# Prevalence and seroepidemiology of *Haemophilus parasuis* in Sichuan province, China

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## ABSTRACT

*Haemophilus parasuis*, the causative agent of Glässer's disease, has been reported as widespread, but little is known about its epidemiology in the Sichuan province of China. The goal of our research is to reveal the prevalence and distribution of *H. parasuis* in this area. Sampling and isolation were performed across Sichuan; isolates were processed using serotyping multiplex PCR (serotyping-mPCR) and agar gel diffusion (AGD) for confirmation of serovar identity. This study was carried out from January 2014 to May 2016 and 254 *H. parasuis* field strains were isolated from 576 clinical samples collected from pigs displaying clinical symptoms. The isolation frequency was 44.10%. Statistically very significant differences of infection incidence were found in three age groups ($P < 0.01$) and different seasons ($P < 0.01$). Serovars 5 (25.98%) and 4 (23.62%) were the most prevalent, however, non-typeable isolates accounted for nearly 7.87%. In terms of geographical distribution, serovars 5 and 4 were mostly prevalent in west and east Sichuan. The results confirmed that the combined approach was dependable and revealed the diversity and distribution of serovars in Sichuan province, which is vital for efforts aimed at developing vaccine candidates allowing for the prevention or control of *H. parasuis* outbreaks.

**Subjects** Microbiology, Veterinary Medicine, Epidemiology
**Keywords** *Haemophilus parasuis*, Seroepidemiology, Serovar

## INTRODUCTION

*Haemophilus parasuis*, a member of the family Pasteurellaceae, not only colonises in the upper respiratory tract of healthy pigs during different breeding periods, but also emerges as the aetiological agent of Glässer's disease in some cases (*Oliveira & Pijoan, 2004*). This organism incurs significant economic losses within the swine industry. Therefore, efforts have been in studying the epidemiology and pathogenesis of this microbe. So far, 15 serovars and a considerable number of non-typeable isolates have been identified. Various molecular approaches have revealed the diversity in genotypic lineage and have shed light on the heterogeneity of pathogenic *H. parasuis* serovars (*Olvera, Calsamiglia & Aragon, 2006*; *Del Río et al., 2006*; *Zhang et al., 2011*).

Traditionally, agar gel diffusion (AGD) and indirect hemagglutination (IHA) are used to serotype *H. parasuis* field isolates (*Kielstein & Rapp-Gabrielson, 1992*; *Del Río,

*Gutiérrez & Rodríguez Ferri, 2003*). In addition, the latter was once proposed as a more discriminatory method for improving the rate of serotyping (*Turni & Blackall, 2005*). Subsequently, a multiplex PCR was reported for rapid molecular serotyping based on the analysis of variation within the capsule loci (*Howell et al., 2015*). Each serovar has its own distinctive markers except serovars 5 and 12, for they share the same marker. AGD using specific hyperimmune antisera may be applied to remedy the serotyping issues seen in distinguishing between serovars 5 and 12.

Although serovars 1, 2, 3, 4, 5, 8, 10, 12, 13, 14 and 15 are considered to be virulent, production of cross-protecting vaccines among different serovars has met with little success (*Rapp-Gabrielson et al., 1997*; *Brockmeier et al., 2013*). Commercial vaccines for use in swine seldom match the causative serovars and inoculation tends to be ineffective, illustrating the fact the commercial vaccines have, thus far, proven incapable of serovar cross protection in China. Detailed information of morbidity and seroepidemiology can play an important role in prevention when applied in conjuction with research aimed at uncovering potential antigenic markers that are highly conserved across multiple serovars. As no detailed report of *H. parasuis* infection was available in Sichuan province, China, we investigated the prevalence and seroepidemiology of *H. parasuis* in Sichuan province from January 2014 to May 2016.

## MATERIALS AND METHODS

### Reference strains and hyperimmune antisera
Reference strains of serovars 5 and 12 were purchased from Bacteriology Research Laboratory, Animal Research Institute, Yeerongpilly 4105, Australia. Subsequently, corresponding hyperimmune reference antisera of rabbit source were prepared (*Kielstein & Rapp-Gabrielson, 1992*).

