

Molecular Serotyping and Resistance of Clinical Strains of *Haemophilus* (*Glaesserella*) *Parasuis* in Chinese Pig Farms From 2016 to 2018.

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Abstract

Haemophilus parasuis (*H. parasuis*) is the etiological agent of Glässer's disease and brings great economic losses to the pig industry. The goal of our research is to reveal the serotypes of *H. parasuis* isolated from large-scale pig farms in China from 2016 to 2018. From 2016 to 2018, 8153 *H. parasuis* field strains were isolated from 14610 clinical samples of sick pigs with clinical symptoms from 26 provinces and cities of China. Among them, 1386 strains were identified as *H. parasuis* by PCR, and the isolation rate was 9.49%. Through multiplex PCR, we showed that type 5/12 and type 4 strains had the highest separation rate, followed by type 13 and type 14 strains. Using disk diffusion method, we found cephalosporin antibiotics and peptide antibiotics all had good inhibitory effect on *H. parasuis*. Our conclusion may play a positive role in the prevention and treatment of *H. parasuis*.

Introduction

Haemophilus parasuis (*H. parasuis*) is a pleomorphic and a member of the family Pasteurellaceae. *H. parasuis* is a NAD-dependent, Gram-negative bacterium (1). *H. parasuis* could cause Glässer's disease, pneumonia and septicemia during different breeding periods (2). *H. parasuis* has brought serious economic losses to the pig industry (3). Scientists have to study the epidemiology and pathogenesis of *H. parasuis*. *H. parasuis* has 15 serovars and a large number of non-typeable (NT) isolates (4). The diversity of the *H. parasuis* genotype makes prevention and treatment particularly difficult, such as the low cross-protection of vaccines and antibiotic resistance (5).

The pathogenesis of *H. parasuis* is very complex, which is related to virulence genes, serum and biofilm production (6). There are many molecular serum-based methods for *H. parasuis*, including agar gel diffusion (AGD), indirect hemagglutination (IHA) (7) and a multiplex PCR (8). To date, a total of 15 serotypes of *H. parasuis* have been identified, including serovars 1 through 15. However, the scientists cannot discriminate between serovars 5 and 12 (8). The serovars 1, 5, 10, 12, 13, and 14 were regarded highly virulent; the serovars 2, 4, 8 and 15 were regarded moderately virulent; and the serovars 3, 6, 7, 9 and 11 were considered low virulence potential (7). The serovars 5 and 4 of *H. parasuis* are widely regarded as pathogenic serums and are the most common serovars isolated from clinically sick pigs worldwide (9).

The abuse of antibiotics and the lack of biosafety knowledge hinder the prevention and control of *H. parasuis* (1). And more Clinical studies have shown that the protection of inactivated vaccine is mainly against isolates of the same serovars, and its cross-protection is extremely limited (10). Therefore, the outbreak of *H. parasuis* due to vaccination failure is a major concern for researchers and pig farmers. In order to prevent and control *H. parasuis* safely and effectively, developing an effective vaccine is still the best choice at present (11, 12). However, the key to the development of a vaccine is to find a highly virulent and widespread strain in the country, so epidemiological investigation is a necessary process.

In this study, we conducted an epidemiological survey of more than 10,000 clinical samples in different provinces of China from 2016 to 2018, and identified 8153 *H. parasuis* strains from 14610 disease materials, among them, 1386 strains were identified as *H. parasuis* by PCR. Besides, we Serotyped 320 *H. parasuis* strains by multiplex PCR, and our results showed that type 5/12 and type 4 strains had the highest proportion, followed by type 13 and type 14 strains.

With the large-scale and intensive development of the pig industry the incidence of *H. parasuis* is increasing year by year, but the vaccine of *H. parasuis* has poor cross-protection between different serotypes. In order to avoid huge economic losses, pig farms have to rely on antibiotics to control this disease. Therefore, it is necessary to screen drugs based on the results of vitro drug sensitivity tests. We studied the drug resistance of *H. parasuis* to 18 common antibiotics by the K-B disc method, and the results showed that the cephalosporin and peptide antibiotics could significantly inhibit the growth of *H. parasuis*.

Our work studied the serotypes of *H. parasuis* isolated from large-scale pig farms in China from 2016 to 2018 and the drug resistance of 166 *H. parasuis* to 18 common antibiotics, which played a vital role for the research of vaccines against prevalent *H. parasuis* strains in our country.

Materials And Methods

Ethics approval and consent to participate

All animal experimental procedures were performed in accordance with the Hubei Regulations for the Administration of Affairs Concerning Experimental Animals. Animal experiments in this study were subject to approval by the Hubei Province Science and Technology Department, concerning experimental animal ethics. The experiments were carried out under the supervision and inspection of the Scientific Ethical Committee for Experimental Animals of Huazhong Agricultural University, Wuhan, China.

Clinical isolates

From January 2016 to December 2018, a total of 14610 clinical samples were collected from 30 to 70 days old pigs suspected of being infected with *H. parasuis*. The clinical samples covered 26 provinces and municipalities. All isolates were from the lungs, heart, brain, joints, or trachea of pigs, and had clinical symptoms similar to *H. parasuis*, such as high fever, arthritis, tremor, incoordination, lying on the side and depression and so on. All clinical samples were stored at -80°C . Detailed information was recorded for each clinical sample, such as viscera, time, location, and clinical symptoms.

Bacterial isolation and identification

Clinical samples were streaked onto tryptic soy agar (TSA) plates (TSA; Becton, Dickinson and Company, Franklin Lakes NJ, USA) containing $10\ \mu\text{g/ml}$ NAD (NAD; Roche, Basel, Switzerland) and 5% newborn calf serum (Gibco, New York, USA), and then incubated at 37°C for 36 h. The suspect colonies should be translucent colonies of 1 mm in diameter and be subjected to further identification by gram staining and PCR (13). Sequences of primers used in the research were showed in table 2.

