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*Haemophilus parasuis*, the causative agent of Glässer’s disease, has been reported widely, but seldom is known about its epidemiology in Sichuan province, China. The objective was to reveal the prevalence and distribution of *H. parasuis* in the area. Widely sampling and isolation was performed initially and following serotyping multiplex PCR (serotyping-mPCR) combined with agar gel diffusion (GD) was subjected to these strains. From January 2014 to May 2016, 254 *H. parasuis* field strains were isolated from 576 pigs with clinical symptoms, for the frequency of 44.10%. Statistically significant differences of infection incidence were found in three age groups and seasons. Serovars 5(25.98%) and 4(23.62%) were the most prevalent and non-typeable isolates accounted for 7.87%. In geographical distribution, serovars 5 and 4 were prepotent in both major two parts of Sichuan province. The results confirmed the compound approach was dependable and revealed the diversity and distribution of serovars in Sichuan province, which was promising to know relevant vaccinal candidates and further prevention.
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 widely, but seldom is known about its epidemiology in Sichuan province, China. The objective was to reveal the prevalence and distribution of H. parasuis in the area. Widely sampling and isolation was performed initially and following serotyping multiplex PCR (serotyping-mPCR) combined with agar gel diffusion (GD) was subjected to these strains. From January 2014 to May 2016, 254 H. parasuis field strains were isolated from 576 pigs with clinical symptoms, for the frequency of 44.10%. Statistically significant differences of infection incidence were found in three age groups and seasons. Serovars 5(25.98%) and 4(23.62%) were the most prevalent and non-typeable isolates accounted for 7.87%. In geographical distribution, serovars 5 and 4 were prepotent in both major two parts of Sichuan province. The results confirmed the compound approach was dependable and revealed the diversity and distribution of serovars in Sichuan province, which was promising to know relevant vaccinal candidates and further prevention.

Key words Haemophilus parasuis, Seroepidemiology, Serovar
Introduction

*Haemophilus parasuis*, a conventional member of the family Pasteurellaceae, not only colonises in the upper respiratory tract of healthy pigs of different breeding periods, but also emerges as the aetiological agent of Glässer’s disease (Oliveira & Pijoan, 2004). The organism has been affecting the pig industry worldwide and causing a mass of economic loss. Consequently, more attention has been paid to study the epidemiology and pathogenesis of the microbe. Come so far, 15 serovars and considerable quantity of non-typeable isolates have manifested the heterogeneity in phenotype and various molecular approaches have revealed the diversity in genotypic lineage (Olvera, Calsamiglia & Aragon, 2006; del Río et al., 2006; Zhang et al., 2011).

Traditionally, agar gel diffusion (GD) and indirect hemagglutination (IHA) are used to serotype *H.parasuis* field isolates (Kielstein & Rapp-Gabrielson, 1992; Tadjine et al., 2004). Moreover, the latter was once proposed as a more discriminatory method for the reason of improving the rate of serotyping (Rafiee & Blackall, 2000). Subsequently, a multiplex PCR was performed for rapid molecular serotyping based on the analysis of variation within the capsule loci (Howell et al., 2015). Each serovar owns its distinction marker except serovars 5 and 12, and they share the same marker. Here, agar gel diffusion using specific hyperimmune serum may be applied to remedy the serotyping of the isolates possessing the mutual marker of serovars 5 and 12.
Although serovars 1, 2, 3, 4, 5, 8, 10, 12, 13, 14 and 15 are considered to be virulent, desired cross protections were hardly detected (Rapp-Gabrielson et al., 1997; Susan et al., 2013). However, commercial vaccines applied in swine production of mainland China seldom match the causative serovars and inoculation tends to be invalid. Thus, detailed information of morbidity and seroepidemiology do play an important role in prevention of H. parasuis. Considering no year-spanning report of H. parasuis infection was approachable in Sichuan province, China, we investigated the prevalence of the microorganism in Sichuan province from January 2014 to May 2016. In addition, data of serovars distribution in the present study could serve as reference upon the prevention of H. parasuis in the area.

