Isolation and characterization of Streptococcus suis from pig farms in Sichuan

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Abstract. In this experiment, bacteria were isolated by aseptically collecting tissue samples of heart, spleen, liver, lymph node, and joint fluid from diseased and dead pigs, and were identified by colony morphology observation, Gram staining microscopy, biochemical experiments, PCR identification, pathogenicity test, and drug sensitivity test. The results showed that the isolated strains were Gram-positive, long-chained, or bipartite coccoid bacteria. It could ferment most sugars, and the VP test and indigo substrate test were negative, consistent with the physiological characteristics of Streptococcus suis. The PCR method was used to identify the serotypes, and about 500 bp of podocarp serotype 9-specific bands could be amplified. Pathogenicity test showed that mice became depressed 6h after intraperitoneal inoculation with the isolate, and died within 24h after rapid breathing, slow movement, and loss of appetite. The results of the drug sensitivity test showed that the isolate was highly sensitive to cefthiophene, florfenicol, cefadroxil, ciprofloxacin, ampicillin, amoxicillin, highly sensitive to sulfisoxazole, polymyxin B, enrofloxacin, doxycycline, and showed resistance to lincomycin, kanamycin, neomycin, gentamicin.

Keywords: Streptococcus suis; isolation; identification;drug sensitivity test.

1. Introduction

Swine streptococcal disease is a zoonosis caused by a variety of pathogenic streptococcal infections, streptococcus once infected pigs, that can cause meningitis, septicemia, pneumonia, endocarditis, and arthritis, etc., but also through wounds and gastrointestinal tract pathway infection of people, causing meningitis and streptococcal toxic shock syndrome. Streptococcus suis is divided into 35 serotypes (1~34 and 1/2[1]). In recent years, serotypes 20, 22, 26, 32, 33, and 34 have been excluded based on sequence analysis of 16SrRNA and other highly conserved housekeeping genes[2],However, at the same time, new serotypes have been identified[3,4], indicating a very complex serotyping of Streptococcus suis. The distribution and range of different serotypes also vary in different parts of the country[5,6,7,8]. Among the clinical isolates of Streptococcus suis of different serotypes, type 2 is the most virulent, followed by type 9[9].

In this research, we collected sick and dead pigs suspected to be infected with streptococcus suis, aseptically took samples of heart, spleen, liver, lymph nodes, joint fluid, and other tissues from the pigs, and determined the serotypes through bacterial isolation, Gram staining microscopy, biochemical test and molecular biology identification. The drug sensitivity test was carried out, and the results of the drug sensitivity test provided the theoretical basis for the scientific selection of antibiotics for the prevention and treatment of the disease in the clinic.

2. Material and methods

Samples of heart, spleen, liver, lymph nodes, joint fluid, and other tissues were taken from the diseased pigs suspected of being infected with Streptococcus suis sent from a large-scale pig farm in Sichuan Province under aseptic conditions, and inoculated onto TSA (Beijing Aoboxing) plates containing 5% calf serum, and then incubated at 37℃ for about 20h in a constant-temperature incubator (Thermo, USA), and then single colonies of these samples were picked for gram staining. Microscopic examination. Gram-positive colonies in chains were inoculated in TSB medium

(Beijing Aooboxing) with 5% newborn bovine serum (Zhejiang Tianhang Biotechnology) and incubated at 37℃ for 20h, then the bacterial solution was preserved with 400mL of glycerol and frozen at -20℃ for subsequent tests. The suspected colonies were cultured for about 20h and then inoculated with lactose, mannitol, sorbitol, sucrose, glucose and maltose, arabinose, salicylic acid, and other biochemical identification tubes, and then placed in the incubator at 37℃ for about 20h, and then the color changes were observed.

According to other research[10,11,12] , universal primers for Streptococcus suis were designed, and the typing primers cps1I, cps2J, cps7H, and cps9H were used for amplification and typing identification of Streptococcus suis, and the sequences of the primers are shown in Table 1.

A single streptococcal colony was inoculated in TSB medium with 5% newborn bovine serum and incubated overnight at 37℃ on a shaker. Pipette 1 mL of the overnight culture solution into a 1.5 ml tube, centrifuge at 4,000 r for 3 min, aspirate the supernatant and retain the precipitate, resuspend it with 200 μL of ddH2O, boil it for 10 min, and then centrifuge it at 16,000 r for 2 min. The aspirated supernatant was used as the template of streptococcal DNA.