DNA preparation

For the serotyping of *H. parasuis*, the bacteria were grown in tryptic soy broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for overnight at 37°C and bacteria suspension was spun down at $10,000 \times g$ for 5 min and then resuspended in phosphate buffered saline (pH: 7.4). The suspension was heated at 100°C for 10 min and followed by a centrifugation under $10,000 \times g$ for 5 min. The supernatant was collected to a nucleic acid-free tube and stored at -20°C .

Serotyping

The serovars of *H. parasuis* were determined by PCR (8). The results were confirmed by two repeated experiments. The PCR products were stained with GelRed™ (Biotium, Fremont, CA, USA) and performed gel electrophoresis in a 1.0% agarose gel in Tris-acetate-EDTA (TAE) buffer at 120 volts for about 20–30 min.

Drug susceptibility test

A disk diffusion method was used to evaluate the drug resistance profiles of 166 isolates, the drug sensitive disks used in this research were purchased from Hangzhou Tianhe Microbial Reagent Company. The drugs used mainly include Cefradine (CEF), Ceftriaxone (CRO), Amoxicillin (AML), Ampicillin (AMP), Streptomycin (STR), Gentamicin (GEN), Spectinomycin (SPE), Kanamycin (KAN), Azithromycin (AZM), Levofloxacin (LEV), Ciprofloxacin (CIP), Enrofloxacin (ENO), Polymyxin B (PB), Cefotaxime (CAZ), Cefotaxime (CTX), Amikacin (AMI), Norfloxacin (NOR) and Florfenicol (FLO).

This study refers to the standard CLSI M2 A12 Ed. 12 (2015) "Performance Standards For Antimicrobial Disk Susceptibility Tests" for drug susceptibility testing. The specific method was picking a purified single colony in the ultra-clean workbench and inoculated in TSB medium and cultured in a shaker at 37°C for about 12h forming bacterial suspension, then dipped a sterile cotton swab into the bacterial suspension and spread on a TSA agar plate containing $10\ \mu\text{L/mL}$ NAD and 5% (v/v) inactivated bovine serum, after the plate dry for 3 min, using sterilized tweezers to pick up the drug sensitive disk and place it on the dried plate. There are no more than 6 drug sensitive disk on each plate. At last, placing the plate at 37°C for 24 to 36 h, and measuring the diameter of the inhibition zone.

Detection of resistance genes in *H. parasuis* by PCR

We detected the three resistance genes of *H. parasuis*, including β -lactam antibiotic resistance gene bla_{ROB-1}, quinolone antibiotic resistance gene Aac (6')-1b, and aminoglycoside antibiotic resistance gene AadA1, primers were showed in table 2.

The PCR reaction system was 2×Easy Taq PCR SuperMix (+ dye) 10.0 μL, primer 1(10 μM) 0.5 μL, primer 2 (10 μM) 0.5 μL, template 2 μL, ddH₂O 7.0 μL, total 20 μL. The PCR amplification program was 95 °C for 10 min;94 °C for 30 s;56 °C for 30 s;72 °C for 1 min—total 35 cycles—then 72 °C for 10 min, at last decreased to 4 °C. The PCR products were stained with GelRed™ (Biotium, Fremont, CA, USA) and performed gel electrophoresis in a 1.0% agarose gel in Tris-acetate-EDTA (TAE) buffer at 120 volts for about 20–30 min.

Data analysis

Data from all samples were analyzed as descriptive statistics. All data were statistically analyzed by GraphPad software (GraphPad software®, La Jolla, CA, USA).

Results

Prevalence of *H. parasuis*

We isolated 8153 strains of bacteria from 14,610 samples in 26 provinces from January 2016 to December 2018. Through morphological observation, gram stain (Fig. 1A) and PCR identification (Fig. 1B), 1386 strains of *H. parasuis* were finally confirmed, and the isolation rate was 9.49%.

The site and geographical distribution of *H. parasuis* isolation

Between 2016 and 2018, we made statistics on the separation rate of samples from different tissues in order to find out the isolation of *H. parasuis* from different tissues. We found that the highest bacterial isolation rate of effusion was 27.27% (3/11), followed by 13.23% (1312/9914) of lung. a small amount of *H. parasuis* could also be isolated from joints, spleen, brain and liver, while kidney and heart could not be isolated (Fig. 2A).

In order to find out the situation of *H. parasuis* infection in different areas of China in recent three years, we counted the isolation rate of samples in various provinces. The majority were from Guangdong province (15.3%), Zhejiang province (11.82%) and Hunan province (9.38%) (Fig. 2B).

The separation rates of different *H. parasuis* serotypes

From 2016 to 2018, we identified a total of 1386 strains of *H. parasuis*, including 446 strains in 2016, 561 strains in 2017 years, and 379 strains in 2018 years. We identified the serotypes of *H. parasuis* isolates by PCR (Fig. 3A), while we did not identified strains of serotype 8.

We found that the serotypes were different in different years. In 2016, *H. parasuis* SV4 was the most common isolate in this study (29.13%), followed by serotype 5/12 (39.81% in total) (Fig. 3B). In 2017, the highest prevalence was serotype 4 (23.48%), followed by serotype 5/12 (33.04% in total) and serotype 14 (13.91%) (Fig. 3B). In 2018, the most common serotypes were serotype 5/12 (43.14%) and serotype 4 (23.53%), followed by serotype 13 (11.76%) (Fig. 3B).

Geographical distribution of different *H. parasuis* serotypes

In order to understand the infection of *H. parasuis* in different regions of China in recent years, we made statistics on the separation rate of samples sent by provinces and municipalities from 2016 to 2018—our results were shown in table 1.

