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Research article

Virulence and molecular epidemiological analysis of three human blood-borne *Streptococcus suis*

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ABSTRACT

Objective: To understand the virulence genes and molecular epidemiological characteristics of human-infected strains of *Streptococcus suis* in Rui'an, Zhejiang Province, from 2021 to 2022, and to provide a scientific basis for diagnosis, treatment, prevention, and control.

Methods: Three blood-borne strains of *Streptococcus suis* were analysed by morphological observation, identification, and drug sensitivity tests. We performed polymerase chain reaction (PCR) amplification of their main seven virulence factors and housekeeping genes. This was followed by virulence analysis and multilocus sequence typing. We analysed their relationships with local pathogens from previous years.

Results: Three *Streptococcus suis* strains were isolated from the blood samples of three patients. From these, the virulence genotypes demonstrated that the two strains were *orf2*+ and *ef*+/*orf2*+/*sly*+, respectively. The Multilocus sequence typing (MLST) typing results demonstrated that the two strains were *ST25* and *ST7,* respectively.

Conclusion: The first isolation of *ST25 Streptococcus suis* in Rui'an was presumed to have a close affinity with the endemic strain in North America. The other strain was an *ST7* clone, consistent with the endemic strain in Sichuan, and which may have originated from Sichuan. Virulence genotype analysis demonstrated that different virulence genes of the pathogens resulted in different clinical manifestations.

1. Introduction

Streptococcus suis (S. suis) is a zoonotic pathogen that can be transmitted to humans through close contact with sick or germ-carrying pigs. Those engaged in swine farming or pork-related work are more exposed to *S. suis*, which can cause septicaemia, meningitis, and other severe symptoms and can even lead to death [1]. There are 29 serotypes of *S. suis* according to their *Capsular Polysaccharide (CPS)*, among which *S. suis* serotype 2 occurs most frequently in the infected population and is the most virulent [2]. In 2014, Goyette-Desjardins et al. found that the most common virulence genotypes of *serotype 2 ST1 S. suis* strains isolated from clinical swine and humans were *sly*+/*mrp*+/*epf*+ and were highly pathogenic [3].

The multilocus sequence typing (MLST) model of *S. suis* was developed by King et al., in 2002 [4]. The model was analysed using seven housekeeping genes (*cpn60*, *dpr*, *recA*, *aroA*, *thrA*, *gki,* and *mutS*). Human infection with porcine streptococcal disease has

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gradually attracted attention globally, and there is currently a risk of misdiagnosis for diagnosing and treating porcine streptococcal disease. Therefore, it is critical to understand the virulence genes and molecular epidemiological characteristics of porcine Streptococci for early diagnosis and treatment of porcine streptococcal disease. Herein, to lay the foundation for further research on porcine Streptococci and provide data support, we performed virulence gene analysis and MLST typing of the collected strains. This was done to analyse the relationships among virulence genes, ST types, and clinical manifestations. The three *S. suis* strains and local pathogens from previous years were also compared.

2. Materials and methods

2.1. Main reagents and materials

1.1 Source of strains The three *S. suis* strains were obtained from the blood samples of three patients at Rui'an People's Hospital. They were named RA4, RA5, and RA6, respectively, in chronological order.

1.2 Main instruments and materials VITEK 2 Compact automatic microbiological analysis system and supporting reagents, VITEK MS automatic rapid microbiological mass spectrometry detection system and supporting reagents, and Bact/ALERT 3D automatic bacterial/mycobacterial culture monitoring system were purchased from Bio-Mérieux, France. The agarose gel electrophoresis instrument was purchased from Bio-rad, USA. AGS4800 real-time fluorescence quantitative PCR instrument was purchased from Hangzhou Anjou Biotechnology Company Limited. Gel imaging system Biosens SC805 was purchased from Shanghai Shanfu Scientific Instrument Co. Ltd. Automatic dyeing machine GSZ-GT116 was purchased from Zhuhai BaSO Biotechnology Co. Ltd. Columbia sheep's blood agar medium and blood Mueller-Hinton (MH) plate were purchased from Guangzhou Dijing Biological Co. Ltd. Agarose powder, 50 × TAE buffer, DNA molecular weight standard marker (100–2000bp), 6 × Loading Buffer, non-toxic nucleic acid staining solution, primers, Taq PCR Mix premix, and ddH_2O were purchased from Sangong Bioengineering (Shanghai) Co. Ltd.

