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

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Haemophilus parasuis , the causative agent of Glässer's disease, has been reported as widespread, but little is known about its epidemiology in 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.

1 **Prevalence and seroepidemiology of *Haemophilus parasuis* in**

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Abstract

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but little is known about its epidemiology in 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

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study was carried out from January 2014 to May 2016 and 254 *H. parasuis* field strains were

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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

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Introduction

45 *Haemophilus parasuis*, a member of the family Pasteurellaceae, not only colonises in the upper

46 respiratory tract of healthy pigs during different breeding periods, but also emerges as the

47 aetiological agent of Glässer's disease in some cases (Oliveira & Pijoan, 2004). This organism

48 incurs significant economic losses within the swine industry. Therefore, efforts have been in

49 studying the epidemiology and pathogenesis of this microbe. So far, 15 serovars and a

50 considerable number of non-typeable isolates have been identified. Varous molecular approaches

51 have revealed the diversity in genotypic lineage and have shed light on the heterogeneity of

52 pathogenic *H. parasuis* serovars (Olvera et al., 2006; Del Río et al., 2006; Zhang et al., 2011).

53   Traditionally, agar gel diffusion (AGD) and indirect hemagglutination (IHA) are used to

54 serotype *H. parasuis* field isolates (Kielstein & Rapp-Gabrielson, 1992; Del Río et al., 2003a). In

55 addition, the latter was once proposed as a more discriminatory method for improving the rate of

56 serotyping (Turni & Blakall, 2005). Subsequently, a multiplex PCR was reported for rapid

57 molecular serotyping based on the analysis of variation within the capsule loci (Howell et al.,

58 2015). Each serovar has its own distinctive markers except serovars 5 and 12, for they share the

59 same marker. AGD using specific hyperimmune antisera may be applied to remedy the

60 serotyping issues seen in distinguishing between serovars 5 and 12.

61   Although serovars 1, 2, 3, 4, 5, 8, 10, 12, 13, 14 and 15 are considered to be virulent,

62   production of cross-protecting vaccines among different serovars has met with little success

63   (Rapp-Gabrielson et al., 1997; Brockmeier et al., 2013). Commercial vaccines for use in swine

64   seldom match the causative serovars and inoculation tends to be ineffective; this illustrating the

65   fact the commercial vaccines have, thus far, proven incapable of serovar cross protection in

66   China. Detailed information of morbidity and seroepidemiology can play an important role in

67   prevention when applied in conjuction with research aimed at uncovering potential antigenic

68   markers that are highly conserved across multiple serovars. As no detailed report of *H. parasuis*

69   infection was available in the Sichuan province, China, we investigated the prevalence and

70   seroepidemiology of *H. parasuis* in Sichuan province from January 2014 to May 2016.

71   **Materials and methods**

72   **Reference strains and hyperimmune antisera**

73   Reference strains of serovars 5 and 12 were purchased from Bacteriology Research Laboratory,

74   Animal Research Institute, Yeerongpilly 4105, Australia. Subsequently, corresponding

75   hyperimmune reference antisera of rabbit source were prepared (Kielstein & Rapp-Gabrielson,

76   1992).

77   **Collection of samples**

78   From January 2014 to May 2016, a total of 576 clinical samples were collected from 576 weak

79   or moribund pigs showing respiratory distress or arthritis. The survey focused on 103 intensive

80   swine farms in the Sichuan province. The farms covered 28 counties of 13 cities of Sichuan

81   province. Incidentally, the province was divided into two parts artificially by climate rather than

82   vertical division. The annual average temperature of the northeast region was a bit higher than

83   that of the southwest region in Sichuan province.

84   All pigs were necropsied humanely. Lungs, heart blood, joints and brain were derived from

85   pigs showing signs of acute infection or septicemia, while lungs, pericardium liquid, pleural

86   effusion, seroperitoneum and joint fluid as well as fibrinous membrane were collected from pigs

87   displaying relatively mild symptoms (Turni & Blackall, 2007). At least two tissues or exudates

88   per pig were analyzed in the present study. Different tissues or exudates from the same pig were

89   considered to be one sample. Additionally, all samples were classified by age groups, seasons

90   and regions.

91   **Isolation and identification**

92   Samples were inoculated on tryptic soy agar (TSA, Becton, Dickinson and Company) with a

93   final concentration of 5% newborn calf serum and 1 μg/ml of nicotinamide adenine dinucleotide

94   (NAD, Roche). The plates were incubated for at least 24 h at 37°C under aerobic conditions.

