Pathogenicity of a strain of *Streptococcus suis Type 2* to mice and swine isolated from Jingzhou city

ABSTRACT

Aims: The objective of this study is to identify *S. suis type 2* and evaluate the virulence of ZHJ01 strain isolation, and verity the clinical and pathological outcome of a systemic infection caused by one serotype 2 when simultaneously inoculated with ZHJ01 strain. We also want to clarify the epidemiologic, clinical, microbiologic characteristics and the pathogenesis mechanism of *S. suis type 2* in Hubei province, China.
Study design: Pigs suspected of being infected with *S. suis* in Jingzhou regions of Hubei province, China were studied. *Swine S. suis type 2* isolation was obtained from the suspicion of diseased pig. The case of *S. suis type 2* was detected by the virulence factor amplification based on PCR detection and bacterial isolation, identification in the laboratory. According to the experimental infections of mice and piglets, pathogenicity of this *S. suis type 2* isolation to mice and swine was monitored. This study was conducted in the key laboratory of pathogenic microbiology, College of Animal Science of Yangtze University, and Institute of Black Pigs Research, Yangtze University.
Methodology: Proper serological typing can be performed using a co-agglutination test. The typical colonies purificated and cultured were inoculated with Glucose, Lactose, Raffinos, Sorbitol, D (+)-sucrose, Trehalose, 6.5%NaCl, D (-)-Salicin, Hippurate, Esculin hydrate, V-p, etc., then the test results were recorded. Detection of virulence factors were performed using PCR amplification and DNA sequencing. *S.suis type 2* isolation was inoculated to mice and piglets for the virulence test, and the observation of the clinical signs and pathological changes.
Results: The virulence factor of extracellular protein factor (EF) was determined from ZHJ01 strain based on PCR detection. Sequence analysis indicated that the isolate was very similar to nucleotide homology with others SS2 strains from different county or contries, and there was not much variation. LD_{50} of *S.suis type 2* for mice was 2.5 x 10^7cfu. LD_{50} of *S. suis type 2* for piglets was 3.92 x 10^9cfu.
Conclusion: The results show that *Swine S. suis type 2* has a relatively strong pathogenicity to pigs in Hubei province, China. This study can be, in part, sufficient to explain the pathogenicity for ZHJ01 strain in area of Zhijing, Jingzhou city, China, which may provide insights into the pathogenesis SS2 and more valid data to support the development of *S. suis* vaccine as well as the epidemiological investigation, further monitoring and effective prevention to *S. suis*.

Keywords: Streptococcus suis type 2 ; Identification ; Biological trait; Experimental model; Median lethal dose (LD_{50})

1. INTRODUCTION

S. suis disease is a zoonotic infectious disease caused by *S. suis*, which can induce the death in humans, and is described as a pathogen for people in contact with swine or raw pork products [1]. *S. suis*, a Gram-positive encapsulated coccus, is a causative agent of serious zoonotic diseases with clinical manifestations of meningitis, septicemia, arthritis, pneumonia, endocarditis, and even acute death in pigs and humans [2]. In the epidemics that occurred in 2005 in the Chinese province of Sichuan, a marked part of patients infected with *S. suis* experienced a shock-like syndrome with a high mortality, that had been described in previous study devoted to epidemiology of the disease caused by this bacterium [3]. *S. suis* has been found in a total of 35 serotypes, the strongest virulence is type 2, type 1, type 9 and type 7. The genotype of *S. suis* isolated in different regions is not exactly same. Although the distribution of different serotypes varies depending on the geographical origins of the strains, *S. suis serotype 2* (SS2) is the most pathogenic of *S. suis* and the most prevalent capsular type among diseased pigs [4, 5].

At present, genetic analysis of virulence and pathogenicity are challenging because *S. suis* produces multifactorial virulence factors [6], and creating many different genotypes because of natural populations are characterized by high rates of recombination [7]. Several virulence factors or candidates have been described, including capsule, muramidase-release protein and extracellular protein factor, suilysin, and adhesins [8]. According to the existence of MRP and the related proteins, *S. suis type 2* has the following phenotypes: MRP+EF+, MRP-EF-, MRP+EF*(EF analogues), MRP+EF-, MRP+EF-, MRP-EF+, MRP-EF+ etc. [9,10]. *S. suis* is one of the economically most important pathogens in the pig industry, which has caused primarily meningitis, arthritis and septicemia mainly in piglets and weaners [11]. Most studies have been studied with the virulence of *S. suis type 2*. *S. suis type 2* outbreaks in pigs between 4 and 10 weeks of age usually, occurs throughout the year without significant seasonality, and have a high morbidity and mortality rates. *S. suis type 2* infection may cause death in weaning piglets as well as growing pigs [12].