2.2. Pathogenicity examination

Blood samples were cultured by blood culture at 35 °C. They were then transferred to Columbia sheep's blood agar medium at 35 °C, 5 % CO_2. Smears were stained with Gram stain for microscopic examination. Pure cultured single colonies were collected and identified using VITEK MS mass spectrometry and a VITEK 2 completely automated microbiological analysis system, according to the instructions of the instruments and kits. Simultaneously, drug sensitivity tests were performed using the paper diffusion (KB) method with a blood MH plate. The operation and results were judged according to the standards of the Clinical and Laboratory Standards Institute (CLSI), USA, version 2022.

2.3. Molecular biology tests

3.1 DNA extraction We add 500 μL of sterile water into the microtube. We then picked the appropriate number of colonies with an inoculating ring and transferred them into the sterile water. Subsequently, we mixed the samples for 30 s and boiled them for 10 min. We then centrifuged the mixture for 5 min at 12000 r/min. We stored the supernatant at −20 °C for spare use.

3.2 PCR amplification Primers for seven virulence genes, such as *cps2j*, *mrp*, *fbps*, *sly*, *ef*, *orf2,* and seven housekeeping genes, such as *mutS*, *cpn60*, *recA*, *gki*, *aroA*, *thrA*, and *dpr* were synthesised according to reference [4–6]. DNA extracted from 1.4.1 was used as a template to amplify the 14 gene fragments. Amplification system (25 μL): 4 μL of DNA template, 1 μL each of upper and lower primers, ddH_2O 6.5 μL, MIX 12.5 μL. Amplification procedure: 94 °C for 5 min; 94 °C for 30 s, annealing (temperature see table) for 30 s, 72 °C for 40 s, for a total of 35 cycles; 72 °C for 10mins, maintained at 4 °C. PCR products were electrophoresed on a 1.5 % agarose gel. Post

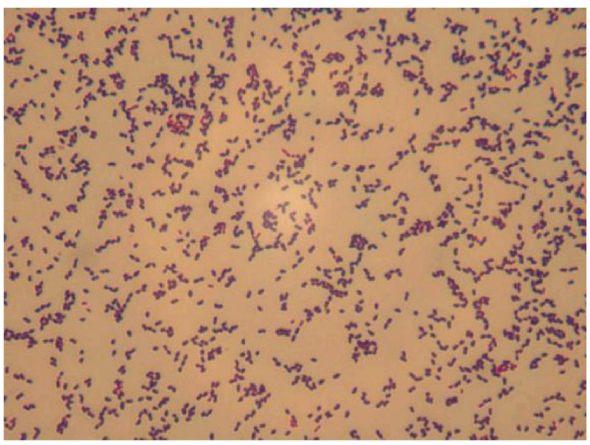

Fig. 1. *S. suis* Gram stain.

electrophoresis, cells were observed and photographed using a gel imaging system. Based on the electrophoresis results, housekeeping gene amplification products with target-sized bands were sent to Sangyo Bioengineering (Shanghai) Co. for sequencing. The sequencing results were uploaded to the *S.suis* MLST database to obtain the sequence typing (sequence type, ST) of the strain.

3. Results

1 **Bacterial culture and morphology** The three strains of pathogenic bacteria grew under both aerobic and anaerobic conditions. They demonstrated α-haemolytic, smooth, moist, and raised grey-white colonies on Columbia blood agar plates. Post Gram staining, purplish-red ovoid or round Gram-positive cocci were visible under the microscope. Some were single, whereas others were in pairs or chains (see Fig. 1).

2 **Identification and drug sensitivity results** VITEK MS mass spectrometry identification results were all *S. suis*: RA4 results with 95 % confidence, RA5 91 %, and RA6 96 %. Gram-positive bacterial (GP) card identification results were obtained for *S. suis*. RA4 biological number was 171011364313671. The confidence in the identification results was 95 %. The biological number for RA5 was 451051364333771, and the confidence level was 91 %. The biological number for RA6 was 171011364333771, and the confidence level was 94 %. The drug sensitivity results demonstrated that the three strains were sensitive to antibiotics, such as penicillin, ceftriaxone, levofloxacin, and vancomycin. The only strains sensitive to chloramphenicol were RA4 and RA5, with RA6 as the intermediary.

3 **Determination of virulence genes** RA4 virulence genotype was *orf2*+, RA5 virulence genotype was *ef*+/*orf2*+/*sly*+ , and the RA6 virulence genotype was *orf2*+. The results of the electrophoresis are depicted in Table 1.