### Collection of samples
From January 2014 to May 2016, a total of 576 clinical samples were collected from 576 weak or moribund pigs showing respiratory distress or arthritis. The survey focused on 103 intensive swine farms in the Sichuan province. The farms covered 28 counties of 13 cities of Sichuan province. Incidentally, the province was divided into two parts artificially by climate rather than vertical division. The annual average temperature of the northeast region was a bit higher than that of the southwest region in Sichuan province.

All pigs were necropsied humanely. Lungs, heart blood, joints and brain were derived from pigs showing signs of acute infection or septicemia, while lungs, pericardium liquid, pleural effusion, seroperitoneum and joint fluid as well as fibrinous membrane were collected from pigs displaying relatively mild symptoms (*Turni & Blackall, 2007*). At least two tissues or exudates per pig were analyzed in the present study. Different tissues or exudates from the same pig were considered to be one sample. Additionally, all samples were classified by age groups, seasons and regions.

### Isolation and identification
Samples were inoculated on tryptic soy agar (TSA; Becton, Dickinson and Company, Franklin Lakes NJ, USA) with a final concentration of 5% newborn calf serum and 1 µg/ml

of nicotinamide adenine dinucleotide (NAD; Roche, Basel, Switzerland). The plates were incubated for at least 24 h at 37 °C under aerobic conditions. Translucent colonies of 1 mm in diameter were further passaged. Subsequently, Gram staining was carried out and culture with a typical morphology of *H. parasuis* such as threadless and medium or short rod was confirmed by PCR as described elsewhere (*Oliveira, Galina & Pijoan, 2001*). Moreover, strains isolated from different tissues of a pig were counted as one isolate.

### Serotyping

First, all the isolates were serotyped by multiplex PCR using molecular markers and reaction procedure as previously described (*Howell et al., 2015*). As noted previously, AGD was performed to differentiate between serovars 5 and 12. Pure cultures of the isolates with the marker of serovars 5 and 12 were grown in tryptic soy broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and cells were harvested by centrifugation at under $8,000 \times$ g for 5 min, and then resuspended in phosphate buffered saline (pH: 7.4); cell concentration was adjusted ($OD_{600} = 1.2 \pm 0.1$). The cells was heated at 121 °C for 2 h and followed by a centrifugation under $6,000 \times g$ for 5 min and supernatant was collected as heat-stable antigen. Using the hyperimmune antisera against serovars 5 and 12 earlier prepared, AGD was performed with 3% agarose by using heat-stable antigens of standard strains as positive controls, ultrapure water as a blank control and *Actinobacillus pleuropneumoniae* as a negative control. A single immune line between antiserum holes and heat-stable antigen holes indicated positives. Strains of the same serovar from a single farm were counted as one isolate.

### Statistical analysis

Data was statistically analyzed by the procedure of IBM SPSS Statistics 19.0, and differences between age groups, seasons and regions were performed by Chi-square analysis. All tests were 2-sided and $P < 0.01$ was defined as a very significant difference while $P < 0.05$ was defined as a significant difference.

## RESULTS

### Bacterial strains

In the present investigation, varying degrees of respiratory symptom and classic Glässer's disease were found in herds of the intensive swine farms (Fig. 1). A total of 254 field *H. parasuis* isolates were confirmed via viewing culture morphology characteristics and 16S rRNA PCR results, with the isolation rate of 44.10%. The infection incidence of *H. parasuis* was 45.98% and 43.77% in 2014 and 2015, respectively, and 41.10% was measured in the first five months of 2016, showing no significant differences ($\chi^2 = 0.531$, $P > 0.05$). However, infection incidence varied with age, season and region In pigs of 3-8 weeks, the general infection incidence was 60.80%, which was much higher than that of pigs of less than three weeks or over eight weeks. Statistically very significant differences were found in three age groups as whole ($\chi^2 = 108.209$, $P < 0.01$).

