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Original Article

Proteomics analysis of important molecules in serum from meningitic piglets caused by *Streptococcus suis* serotype 2

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Abstract

Introduction*: Streptococcus suis serotype 2* (SS2) is an important zoonotic pathogen that causes meningitis in China. This study's aim was comparative analysis of serum proteomics from meningitis and non-meningitis piglets.

Methodology: SS2 meningitis and non-meningitis piglet models were established. The serum samples were collected and analyzed by label-free LC-MS/MS proteomics technology. Differentially expressed proteins (DEPs) from serum were screened out by comparing the meningitis group and non-meningitis group to the healthy group (M/C; N/C), respectively. And then, globally and comparative analysis of DEPs in "M/C" and "N/C" in serum were performed using bioinformatics method. Finally, we comparatively analyzed the serum and cerebrospinal fluid proteomics in piglets that lived with meningitis.

Results: We obtained 316 and 191 DEPs from "M/C" and "N/C" which classification visualizations were established. 157 DEPs were common in both groups and 159 DEPs were unique to the "M/C". These DEPs and the signaling pathways which they participated in were visualized. Moreover, some DEPs which participated in multiple pathways were discovered and the interaction between 159 DEPs was also mapped. 39 common DEPs were also screened out in serum and cerebrospinal fluid during meningitis, and signaling pathways associated with these DEPs were further visualized.

Conclusions: DEPs in "M/C" and "N/C" were comparatively analyzed and the similarities and differences of these DEPS which were involved in signal pathways were summarized. Moreover, several important molecules were screened out.

Key words: *Streptococcus suis*; meningitis; non-meningitis; serum; proteomics.

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Introduction

Streptococcus suis serotype *2* (SS2) is a major pathogen, which can cause several symptoms such as arthritis, pneumonia, endocarditis, and sepsis in swine and humans [1]. Although many other clinical manifestations have been reported, meningitis and septic shock are the most common symptoms in humans [2]. SS2 meningitis is a severe and frequent evolution of SS2 infection [1]. Meningitis can be fatal if leaving untreated or delayed treatment. Besides, patients with surviving streptococcus meningitis are often accompanied by irreversible sequelae such as hearing loss [2]. Since the pathogen was discovered, approximately 1,600 cases of *Streptococcus suis* (SS) infection have been declared in the world [3]. In the past few years, infection in humans has attracted a high level of attention, with deadly outbreaks in Asian countries [4], where meningitis is one of the most common causes of death from SS2 infection [2,4]. Therefore, understanding the infection mechanism of meningitis and non-meningitis SS2 and the host's response characteristic is particularly important for the treatment of SS2 infection.

The interaction between bacteria in the blood and blood-brain barrier (BBB) is a precondition for meningitis. Bacteria can invade into BBB with the help of some molecules in the blood. Numerous studies have been reported that some significantly changed molecules in serum could be used as potential biomarkers of some brain diseases and bio-targets of pathogenesis research [5,6]. For example, the SPC25 as a diagnostic biomarker of patients with Alzheimer's disease and/or mild cognitive impairment (MCI) [6]. Significantly increased serum concentrations of

NADPH oxidase 1, ferritin and selenium can be used as potential diagnostic and monitoring biomarkers for Parkinson's disease (PD) patients [7].

Nevertheless, to our knowledge, there have been no previous reports about the serum protein profiling of meningitis piglets. For this purpose, SS2 meningitis and non-meningitis piglet models were established in this study. Serum from different piglet models was collected and analyzed by protein profiling using label-free Liquid chromatography-mass Spectrometry (LFQ LC-MS/MS) proteomics technology. Data on proteomics analysis are useful for understanding the differences in the mechanisms of the host response to meningitis SS2 and non-meningitis SS2. It also helps to screen for therapeutic targets after infection with SS2 infection in meningitis.

Methodology

Serum sample collection and LFQ LC-MS/MS Measurements

Serum samples were collected from infected and uninfected Bama miniature pig models used in our previous study [8]. Bama miniature pigs used for infection experiments were purchased from Shandong Binzhou Animal Science Veterinary Medicine Academy. Eighteen piglets were divided into three groups evenly (n = 6), healthy control group, meningitis group, and non-meningitis group. The sample collection process was described below briefly. JZLQ022 and JZLQ001 strains that used for the establishment of porcine models of SS2 infection in this study were isolated from brain tissue of meningitis pig and lymphonodi mandibular of arthritis pig respectively. The culture method of strains and the method establishment of the infection model refers to our previous research [8].