4 MLST typing By comparing the housekeeping genes of each strain with standard sequences in the database, RA5 was *ST7*, the housekeeping gene loci of RA4 and RA6 were identical, and both were *ST25*. The housekeeping gene loci of each strain of *S. suis* are listed in Table 2. Comparing the housekeeping gene loci of the three strains of *S. suis* detected in our hospital in 2012 and the MLST database for each subtype, it can be seen that the *ST1* and *ST7* are closer, with a difference of only one allele. The *ST25* and *ST29* are closer, with only one allele difference. We obtained the same gene loci using the goeBURST and MEGA7 software for graphical analysis. We obtained the same results by using the goeBURST and MEGA7 software. The results were corroborated by each other (the results are depicted in Figs. 2 and 3).

4. Discussion

S. suis are zoonotic pathogens that can infect humans via the digestive tract, respiratory tract, and wounds. It can cause severe systemic infections in swine herds and occupation-related infections in people working with pigs and pork products. Human infections primarily manifest as fever. Resulting from the different sites of infection, it can cause different clinical manifestations, such as arthritis, pneumonia, meningitis, and intraocular inflammation etc. [3] It can easily be confused with other clinical causes, leading to misdiagnosis. Human infections caused by this organism are rare but life-threatening. The bacterium has attracted global attention, and according to the MLST database, 3474 isolates of the bacterium have been enumerated. More than 50 % of *S. suis* originated from Asia, of which approximately 26.6 % were isolated from China. It depicts that China is a highly prevalent area for *S. suis* in comparison with other regions. Except for two large-scale epidemics in China in 1998 and 2005, all human infections were disseminated. In 2012, three *S. suis* strains were also found in Rui'an, Zhejiang Province, of which two were *ST7* and *ST1,* respectively. Currently, human infections with *S. suis* in China are dominated by *ST1* and *ST7* [7]. Diverse *S. suis* strains have notably different virulence, which is related to the virulence factors that they carry. Goyette-Desjardins et al. (2014) found that the virulence genes of serotype 2 *ST1 S. suis* strains, which were isolated from diseased pigs and patients, were mostly sly+/mrp+/epf+. They were highly pathogenic [8].

Herein, after sequencing comparison of the housekeeping genes, the MLST typing of RA5 was *ST7*, consistent with the Sichuan epidemic strain. The virulence genotypes, including the MLST typing of RA4 and RA6, were identical, and the clinical phenotypes were basically the same, *ST25*. The virulence genes of RA5 were identified as *ef*+/*orf2*+/*sly*+, and those of RA4 and RA6 were identified as *orf2*+. Currently, the highest detection rate of porcine streptococci in China is *ST7*. A virulence factor study on Sichuan and Jiangsu strains in 2014 found that the genotype of most *ST7* strains was *ef*+/*mrp*+. *mrp* is a virulence factor that is highly associated with meningitis. While our RA5 was *ST7*, patients infected with this strain did not have the *mrp* gene detected. They did not have meningitis-related symptoms. These patients presented with acute cholecystitis only. In a genome-wide study of *ST25*, the genome of *Streptococcus toxicus* strain *ST25* was found to lack the *sly* and *ef* genes [9]. In 2011, there were 28 *ST25* strains out of 64 *S. suis* strains from Canada and the United States. All of them had the genotype of *sly/mrp/ef-*, similar to the two strains in this study and consistent with the *ST25* strains. Combined with clustering analysis, we hypothesised that local *ST25* may be closely related to the second epidemic in North America and the endemic strains in Canada [10]. The *ST25* strain induced a higher incidence of meningitis in a mouse model and was

Table 1
Virulence loci for each *S. suis* strain.

	cps2j	mrp	fbps	sly	ef	orf2
RA4	Neg	Neg	Neg	Neg	Neg	Pos
RA5	Neg	Neg	Neg	Pos	Pos	Pos
RA6	Neg	Neg	Neg	Neg	Neg	Pos

Table 2
Housekeeping loci for each *S. suis* strain.

	mutS	*cpn60*	*recA*	*gki*	*aroA*	*thrA*	*dpr*	MLST typing
RA4	30	30	3	34	9	25	5	*ST25*
RA5	1	1	1	1	1	3	1	*ST7*
RA6	30	30	3	34	9	25	5	*ST25*