Moreover, infection incidence in winter and spring were much higher than the other two seasons, and statistical differences were very significant ($\chi^2 = 27.854$, $P < 0.01$)
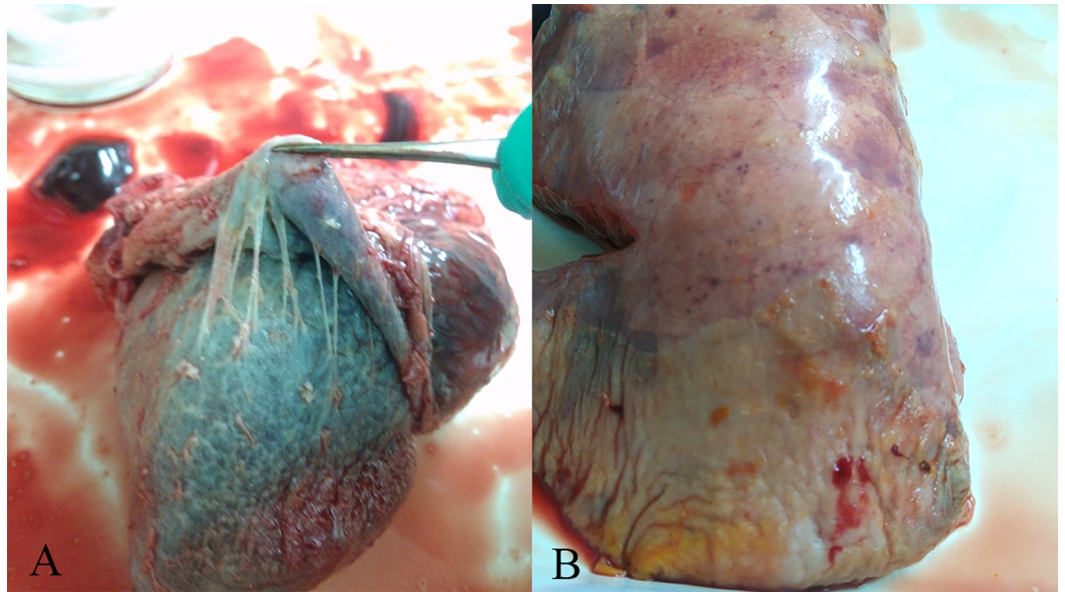
**Figure 1** **Gross lesions in *H. parasuis* infected pigs.** (A) Fibrinous pericarditis. (B) Fibrinous pleuritis.

**Table 1** *Haemophilus parasuis* infections in Sichuan province, China (January, 2014–May, 2016).

| Year | Total | Age groups (weeks) | | | Seasons | | | | Regions | |
|------|-------|------|------|------|--------|--------|--------|--------|------|------|
| | | <3 | 3–8 | >8 | Winter | Spring | Summer | Autumn | East | West |
| 2014 | 80/174[*] | 2/24 | 65/104 | 13/46 | 29/61 | 45/86 | 6/19 | 0/8 | 20/45 | 60/129 |
| 2015 | 144/329 | 3/51 | 120/186 | 21/92 | 67/136 | 52/93 | 7/47 | 18/53 | 32/83 | 112/246 |
| 2016 | 30/73 | 1/7 | 29/62 | 0/4 | 9/25 | 21/48 | / | / | 0/10 | 30/63 |
| Total | 254/576 | 6/82 | 214/352 | 34/142 | 105/222 | 118/227 | 13/66 | 18/61 | 52/138 | 202/438 |

**Notes.**
*A/B, A represents number of strains, and B represents number of samples.

which indicated winter and spring were the major epidemic seasons of *H. parasuis* in Sichuan province. However, no significant differences were seen in two regions as whole ($\chi^2 = 3.031$, $P > 0.05$) (Table 1).

## mPCR and AGD for serotyping

Follow-up serotype-mPCR and complementary AGD examination classified them into 11 serovars, which showed a considerable serovar diversity (Fig. 2; Fig. 3). Serovar 5 (66 of 254, 25.98%) and serovar 4 (60 of 254, 23.62%) were most prevalent. However, serovars 3, 6, 10 and 15 were not found. The typeable and non-typeable isolates were 234 (92.13%) and 20 (7.87%) out of total 254 respectively.