As described in our previous research [8], heparinized blood was collected from Bama miniature pigs via ear veins and serum was further separated. The serum samples which were collected at 3d were added to appropriate doses of lysis buffer (7 M Urea, 2 M Thiourea, 0.1% CHAPS) and vortex blended followed by incubation for 30 minutes at 25°C. Then, the sample was centrifuged at 15000 g for 20 minutes at 4°C. The supernatant was sub packaged into a 1.5 mL tube and store at -80 °C. The concentration of total protein in the supernatant was determined by the Bradford method (Bradford 1976). The serum of pigs from the two infection groups (meningitis group; non-meningitis group) and one control group was collected and analyzed by LFQ LC-MS/MS proteomics technology using the Thermo Scientific EASY-nLC 1000 System and Thermo Q-Exactive MS system at the QL-bio Biotechnology Corporation (Beijing, China). Each sample was checked in triplicate.

Proteomic Data Analysis

The identified proteins from the meningitis group and the non-meningitis group were compared to proteins from the control group and defined as the "M/C" group and "N/C" group, respectively. Differentially expressed proteins (DEPs) from serum could be further screened out from the "M/C" group and "N/C" group, respectively. Fold changes in proteins were determined according to LFQ quantitative intensity. Proteins with a difference > 1.5-fold or < 0.66-fold, p values< 0.05 were regarded as DEPs [8]. We first used UNIPORT (http://www.uniprot.org/) to convert the obtained protein IDs from serum into their respective gene symbols and further define these genes as differentially expressed genes (DEGs). Gene Ontology (GO) biological classification (BP) was performed using Metascape software [9]. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was further performed using the Database of Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources 6.8 [10]. DEPs were visualized separately using Cytoscape ClueGO [11], to identify several cross-talk (associated with two or more pathways) genes that can serve as a "bridge" between different pathways. Finally, the interaction of these DEPs was further analyzed through the STRING database (https://string-db.org/) and was visualized by Cytoscape [12]. Molecular Complex in large protein interaction networks was further analyzed using Cytoscape MCODE [13].

To further understand the pathogenesis of meningitis after SS2 infection, we compared the data used in this study with our previous cerebrospinal fluid data [8]. Screening for common DEPs only in cerebrospinal fluid and serum during meningitis infection, and analyzing the biological processes involved in these proteins using Metascape software [9]. These proteins may have an important role in meningitis.

Validation with Enzyme-linked Immunosorbent Assay (ELISA) Analyses

Ten DEPs in serum from two infected groups (n = 6), including APOD, FGA, H2B, HRG, HSP90AB1, MANF, MSN, PARK7, PGAM1, and PPP1CA, were detected by double-antibody sandwich ELISA kits (Jin Ma Biological Technology co., LTD, Shanghai, China).

Table 1. 10 proteins with significant differences in meningitis and non-meningitis.

group	DEPs
9 elements in "M/C (UP)" and "N/C (Down)" in serum	ARF4, LOC100523213, PGAM1, PPBP, TRX1, m6p/igf2r, ANGI, CXCL7, CNN2
1 element in "M/C (Down)" and "N/C (UP)" in serum	C4BPA

Statistical analyses

Metascape uses the well-adopted hypergeometric test and Benjamini-Hochberg p-value correction algorithm to identify all ontology terms. Enriched terms are computed based on a Kappa-test score [9]. KEGG terms for individual comparisons were performed by the Benjamin method using DAVID [10]. KEGG enrichment analysis is based on a Two-side hypergeometric test and Bonferroni p-value correction using Cytoscape ClueGO [11]. Statistical analysis for ELISA results was subjected to one-way analysis of variance (ANOVA) and means were compared by an LSD test using an IBM SPSS Statistics 19.0 software (SPSS Inc., USA). A significant difference was considered when P values < 0.05.

Results

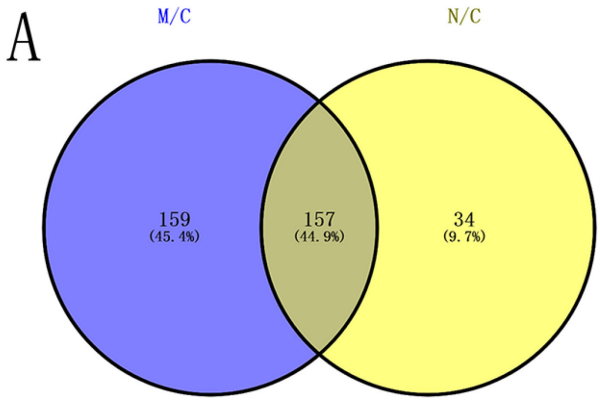
Screening for DEPs by Comprehensive Analysis of Proteomic Data from serum