Among all provinces with no less than 100 samples submitted for detection in 2016, Guangdong Province accounted for the highest separation rate (16.42%, 146/1941), followed by Hubei Province (9.07%, 176/1941) and Henan Province (8.64%, 28/ 324), In addition, the separation rates of Hunan Province (8.05%, 24/298), Zhejiang Province (7.77%, 8/103), and Sichuan Province (6.36%, 14/220) are successively reduced. In 2017, Guangdong Province accounted for the highest separation rate(16.11%, 168/1043), followed by Shandong Province (12.5%, 23/184) and Hunan Province (12.03%, 35/291), then the separation rate of Guangxi Province (11.88%, 12/101), Hubei Province (8.8%, 161/1829), Henan Province (8.58%, 79/921), Sichuan Province (7.41%, 16/216), Fujian Province (6.06, 12/198), Anhui Province (5.04%, 7/139), Shaanxi Province (4.23%, 3/71), Jiangsu Province (4.1%, 5/122) and Shanxi Province (3.92%, 2/51) decreased in turn. In 2018, Zhejiang Province accounted for the highest separation rate (20.34%,

12/59), followed by Guangdong Province (13.11%, 110/839), Besides, the separation rate of Fujian Province (10.98%, 19/173), Guangxi Province (9.02%, 12/133), Henan Province (7.58%, 75/990), Hunan Province (7.14%, 12/168), Hubei Province (5.41%, 91/1682) and Shandong Province (5.17%, 6/116) decreased in turn.

Among all provinces with no less than 200 samples submitted for detection in three years, Guangdong Province accounted for the highest separation rate (16.42%, 146/1941), followed by Zhejiang Province (11.82%, 26/220), besides, Hunan Province (9.38%, 71/757), Shandong Province (9.12%, 31/340), Guangxi Province (8.93%, 26/291), Fujian Province (8.69%, 39/449), Henan Province (8.14%, 182/2235), Anhui Province (7.88%, 26/330), Hubei Province (7.85%, 428/5452), Sichuan Province (6.9%, 35/507) and Jiangsu Province (6.86%, 19/277) decreased in turn.

The susceptibility Testing Results of *H. parasuis*

The susceptibility Testing Results of *H. parasuis* were shown in table 3, as seen from this table, *H. parasuis* was more sensitivity to macrolide antibiotics, polypeptide antibiotics, chloromycetin, and β -lactam antibiotics (except ampicillin), among the 18 selected drugs tested, *H. parasuis* showed the highest sensitivity to polymyxin B (96.99%, 161/166) and cefradine (96.39%, 160/166), followed by ceftriaxone (92.17%, 153/166), florfenicol (91.57%, 152/166), cefotaxime (84.34%, 140/166), ceftazidime (82.53%, 137/166) and azithromycin (80.72%, 134/166). At the same time, *H. parasuis* was resistant to ciprofloxacin (54.82%, 91/166), streptomycin (51.20%, 85/166), ampicillin (48.80%, 81/166), norfloxacin (36.14%, 60/166), amikacin (35.54%, 59/166) and levofloxacin (33.73%, 56/166).

Drug resistance profiles of *H. parasuis* isolates

The drug sensitivity analysis of 166 *H. parasuis* isolates found that 18 tested drugs included a total of 94 drug resistance profiles. As shown in table 4, only 5 isolates were sensitive to all tested drugs, 10 isolates were resistant to 1 tested drug, 15 isolates were resistant to 2 tested drugs, 33 isolates were resistant to 3 tested drugs, and 39 isolates were resistant to 4 tested drugs resistance, 21 isolates were resistant to 5 tested drugs, 16 isolates were resistant to 6 tested drugs, 13 isolates were resistant to 7 tested drugs, 10 The isolates were resistant to 8 tested drugs, the tested isolates were mainly resistant to 3-5 tested drugs.

Discussion

H. parasuis is a Gram-negative, nicotinamide adenine dinucleotide dependent bacterium. Who can cause Glässer's disease in pigs, (14). *H. parasuis* usually appear in swine respiratory tract, causes systemic infections, pneumonia, fibrin polyserositis, polyarthritis and meningitis (15). This bacterial infectious disease can infect pigs of any age, which brings serious influences to the pig breeding industry. Although the mortality rate of pigs is relatively low, it will seriously affect the disease resistance of the pig herd, resulting a decline of the immune abilities, and it is easy to be infected with a variety of infectious diseases, causing complex clinical symptoms, and bringing great difficulty to disease diagnosis. At present, prevention and control of *H. parasuis* are difficult, the trend of large-scale and intensive development of the pig industry in our country is becoming more and more obvious recent years, and the prevalence of *H. parasuis* diseases is becoming diversified and complicated, which often presents as a secondary or mixed infection, and brings great difficulties to the diagnosis and treatment of diseases. In order to reduce the economic damage caused by Glässer's disease, a kind of inactivated whole cell vaccine is widely used in the world (16). While inactivated whole cell vaccine does not produce local immunity and cell-mediated immunity ability is weak, so the immunity is slower, and good immunity is usually obtained 2 weeks after vaccination. Now, subunit vaccine is currently the best research direction(14).

Our work studied the serotypes of *H. parasuis* isolated from large-scale pig farms in China from 2016 to 2018, which played a vital role for the research of vaccines against prevalent *H. parasuis* strains in our country. *H. parasuis* isolates accurately would help to prevent and control Glässer's disease through appropriate vaccination in specific geographic areas.

we first identified 8153 strains of *H. parasuis* from 14610 disease materials derived from 26 provinces and cities of China, our sample size and typing method are larger and more representative. We Serotyped 320 *H. parasuis* strains by multiplex PCR. Molecular typing is an excellent alternative test compared to regular serotyping (gel immunodiffusion, Kielstein and Rapp-Gabrielson scheme), which is very cumbersome to perform because of the necessity of producing specific anti sera (16). The Kielstein-Rapp-Gabrielson agar diffusion method for serotyping of *H. parasuis* is classic serological typing method, which can identify 15 serotypes, but 15 high immune serums are required, and about 20% of strains cannot be typed.