## Distribution and tendency of serovars in Sichuan province

In the present study, geographical distribution of serovars indicated that serovars 5 and 4 were the most prevalent in Sichuan province (Fig. 4A). In addition, serovars 1, 7 and 12 were only found in west Sichuan and serovar 8 was only found in east Sichuan
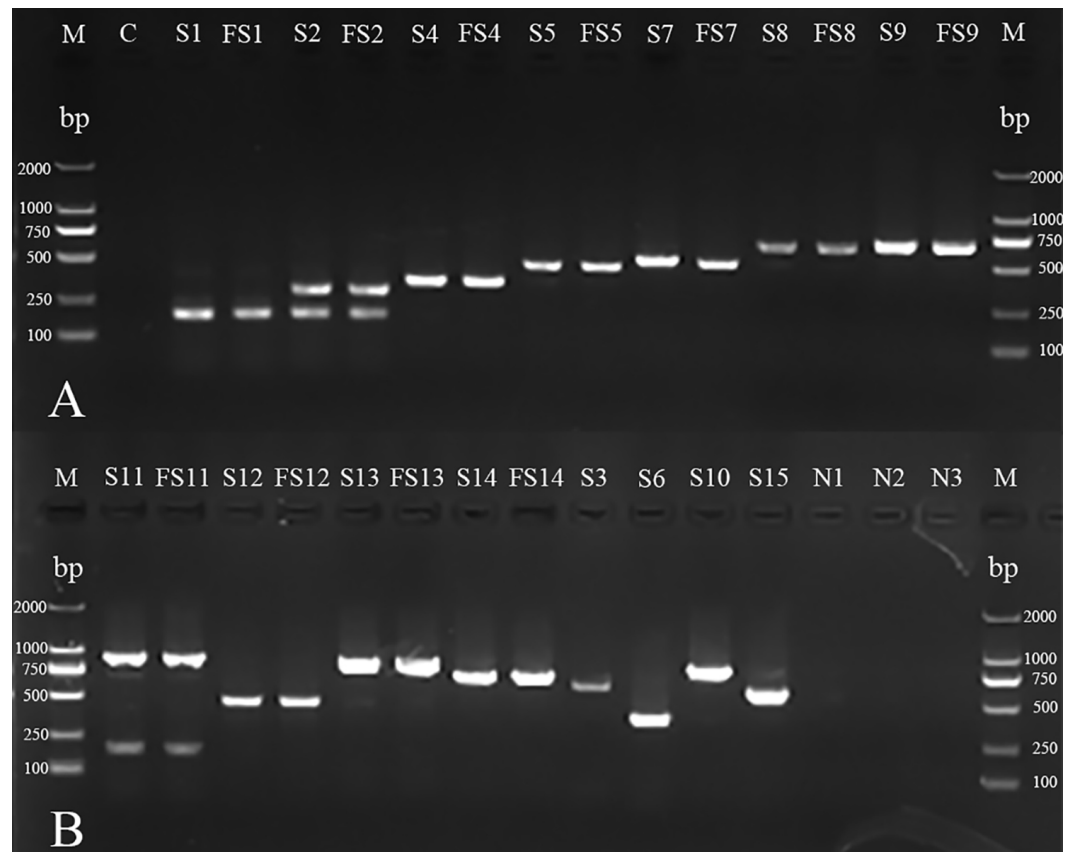
**Figure 2** **mPCR of serotyping for *H. parasuis* field isolates and standard strains.** (A) M: DL 2000 DNA Marker (Takara); C: blank control; S1, S2, S4, S5, S7, S8 and S9: standard strains of serovars 1, 2, 4, 5, 7, 8 and 9[8];FS1, FS2, FS4, FS5, FS7, FS8 and FS9: field isolates AB14011, GA14051, YA14041, GA14032, LS14031, GA15013, NC14041 (There were no field isolates of serovars 3, 6, 10 or 15 obtained in the investigation). (B) M: DL 2000 DNA Marker (Takara, Shiga, Japan); S11, S12, S13, S14, S3, S6, S10 and S15: standard strains of serovars 11, 12, 13, 14, 3,6,10 and 15; FS11, FS12, FS13 and FS14: field isolates CD14021, LS15044, NJ14021, MY14021; N1: non-typeable strain; N2: *A. pleuropneumoniae*; N3: *Streptococcus suis*.