S. suis type 2 has a certain correlation with the clinical diseases of pigs. Pigs can be infected by *S. suis type 2* in all ages, but most of them are epidemic between 3 and 12 weeks old in piglets, especially at the peak of weaning and mixed groups [4]. The mouth and nasal cavity are the main invasive tissues of *S. suis type 2*, which are settled and bred in the tonsils. In cases of arthritis, the earliest changes can be seen from the dilation and congestion of synovial vessels. The surface of the joints might appear fibrin multiple serous inflammation, and the infection of joint cystic wall might thicken, and synovial membrane could form the signs of erythema, and the amount of synovial fluid were increased, meanwhile, contains the inflammatory cells. *S. suis type 2* could cause bronchial pneumonia, meningitis, endocarditis, arthritis, arteritis and abscess in patients with diseased pigs. Diagnosis of *S. suis type 2* infection is based on clinical symptoms and pathological changes to confirm that the infection can to be determined by pathogen separation and tissue examination. At present, the detection methods are mainly isolation culture and biochemical identification, serological identification, molecular level identification, stochastic amplification of polymorphic DNA analysis (RAPD) method [13]. According to the bacterial morphology, *S. suis* can be preliminarily identified, but it is difficult to determine the serotype, and the type of diagnostic serum can be used for PCR detection of latex or slide agglutination experiment [14].

The distribution of *S. suis serotypes* among clinical isolates differs between regions, and may also vary with time in the previous study. Moreover, *S. suis serotype 2* strains are heterogeneous, composed of a multitude of sequence types (STs) whose distribution greatly varies worldwide. That is endemically present and seems to be emerging in pigs [15]. The pathogenic mechanisms of *S. suis* is not completely defined previously. The objective of this study is to identify SS2, and evaluate the virulence of ZHJ01 strain isolate, and to verity the clinical outcome of a systemic infection caused by one serotype 2 when inoculated simultaneously. We also want to clarify its epidemiologic, clinical, microbiologic traits and the pathogenesis mechanism of *S. suis type 2* in Hubei province, China.

2. MATERIAL AND METHODS

2.1 Standard serum

S. suis type 2 standard serum prepared by the key laboratory of pathogenic microbiology of the College of Animal Science of Yangtze University [16].

2.2 Isolation, Biochemical identification and Serotyping of Bacteria

A new *S. suis* isolate was collected from palatine tonsils of a sick pig housed in a pig farm in the infection area of Zhijiang, Jingzhou city, Hubei Province, China. The strain isolate was designated as ZHJ01. The strain isolate was grown on Todd-Hewitt broth supplemented with 1% yeast extract (THY), and incubated at 37℃ without agitation [17]. If required, strains were cultured on THY agar plate (1.5% w/v) containing 6% (v/v) sheep blood and incubated at 37℃ for 48 h.

The biological properties of ZHJ01 strain were identified. The typical colonies purificated and cultured were inoculated with Glucose, Lactose, Raffinos, Sorbitol, D (+)-sucrose, Trehalose, 6.5%NaCl, D (-)-Salicin, Hippurate, Esculin hydrate, V-p, etc., then cultured 24 h at 37 ℃, and the test results were recorded. Gram staining was performed to identified the morphologies of ZHJ01 strain, and the bacterial capsule was observed through transmission electron microscopy as described by Charland *et al* [18].

Proper serological typing, which is one of the most important features of the *S. suis* infection diagnosis, can be performed using a co-agglutination test. The operation details were described as previously published [17].

2.2 DNA extraction and virulence factor amplification

Genomic DNA of ZHJ01 strain was extracted according to the manufacturer's instructions as the previously described method [19]. The quality and quantity of extracted DNA were measured by using a NanoDrop ND2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only the sample that had an OD260/OD280 ratio of approximately 2.0 and showed no degradation were used for PCR verification. DNA samples were stored at -20℃. The primers of three main virulence factors were designed according to the sequence of GenBank (shown in Table 1). Detection of virulence factors were performed using PCR amplification and DNA sequencing as previously described [20].

Table 1. PCR Primer set amplification conditions.