Materials and methods

Reference strains and hyperimmune antiserum

Fifteen serovars of reference strains were purchased from Bacteriology Research Laboratory, Animal Research Institute, Yeerongpilly, Australia. Subsequently, corresponding hyperimmune reference antisera of rabbit source were prepared (Kielstein & Rapp-Gabrielson, 1992).

Collection of samples

From January 2014 to May 2016, total 576 clinical samples of 576 weak or moribund pigs showing respiratory distress or arthrocele from 103 intensive swine farms in Sichuan province were collected aseptically. And all the farms covered 20 counties of 13 cities or autonomous prefectures of Sichuan province. Incidentally, we divided the province into two parts artificially and the two parts were divided by climate rather than vertical division. The annual average temperature of region northeast by east was a bit higher than that of region southwest by west.
All pigs were necropsied humanely. Lungs, heart blood, infectious joints and brain were derived from pigs with acute infection or septicemia, while lungs, pericardium liquid, pleural effusion, seroperitoneum and joint fluid as well as fibrous membrane were derived from relatively mild infection (Turni & Blackall, 2007). Additionally, all samples were classified by age groups, seasons and regions.

**Isolation and identification**

Samples were routinely inoculated onto tryptic soy agar (TSA, Becton, Dickinson and Company) with a final concentration of 5% for newborn calf serum and 1 ug/ml for nicotinamide adenine dinucleotide (NAD, Roche), respectively. The plates were incubated for at least 24 h at 37°C under aerobic conditions. Colonies appeared to be translucent and 1mm-diameter sized were suspicious and then passaged overnight. Subsequently, the pure culture were observed under optical microscope after Gram stain and the gram negative one showing threadiness, rhabditiform or short rod was disposed to further identification. The PCR was implemented as previously described and the colony generating a product with 821bp of 16S rRNA was affirmed to be *H.parasuis* (Oliveira, Galina & Pijoan, 2001). Moreover, strains from different tissues of the same pig were counted as one isolate.

**Serotyping**

Firstly, all the isolates were serotyped by multiplex PCR using molecular markers and reaction procedure proved by previous report (Howell et al., 2015). As noted previously, GD, which was conducted as a supplement, was put into practice to differentiate serovars 5 and 12. Pure culture of the isolates own the marker of serovars 5 and 12 in tryptic soy broth (TSB, Becton, Dickinson
and Company) was centrifuged under 8,000×g for 5 min and then suspended by phosphate buffered saline into an appropriate concentration (OD$_{600}$=1.2±0.1). The suspension was inactivated under 121°C for 2 h and followed by a centrifuge under 6,000×g for 5 min. Whereafter, supernate was collected to be the heat-stable antigen. Using the hyperimmune-serovar-5 reference antiserum of rabbit source preserved by our laboratory, GD was performed within 3% agar accompanied with corresponding heat-stable antigen of standard strains as positive control and aqua pura as blank control as well as *Actinobacillus pleuropneumoniae* as negative control. Legible and single immune line between antiserum hole and heat-stable antigen hole indicated positive when three controls came through. It’s worth nothing that strains of the same serovar from a single farm were counted as one isolate.

**Statistical analysis**

Data were analyzed by the procedure of IBM SPSS Statistics 19, and differences between pairs of interesting measures were performed by Chi-square analysis. All tests were 2-sided and $P<0.01$ was defined as very significant difference while $P<0.05$ was defined as significant difference.

**Results**

**Bacterial strains**

In our investigation, varying degrees of respiratory symptom and classic Glässer’s disease were found in herbs of the intensive swine farms (Fig. 1). Total 254 field *H.parasuis* isolates were finally affirmed with strict culture and 16S rRNA PCR, and the isolation rate was 44.10% in these pigs showing suspicious symptoms. The infection incidence of *H.parasuis* was 45.98% and
43.16% in 2014 and 2015, respectively, and 43.84% was measured in the first 5 months of 2016, showing no significant differences ($\chi^2=0.368, P=0.832$). However, infection incidence varied with ages, seasons and regions. In nursery pathogenetic pigs, the general infection incidence was 60.80%, and statistically very significant differences were found in three age groups on the whole ($\chi^2=108.209, P=0.000$) and in 2014($\chi^2=30.936, P=0.000$) as well as 2015($\chi^2=75.580, P=0.000$), while statistically significant differences were seen in 2016 ($\chi^2=6.562, P=0.038$).