(Fig. 4A). On the other hand, serovars 4 and 5 were prevalent in both 2014 and 2015, however, serovar 7 was the lead in prevalence for the first five months of 2016 (Fig. 4B).

## DISCUSSION

Isolation and identification of *H. parasuis* is the "gold standard" for diagnosis, but it requires that the samples to be collected from acute or typical infection cases without antimicrobial treatment as possible. Furthermore, rigorous nutritional demands and fragility of *H. parasuis* slow down the culture process and increase the probability of false negatives (*Del Río et al., 2003*). The isolation rate of previous studies ranged from 0.99% to 21% (*Oliveira, Blackall & Pijoan, 2003*; *Fablet et al., 2012*; *Zhang et al., 2012*). In the present study, the isolation rate was 44.10% in the Sichuan province (Table 1), and revealed a mark differences in prevalence in different areas. Differences of infection incidence among different age groups were in accordance with previous reports, which reconfirmed that
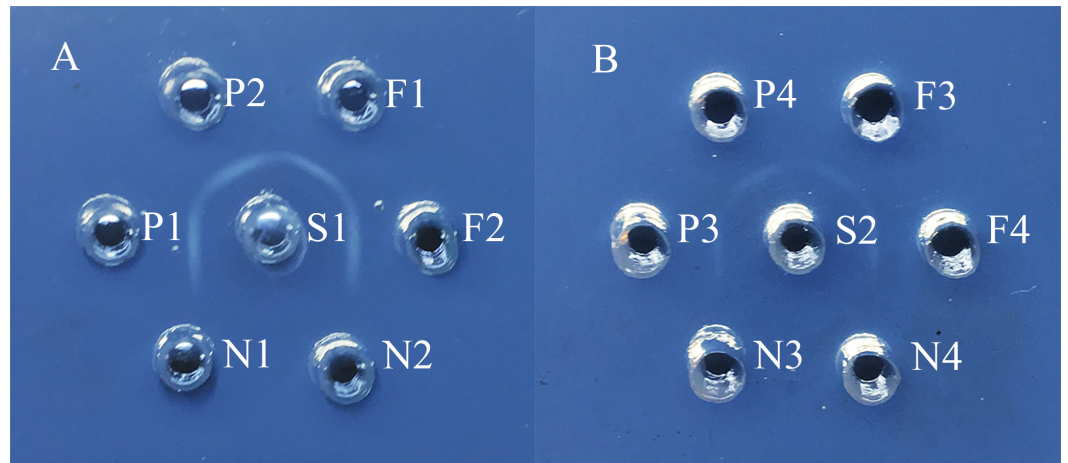
**Figure 3** **Agar gel diffusion (AGD) of serotyping for *H. parasuis* field isolates.** (A) S1: hyperimmune antiserum of serovar 5; F1: *H. parasuis* field isolates GA14032; F2: *H. parasuis* field isolates CD14041; P1 and P2: positive control of reference strain for serovar 5; N1: negative control of *A. pleuropneumoniae*; N2: blank control. (B) S2: hyperimmune antiserum of serovar 12; F3: *H. parasuis* field isolates CD14051; F4: *H. parasuis* field isolates LS15044; P3 and P4: positive control of reference strain for serovar 12; N3: negative control of *A. pleuropneumoniae*; N4: blank control.
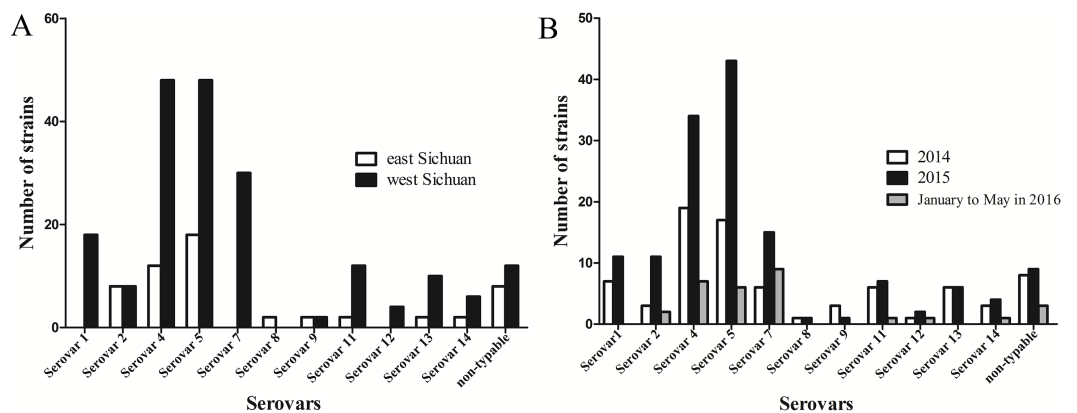