Gene	Primer	Amplicon/bp	Sequence (5'—3')	GenBank number	Reference
MRP	mrp-1	1094	TTTATGCGTAGATCAAAT	AM946016	[22]
	mrp-2		GAGTTGTATTAATCTGATAAGCAGG		
EF	epf-1	1162	AACACAGTGACAGAAGCAGAGACAG	CP000407	[23]
	epf-2		CAAATTCCAAGTTACGTTTAGCAAG		
Sly	sly-1	1400	AAACTTATGAGAAAAAGTTCGCACT	NC009443	[24]
	sly-2		GCCAGATTACTCTATCACCTCATCC		

2.3 Bacterial strains cultivation and CFU counts

Bacteria were grown overnight on sheep blood agar plates at 37℃, and an isolate was inoculated into 5 ml cultures of Todd-Hewitt broth (THB) (Oxoid), which were incubated in incubator with 12 h at 37℃. Working cultures were prepared by transferring 3 ml of the 12 h cultures into 30 ml of THB, which were further incubated in incubator with 3–4 h at 37℃. Late log phase bacteria were washed twice in phosphate-buffered saline (PBS) (pH 7.4). The pelleted bacteria were then resuspended and adjusted to a concentration of 5.6 x 10^{10} CFU/ml approximately. The inoculum for experimental infection was diluted in THB to obtain a final concentration according to the experimental Infection of mice and piglets. This final suspension was plated onto agar to accurately determine the CFU/ml as previously described [16].

The cultures of ZHJ01 strain were centrifuged at 10000g for 15 min at 4 ℃. The supernatant was removed and the precipitation was resuspended to the original volume with sterile phosphate buffered saline (PBS). From the bacterial suspension a 10-fold serial dilution was prepared in PBS, inoculated into THB (preheated at 37 ℃) resulting in start inoculum of approximately 10^1, 10^3, and 10^6 CFU/ml. Broths were all incubated in incubator overnight at 37 ℃. The counts were performed after 24 h of incubation and 10-fold diluting it in PBS. Colonies were counted after incubation overnight at 37 ℃. The number of CFUs per ml of sample was equal to the average number of colonies (plate containing 20–200 colonies). The dilution factor of the plate counted [25].

2. 4 Experimental infection of mice and piglet

All experimental procedures involving animals were approved by the Laboratory Animal Monitoring Committee of Yangtze University. All efforts were made to minimize suffering. The signs of lethal disease were recorded before surviving mice and piglets were euthanized [28]. LD50 of SS2 for mice and piglets was calculated by using the Reed-muench method [26].

For mice infection, mice of 6 weeks of age were acclimated to standard laboratory conditions of a 12-h light/12-h dark cycle with free access to rodent chow and water. A preliminary study was performed to verify the 50% lethal dose (LD_{50}) of the ZHJ01 strain, and to determine the optimal bacterial dose with time points. Six-week old mice (six mice in each group), purchased from Hubei Province Animal Disease Control Center, were randomly divided into 5 groups, and infected by intraperitoneal injection with SS2 in 1 ml volume. Three control mice (group 6) were each injected with a 1 ml volume of the vehicle solution (sterile THB). The inoculum for experimental infection was diluted in THB to obtain a final concentration of 2.5 x 10^8 CFU/ml according to be adjusted to a concentration of 5.6 x 10^{10} CFU/ml described above. Experimental mice were inoculated by intraperitoneal (ip.) injection with 1 ml of a suspension of strain at the following concentrations: 2.5 x 10^8 CFU/ml, 5 x 10^7 CFU/ml, 1 x 10^7 CFU/ml, 2 x 10^6 CFU/ml, 2 x 10^5 CFU/ml. The clinical symptoms, including activity, and the course of disease, were monitored daily after infection for 14 days post infection (d.p.i.). All mice were monitored once per two days for two weeks for mortality, clinical signs and assigned clinical scores and pathological scores as described [21, 27].

For piglet infection, 30 piglets (four-week-old), obtained from the experimental swine herd of Institute of Black Pigs Research, Yangtze University, were randomly divided into five groups. Piglets were housed in isolated rooms in experimental swine herd, and were fed with non-medicated feed and water and libitum. The control group (group 6) contained three piglets. As mentioned above, the inoculum for experimental infection was diluted in THB to obtain a final concentration of 2.2 x 10^{10} CFU/ml according to be adjusted to a concentration of 5.6 x 10^{10} CFU/ml. Piglets were injected intramuscularly with a bacterial suspension of strain at the following concentrations: 2.2 x 10^{10} CFU/ml, 2.2 x 10^9 CFU/ml, 2.2 x 10^8 CFU/ml, 2.2 x 10^7 CFU/ml, 2.2 x 10^6 CFU/ml. Three control piglets were each injected with the vehicle solution (sterile THB). The infected piglets were carefully monitored for clinical symptoms, such as body temperature, appetite, daily activity, appetite, lethargy, movements, joints and lameness. Death piglets were immediately dissected. The score of pathological changes and clinical signs were observed [28], and the main tissues, organs and blood were isolated and collected in order to evaluate bacterial load.