Moreover, infection incidence winter and spring were much higher than that in summer and autumn, and statistically very significant differences were also found in seasons on the whole ($\chi^2=29.904, P=0.000$) and in 2015 ($\chi^2=23.463, P=0.000$), while statistically significant differences were seen in 2014 ($\chi^2=9.850, P=0.02$). Last but not least, no significant differences were seen in two regions on the whole ($\chi^2=0.911, P=0.340$) (Table 1).

**mPCR and agar gel diffusion for serotyping**

Follow-up distinguishing serotype-mPCR and complementary GD examination classified them into 12 serovars, which showing a considerable diversity, while serovars 3, 6, 10 and 15 did not show up (Fig. 2; Fig. 3). Serovar 5 (66 of 254, 25.98%) and serovar 4 (60 of 254, 23.62%) were most prevalent, and the typeable isolates (234 of 254, 92.13%) was almost 12 times the non-typeable isolates (20 of 254, 7.87%). The other typeable isolates were consisted of serovars 7 (30 of 254, 11.81%), 1 (18 of 254, 7.09%), 2 (16 of 254, 6.30%), 11 (14 of 254, 5.51%), 13 (12 of 254, 4.72%), 14 (8 of 254, 3.15%), 12 (4 of 254, 1.57%), 9 (4 of 254, 1.57%) and 8 (2 of 254, 0.80%) (Fig. 4).

**Distribution of serovars in Sichuan province**
In the investigation, geographical distribution of serovars indicated serovars 5 and 4 were the most prevalent in Sichuan province. However, unlike serovars 7 and 1 were followed by serovars 4 and 5 in west Sichuan, followed by serovars 4 and 5 were serovar 2 in west Sichuan. In addition, serovars 1, 7 and 12 were only found in west Sichuan and serovar 8 was only found in east Sichuan (Fig. 5).

**DISCUSSION**

Bacterium isolation is the golden standard of *H. parasuis* laboratory diagnosis, but it requires that samples come from acute or typical infection cases without antimicrobial treatment as much as possible. Furthermore, rigorous nutritional demands and fragility of *H. parasuis* slow the culture and reduce positive incidence artificially (del Río et al., 2003). The isolation ratio 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 frequency was 44.10% on the whole (Table 1), which revealed a big discrepancy in different areas. Differences of infection incidence among age groups were in accordance with previous reports, which reconfirmed *H. parasuis* infection was a big threat to nursery pigs. In addition, an apparently improving infection incidence in winter and spring was mainly caused by the sudden change of temperature and poor ventilation. And poor ventilation in winter and spring was the result of improper insulation in nursery house.

Before 2015, studies on epidemiology of *H. parasuis* always applied traditional approaches to finish serotyping. In 2012, 112 *H. parasuis* strains were derived from 536 pigs with clinical signs in southern China, and combination of GD and IHA revealed that serovars 5 and 4 were the most prevalent, while non-typeable strains accounted for 20% (Zhang et al., 2012). In Northern
Italy, serovars 4, 13 and 5 were demonstrated as the top three in epidemic *H. parasuis* strains using GD, however, non-typeable strains accounted for 27.3% (Luppi et al., 2013). Simplex method is always subjected to susceptibility and improper preparations of hyperimmune antisera. With combination of mPCR and complementary GD, a novel profile of prepotent serovars was revealed in Sichuan province and non-typeable strains were 7.87% in the present study (Fig. 4), which confirmed this compound approach was efficient and dependable. Considering the existence of non-typeable strains validated by mPCR and how the approach comes, the most convincing hypothesis is the deletion or mutation of serotype specific genes on capsule loci. Secondly, there is difference between phenotypic and genotypic serotype in some certain bacteria due to insertions or other alterations (Gentle et al., 2016). According to the previous report, the two *in silico*-nontypeable isolates were typeable using the mPCR manifested a not absolute concordance between mPCR and *in silico* serotype analysis, and further suggested strains corroborated by *in silico* analysis probably proved to be non-typeable by mPCR (Howell et al., 2015). Thus, more efforts have to make to explain the fluctuation. Moreover, some serovars once believed nonvirulent were also isolated for the frequency of 18.89%, and the discrepancy is due, at least in part, to the immune capacities, feeding conditions and supervision (Kielstein & Rapp-Gabrielson, 1992; Brockmeier et al., 2013; Yu et al., 2014).