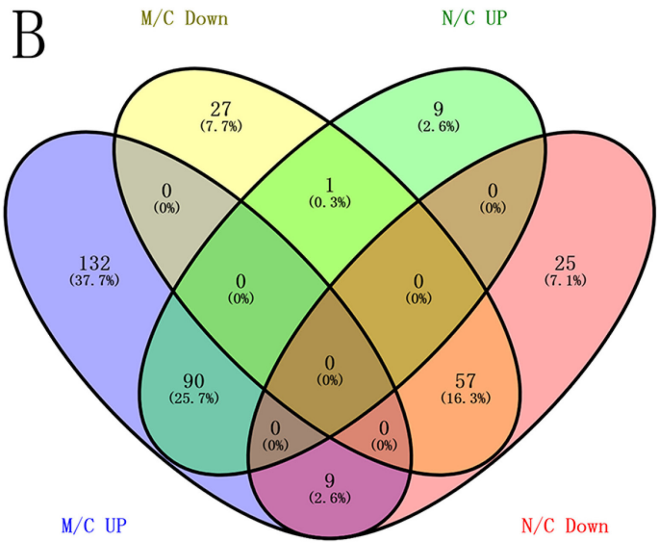
As a result, 316 DEPs and 191 DEPs were identified in “M/C” and “N/C”. Among them, 159 elements included exclusively in "M/C" and 157 common elements in "M/C" and "N/C"(Figure 1A). The up-regulated DEPs and down-regulated DEPs of each group were classified, we found 9 common elements in "M/C_UP" and "N/C_DOWN" and 1 common element in "M/C_DOWN_" and "N/C_UP" (Figure 1B, Table 1). These DEPs may be as a marker for identifying meningitis infections or non-meningitis infections.

Globally analysis of DEPs in “M/C” and “N/C” in serum

GO classification was performed using Metascape software, and we found DEPs from the “M/C” group and “N/C” group all significantly enriched in biological pathways associated with complement and coagulation cascades(hsa04610), platelet activation, signaling and aggregation (R-HSA-76002), regulated exocytosis (GO: 0045055), and cell-extracellular matrix interactions (R-HSA-446353) (Figure 2A). It was noted that DEPs related to muscle cell migration (GO: 0014812), ECM proteoglycans (R-HSA-3000178), and regulation of blood vessel remodeling (GO: 0060312) were significantly down-regulated in both “M/C” group and “N/C” group (Figure 2A). Then we further compared two groups (“M/C” and “N/C”) of GO clusters and found differences in meningitis and non-meningitis. DEPs associated with long-chain fatty acid transport (GO: 0015909), maintenance of location (GO: 0051235), production of molecular mediator involved in the inflammatory response (GO: 0002532), and regulation of apoptotic signaling pathway (GO: 2001233) were more significantly up-regulated when “M/C” group were compared to “N/C” (Figure 2A).

Figure 1. Venn diagram shows DEPs in serum from meningitis infection and non-meningitis infection. A: M/C, DEPs in meningitis infection group compared to the control group. N/C, DEPs in non-meningitis infection group compared to the control group. B: The up-regulated or down-regulated DEPs in each group were displayed by the Venn diagram. (The number in the Venn diagram shows the number of DEPs in each group, and the intersection represents the DEPs shared by each group).





However, DEPs only in the "N/C" group were significantly enriched in the regulation of plasma lipoprotein oxidation (GO: 0034444), and most of these proteins were significantly down-regulated (Figure 2A). KEGG enrichment analysis of DEPs using DAVID, the results of KEGG analysis were consistent with the results of GO analysis. We found that the DEPs from the "M/C" and "N/C" group were also significantly enriched in complement and coagulation cascades, focal adhesion, platelet activation, etc. (Figure 2B). DEPs were more abundant in the ECM-receptor interaction pathway in the "M/C" group (Figure 2B), and related proteins were significantly down-regulated. However, DEPs in the "N/C" group were more enriched in the proteasome pathway than in the "M/C" group, and related proteins were also significantly down-regulated compared to uninfected.

Figure 2. GO terms and KEGG term classification visualizations of DEPs in serum from meningitis and non-meningitis models. (A) Heatmap showing the top 100 enrichment clusters based on Metascape, one row per cluster, using a discrete color scale to represent statistical significance. Gray color indicates a lack of significance; (B) KEGG enrichment analysis based on DAVID. (The X-axis indicates the ratio of the DEPs enriched in this pathway to the total DEGs. The Y-axis indicates the KEGG term. The count and negLog10_qValue indicate the number and degree of enrichment of genes in a category, respectively. *negLog10_qValue > 1.3 were considered significantly enriched by the DEPs).