The total PCR system was designed to be 15 μL, including 7.5 μL of 2×TaqMix enzyme, 1 μL of upstream primer, 1 μL of downstream primer, 1 μL of DNA template, and then made up to 15 μL of ddH2O, and ddH2O was used as a blank control at the same time. The PCR amplification program was as follows: 3 min of pre-denaturation at 95 ℃, 15s of denaturation at 95 ℃, 15s of annealing at 55 ℃, 15 s of extension at 72 ℃, with a total of 34 cycles. A total of 34 cycles; extension at 72 ℃ for 5 min, stored at 12 ℃, 6 μL of the PCR reaction product was detected by 1.0% agarose gel electrophoresis, and the results were observed under UV transmission analyzer. The positive PCR products were sent to Sangong Bioengineering (Shanghai) Co., Ltd. for sequencing, and the sample sequences were compared with the database using BLAST software.

The strains identified as Streptococcus suis were amplified and serotyped with primers specific for Streptococcus suis type l, type 2, type 7, and type 9, cps1I, cps2J, cps7H, and cps9H, using the same method as above except that the annealing temperatures were altered to 64℃ for type 1, 59℃ for type 2, 63℃ for type 7, and 61℃ for type 9.

Table 1. PCR Primers

Target gene(s)	primer nucleotide sequence（5’→3’）	Annealing temperature（℃）	Product length（bp）
16S rDNA	F:AAGACGAACCAAACGTGCAGG R:GAAGACTGACGTACAGGTTTCCATTT	55	1466
cps1I	F:CTGAGTATAATGCCGATATAG R:CTTCTCTTTAATAACTTTTGC	64	438
cps2J	F:CAAACGCAAGGAATTACGGATTC R:GAGTAXTAAAGAATGAATATTG	59	675
cps7H	F:TGCCCTCGTCCAATACAGCTA R:GATAACAGTCATACTTCTTACAC	63	540
cps9H	F:GATGGCTACATATAATGGAAG R:ATCCGAAGTATCTGGGCTAC	61	394

The cultured Streptococcus suis bacterial fluid was taken, and centrifuged at 4000r for 3min, the supernatant was aspirated, the precipitate was retained, and PBS resuspended the bacteria and the concentration of the resuspended bacterial fluid was adjusted to 2×107CFU/mL[13]. BALB/c mice were randomly divided into 2 groups (I, II), with 3 mice in each group. Mice in group I were injected intraperitoneally with 0.2mL of Streptococcus suis suspension; mice in group II were injected intraperitoneally with 0.2mL of physiological saline as a control group. Observe the mice for 7d after injection, and record their symptoms and survival every 12h. The dead mice were promptly dissected, and the diseased tissues of the liver, lung, and spleen were collected aseptically

and isolated for culture, staining, microscopic examination and PCR identification of Streptococcus type 9 gene.

The single colony on the plate was picked and inoculated in TSB medium with 5% newborn bovine serum and shaken at 37℃ overnight. Dilute the bacterial solution with PBS to 0.5 Mack's turbidity standard, take 100 μL, and spread it evenly on the TSA plate added with 5% newborn bovine serum. Selected cefthiophene, florfenicol, cefadroxil, ciprofloxacin, ampicillin, amoxicillin, sulfisoxazole, polymyxin B, enrofloxacin and doxycycline, and drug-sensitive paper tablets (Hangzhou Microbiology Reagent Co., Ltd.) of lincomycin, kanamycin, neomycin and gentamicin were affixed to the bacteria-coated plate, placed in the incubator at a constant temperature of 37 ℃ for overnight cultivation, and then taken out and the diameter of the ring of inhibition was measured. The results of drug sensitization were determined by referring to the instructions of the drug sensitization paper tablets.

3. Results

Bacteria isolated from the heart, spleen, liver, lymph nodes, joint fluids and other tissues were seen on TSA plates with 5% calf serum added as grayish-white semi-transparent, moist, with smooth surface elevated in the middle and neat edges, ovoid or rounded protruding fine colonies (Fig. 1), and Gram-stained as Gram-positive chains or arranged in pairs, with varying lengths of the chains (Fig. 2).

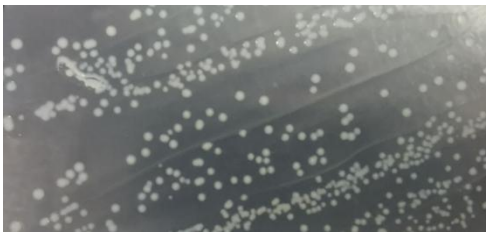

Fig. 1 Colony samples from isolated cultures

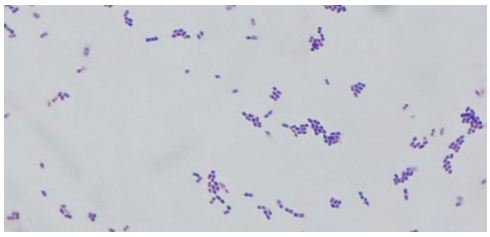

Fig. 2 Gram staining results

The results of biochemical tests showed that the isolates fermented carbohydrates such as maltose, salicylic acid, glucose, sucrose, and lactose positively, and did not ferment sorbitol, mannitol, and arabinose negatively.The VP assay and the indigo substrate assay were also negative (Table 2).