2.5 Histopathology and bacteriology identification

The presence of bacteriological distribution in the tissues and organs from animal were analyzed by classical bacteriological analysis and by PCR due to the infection of ZHJ01 strain. We evaluated bacterial colonization of blood samples from the liver, spleen, kidney, Peritoneum and brain. Small samples of these tissues and organs were trimmed, placed in 2 ml of PBS, at pH 7.4, and then homogenized. we prepared dilutions of each homogenate in PBS, and plated the suspensions onto THB agar [29]. Blood samples were also plated on TSA agar. Colonies were counted and expressed as CFU/g, for organ samples, and CFU/ml, for blood samples.

2. 6 Statistical Analysis

Statistical analysis was performed using the SPSS 21.0 program. Data is displayed mean ± SD. The statistical method of one-way analysis of variance (ANOVA) was used to compare the mean values obtained among different groups. Di ▪erences were considered to be significant for a $P<0.05$.

3. RESULTS AND DISCUSSION

3.1 Morphology and culture characteristics of ZHJ01 strain

Gram staining showed that ZHJ01 strain is a Gram-positive coccus appearing in short chains under an optical microscope. Moreover, serologic tests indicated that the strain isolate belongs to the *S. suis type 2*. When cultured on sheep blood agar, ZHJ01 strain formed slightly gray or semitransparent, wet, smooth, and glossy colonies. The strain showed a hemolysis, as reflected by the 2 mm diameter hemolytic rings on the plates.

3.2 Biochemical Results

The strain isolate was inoculated with Glucose, Lactose, Raffinos, Sorbitol, D (+)-sucrose, Trehalose dihydrate, 6.5%NaCl, D (-)-Salicin, Hippurate, Esculin Hydrate, V-p, etc. in order to observe their biochemical characteristics. The biochemical test results of the bacterial isolate are shown in table 2. In the current study, we assessed the properties of morphology and biological, and the results would offer more information, and might explain the distinction of the virulence of *S. suis type 2*.

Table 2. Biochemical test results of bacterial isolate

Content	Bacterial isolates	Content	Bacterial isolates
Lactose	+	Raffinose	–
Glucose	+	Sorbitol	–
D (+)-sucrose	+	6.5%NaCl	–
Esculin	+	Hippurate	–
Trehalose dihydrate	(+)	V-P	–
D (-)-salicin	(+)		

+, means positive; -, means negative; (+), means positive and aerogenesis.

3.3 PCR results of Virulence factor

The *S. suis type 2* virulence factor of the extracellular factor was previously developed to detect by PCR test [31]. In the experiments, the PCR products were electrophoresed on a 2% agarose gel, stained with golden-view, and photographed under UV light, and derived from the sequences of a sick pig isolate of *S. suis type 2* producing the expected 1162-bp product of EF gene (shown in Figure 1). The PCR product of the *epf*-positive isolate was sequenced by Sangon Biotech (Shanghai) Co., Ltd. The results showed that the homology with the gene sequence of *S. suis type 2* published on GenBank (Accession number: AY341262) [31] was more than 99.8% (data not shown). ZHJ01 strain maybe not much variation in the area of Zhijing, Hubei province.

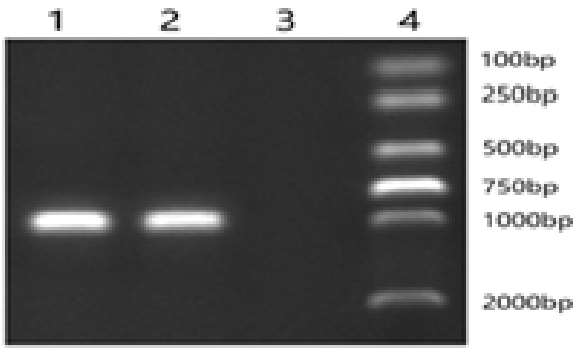

Fig. 1. Results of PCR amplification of EF gene.