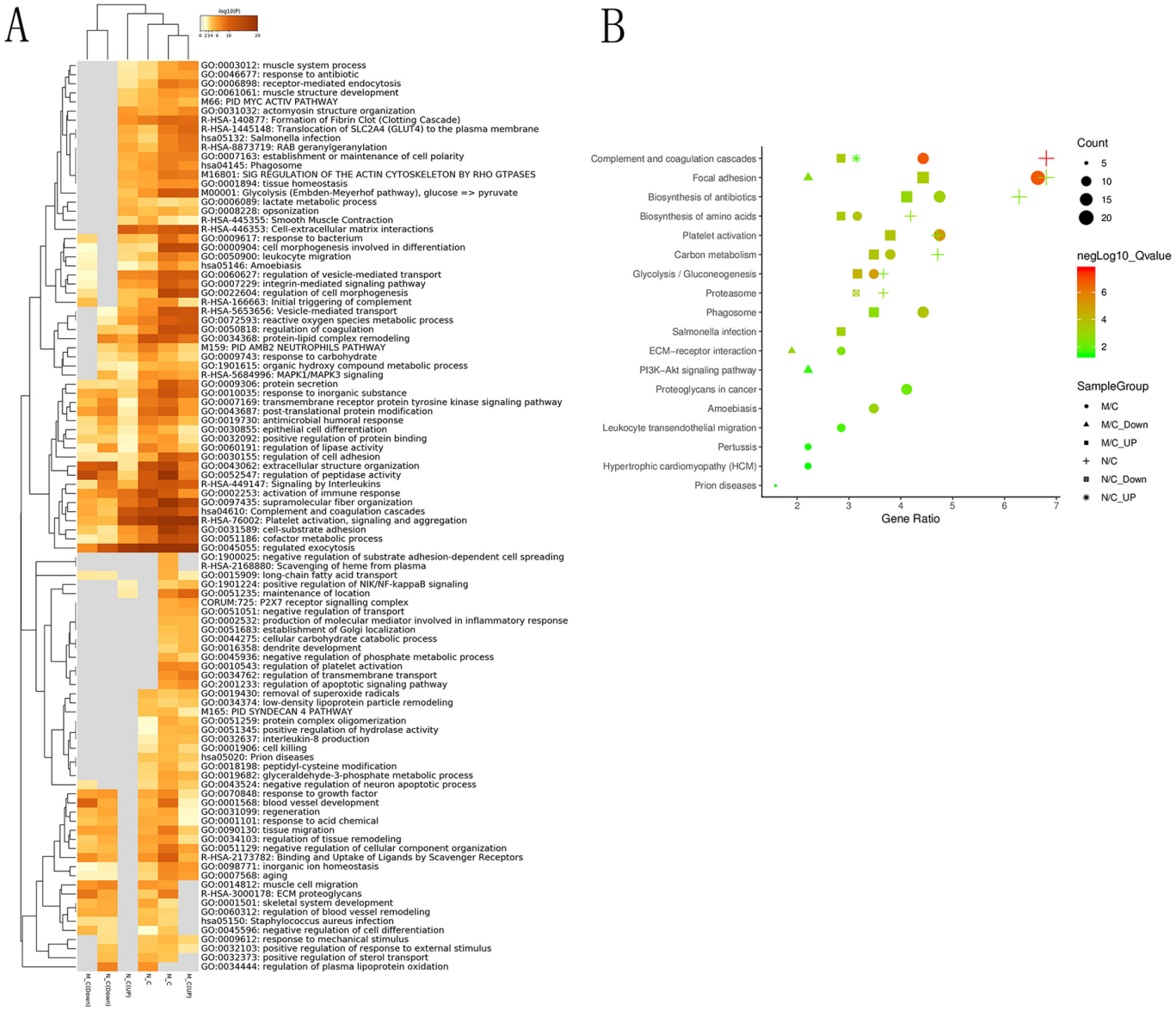