Table 2. Results of biochemical characterization of isolated strains

Reagent	Result	Reagent	Result
Maltose	+	VP test	-
Salicylate acid	+	Indigo substrate test	-
Glucose	+	Sorbitol	-
Fructose	+	Mannitol	-
Lactose	+	Arabinose	-

Operate PCR process on the genomic DNA of the isolate using universal primers for Streptococcus suis. The reaction products were detected by 1.0% agarose gel electrophoresis, and the results were observed under a UV-transmission analyzer, which showed a clear band. The positive PCR product was sent to Bioengineering (Shanghai) Co., Ltd. for sequencing, and the isolate was identified as Streptococcus spp. by BLAST software (Figure 3). The genomic DNA of the isolate was used as a template for PCR using primers specific for Streptococcus suis types 1, 2, 7 and 9, and the results of the genotyping are shown in Fig. 4. As can be seen in Fig. 4, the PCR amplified a clear target fragment of the cps9H gene, whereas the PCR products of cps1I, cps2J and cps7H were negative, and the positive products were sequenced by Bioengineering (Shanghai) Co.

Ltd. for sequencing and compared with BLAST software, and the isolate was determined to be Streptococcus, thus indicating that the strain was Streptococcus suis type 9.

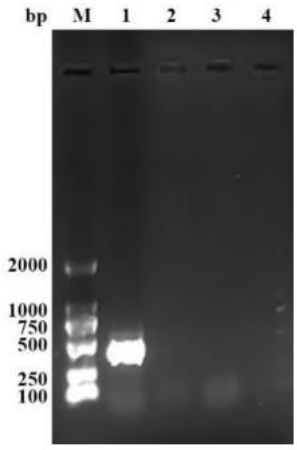

Fig. 3 Identification of isolated strains
Note: M is 2000bp DNAMarker, 1 is sample,2 is negative control

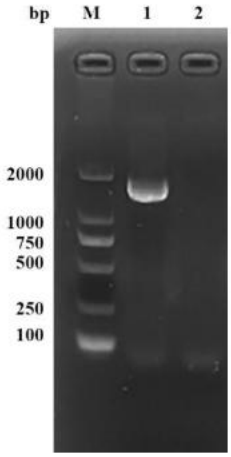

Fig.4 Serotyping of isolates
Note: M is 2000bp DNAMarker, 1 is cps9H,2 is cps7H,3 is cps2J, 4 is cps1I

At the dose of 2×107CFU/mL CFU/mL, the mice in the experimental group showed depression and piling up 6h after streptococcal infection and died within 24h. Before death, the mice showed disheveled coat, shortness of breath (Fig. 5), shivering, slow movement, and loss of appetite; timely autopsy of the dead mice showed swollen, congested and hemorrhagic livers, bruised spleens, and a large number of germs with a single colony morphology could be isolated and cultivated in hearts, livers, spleens, lungs, and kidneys, which were stained and microscopically examined as streptococci. The detection of the cps9H gene by the PCR method was positive (Fig. 6).

Fig. 5 Mice showed bunching, disorganized coat

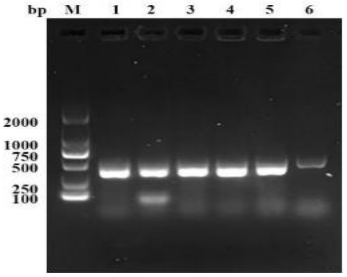

Fig. 6PCR identification of dissected mice

As shown in Table 4, the isolate was highly sensitive to cefthiophene, florfenicol, cefadroxil, ciprofloxacin, ampicillin, amoxicillin, highly sensitive to sulfisoxazole, polymyxin B, enrofloxacin, doxycycline, and resistant to lincomycin, kanamycin, neomycin, gentamicin.