Although there are three bivalent and two multivalent inactivated vaccines authorized in mainland China, desired protection are hardly obtained on account of the poor cross protection among these epidemic serovars. Therefore, vaccine corresponding to serovars fits the region is optimal. However, no latest seroepidemiology reports were approachable in Sichuan province.
and prepotent serovars may change over time, hence we developed the investigation. The serovar profile in the present investigation was partly consistent with previous reports in China mainland, however, followed by serovars 4 and 5 were serovars 7, 1 and 2 rather than serovars 14, 13 and 12 (Cai et al., 2005; Zhang et al., 2012; Chen et al., 2015). Furthermore, despite the most two epidemic serovars were same in the two parts of Sichuan province, these secondary serovars may interfere in the control of \textit{H. parasuis} infection. Consequently, seroepidemiology investigation was the key to \textit{H. parasuis} prevention. The present study revealed the seroepidemiology in Sichuan province, China. Thus, existing vaccines and vaccinal candidates can take into consideration to control the disease. Subsequent investigation should be performed annually to understand the variation tendency in seroepidemiology of \textit{H. parasuis} in Sichuan province and to enrich the pathogen library.

**CONCLUSION**

We reported the infection incidence of \textit{H. parasuis} in Sichuan province from January 2014 to May 2016, and revealed that \textit{H. parasuis} incidence was much higher in nursery pigs and in winter and spring. In addition, we demonstrated serovars 4 and 5 were the most prevalent in Sichuan province, and validated that mPCR combined with GD was dependable in \textit{H. parasuis} serotyping.

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Figure 1 Gross lesions in *H. parasuis* infected pigs. (A) Trichocardia. (B) Fibrous membrane in lung.
Table 1 *Haemophilus parasuis* infections in Sichuan province, China (January, 2014-May, 2016).

<table>
<thead>
<tr>
<th>Year</th>
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<td></td>
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<td>3-8</td>
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<td>80/174*</td>
<td>2/24</td>
<td>65/104</td>
<td>13/46</td>
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<td>142/329</td>
<td>3/51</td>
<td>118/186</td>
<td>21/92</td>
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<tr>
<td>2016</td>
<td>32/73</td>
<td>1/7</td>
<td>31/62</td>
<td>0/4</td>
</tr>
</tbody>
</table>

*: A/B, A represents number of strains, and B represents number of samples.
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; FS1, FS2, FS4, FS5, FS7, FS8 and FS9: field isolates of serovars 1, 2, 4, 5, 7, 8 and 9 (There were no field isolates of serovars 3, 6, 10 or 15 obtained in the investigation). (B) M: DL 2000 DNA Marker (Takara); 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 of serovars 11, 12, 13 and 14; N1: non-typeable strain; N2: *Actinobacillus pleuropneumoniae*; N3: *Streptococcus suis*. 
Figure 3 Agar gel diffusion (GD) of serotyping for *H. parasuis* field isolates. S: hyperimmune antiserum of serovar 5; F1-F3: *H. parasuis* field isolates GA1402, QL1504, SL1505; P: positive control of standard strain for serovar 5; N: negative control of *A. pleuropneumoniae*; B: blank control.
Figure 4 Numbers of field strains for different serovars from January 2014 to May 2016.
Figure 5: Distribution of different serovars of *H. parasuis* in major two parts of Sichuan province, China.