Lane 1: Positive control ; Lane 2: Isolated strains ; Lane 3: Negative control ; Lane 4: Marker 2000b

3.4 Animal experimental model.

3.4.1 Mice trail

Mice were used to determine mortality and clinical signs after infection with ZHJ01 strain. The data of clinical signs and macroscopic lesions are summarized in Table 3 and Table 4. The mice injected with 10^8,$10^7$and 10^6CFU bacteria were inactive at 3 days post-injection. Group 1 (a high dose of 10^8 CFU) was shown 100% mortality. All mice (10^8,$10^7$and 10^6CFU) presented severe clinical symptoms associated with septicemia during the first 36 h post-inoculation (pi), including depression, swollen eyes, weakness and prostration. The inoculated mice showed expected clinical signs of disease such as depression-like behavior, rough appearance of hair coat and swollen eyes [32].

The score of clinical signs are shown in Table 3. The mice injected with 10^8 CFU bacteria were died from septicemia at one day post-injection, and injected with 10^7 CFU bacteria were died at 2 days post-injection. At an intermediate dose (10^7 CFU per animal), most mice also presented severe clinical symptoms and prostration during the first 72 h pi. At a low dose (10^6 CFU per animal), the mice presented moderate clinical symptoms Finally, no mice in the lowest group (10^5 CFU per animal) showed clinical signs associated with *S. suis type 2* infection, with the exception of slight depression immediately after inoculation, which subsided after 3 days post-inoculation.

There was no death record in the control group, and the rest of groups showed different mortality rates with different concentration of bacteria liquid. They caused moderate clinical signs associated with *S. suis type 2* infection, and relatively high mortality among inoculated mice. the score of pathological changes were gradually decreased according to

challenge dose in the test groups, and the data was shown in Table 4. According to the proportion of death within one week, LD_{50} was calculated according to Reed-muench method [26], and the value of five test groups was calculated. LD_{50} was 2.5×107cfu (shown in Table 5). The bacteriological distribution in the organs from mice are shown in table 6. Difference was obtained between the brain and the blood ($P < 0.05$). Organs showed a pattern more or less homogeneous in bacterial counts, where more individual variability was observed, as previously reported [33].

Table 3. Score of clinical symptoms in mice

Challenge dose/CFU	Number of infected mice/ Total	Score
2.5×10^8	6/6	3.33±0.41
5×10^7	6/6	3.00±0.55
1×10^7	5/6	2.67±0.75
2×10^6	4/6	1.67±1.17
4×10^5	0/6	0.50±0.00
Control	0/3	0.50±0.00

The score of clinical symptoms are expressed as mean ± SD obtained from three independent experiments, and following: 0.5, means normal; 1.5, means mild (dysregulation, difficulty walking, unstable standing) ; 2.5, means moderate clinical symptoms (trembling, twitching, drowsiness, blindness, diarrhea and poor appetite) ; 3.5, means dying or dead.

Table 4. Score of pathological changes in mice

Challenge dose	Mice with pathological changes/ Total (Score of pathological changes)											
	Brain		Lung		Liver		Spleen		Kidney		Peritoneum	
2.5×10^8	6/6	3.0±0.0	6/6	2.0±0.0	6/6	2.8±0.4	6/6	3.8±0.4	6/6	3.7±0.5	6/6	3.8±0.4
5×10^7	6/6	2.8±0.4	6/6	1.7±0.5	6/6	2.7±0.5	5/6	3.2±1.2	4/6	2.5±1.2	6/6	3.3±0.5
1×10^7	1/6	1.2±0.4	1/6	1.2±0.4	2/6	1.3±0.5	1/6	1.2±0.4	0/6	1.0±0.0	5/6	2.0±0.6
2×10^6	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0
4×10^5	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0
Control	0/3	1.0±0.0	0/3	1.0±0.0	0/3	1.0±0.0	0/3	1.0±0.0	0/3	1.0±0.0	0/3	1.0±0.0

The score of pathological are expressed as mean ± SD obtained from three independent experiments in every group, and following: 4, means severe diffuse injury; 3, means moderate injury with many lesions; 2, means between normal and mild lesions; 1, means normal.

Table 5. LD_{50} determinations of mice challenged with SS2

Challenge dose/ CFU	Number of dead mice/ Total
2.5×10^8	6/6
5×10^7	3/6
1×10^7	0/6
2×10^6	0/6
4×10^5	0/6

Control	0/3

Mice were injected with S.suis type 2. According to Reed-Muench method, LD_{50} of S.suis type 2 for mice was 2. 5 x 10^7cfu.

