)/pI[a]	Experimental MW (Da)/pI[b]	Mascot scores[c]	No. of peptides matched[d]	Coverage (%)[e]
2	gi|223933844	Ribosome recycling factor/*S. suis* 89/1591	20637/6.78	43000/4.52	302	21	2
3	gi|223932329	Ribose-phosphate pyrophosphokinase/*S. suis* 89/1591	35512/5.16	27000/4.35	220	15	71
6	gi|22393408	Sulfatase/*S. suis* 89/1591	93134/8.98	52000/5.15	347	29	55
7	gi|146318980	Ribosome recycling factor/*S. suis* 05ZYH33	20651/ 6.78	55000/4.91	89	10	56
9	gi|146318916	Chorismate synthase/*S. suis* 05ZYH33	42471/5.88	12000/4.95	324	19	72
10	gi|146321680	30S ribosomal protein S6/*S. suis* 98HAH33	11173/5.39	11000/5.51	359	12	83
11	gi|223933844	Ribosome recycling factor/*S. suis* 89/1591	20637/6.78	20000/6.83	525	14	58
12	gi|146319260	HPr kinase/phosphorylase/*S. suis* 05ZYH33	41297/5.98	13000/4.68	262	20	77
13	gi|146318185	Triosephosphate isomerase/*S. suis* 05ZYH33	26622/4.68	26000/4.62	352	14	45
14	gi|225624951	Muramidase-released protein/*S. suis*	97457/4.84	37000/5.80	418	24	24
15	gi|225624911	Muramidase-released protein/*S. suis*	75867/4.93	26000/6.23	493	25	27
16	gi|223933667	Extracellular solute-binding protein family 5/*S. suis* 89/1591	64556/4.82	65000/4.85	180	24	39
17	gi|223933541	Translation elongation factor Ts/*S. suis* 89/1591	37179/4.72	38000/4.73	495	21	77
18	gi|146318198	Pyruvate kinase/*S. suis* 05ZYH33	54596/5.12	55000/5.34	123	15	32
19	gi|225624915	Muramidase-released protein/*S. suis*	121612/4.95	19000/5.71	383	22	22
20	gi|223932492	6-phosphofructokinase/*S. suis* 89/1591	35437/5.41	34000/5.65	267	22	72
22	gi|225624951	Muramidase-released protein/*S. suis*	97457/4.84	44000/4.62	89	17	30
23	gi|223932376	Type II site-specific deoxyribonuclease/*S. suis* 89/1591	35956/5.32	28000/4.45	195	17	71

[a]gi number in NCBI. [b]Observed MW and pI of protein spot in the 2D gel. [c]Protein score greater than 83 is significant in this study ($p<0.05$). [d]Number of peptides that match the predicted protein sequence. [e]Percentage of predicted protein sequence covered by matched peptides

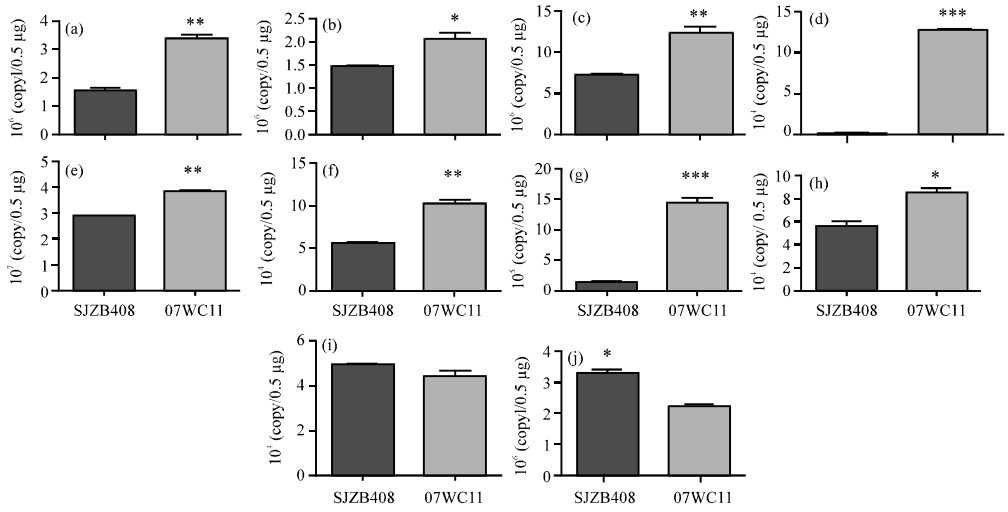

Fig. 2: RT-qPCR analysis of ten secreted genes mRNA in strain 07WC11 derived from strain SJZB408; a) *rpp*; b) *tef*; c) *rrf*; d) *esp*; e) *rsp*; f) *pft*; g) *cms*; h) *pk*; i) *hpk*; j) *tis* (*p<0.05; **p<0.01; ***p<0.0001)

researchers defined these eight proteins as different expression proteins between low pathogenic and high pathogenic SS7 strain.

Among of them, the mRNA expression amount of ESP and CMS were significantly different (p<0.0001). In earlier reports, ESP has a chaperone activity and has been reported as a putative virulence-associated factor in other microorganisms. The expression of molecular chaperones is induced during the replication of several viruses and they facilitate the correct folding and assembly of viral proteins. Recently, chaperone proteins were found to transiently associate with nascent viral proteins (Cho *et al*., 2002). In the present result, *esp* gene sequence of SS7 was cloned from the genome of strain 07WC11 by Homologization Clone Method. The ESP protein is composed of 508 Amino Acid which contain three transmembrane domains in 3' terminal through TMpred prediction. Thus, it indicated that ESP is a transmembrane protein and its molecular function is enables the directed movement of substances such as toxins into, out of or within a cell or between cells. CMS is a key enzyme of the shikimic acid pathway (Song *et al*., 2012) and essential for the synthesis of aromatic amino acids in *Mycobacterium tuberculosis* (Fernandes *et al*., 2007).

CONCLUSION

The clone result showed that the ORF of *cms* gene is composed of 1167 nucleotide in SS7. There are transmembrane domains in 3' terminal by TMpred Software prediction. Therefore, CMS is infered to also be a transmembrane protein. If the different amount of these eight proteins gene expression affect the pathogenicity for SS7 will be verified using gene knockout technology in the next study.

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