95   Colonies with translucent and 1mm-diameter size were further passaged. Subsequently, Gram

96   staining was carried out and culture with a typical morphology of *H. parasuis* such as threadless

97   and medium or short rod was confirmed by PCR as described elsewhere (Oliveira et al., 2001).

98   Moreover, strains isolated from different tissues of a pig were counted as one isolate.

99   **Serotyping**

100   First, all the isolates were serotyped by multiplex PCR using molecular markers and reaction

101   procedure as previously described (Howell et al., 2015). As noted previously, AGD, was

102   performed to differentiate between serovars 5 and 12. Pure cultures of the isolates with the

103   marker of serovars 5 and 12 were grown in tryptic soy broth (TSB, Becton, Dickinson and

104   Company) and cells were harvested by centrifugation at under 8,000 ×g for 5 min, and then

105   resuspended in phosphate buffered saline (pH: 7.4); cell concentration was adjusted

106   (OD_{600}=1.2±0.1). The cells was heated at 121°C for 2 h and followed by a centrifugation under

107   6,000 ×g for 5 min and supernatant was collected as heat-stable antigen. Using the hyperimmune

108   antisera against serovars 5 and 12 earlier prepared, AGD was performed with 3% agarose by

109   using heat-stable antigens of standard strains as positive controls, ultrapure water as a blank

110   control and *Actinobacillus pleuropneumoniae* as a negative control. A single immune line

111   between antiserum holes and heat-stable antigen holes indicated positives. Strains of the same

112   serovar from a single farm were counted as one isolate.

113   **Statistical analysis**

114   Data was statistically analyzed by the procedure of IBM SPSS Statistics 19.0, and differences

115   between age groups, seasons and regions were performed by Chi-square analysis. All tests were

116   2-sided and $P < 0.01$ was defined as a very significant difference while $P < 0.05$ was defined as a

117   significant difference.

118   **Results**

119   **Bacterial strains**

120   In the present investigation, varying degrees of respiratory symptom and classic Glässer's

121   disease were found in herds of the intensive swine farms (Fig. 1). 254 field *H.parasuis* isolates

122   were confirmed via viewing culture morphology characteristics and 16S rRNA PCR results, with

123   the isolation rate of 44.10%. The infection incidence of *H. parasuis* was 45.98% and 43.77% in

124   2014 and 2015, respectively, and 41.10% was measured in the first five months of 2016,

125   showing no significant differences (χ^2=0.531, P >0.05). However, infection incidence varied

126   with age, season and region. In pigs of 3-8 weeks, the general infection incidence was 60.80%,

127   which was much higher than that of pigs of less than 3 weeks or over 8 weeks. Statistically very

128   significant differences were found in three age groups as whole (χ^2=108.209, P <0.01).

129   Moreover, infection incidence in winter and spring were much higher than the other two seasons,

130   and statistical differences were very significant (χ^2=27.854, P<0.01) which indicated winter and

131   spring were the major epidemic seasons of $H.parasuis$ in Sichuan province. However, no

132   significant differences were seen in two regions as whole (χ^2=3.031, P >0.05) (Table 1).

133   **mPCR and AGD for serotyping**

134   Follow-up serotype-mPCR and complementary AGD examination classified them into 11

135   serovars, which showed a considerable serovar diversity (Fig. 2; Fig. 3). Serovar 5 (66 of 254,

136   25.98%) and serovar 4 (60 of 254, 23.62%) were most prevalent. However, serovars 3, 6, 10 and

137   15 were not found. The typeable and non-typeable isolates were 234 (92.13%) and 20 (7.87%)

138   out of total 254 respectively.

139   **Distribution and tendency of serovars in Sichuan province**

140   In the present study, geographical distribution of serovars indicated that serovars 5 and 4 were

141   the most prevalent in Sichuan province (Fig. 4A). In addition, serovars 1, 7 and 12 were only

142   found in west Sichuan and serovar 8 was only found in east Sichuan (Fig. 4A). On the other hand,

143   serovars 4 and 5 were prevalent in both 2014 and 2015, however, serovar 7 was the lead in

144   prevalence for the first five months of 2016 (Fig. 4B).