This study used the multiple PCR molecular serotyping method established by Howell et al in 2015 to serotype 320 *H. parasuis* isolates. The results showed that only 10% of the isolates could not be typed. The molecular method has the advantages of simple operation and less time-consuming in the mass identification of serotypes, but it is worth noting that this method cannot distinguish between serotype 5 and serotype 12. Using a multiplex PCR assay (8) and a specific PCR reaction for the *H. parasuis* serotyping were more precise.

According to the research on the serotype of *H. parasuis*, the most prevalent serotypes were serotype 5, followed by serotype 2 and serotype 4 in Quang Binh and Thua Thien Hue provinces in Central Vietnam (17). One study about the prevalence and characteristics of *H. parasuis* from healthy pigs in China from 2016 to 2017 showed that the most prevalent serovars were 7, followed by 3, 2, 11, 5/12 and 4 (18). Besides, there were other research about the most prevalent serotypes of *H. parasuis*, 4, 5, 12, 13, NT (nontypeable isolates), and 2 were the most prevalent strains in southern China(19). One research in Sichuan province of China showed that Serovars 5 (25.98%) and 4 (23.62%) were the most prevalent Serotypes (3). Our results of serotyping showed that serotype 4 (25.31%) and serotype 5/12 (38.44%) were the most popular strains in China, followed by serotype 13 (7.81%) and serotype 14 (6.56%). Only a few strains of types 1, 2, 6, 7, 9, 10, and 15 had been identified. Serotypes 3, 8, and 11 were not identified in this study. This might be because the collected disease materials mainly come from sick pigs with suspected symptoms of *H. parasuis*. In the research of Cai Xuwang, KPG agar diffusion test identified the existence of *H. parasuis* serotype 11 isolates in China from 2002 to 2004. Zhou Xueli used the same method identified the existence of *H. parasuis* serotype 3 and serotype 8 in China from 2007 to 2008. Up to now, 15 serotypes of *H. parasuis* were distributed in China. Most of the isolates used for serotyping in this study were from Guangdong, Hubei and Henan provinces, while the number of samples submitted for inspection in other provinces was relatively small and not representative. In these three provinces, serotype 4 and serotype 5/12 were the most prevalent. In addition, serotypes 1, 13, and 14 were prevalent in Guangdong and Henan provinces; serotypes 1, 13, 14, and 15 were prevalent in Hubei province. From the statistical results, the main serotypes in Henan, Hubei provinces and Guangdong Province were roughly same, and the percentages of each serotype were slightly different, but the most prevalent serotypes in China were types 4 and 5(20). While one previous study also studied the isolation of *H. parasuis* in China from September 2016 to October 2017, they obtained 244 isolates from 1675 nasal samples from 6 provinces, *H. parasuis* isolation was more successful in weaner pigs (22.6%, 192/849), followed by finisher pigs (9.3%, 43/463), and sows (2.5%, 9/363). The most prevalent serovar was type 7 (20.1%, 49/244), followed by type 3 (14.8%, 36/244), type 2 (14.3%, 35/244), type 11 (12.7%, 31/244), type 5/12 (5.7%, 14/244) and type 4 (2.5%, 6/244)(21).

Our research also showed that annual isolation rate of *H. parasuis* ranged from 7.36–12.34%. The months with higher isolation rates were December, January, February, March, and April, and the isolation rates showed a clear upward trend in October and November.

Combined with data analysis over the past three years, the incidence of *H. parasuis* in coastal areas of high temperature and humidity such as Guangdong, Fujian, Zhejiang and Guangxi were relatively high. This may be due to the local climate was suitable for bacterial growth and reproduction, because climate was an important factor affecting the growth of bacteria(22).

Most Clinical studies had shown that the protection of inactivated vaccine was mainly against isolates of the same serovars, and its cross-protection was extremely limited (10), for this reason, choosing inactivated vaccines of local epidemic serotypes was more beneficial to defend against *H. parasuis*. In different regions and a combination of protective antigens might be able to provide effective protection against multiple *H. parasuis* serovars(23).

The susceptibility testing results showed that *H. parasuis* was more sensitivity to macrolide antibiotics, polypeptide antibiotics, chloromycetin, and β -lactam antibiotics (except ampicillin) among all 18 selected drugs tested, *H. parasuis* showed the highest sensitivity to polymyxin B and cefradine, followed by ceftriaxone, florfenicol, cefotaxime, ceftazidime and azithromycin. At the same time, *H. parasuis* was resistant to ciprofloxacin, streptomycin, ampicillin, norfloxacin, amikacin and levofloxacin. The drug sensitivity analysis of 166 *H. parasuis* isolates found that 18 drugs tested included a total of 94 drug resistance profiles. Only 5 isolates were sensitive to all tested drugs.

Now, abuse of antimicrobials in farmed animals was a hazard to humans, besides, *H. parasuis* clinical isolates had been reported to exhibit resistance to several antibiotics (24). So, vaccines were preferred method in control and elimination of Glässer Disease. However, the current vaccines of *H. parasuis* were too poor, and cannot prevented *H. parasuis* disease. Recently, it was reported that

subunit vaccines were better choice for treating diseases, However, in contrast to other diseases of similar importance, there were few effective subunit vaccines(25), in the light of our work could provide reference and basis for prevention and control of *H. parasuis*, the follow-up researcher could combine the prevailing trend of the dominant strains in this article, and prepared targeted vaccines.