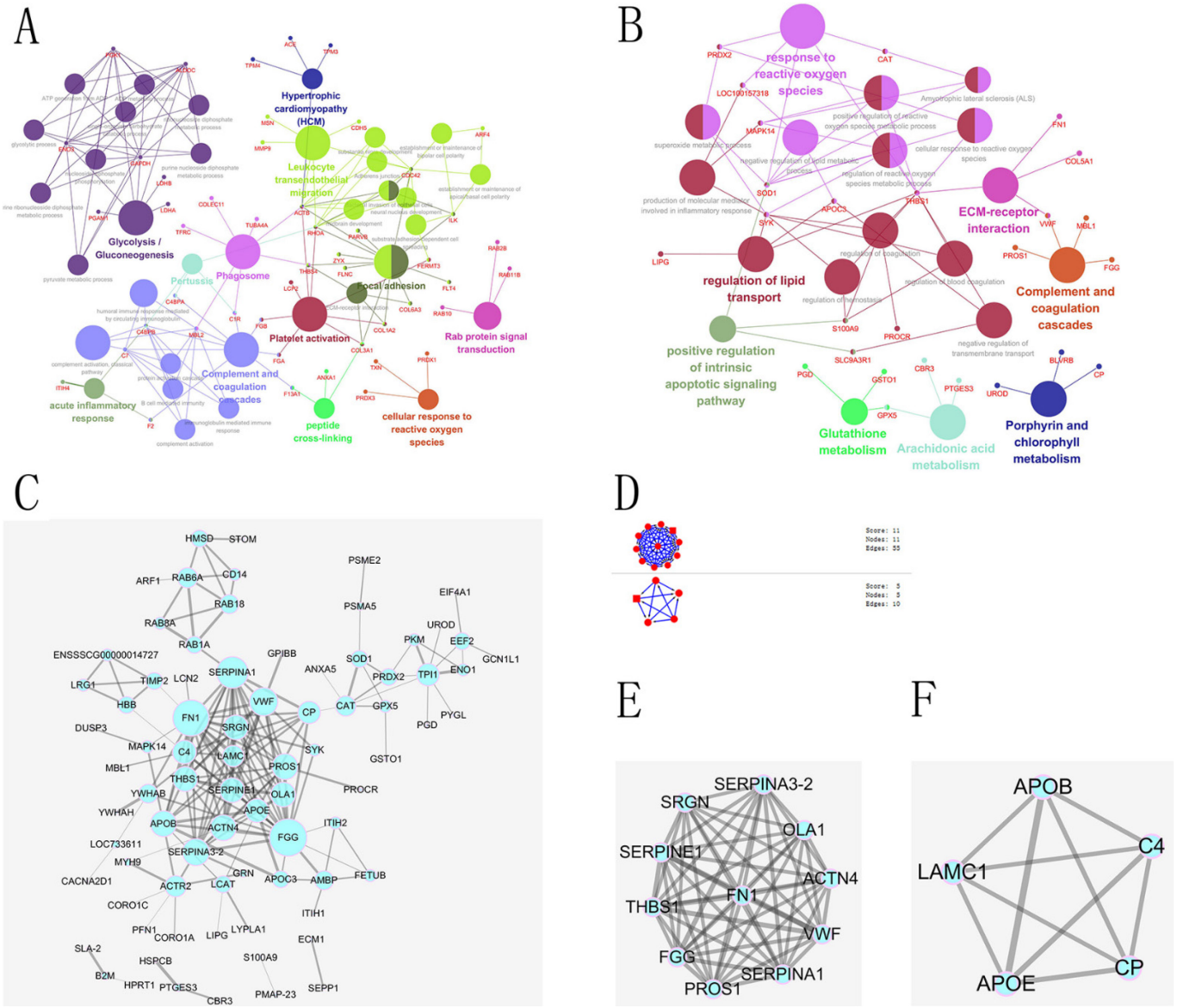