**Figure 4** **Distribution and variation of different serovars of *H .parasuis* in Sichuan province, China.** (A) Distribution of different serovars of *H. parasuis* in east and west Sichuan. (B) Variation of different serovars of *H. parasuis* during a two year five months survey period.

*H. parasuis* infection was a big threat to piglets. In addition, an apparently increased infection rate in winter and spring was due mainly to the sudden change of temperature and poor ventilation.

Before 2015, studies on epidemiology of *H. parasuis* usually depended on traditional approaches for serotyping. In 2012, 112 (out of 536 pigs) *H. parasuis* strains were isolated in southern China, and combination of AGD and IHA revealed that serovars 5 and 4 were the most prevalent serovars, whereas non-typeable strains accounted for nearly 20% (*Zhang et al., 2012*). In Northern Italy, serovars 4, 13 and 5 were demonstrated as the top three serovars in epidemic *H. parasuis* strains using AGD, however, non-typeable

strains accounted for 27.3% (*Luppi et al., 2013*). The simplex method is always subjected to susceptibility and improper preparations of hyperimmune antisera. By the combination of mPCR and complementary AGD, the profile of prevalent serovars in the Sichuan province has been revealed in the present study (Fig. 4), which confirmed that the combined approach proposed here is efficient and reliable. Considering the existence of non-typeable strains, the most convincing hypothesis is that there is deletion or insertion or mutation in serotype specific genes on the capsule loci. Secondly, there are differences between phenotypic and genotypic serotype in certain bacteria due to other alterations (*Gentle et al., 2016*). According to the previous report, the two *in silico*-nontypeable isolates can be differentiate by mPCR which indicated a non-absolute concordance between mPCR and *in silico* serotype analysis. We also found that some strains cannot be identified by mPCR, but the *in silico* serotype analysis is able to differrntiate them (*Howell et al., 2015*). Thus, more efforts have to be made to elucidate the differences. Moreover, some serovars previously believed to be nonvirulent or hypervirulent were also isolated with a frequency of 18.89%, and the discrepancy is due, to some extent, to the immune capacities, feeding conditions and supervision, and so forth (*Kielstein & Rapp-Gabrielson, 1992*; *Brockmeier et al., 2013*; *Yu et al., 2014*).

There are four bivalent and one monovalent inactivated vaccines for *H. parasuis* authorized in mainland China; however, desired protection are seldom obtained on account of the poor cross protection among these endemic serovars. Therefore, vaccines corresponding to specific serovars fits the region are optimal (*Yu et al., 2014*). However, no recent seroepidemiology reports were accessible for the Sichuan province and the prevalent serovars may change over time. The serovar profile in the present investigation was partly consistent with previous reports in China mainland, however, following serovars 4 and 5 were serovars 7, 1 and 2 rather than serovars 14, 13 and 12 as previously reported (*Cai et al., 2005*; *Zhang et al., 2012*; *Chen et al., 2015*). Furthermore, despite the most two epidemic serovars being the same in the two parts of the Sichuan province, the secondary serovars may interfere in the control of *H. parasuis* infection. The present study revealed the seroepidemiology of *H. parasuis* in Sichuan province, China. Thus, existing vaccines and vaccine candidate strains should be taken into consideration to control the disease. To our knowledge, several vaccine manufacturers and laboratories were considering more appropriate adjuvant candidates to reduce the irritation generated by inactivated vaccines. Furthermore, genetically engineered and attenuated vaccines against virulent strains of different serovars might be promising (*Oliveira, Blackall & Pijoan, 2003*). Although the prevalent serovars in the present study in a given area may change over a prolonged time span, throughout a two and a half year period, we believe that the serovars percentage profiles remained stable. Subsequent investigation should be carried out on annual basis to understand the variation tendency in seroepidemiology of *H. parasuis* in Sichuan province.