Our work studied the serotypes of *H. parasuis* isolated from large-scale pig farms in China from 2016 to 2018 and the resistance characteristics of isolates, which played a vital role for the research of vaccines against prevalent *H. parasuis* strains in our country, and serotype *H. parasuis* isolates accurately would help to prevent and control Glässer's disease outbreaks through appropriate vaccination.

Conclusion

Haemophilus parasuis (*H. parasuis*), the causative agent of Glässer's disease, which seriously affected the global pig breeding industry. Our research showed a national trend of *H. parasuis* in China, our study was carried on from 2016 to 2018 and 8153 *H. parasuis* field strains were isolated from 14610 clinical samples collected from sick pigs with clinical symptoms in 26 provinces and cities of China, among them, 1386 strains were identified as *H. parasuis* by PCR, and the isolation rate was 9.49%.

We Serotyped 320 *H. parasuis* strains by multiplex PCR, and our results showed that type 5/12 and type 4 strains had the highest proportion respectively were 38.44% and 25.31%, followed by type 13 and type 14 strains respectively were 7.81% and 6.56%, besides, 10% of isolates cannot be typed by this method.

Our antimicrobial susceptibility test showed that *H. parasuis* was very sensitivity to polymyxin B and cefradine, then ceftriaxone, florfenicol, cefotaxime, ceftazidime and azithromycin. At the same time, *H. parasuis* was resistant to ciprofloxacin, streptomycin, ampicillin, norfloxacin, amikacin and levofloxacin.

In general, our results revealed the diversity and distribution of different serotypes of *H. parasuis* across the country and the resistance characteristics of isolates, which were essential for the prevention and treatment of *H. parasuis* in our country.

Declarations

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ADDITIONAL INFORMATION AND DECLARATIONS

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Author Contributions

Jinjin Liu designed and performed the experiments, analyzed the data, and wrote the manuscript. Long Guo, Yi Yuan, Wenbo Song, Qianqian Li, Ying Huang, Yunzhi Long, Liu Yang and Gong Liang performed the experiments. Chao Huang and Xibiao Tang conceived the project, analyzed the data, and wrote the manuscript.

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Tables

Table 1 Isolation rate of *Haemophilus parasuis* in different provinces of China from 2016 to 2018

Province	Number of samples				Number of positive samples				Isolation rate(%)			
	2016	2017	2018	Total	2016	2017	2018	Total	2016	2017	2018	Total
Hu Bei	1941	1829	1682	5452	176	161	91	428	9.07	8.80	5.41	7.85
GuangDong	889	1043	839	2771	146	168	110	424	16.42	16.11	13.11	15.30
He Nan	324	921	990	2235	28	79	75	182	8.64	8.58	7.58	8.14
Hu Nan	298	291	168	757	24	35	12	71	8.05	12.03	7.14	9.38
Si Chuan	220	216	71	507	14	16	5	35	6.36	7.41	7.04	6.90
Fu Jian	78	198	173	449	8	12	19	39	10.26	6.06	10.98	8.69
Shan Dong	40	184	116	340	2	23	6	31	5.00	12.50	5.17	9.12
An Hui	97	139	94	330	7	7	12	26	7.22	5.04	12.77	7.88
Guang Xi	57	101	133	291	2	12	12	26	3.51	11.88	9.02	8.93
Jiang Su	63	122	92	277	4	5	10	19	6.35	4.10	10.87	6.86
Zhe Jiang	103	58	59	220	8	6	12	26	7.77	10.34	20.34	11.82
Jiang Xi	85	58	45	188	7	3	5	15	8.24	5.17	11.11	7.98
He Bei	20	77	90	187	2	9	8	19	10.00	11.69	8.89	10.16
Shan Xi	73	51	15	139	10	2	1	13	13.70	3.92	6.67	9.35
San Xi	54	71	0	125	4	3	0	7	7.41	4.23	0	5.60
Gui Zhou	2	39	18	59	0	4	0	4	0	10.26	0	6.78
Xin Jiang	2	35	22	59	0	5	0	5	0	14.29	0	8.47
Nei Meng	20	24	11	55	0	5	0	5	0	20.83	0	9.09
Liao Ning	16	17	6	39	1	3	0	4	6.25	17.65	0	10.26
Hai Nan	10	13	12	35	0	2	0	2	0	15.38	0	5.71
Chong Qing	9	20	0	29	0	0	0	0	0	0	0	0
Shang Hai	6	12	6	24	1	1	1	3	16.67	8.33	16.67	12.50
Gan Su	20	3	0	23	2	0	0	2	10.00	0	0	8.70
Bei Jing	0	7	2	9	0	0	0	0	0	0	0	0
Tian Jin	8	0	0	8	0	0	0	0	0	0	0	0
Ji Lin	2	0	0	2	0	0	0	0	0	0	0	0