Table 2. 39 common DEPs in serum and cerebrospinal fluid during meningitis.

group	DEPs
4 common elements in "M/C Down in serum" and "M/C UP in CSF"	C4A; ENPP2; FBLN1; LCAT
5 common elements in "M/C UP in serum" and "M/C UP in CSF"	APOD; CAT; CP; LOC396684; PRDX2
30 common elements in "M/C UP in serum" and "M/C Down in CSF"	ACTN4; ACTR2; ALDOA; ARHGDIA; CACNA2D1; CNRIP1; CORO1C; DUSP3; EEF2; ENO1; HPRT1; HSP90AB1; KCTD12; LIMS1; MANF; NCAM1; PEBP1; PKM; PPP1CA; PSMA5; PYGL; RAB18; RAB1A; RAB6A; RIDA; SPR; TPI1; WDR1; YWHAB; YWHAH;

Figure 3. (A), (B): KEGG terms and their DEPs visualization in serum from meningitis and non-meningitis models. (A) Cytoscape ClueGO analysis identified the link between the signaling pathway and its related cross-talk DEPs which from 157 common elements in "M/C" and "N/C". (B) Cytoscape ClueGO analysis identified the link between the signaling pathway and its related cross-talk DEPs which from 159 elements included exclusively in "M/C". Each cross-node represents a cross-talk protein (Different circles indicate signal pathways where DEPs are enriched and proteins on branches indicate DEPs involved in the signaling pathway). (C) Interaction network of DEPs which from 159 elements included exclusively in "M/C" based on Cytoscape. (The larger the area of the circle, the more protein it interacts with. The thicker the connection between the two proteins, the greater their combine score.) (D) Molecular complex regions were screened from the protein interaction network map by Molecular Complex Detection (MCODE) method based on Cytoscape MCODE. (E) (F) Protein interactions involved in each molecular complex.



Screening for DEPs by Comparative analysis of DEPs in "M/C" and "N/C" in serum

As result one, 157 common elements in "M/C" and "N/C" were obtained. We then visualized these proteins and the signaling pathways in which they were involved by Cytoscape software. These proteins were involved in glycolysis/gluconeogenesis, complement and coagulation cascades, platelet activation, focal adhesion, etc. (Figure 3A). At the same time, 159 DEPs included exclusively in "M/C" were visualized by using the same method (Figure 3B). These proteins were also involved in not only these signaling pathways such as the complement and coagulation cascades, ECM receptor interactions and response to reactive oxygen species but also affected the positive regulation of intrinsic apoptotic signaling pathway (Figure 3B). DEPs involved in multiple pathways were discovered, such as THBS1, SOD, S100A9, which linked with different signaling pathways during infection (Figure 3B). For example, S100A9 participated not only in positive regulation of intrinsic apoptotic signaling pathway but also in the regulation of lipid transport (Figure 3B). To find out the relationship between 159 DEPs which included exclusively in "M/C", the interaction network was mapped (Figure 3C). Two molecular complex regions were mainly screened out through Molecular Complex Detection (MCODE) (Figure 3D). Several important DEPs were screened out, such as THBS1, FN1, LAMC1, etc. (Figure 3E).

Screening DEPs between serum and cerebrospinal fluid proteomics in piglets with meningitis by Comparative analysis

We screened out 39 common DEPs in serum and cerebrospinal fluid during meningitis (Figure 4A Table 2). These DEPs were significantly enriched in regulated endocytosis (GO: 0045055), cell morphogenesis involved in differentiation (GO: 0000904), Glycolysis (Embden-Meyerhof pathway), glucose → pyruvate (M00001), etc. (Figure 4B, Table S1).

Verification of a Subset of Differential Proteins by ELISA

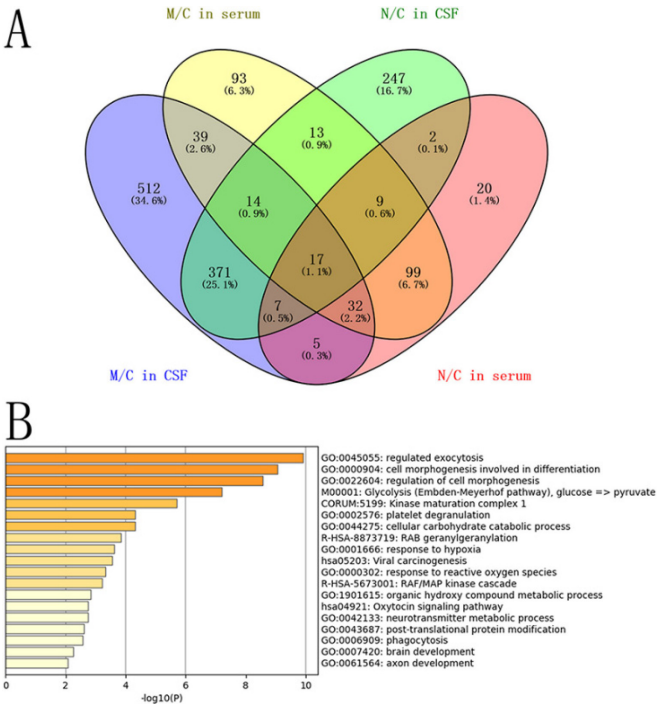
According to the previous reports [8], some DEPs were selected to be identified by ELISA (n = 6). The expression levels of the ten proteins including APOD, FGA, H2B, HRG, HSP90AB1, MANF, MSN, PARK7, PGAM1, and PPP1CA in the serum of piglets with meningitis infection were significantly higher than those in non-meningitis piglets. (Figure S1).