Table 6. The numbers of bacterial count test in each organs (×10^7CFU)

Organ	Brain	Liver	Spleen	Lungs	Kidney	Blood
Intraperitoneal	0.27±0.14*	1.46±0.10	2.26±0.08	1.72±0.46	1.85±0.08	2.60±0.03*

*Data are represented as mean ± SD of three independent experiments based on bacterial count test, and the number of SS2 is detected in each organ. * indicates a significant difference (p <0.01).*

3.4.2 Piglets trail

Clinical symptoms of the piglets after infection with *S. suis type 2*. The typical course of disease could be divided into three phases: the early stage, acute progress phase, and relatively convalescent period. The body temperatures of each piglet were monitored continuously once per two days during the experimental period after intramuscular injection with ZHJ01 strain (shown in Figure 2).

The piglets, which were challenged with a dose from 10^{10} to10^8, presented clear clinical symptoms (100% morbidity), developed slight fevers (38.5 ℃to 39.6 ℃), and exhibited significant levels of respiratory disease characterized by rapid and labored respiration at 3-6 d.p.i. Nine piglets displayed joint swelling, lameness, and crouching from 1–4 d.p.i. in the acute progress phase (5 d.p.i. to 11 d.p.i.), generally speaking 1–3 days after the emergence of early symptoms. Eight piglets died with respiratory distress soon after developing the syndrome with joint swelling, lameness, and crouch, and two defecated flavo-green loose stool. Furthermore, sixteen piglets exhibited crouching with joint swelling and stiff limbs, and unable to raise their heads (appeared at 5–7 d.p.i.) in table 8. The infected piglets showed joint swelling, lameness, and crouch at beginning, then developed into septic-like shock syndrome (SLSS) syndrome, at last the survivals showed physical activity impairment. Brain lesions, such as venous thrombosis, which maybe also directly contribute to the sequelae in human cases, were identified in the pigs [34]. The morbidity, mortality, and incidence rates of the severe and relatively ligter syndromes have changed via the clinical presentation for epidemiological investigation of *S. suis*. At 7 DPI, the body temperatures of piglets challenged with SS2 risen to 40.0 ℃. All piglets in group 2 exhibited roughened hair coats at 9 DPI. These pigs had loss of appetite, elevated body temperatures (40.0 ℃ to 41.5 ℃), and were reluctant to rise and were lame in one or more legs. Four pigs exhibited severe signs such as head tilt, nystagmus, tremors, ataxia, prostration and opisthotonus, and had fevers (>41 ℃) at 11 DPI. They were euthanized immediately. All of the piglets injected with SS2 strain developed most of the typical disease symptoms, including high fever, poor appetite, limping, shivering and dyspnea. Negative control animals did not exhibit any clinical signs of *S. suis* infection. Postmortem examinations did not reveal any lesions in these pigs.

LD_{50} values of both strains had to be determined in series of experiments for which a large number of piglets were required. The determination of LD_{50} of *S. suis type 2*infected with piglet was 3.92 x 10^9cfu (shown in Table 9). Determination of bacteria in tissues and organs in table 10. The bacteriological distribution in the tissues and organs from the eight dead animals with 3–4 d.p.i. were identified by bacterial culturing and counting after the infection with ZHJ01 strain. The difference was shown between the brain and the blood ($P < 0.05$). The bacterial load of lungs was relatively high compared to the liver, spleen, kidney.

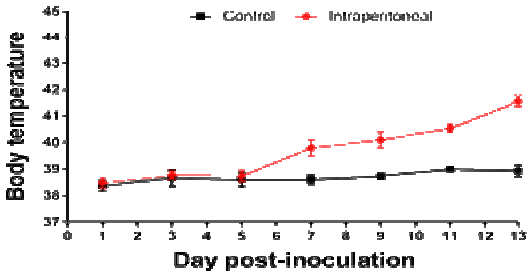

Fig. 2. The changing temperature of each piglet in the infection periods

Table 7. Score of clinical symptoms in piglets

Challenge dose/CFU	Number of infected piglets/ Total	Score
2.2 x 10^{10}	6/6	5.7±0.8
2.2 x 10^{9}	6/6	5.0±1.1
2.2 x 10^{8}	5/6	3.8±2.0
2.2 x 10^{7}	2/6	1.7±1.2
2.2 x 10^{6}	0/6	1.0±0.0
Control	0/3	1.0±0.0

The score of clinical symptoms are expressed as means ± SD obtained from three independent experiments, and following: 0.5, means normal; 1.5, means mild (drowsiness, limping, swollen eyes; 2.5, means moderate clinical symptoms (poor appetite, shivering, dyspnea, blindness and prostration) ; 3.5, means dying or dead.