Table 2 Sequences of primers used in this research

Primers	Primer sequence (5'→3')	PCR product / bp	Annealing temperature (°C)
Hps-F	ACAACCTGCAAGTACTTATCGGGAT	275	58
Hps-R	TAGCCTCCTGTCTGATATCCCACG		
<i>funB</i> -F	CTGTGTATAATCTATCCCCGATCATCAGC	180	58
<i>funB</i> -R	GTCCAACAGAATTTGGACCAATTCCTG		
<i>wzx</i> -F	CTAACAAGTTAGGTATGGAGGGTTTTGGTG	295	58
<i>wzx</i> -R	GGCACTGAATAAGGGATAATTGTACTG		
<i>glyC</i> -F	CATGGTGTTTATCCTGACTTGGCTGT	650	58
<i>glyC</i> -R	TCCACATGAGGCCGCTTCTAATATACT		
<i>wciP</i> -F	GGTTAAGAGGTAGAGCTAAGAATAGAGG	320	58
<i>wciP</i> -R	CTTTCCACAACAGCTCTAGAAACC		
<i>wcwK</i> -F	CCACTGGATAGAGAGTGGCAGG	450	58
<i>wcwK</i> -R	CCATACATCTGAATTCCTAAGC		
<i>gltI</i> -F	GATTCTGATGATTTTTGGCTGACGGAACG	360	58
<i>gltI</i> -R	CCTATTCTGTCTATAAGCATAGACAGGAC		
<i>funQ</i> -F	CTCCGATTTTCATCTTTTCTATGTGG	490	58
<i>funQ</i> -R	CGATAAACCATACAATTCTGGCAC		
<i>scdA</i> -F	GGAAGGGGATTACTACTACCTGAAAG	650	58
<i>scdA</i> -R	CTCCATAGAACCTGCTGCTTGAG		
<i>funV</i> -F	AGCCACATCAATTTTAGCCTCATCA	710	58
<i>funV</i> -R	CCTTAAATAGCCTATGTCTGTACC		
<i>funX</i> -F	GGTGACATTTATGGGCGAGTAAGTC	790	58
<i>funX</i> -R	GCACTGTCATCAATAACAATCTTAAGACG		
<i>amtA</i> -F	CCATCTCTTTAACTAATGGGACTG	890	58
<i>amtA</i> -R	GGACGCCAAGGAGTATTATCAAATG		
<i>gltP</i> -F	GCTGGAGGAGTTGAAAGAGTTGTTAC	840	58
<i>gltP</i> -R	CAATCAAATGAAACAACAGGAAGC		
<i>funAB</i> -F	GCTGGTTATGACTATTTCTTTTCGCG	730	58
<i>funAB</i> -R	GCTCCCAAGATTAAACCACAAGCAAG		
<i>funI</i> -F	CAAGTTCGGATTGGGAGCATATATC	550	58
<i>funI</i> -R	CCTATATCATTGTTGGATGTACG		
<i>bla_{ROB-1}</i> -F	CATTAACGGCTTGTTCCG	852	56
<i>bla_{ROB-1}</i> -R	CTTGCTTTGCTGCATCTTC		
<i>Aac⁶⁷-1b</i> -F	TATGAGTGGCTAAATCGA	394	54

<i>Aac</i> 67-1b-R	CCCGCTTTCTCGTAGCA		
<i>AadA</i> I-F	TTTGCTGGTTACGGTGAC	497	54
<i>AadA</i> I-R	GCTCCATTGCCAGTCG		
ERIC-F	ATGTAAGCTCCTGGGATTAC	/	52
ERIC-R	AAGTAAGTGACTGGGGTGAGCG		

Table 3 Drug sensitivity of *H. parasuis* isolates in China

Antibiotics category	Drug sensitive slips	Sensitive rates	Resistance rates (%)
β-lactams	Amoxicillin	79.52	9.64
	Ampicillin	43.98	48.80
	Cefradine	96.39	3.01
	Ceftriaxone	92.17	7.83
	Cefotaxime	84.34	12.05
	Cefotaxime	82.53	8.43
aminoglycosides	Amikacin	50.60	35.54
	Kanamycin	45.18	29.52
	Streptomycin	36.75	51.20
	Gentamicin	71.69	21.08
	Spectinomycin	60.84	29.52
macrolides	Azithromycin	80.72	13.86
polypeptides	Polymyxin B	96.99	3.01
quinolones	Enrofloxacin	78.31	21.08
	Ciprofloxacin	44.58	54.82
	Norfloxacin	59.04	36.14
	Levofloxacin	63.86	33.73
chloramphenicol	Florfenicol	91.57	8.43

Table 4 Antimicrobial resistance profiles of *H. parasuis* isolates (2016-2018)

Number of isolates	Number of antimicrobial agents	Resistance phenotype
0	No antimicrobial resistance	5
1	AMI	1
1	AMP	2
1	KAN	2
1	STR	3
1	NOR	1
1	CRO	1
2	ENO+LEV	1
2	CIP+STR	1
2	KAN+AMP	1
2	STR+AMI	1
2	GEN+AMI	2
2	GEN+SPE	1
2	CRO+AMP	1
2	CAZ+STR	1
2	LEV+NOR	5
2	SPE+GEN	1
3	AMP+CIP+STR	2
3	AMP+NOR+AZM	1
3	ENO+CIP+STR	1
3	ENO+CIP+NOR	4
3	CIP+STR+AMI	1
3	KAN+AMP+AML	2
3	KAN+AMP+STR	1
3	KAN+CIP+STR	1
3	KAN+GEN+AZM	1
3	KAN+GEN+STR	2
3	STR+AZM+AMI	1
3	LEV+AMP+CIP	5
3	LEV+AMP+NOR	1
3	SPE+AMP+AMI	1
3	SPE+AMP+CIP	3
3	SPE+AMP+STR	6
4	AMP+CIP+NOR+AZM	2
4	AMP+SPE+CAZ+NOR	1
4	ENO+LEV+AMP+AMI	1