Discussion

In this study, DEPs profiling of serum in meningitis and non-meningitis piglets infected by SS2 strains was analyzed by LFQ LC-MS/MS proteomics technology.

In our data, we characterized several important proteins, such as HSP90AB1, PPP1CA, THBS1, FN1. These proteins were significantly changed in the "M/C" group rather than in the "N/C" group. Similarly, transcriptomics research of SS2-infected brain tissue also revealed that these DEPs showed the same change trend compared to the normal group [14]. For example, the transcription level of HSP90AB1, PPP1CA, and MSN in brain tissue were all considerably increased [14]. Ernst JT's studies have also shown that HSP90AB1 inhibitors were well effective in the treatment of CNS disorders such as Huntington's disease [15]. Our data and Liu's data both showed that the expression of S100A9 in the meningitis group was greater than that of the control group [14]. Gene expression profiling was utilized to study meningitis caused by *Streptococcus pneumoniae*, which showed that S100A9 protein was also up-regulated in peripheral blood [16]. Moreover, relevant reports revealed that

Figure 4. 39 common DEPs in serum and cerebrospinal fluid were screened and analyzed during meningitis (A) Venn diagram shows differentially significant proteins (DEPs) in serum and Cerebrospinal fluid from a meningitis infection. (B) Heatmap showing the top enrichment clusters based on Metascape, one row per cluster, using a discrete color scale to represent statistical significance. Gray color indicates a lack of significance.



S100A9 was significantly increased in the serum of multiple sclerosis (MS) patients. S100A9 could promote the microglial secretion of inflammatory factors and induced apoptosis of OPCs [17]. S100A9 is involved in the apoptotic pathway, which may be the potential mechanism that SS2 disrupts BBB and induces meningitis in our study. A large number of studies have reported that S100 protein levels in serum can be used as an early indicator for diagnosis and prognosis of brain injury [18,19]. SS2 infection causes to elevate expression of S1009 protein in the serum of meningitic piglets, which may be employed as a marker to identify meningitis. Also, studies have reported that THSB1 and its receptor CD47 play an important role in meningitis caused by E. coli K1. It can effectively protect animals from meningitis when its expression has been disturbed, and it may be a novel target for designing preventive approaches for E. coli K1 meningitis [20].

Cell wall (CW) and extracellular (EC) proteins from SS2 are often involved in interactions with the extracellular matrix (ECM) proteins such as fibronectin (FN), which play important roles in adhesion and invasion [21-23]. Similarly, DEPs were significantly enriched in ECM receptor interaction signaling pathways in our study. FN1, as a kind of FN, also participates in the ECM interaction process in our research. Moreover, FN1 has been proposed as a potential therapeutic target in late-delayed radiation-induced brain injury (RIBI) [24]. The signaling pathway of platelet activation is a key event in the pathogenesis of streptococcal infection [25]. CDC42 and RhoA participate in multiple signaling pathways in our research, such as platelet activation, bacterial invasion of epithelial cells, and focal adhesion. Some studies have reported that Rho and CDC42 are involved in disrupting the BBB [26]. Our previous data also showed that RhoA differentially expressed in cerebrospinal fluid from piglets infected with SS2 [8]. Therefore, the signal pathway and the proteins involved in this pathway, such as CDC42, PPP1CA, ACTG1, may also play an important role in meningitis SS2 infection.

Studies have shown that the complement system is important for host resistance to SS infection [27] and SS has developed a mechanism to escape the host complement response to achieve immune escape [28]. Transcriptome profiling of zebrafish infected with SS showed that complement activation-related genes were significantly up-regulated during SS infection [29]. Our data also suggested that DEPs were significantly enriched in complement responses at “M/C” group and “N/C” group. These proteins were involved in the complement and coagulation cascade, such as C4BPA, C4A, which may have a very important role. Studies had demonstrated that the deposition of complement proteins from adult serum prevented the invasion of *E. coli K1* into human brain microvascular endothelial cells, whereas the invasion of *Group B Streptococcus* was enhanced, and further demonstrated that the inhibitory effect of complement proteins is the result of the complement inhibitors C4b-binding protein, in the case of *E. coli K1* [30]. After SS2 infection, the expression trend of C4BPA in the serum of meningitis and non-meningitis is significantly different from that of the control group. It is down-regulated in meningitis and up-regulated in non-meningitis, which may serve as a potential target for studying the pathogenesis of meningitis.