Table 8. Score of pathological changes in piglets

Challenge dose	Piglets with pathological changes/ Total (Score of pathological changes)											
	Liver		Spleen		Lung		Brain		Kidney		Peritoneum	
2.2 x 10^{10}	6/6	6.0±0.0	6/6	5.7±0.8	6/6	6.0±0.0	6/6	5.7±0.8	6/6	5.3±1.0	6/6	5.7±0.8
2.2 x 10^{9}	6/6	5.3±1.0	6/6	5.7±0.8	6/6	5.3±1.0	5/6	4.5±2.0	4/6	3.3±2.0	5/6	4.5±2.0
2.2 x 10^{8}	2/6	1.7±1.2	2/6	2.0±1.5	3/6	2.2±1.5	2/6	1.7±1.2	1/6	1.2±0.4	4/6	3.7±2.3
2.2 x 10^{7}	0/6	1.0±0.0	0/6	1.0±0.0	1/6	1.2±0.4	0/6	1.0±0.0	0/6	1.0±0.0	1/6	1.2±0.4
2.2 x 10^{6}	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0	0/6	1.0±0.0
Control	0/3	1.0±0.0	0/3	1.0±0.0	0/3	1.0±0.0	0/3	1.0±0.0	0/3	1.0±0.0	0/3	1.0±0.0

The score of pathological are expressed as mean ± SD obtained from three independent experiments in every group, and following: 4, means severe diffuse injury; 3, means moderate injury with many lesions; 2, means between normal and mild lesions; 1, means normal.

Table 9. LD_{50} determinations of piglets challenged with SS2

Challenge dose/ CFU	Number of dead piglets/ Total
2.2 x 10^{10}	6/6
2.2 x 10^{9}	2/6
2.2 x 10^{8}	0/6
2.2 x 10^{7}	0/6
2.2 x 10^{6}	0/6
Control	0/3

Piglets were injected with S.suis type 2. According to Reed-Muench method, LD_{50} of S.suis type 2 for mice was 3. 92 x 10^{9}cfu.

Table 10. The numbers of bacterial count test in each organs (×10^{9}CFU)

Organ	Brain	Liver	Spleen	Lungs	Kidney	Blood
Intramuscularly	8.05±0.22*	0.23±0.09	0.25±0.10	9.95±0.12	0.23±0.09	13.0±0.13*

*Data are represented as mean ± SD of three independent experiments based on bacterial count test, and the number of SS2 is detected in each organ. Significant differences in levels between tissues and organ are marked. * indicates a significant difference (p <0.01).*

3.5 Histopathological

Death piglets were histopathologically analyzed by necropsy. Gross lesions were noted in six pigs, in which a fibrinopurulent polyserositis was seen, and in four piglets, which had an exudative meningitis and arthritis. Brain, spleen, liver, kidney, heart, lung, joint, intestine and lymph node from the piglets infected with *S. suis type 2* displayed conspicuous macroscopic lesions (Fig.3), such as encephalemia and encephaledema (Fig. 3-A), kidney swelling and nephremia (Fig. 3-F). The histology of macroscopic lesions could be shown such as Cerebral congestion (n=13), Pulmonary congestion (n=16), Hepatic congestion (n=15), Polyserositis (n=11), Purulent arthritis (n=22). "n" indicate the number of the lesion.

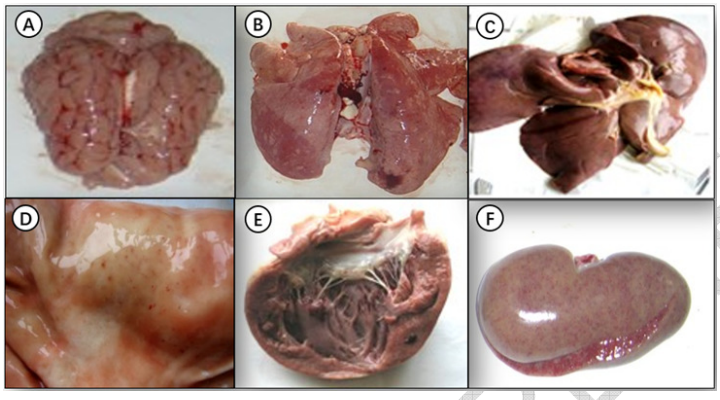

Fig. 3. Histopathology of *S.suis type 2* (SS2) disease caused by ZHJ01 strain.