## CONCLUSION

This study confirmed that there was a high infection rate of *H. parasuis* in Sichuan province from January 2014 to May 2016. The present research revealed that *H. parasuis* incidence

was the highest in nursery pigs and infection rates in winter and spring were much higher than during the other two seasons. In addition, it is also demonstrated serovars 4 and 5 were the most prevalent in the Sichuan province, and mPCR combined with AGD was dependable for *H. parasuis* serotyping.

## ADDITIONAL INFORMATION AND DECLARATIONS

### Competing Interests

The authors declare there are no competing interests.

### Author Contributions

- Zhenghao Wang conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper.
- Qin Zhao and Hailin Wei performed the experiments, prepared figures and/or tables.
- Xintian Wen and Sanjie Cao contributed reagents/materials/analysis tools.
- Xiaobo Huang, Rui Wu, Qigui Yan and Yong Huang reviewed drafts of the paper.
- Yiping Wen analyzed the data, reviewed drafts of the paper.

### Data Availability

The following information was supplied regarding data availability:
Raw data can be found in the Supplemental Information.

### Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3379#supplemental-information.

# REFERENCES

**Brockmeier SL, Loving CL, Mullins MA, Register KB, Nicholson TL, Wiseman BS, Baker RB, Kehrli ME. 2013.** Virulence, transmission, and heterologous protection of four isolates of *Haemophilus parasuis*. *Clinical and Vaccine Immunology* **20(9)**:1466–1472 DOI 10.1128/CVI.00168-13.

**Cai X, Chen H, Blackall PJ, Yin Z, Wang L, Liu Z, Jin M. 2005.** Serologicalcharacterization of *Haemophilus parasuis* isolates from China. *Veterinary Microbiology* **111(3–4)**:231–236 DOI 10.1016/j.vetmic.2005.07.007.

**Chen S, Chu Y, Zhao P, He Y, Jian Y, Liu Y, Lu Z. 2015.** Development of arecombinant OppA-based indirect hemagglutination test for the detection ofantibodies against *Haemophilus parasuis*. *Acta Tropica* **148**:8–12 DOI 10.1016/j.actatropica.2015.04.009.

**Del Río ML, Gutiérrez B, Gutiérrez CB, Monter JL, Rodríguez Ferri EF. 2003.** Evaluation of survival of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* in four liquid media and two swab specimen transport systems. *American Journal Veterinary Research* **64(9)**:1176–1180 DOI 10.2460/ajvr.2003.64.1176.

**Del Río ML, Gutiérrez CB, Rodríguez Ferri EF. 2003.** Value of indirect hemagglutination and coagglutination tests for serotyping *Haemophilus parasuis*. *Journal of Clinical Microbiology* **41(2)**:880–882 DOI 10.1128/JCM.41.2.880-882.2003.

**Del Río ML, Martín CB, Navas J, Gutiérrez-Muñiz B, Rodríguez-Barbosa JI, Rodríguez Ferri EF. 2006.** aroA gene PCR-RFLP diversity patterns in *Haemophilus parasuis* and Actinobacillus species. *Research in Veterinary Science* **80(1)**:55–61 DOI 10.1016/j.rvsc.2005.03.004.

**Fablet C, Marois C, Dorenlor V, Eono E, Eveno E, Jolly JP, Le Devendec L, Kobisch M, Madec F, Rose N. 2012.** Bacterial pathogens associated with lung lesions in slaughter pigs from 125 herds. *Research in Veterinary Science* **93(2)**:627–630 DOI 10.1016/j.rvsc.2011.11.002.