SS2 is vulnerable to a hostile environment with various stress factors, including reactive oxygen species (ROS) generated by host phagocytes. Zhu et al.'s study have found that the virulence factor redox-sensing regulator Rex of SS2 plays a major role in oxidative stress tolerance [31]. In our study, the reactive oxygen species metabolic process (GO: 0072593) was more significant in the “M/C” group than in the “N/C” group. However, the number of colonies of meningitis strain JZLQ022 in blood was greater than that of non-meningitis strain JZLQ001 in our previous study [8]. So, we speculated that the ability to resist ROS varies among different strains. Rex can also significantly regulate the expression of adhE that has been proved to interact with mammalian heat shock protein 60 (Hsp60), which is an essential chaperone to assist in proper protein folding and configuration [31,32]. Therefore, SS2 may affect the proper folding of host functional proteins. In our data, DEPs related to the proteasome, which an important way to degrade misfolded proteins was more significantly down-regulated in the “N/C” group than that in the “M/C” group. There may be differences in the host's ability to respond to misfolded proteins after infection with meningitis strain and non-meningitis strain.

Conclusion

In conclusion, the present work is the first study, to analyze the differential protein profiling in the serum of SS2 meningitis piglets compared to non-meningitic piglets. The data provide a theoretical basis for the diagnosis and pathogenesis of SS2 infection and meningitis. Further studies need to clarify the role of these DEPs in the process of SS2 meningitis, though

some of these proteins have been reported to be associated with neurological diseases.

Author contributions

Liancheng Lei and Hongtao Liu designed the experiments and provide research funding. Hexiang Jiang conducts data analysis and writing articles. Junjie Wang, Qiang Sun, Jianan Liu, Tong Wu, Mengmeng Liu, and Rining Zhu are responsible for model building and organizing data. Guanggang Qu and Shuguang Li provide experimental animals and feeding facilities and places.

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Ethical approval

All animal experimental procedures were performed in strict accordance with Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China (1988.11.1) [8] and meet the Laboratory animal—Guideline for ethical review of animal welfare by National Standardization Administration Committee of China (Standard Number: GB/T 35892). The protocol was reviewed and approved by the Institutional Animal Care and the Committee of Jilin University (Changchun, China).

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Annex – Supplementary Items

Supplementary Table 1. The signaling pathways involved in 39 common DEPs in serum and cerebrospinal fluid.

Signaling pathways	DEPs
regulated exocytosis (GO:0045055)	ACTN4, ALDOA, CAT, EEF2, HSP90AB1, PKM, PSMA5, PYGL, RAB6A, MANF, WDR1, ACTR2, RAB18
Cell morphogenesis involved in differentiation (GO:0000904)	ACTN4, ARHGDIA, FBLN1, HPRT1, HSP90AB1, LIMS1, NCAM1, RAB1A, YWHAH, WDR1, ACTR2, CORO1C, YWHAB, ENPP2, PEBP1
regulation of cell morphogenesis (GO:0022604)	ACTN4, ALDOA, ARHGDIA, FBLN1, LIMS1, ENPP2, YWHAH, WDR1, ACTR2, CORO1C, APOD, DUSP3, PRDX2, RAB1A, PEBP1, YWHAB, HSP90AB1
Glycolysis (Embden-Meyerhof pathway), glucose → pyruvate (KEGG:M00001)	ALDOA, ENO1, PKM, TPI1, PPP1CA, PYGL, APOD, CAT, HPRT1, PSMA5, YWHAB, YWHAH, RIDA, ENPP2, CP, LIMS1, KCTD12, SPR, PRDX2, LCAT
Kinase maturation complex 1 (CORUM:5199)	HSP90AB1, YWHAB, YWHAH, CAT, EEF2, PRDX2, ENO1, PPP1CA, DUSP3, PSMA5, RAB1A, APOD
platelet degranulation (GO:0002576)	ACTN4, ALDOA, MANF, WDR1, ARHGDIA, CACNA2D1, ACTR2, CORO1C, HSP90AB1
cellular carbohydrate catabolic process (GO:0044275)	PPP1CA, PYGL, TPI1
RAB geranylgeranylation (R-HSA-8873719)	RAB1A, RAB6A, RAB18, YWHAB, YWHAH, ACTR2, ACTN4, ARHGDIA
response to hypoxia (GO:0001666)	ACTN4, CAT, ENO1, PKM, PSMA5, LIMS1, WDR1, HSP90AB1, NCAM1
Viral carcinogenesis (hsa05203)	ACTN4, PKM, YWHAB, YWHAH, CACNA2D1, WDR1

Supplementary Figure 1. Detection results of differential proteins in serum of meningitis compared with non-meningitis by ELISA. (**p < 0.01).