Piglets were intramuscularly inoculated with S. suis type 2 strain. Four piglets that died from SS2 were dissected and histopathological analysis. (Figure 3) Gross pathology of the piglets at 4 d.p.i (day post-infectio). (A) Encephalemia and encephaledema; (B) Pulmonary congestion; (C) Light liver degeneration; (D) The petechia emerging on the surface of the stomach tract; (E) Cardipericarditis; (F) Kidney swelling and nephremia;

3.5 Discussion

Streptococcus suis is a very important Gram-positive bacterium considered worldwide to be one of the most important pathogens in the swine industry. Bacterioscopical and bacteriological examination together with the biochemical characteristics allowed classification of the isolates into *Streptococcus suis* species [35]. The results indicate that ZHJ01 strain can be confirmed by the test of Biochemical identification, Serotyping and CFU counts. Moreover, the presence of *mrp*, *epf,* and *sly* genes, considered "classical" virulence markers mostly described for serotype 2 strains were studied previously [36]. The *epf* gene was detected in ZHJ01 strain from clinical isolate, which maybe support their association with virulence. It is better collecting pathogens from parts such as tonsils, and the results were accurate [36]. The results show that EF genes can be a critical virulence factor for *S. suis type 2* infection, and EF genes seems to be strongly correlated to invasiveness of ZHJ01 strain in serotype-2.

Furthermore, the *epf*-positive isolate was detected in the diseased pigs, indicating that expression of *epf* is possibly associated with actual virulence. these virulence-associated genes determine the actual virulence of strains is currently unclear [37]. The genome of SS2-1 harbors some virulence gene candidates, such as *epf* [38]. The results showed that the homology with the gene sequence of *S. suis type 2* published on GenBank (Accession number: AY341262) was more than 99.8%. Two large-scale outbreaks of SS2 in China in 1998 and in 2005 have posed public health concerns worldwide. The genomic features of SS2-1 will support the analysis of comparative genomic and pathogenic characteristics of *S. suis* and development of therapeutic agents and vaccines. The SS2-1 was confirmed a relatively strong pathogenicity to pigs and human described previous study. The LD50 values were 4.26 x 10^7 CFU/mouse in SS2-1. Piglet infection experiments showed that all of the piglets infected with SS2-1 at the dose of 5.6 x 10^6 CFU/piglet. Similar results have been reported for SS2 for all piglets died within 5 days PI [38]. In this trail, LD_{50} of *S. suis type 2* for mice was 2.5 x 10^7cfu. LD_{50} of *S.suis type 2* for piglets was 3.92 x 10^9cfu. This might indicate its more pathogenic nature of ZHJ01 than SS2-1 with its LD50 of about less. The value of LD50 of strain ZHJ01, together with previous study, might also show that the virulence of strain ZHJ01 was not very stable in the cultivation process. Thus, the results show that ZHJ01 has a relatively strong pathogenicity to pigs in the area of Jingzhou city.

In the present study, we sought to determine the virulence of ZHJ01 strain based on the infection experiments of mice and piglets. These results are different from those of Knuradeszczka' previous trials carried out under these standardized

conditions with different strains of *S. suis type 2* [39]. The clinical signs and macroscopic lesions induced in piglets were relatively high in this study. It is of high importance for gaining insight into the process of pathogenesis, identification and characterization of virulence factors of *S. suis* [40]. More studies needed to confirm their virulence potential and pathogenicity in several regions/countries. These results might be not sufficient to explain the pathogenicity for ZHJ01 strain in area of Zhijing, Jingzhou city, China, which may provide insights into the pathogenesis SS2 as well as the prevention and treatment of its resulting disease.

4. CONCLUSION

ZHJ01 strain can cause a relatively high occurrence and mortality on pathogenicity test of mice and piglets. Through the results of bacterial isolation, culturing, biochemical identification, serological test, PCR assay and sequencing, the pathogen of the sick pig is identified as *S. suis type 2.* As reflected by our results, *S. suis type 2* strain can display virulence in the mice and piglet infection experiments. LD_{50} of *S. suis type 2* for mice is 2.5 x 10^7cfu. LD_{50} of *S. suis type 2* for piglets is 3.92 x 10^9cfu. ZHJ01 strain (one isolated from a diseased pig from Jingzhou area of Hubei province, China) clearly present a relatively high virulence potential, and maybe cause a high infection rate for zoonotic disease. This study may provide information for epidemiological investigation of *S. suis type 2* and useful data for future bacteriological studies as well as aid in developing new vaccines for the control, and has great significance for further monitoring and effective prevention to *S. suis*.

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