4	FLO+STR+AZM+AMI	4
4	KAN+CIP+AMP+STR	1
4	KAN+STR+AZM+AMI	1
4	KAN+GEN+STR+AMI	4
4	KAN+LEV+AMP+CIP	1
4	CRO+CIP+AMI+AML	1
4	CRO+LEV+AMP+CIP	1
4	CTX+SPE+CRO+AMP	1
4	LEV+AMP+CIP+STR	1
4	LEV+CIP+STR+AMI	2
4	LEV+CIP+NOR+AMI	1
4	LEV+CIP+NOR+AZM	4
4	LEV+STR+CIP+AMI	2
4	SPE+AMP+CIP+STR	10
4	SPE+GEN+LEV+CIP	1
5	AMP+CIP+NOR+AZM+AML	1
5	ENO+LEV+AMP+CIP+AMI	2
5	ENO+LEV+CIP+NOR+AMI	4
5	KAN+AMI+NOR+LEV+CIP	1
5	KAN+FLO+STR+AZM+AMI	3
5	KAN+GEN+CIP+STR+AMI	1
5	KAN+GEN+CRO+STR+AML	1
5	KAN+LEV+CIP+NOR+AMI	3
5	KAN+SPE+LEV+AMP+STR	1
5	GEN+ENO+AMP+CIP+AMI	1
5	CTX+FLO+CIP+NOR+AMI	1
5	LEV+CIP+NOR+AZM+AMI	1
5	SPE+ENO+FLO+NOR+AMI	1
6	AMP+SPE+KAN+CTX+NOR+CIP	1
6	KAN+CEF+AMP+CIP+STR+AML	1
6	KAN+GEN+LEV+STR+AZM+AMI	1
6	KAN+CTX+SPE+AMP+CIP+NOR	3
6	KAN+SPE+PB+AMP+STR+AMI	1
6	KAN+SPE+GEN+NOR+STR+AMI	2
6	GEN+ENO+LEV+CIP+STR+AMI	1
6	CTX+ENO+LEV+AMP+CIP+NOR	1

6	CAZ+KAN+GEN+LEV+AMP+STR	2
6	SPE+ENO+AMP+CIP+NOR+STR	2
6	SPE+ENO+CRO+AMP+STR+AMI	1
7	KAN+CEF+AMP+CIP+NOR+STR+AMI	1
7	KAN+GEN+ENO+LEV+AMP+CIP+STR	1
7	GEN+ENO+LEV+CIP+NOR+STR+AMI	4
7	CAZ+KAN+GEN+CRO+LEV+AMP+STR	1
7	CAZ+KAN+SPE+CRO+AMP+NOR+STR	1
7	CAZ+CTX+SPE+GEN+AMP+CIP+NOR	1
7	CAZ+CTX+SPE+LEV+AMP+CIP+AML	2
7	CAZ+KAN+SPE+LEV+AMP+STR+AML	1
7	SPE+GEN+ENO+CIP+NOR+STR+AMI	1
8	KAN+PB+CEF+CRO+AMP+NOR+STR+AMI	1
8	KAN+GEN+ENO+LEV+AMP+CIP+STR+AML	2
8	KAN+SPE+ENO+CRO+AMP+CIP+NOR+AML	1
8	CTX+PB+ENO+FLO+CIP+NOR+STR+AMI	3
8	CTX+SPE+GEN+AMP+CIP+NOR+STR+AZM	2
8	CAZ+CTX+SPE+CEF+AMP+CIP+NOR+AML	1
9	KAN+CTX+GEN+ENO+AMP+CIP+NOR+STR+AML	1
10	CAZ+CTX+GEN+ENO+LEV+AMP+CIP+NOR+STR+AMI	1
11	CAZ+CTX+SPE+ENO+CEF+CRO+FLO+AMP+NOR+STR+AML	1
13	CAZ+KAN+CTX+SPE+CRO+FLO+LEV+AMP+CIP+STR+AMI+AZM+AML	1

Table note: CEF, Cefradine; CRP, Ceftriaxone; AML, Amoxicillin; AMP, Ampicillin; STR, Streptomycin; GEN, Gentamicin; SPE, Spectinomycin; KAN, Kanamycin; AMI, Amikacin; AZM, Azithromycin; LEV, Levofloxacin; CIP, Ciprofloxacin; ENO, Enrofloxacin; PB, Polymyxin B; CAZ, Ceftazidime; CTX, Cefotaxime; NOR, Norfloxacin; FLO, Florfenicol.