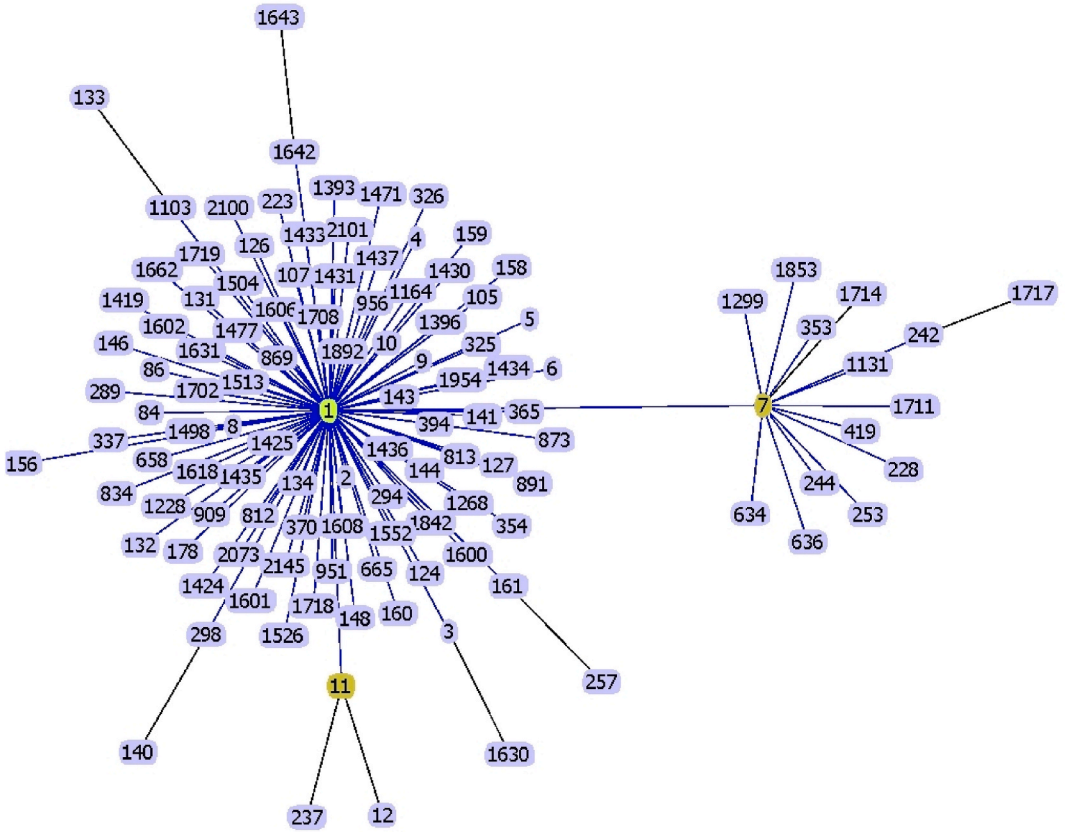

Fig. 2. Plot of the results of the goeBURST analysis of *ST7* affinities.

associated with persistent bacteraemia [11]. Herein, both RA4 and RA6 pathogens were detected in the blood of patients. RA6 demonstrated pyogenic meningitis, which is consistent with the results of the mouse study.

RA4 and RA6 lack two common virulence factors, *ef* and *sly*, compared to RA5, and it has been reported that *sly* + strains are highly virulent [12]. However, it is puzzling that the severity of infection symptoms in the three cases was reversed. RA4 was older and presented with hyperthermia, septic shock, and infectious multiorgan dysfunction syndrome. RA6 had a history of falling from a height, burns, hypertension, and presented with hyperthermia, sepsis, and septic meningitis. RA5 was diagnosed with acute cholecystitis due to the clinical manifestations of fever and vague pain in the right epigastric region. Based on the clinical manifestations, RA4 and RA6 were more severe, developed more rapidly, and had a more prolonged illness duration. We speculate that the virulence factor is not completely compatible with the disease and may be related to the patient's age, immune status, and presence or absence of underlying diseases. Currently, 70 virulence factors have been reported. Only the seven most important of them were detected in this study. In-depth studies, such as whole-genome sequencing are required further to elucidate the relationship between virulence factors and clinical manifestations.

Among the three *S. suis* strains that were detected in the Rui'an area in 2012, the virulence genotypes of the two *ST7* strains were *ef*+/*sly*+. These were highly similar to the genotypes of the *ST7 S. suis* detected in this study. Only the *ST1* strain had the virulence genotypes *ef*+/*mrp*+/*sly*+. Ten years later, with the emergence of porcine streptococci of *ST7* and *ST25*, we presume that the prevalence of porcine streptococci MLST typing will also change over time. The causes of this change may be the environment, climate, source of pork, and migration of people, which will require further study.