**Gentle A, Ashton PM, Dallman TJ, Jenkins C. 2016.** Evaluation of molecular methods for serotyping *Shigella flexneri*. *Journal of Clinical Microbiology* **54(6)**:1456–1461 DOI 10.1128/JCM.03386-15.

**Howell KJ, Peters SE, Wang J, Hernandez-Garcia J, Weinert LA, Luan SL, Chaudhuri RR, Angen Ø, Aragon V, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Maskell DJ, Tucker AW. BRaDP1T Consortium. 2015.** Development of a multiplex PCR for rapid molecular serotyping of *Haemophilus parasuis*. *Journal of Clinical Microbiology* **53(12)**:3812–3821 DOI 10.1128/JCM.01991-15.

**Kielstein P, Rapp-Gabrielson VJ. 1992.** Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. *Journal of Clinical Microbiology* **30(4)**:862–865.

**Luppi A, Bonilauri P, Dottori M, Iodice G, Gherpelli Y, Merialdi G, Maioli G, Martelli P. 2013.** *Haemophilus parasuis* serovars isolated from pathological samples in Northern Italy. *Transboundary and Emerging Disease* **60(2)**:140–142 DOI 10.1111/j.1865-1682.2012.01326.x.

**Oliveira S, Blackall PJ, Pijoan C. 2003.** Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping. *American Journal of Veterinary Research* **64(4)**:435–442 DOI 10.2460/ajvr.2003.64.435.

**Olvera A, Calsamiglia M, Aragon V. 2006.** Genotypic diversity of *Haemophilus parasuis* field strains. *Applied and Environmental Microbiology* **72(1)**:3984–3992 DOI 10.1128/AEM.02834-05.

**Oliveira S, Galina L, Pijoan C. 2001.** Development of a PCR test to diagnose *Haemophilus parasuis* infections. *Journal of Veterinary Diagnostic Investigation* **13(6)**:495–501 DOI 10.1177/104063870101300607.

**Oliveira S, Pijoan C. 2004.** *Haemophilus parasuis* : new trends on diagnosis, epidemiology and control. *Veterinary Microbiology* **99(1)**:1–12 DOI 10.1016/j.vetmic.2003.12.001.

**Rapp-Gabrielson VJ, Gordon JK, Jeffrey TC, Stephen KM. 1997.** *Haemophilus parasuis*: immunity in swine after vaccination. *Veterinay Medicine* **92**:83–90.

**Turni C, Blackall PJ. 2005.** Comparison of the indirect haemagglutination and gel diffusion test for serotyping *Haemophilus parasuis*. *Veterinary Microbiology* **106(1–2)**:145–151 DOI 10.1016/j.vetmic.2004.12.019.

**Turni C, Blackall PJ. 2007.** Comparison of sampling sites and detection methods for *Haemophilus parasuis*. *Australian Veterinary Journal* **85(5)**:177–184 DOI 10.1111/j.1751-0813.2007.00136.x.

**Yu J, Wu J, Zhang Y, Du Y, Peng J, Chen L, Sun W, Cong X, Xu S, Shi J, Li J, Huang B, Zhu X, Wang J. 2014.** Identification of putative virulence-associated genes among *Haemophilus parasuis* strains and the virulence difference of different serovars. *Microbial Pathogenesis* **77**:17–23 DOI 10.1016/j.micpath.2014.10.001.

**Zhang J, Xu C, Guo L, Ke B, Ke C, Zhang B, Deng XL, Liao M. 2011.** A rapid pulsed-field gel electrophoresis method of genotyping *Haemophilus parasuis* isolates. *Letters in Applied Microbiology* **52(6)**:589–595 DOI 10.1111/j.1472-765X.2011.03048.x.

**Zhang J, Xu C, Guo L, Shen H, Deng X, Ke C, Ke B, Zhang B, Li A, Ren T, Liao M. 2012.** Prevalence and characterization of genotypic diversity of *Haemophilus parasuis* isolates from southern China. *Canadian Journal of Veterinary Research* **76(3)**:224–229.