Figures

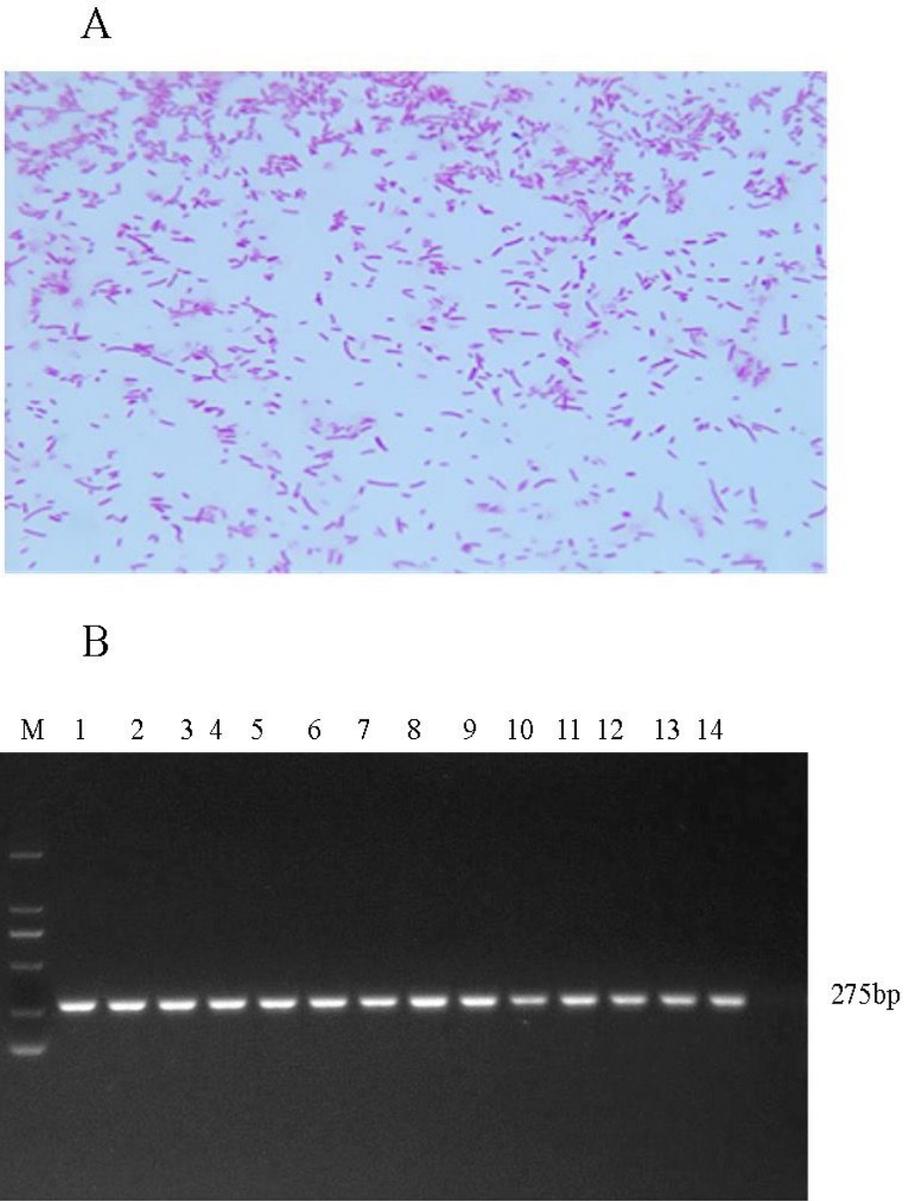


Figure 1

Prevalence of *H. parasuis*. The *H. parasuis* was identified by gram stain (A) and PCR (B).

A

	Lung	Joint	Effusion	Spleen	Brain	Liver	Kidney	Heart
Number of sick materials	9914	1638	11	265	1914	554	189	125
Number of positive disease materials	1312	38	3	2	29	2	0	0
Separation rate (%)	13.23	2.32	27.27	0.75	1.52	0.36	0	0

B

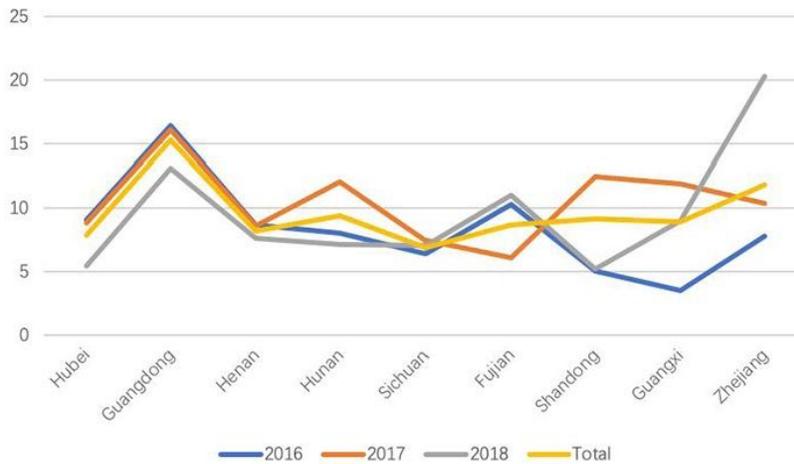
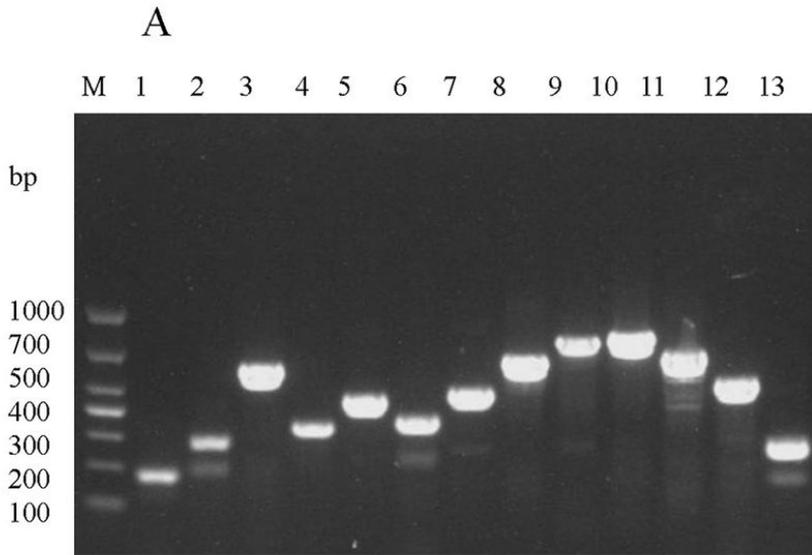


Figure 2

The site and geographical distribution of *H. parasuis* isolation. The *H. parasuis* was separated from different organs, such lung, joint, effusion, spleen, brain, liver, kidney and heart (A). The nine provinces in China where *H. parasuis* are most commonly distributed (B).



B

serovars	Number of bacteria				Percent (%)			
	2016	2017	2018	Total	2016	2017	2018	Total
1	5	7	5	17	4.85	6.09	4.90	5.31
2	5	2	0	7	4.85	1.74	0.00	2.19
4	30	27	24	81	29.13	23.48	23.53	25.31
5/12	41	38	44	123	39.81	33.04	43.14	38.44
6	1	1	0	2	0.97	0.87	0.00	0.63
7	0	2	0	2	0.00	1.74	0.00	0.63
9	1	1	1	3	0.97	0.87	0.98	0.94
10	0	1	0	1	0.00	0.87	0.00	0.31
13	6	7	12	25	5.83	6.09	11.76	7.81
14	1	16	4	21	0.97	13.91	3.92	6.56
15	1	1	4	6	0.97	0.87	3.92	1.88
NT ^a	12	12	8	32	11.65	10.43	7.84	10.00

Figure 3

The separation rates of different *H. parasuis* serotypes. The *H. parasuis* serovars were identified by PCR (A). Distribution of 13 *H. parasuis* serovars in China from 2016 to 2018 (B).