Of the three cases in this study, one patient had a clear history of pork exposure. His occupation was a pork butcher who had cut his finger accidently while slaughtering pigs before consultation, which resulted in his fever. The other two cases had an unclear route of

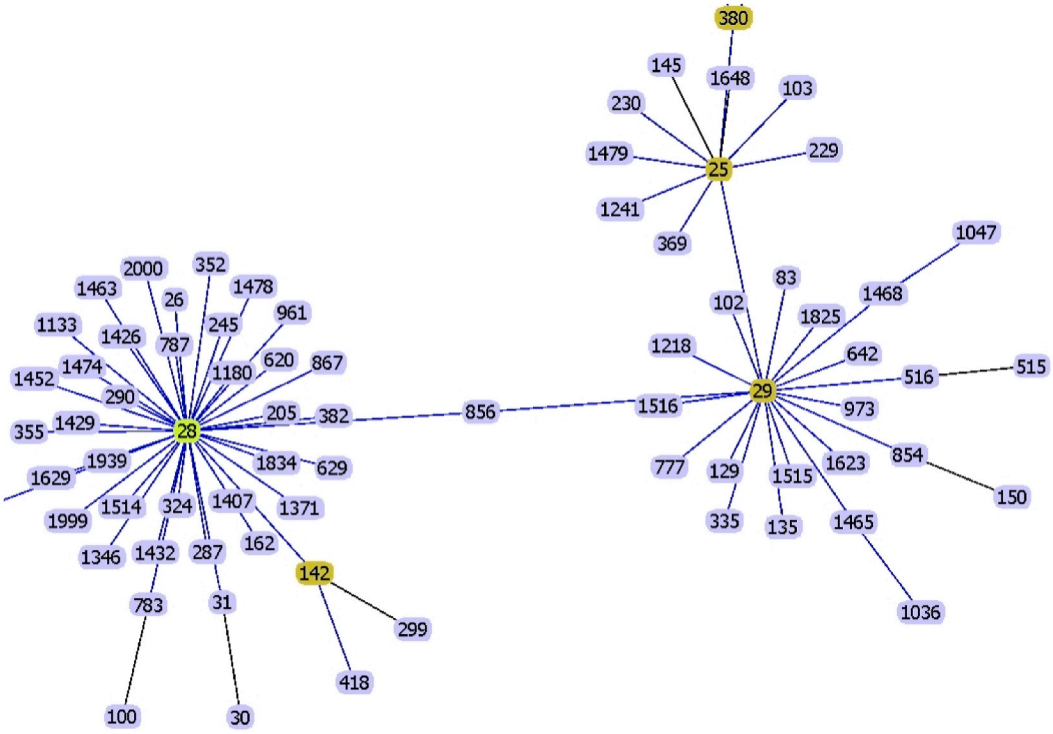

Fig. 3. Plot of the results of the goeBURST analysis of *ST25* affinities.

infection; one of the local cases in 2012 was a pork salesperson who often cut raw food, such as pork and pig offal, and his finger was cut before the onset of the disease. Therefore, such workers need to be provided with proper personal protection. Pigs are the primary hosts of *S. suis*. The outbreaks of *S. suis* in Sichuan in 2005 and *S. suis* in Nantong, Jiangsu Province, in 1998 were caused by pigs. Because pork is the most common meat people consume daily, it is important to strengthen local inspections and quarantine pork. Currently, there is a lack of epidemiological data on *S. suis*. Clinicians lack knowledge of this disease, which not only causes a detection lag but is also very easy for them to miss and misdiagnose. Therefore, expanding the flow database to improve detection rates is critical.

CRediT authorship contribution statement

Qianying Zhu: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Fei Xia:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Zhe Chen:** Project administration, Methodology, Investigation. **Sen Lin:** Resources, Project administration, Methodology. **Qing Zhang:** Supervision, Software. **Bingru Xue:** Visualization, Validation. **Weisi Dai:** Writing – review & editing, Writing – original draft.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets used and analysed in the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All methods were performed in accordance with approved guidelines and regulations. The need for ethical approval and consent was deemed unnecessary for this study according to the Ethics Committee of the Third Affiliated Hospital of Wenzhou Medical University.

Consent for publication

Not applicable.

Data availability statement

We confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Qianying Zhu reports financial support was provided by Ruian Municipal Science and Technology Bureau. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39978.

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