4. Summary

The results of biochemical tests showed that the isolates could ferment maltose, salicylic acid, glucose, sucrose, lactose and other carbohydrates, which was positive; they did not ferment sorbitol, mannitol and arabinose, which was negative, and the VP and indocyanine substrate tests were negative, which was consistent with the biochemical characteristics of streptococci. Still, the results of the biochemical tests showed slight differences due to the difference in serotypes of different

streptococci. Due to the different serotypes of streptococci, the results of biochemical tests were slightly different, and it was difficult to accurately categorize the isolated strains according to their phenotypic characteristics such as morphology, Gram staining characteristics and biochemical tests, so the identification should be carried out by serological methods.

Table 4. Results of drug sensitivity tests for isolates

Drug name	Antibacterial ring diameter	Circle of inhibition diameters of drug-resistant bacteria（mm）			Sensitivity
	（mm）	Resistance（R）	Medium（M）	Sensitive（S）	
Cephalothin	25	≤14	15～17	≥18	S
Sulfisozole	15	≤12	13～16	≥17	M
Frobenicol	23	≤12	13～17	≥18	S
Cefadroxil	22	≤14	15～17	≥18	S
Ciprofloxacin	20	≤15	16～20	≥21	S
Ampicillin	30	≤18	19～25	≥26	S
Lincomycin	0	≤2	4	≥8	R
Kanamycin	13	≤13	14～17	≥18	R
Amoxicillin	40	≤13	14～17	≥18	S
Neomycin	8	≤12	13～16	≥17	R
Polymyxin B	12	≤8	8～11	≥12	M
Enrofloxacin	20	≤18	19～22	≥23	M
Doxycycline	15	≤12	13～16	≥17	M
Gentamycin	0	≤12	13～14	≥15	R

It is known from the literature that the primers of 1, 2, 7 and 9 types of streptococci are commonly used to determine streptococcal serotypes. Therefore, the primers of 1, 2, 7 and 9 types were mainly used to identify the serotypes of streptococci in this experiment. The results showed that the serotype of the isolate was Streptococcus 9. Currently, types 1, 2, 1/2, 7 and 9 are more pathogenic to pigs. Streptococcus suis type 2 is the most prevalent and pathogenic serotype, followed by type 9, type 7 and type 1. In this experiment, the PCR identification of the bacterial podoplanoside gene was used, i.e., different serotype-specific primers of Streptococcus carried out PCR amplification, and it was observed whether different serotype-specific primers of Streptococcus amplified the target bands or not. Finally, the band was found in the primers specific for Streptococcus type 9.

References

[1] Windsor RS, Elliott SD. Streptococcusal infecton in young pigs. IuL.An outbreak of streptococcal meningitis in weaned pigs[J] J Hyg(Lond), 1975, 75(1): 69-78.

[2] Kerdsin A , Akeda Y , Hatrongjit R et al , Oishi K Streptococcus suis serotyping by a new multiplex PCR [J] Med Microbiol , 2014 , 63(Pt6): 824-830(5).

[3] Pan Z , Ma J , Dong W et al . NouLel uLariant serotype of streptococcus suis isolated from piglets with meningitis [J]. Appl EnuLiron Microbiol , 2015 , 81 (3): 976-985.

[4] Ma Kunlong. Short term distributed load forecasting method based on big data. Changsha: Hunan University, 2014.

[5] SHI Biao, LI Yu Xia, YU Xhua, YAN Wang. Short-term load forecasting based on modified particle swarm optimizer and fuzzy neural network model. Systems Engineering-Theory and Practice, 2010, 30(1): 158-160.

[6] Fangfang. Research on power load forecasting based on Improved BP neural network. Harbin Institute of Technology, 2011.

[7] Amjady N. Short-term hourly load forecasting using time series modeling with peak load estimation capability. IEEE Transactions on Power Systems, 2001, 16(4): 798-805.

[8] Ma Kunlong. Short term distributed load forecasting method based on big data. Changsha: Hunan University, 2014.

[9] SHI Biao, LI Yu Xia, YU Xhua, YAN Wang. Short-term load forecasting based on modified particle swarm optimizer and fuzzy neural network model. Systems Engineering-Theory and Practice, 2010, 30(1): 158-160.

[10] Fangfang. Research on power load forecasting based on Improved BP neural network. Harbin Institute of Technology, 2011.

[11] Amjady N. Short-term hourly load forecasting using time series modeling with peak load estimation capability. IEEE Transactions on Power Systems, 2001, 16(4): 798-805.

[12] Ma Kunlong. Short term distributed load forecasting method based on big data. Changsha: Hunan University, 2014.

[13] SHI Biao, LI Yu Xia, YU Xhua, YAN Wang. Short-term load forecasting based on modified particle swarm optimizer and fuzzy neural network model. Systems Engineering-Theory and Practice, 2010, 30(1): 158-160.