145   **DISCUSSION**

146 Isolation and identification of *H. parasuis* is the "gold standard" for diagnosis, but it requires that

147 the samples to be collected from acute or typical infection cases without antimicrobial treatment

148 as possible. Furthermore, rigorous nutritional demands and fragility of *H. parasuis* slows down

149 the culture process and increases the probability of false negatives (Del Río et al., 2003b). The

150 isolation rate of previous studies ranged from 0.99% to 21% (Oliveira et al., 2003; Fablet et al.,

151 2012; Zhang et al., 2012). In the present study, the isolation rate was 44.10% in the Sichuan

152 province (Table 1), and revealed a mark differences in prevalence in different areas. Differences

153 of infection incidence among different age groups were in accordance with previous reports,

154 which reconfirmed that *H. parasuis* infection was a big threat to piglets. In addition, an

155 apparently increased infection rate in winter and spring was due mainly to the sudden change of

156 temperature and poor ventilation.

157   Before 2015, studies on epidemiology of *H. parasuis* usually depended on traditional

158 approaches for serotyping. In 2012, 112 (out of 536 pigs) *H. parasuis* strains were isolated in

159 southern China, and combination of AGD and IHA revealed that serovars 5 and 4 were the most

160 prevalent serovars, whereas non-typeable strains accounted for nearly 20% (Zhang et al., 2012).

161 In Northern Italy, serovars 4, 13 and 5 were demonstrated as the top three serovars in epidemic *H.*

162 *parasuis* strains using AGD, however, non-typeable strains accounted for 27.3% (Luppi et al.,

163 2013). The simplex method is always subjected to susceptibility and improper preparations of

164 hyperimmune antisera. By combination of mPCR and complementary AGD, the profile of

165 prevalent serovars in the Sichuan province has been revealed in the present study (Fig. 4), which

166 confirmed that the combined approach proposed here is efficient and reliable. Considering the

167 existence of non-typeable strains, the most convincing hypothesis is that there is deletion or

168 insertion or mutation in serotype specific genes on the capsule loci. Secondly, there are

169 differences between phenotypic and genotypic serotype in certain bacteria due to other

170 alterations (Gentle et al., 2016). According to the previous report, the two *in silico*-nontypeable

171 isolates can be differentiate by mPCR which indicated a non-absolute concordance between

172 mPCR and *in silico* serotype analysis. We also found that some strains cannot be identified by

173 mPCR, but the *in silico* serotype analysis is able to differrntiate them (Howell et al., 2015). Thus,

174 more efforts have to be made to elucidate the differences. Moreover, some serovars previously

175 believed to be nonvirulent or hypervirulent were also isolated with a frequency of 18.89%, and

176 the discrepancy is due, to some extent, to the immune capacities, feeding conditions and

177 supervision, and so forth (Kielstein & Rapp-Gabrielson, 1992; Brockmeier et al., 2013; Yu et al.,

178 2014).

179   There are four bivalent and one monovalent inactivated vaccines for *H. parasuis* authorized

180 in mainland China, however, desired protection are seldom obtained on account of the poor cross

181 protection among these endemic serovars. Therefore, vaccines corresponding to specific serovars

182 fits the region are optimal (Yu et al., 2014). However, no recent seroepidemiology reports were

183 accessible for the Sichuan province and the prevalent serovars may change over time. The

184 serovar profile in the present investigation was partly consistent with previous reports in China

185 mainland, however, following serovars 4 and 5 were serovars 7, 1 and 2 rather than serovars 14,

186 13 and 12 as previously reported (Cai et al., 2005; Zhang et al., 2012; Chen et al., 2015).

187 Furthermore, despite the most two epidemic serovars being the same in the two parts of the

188 Sichuan province, the secondary serovars may interfere in the control of *H. parasuis* infection.

189 The present study revealed the seroepidemiology of *H. parasuis* in Sichuan province, China.

190 Thus, existing vaccines and vaccine candidate strains should be taken into consideration to

191 control the disease. To our knowledge, several vaccine manufacturers and laboratories were

192 considering more appropriate adjuvant candidates to reduce the irritation generated by

193 inactivated vaccines. Furthermore, genetically engineered and attenuated vaccines against

194 virulent strains of different serovars might be promising (Oliveira et al., 2003). Although the

195 prevalent serovars in the present study in a given area may change over a prolonged time span,

196 throughout a two and a half year period, we believe that the serovars percentage profiles

197 remained stable. Subsequent investigation should be carried out on annual basis to understand

198 the variation tendency in seroepidemiology of *H.parasuis* in Sichuan province.

199 **CONCLUSION**

200 This study confirmed that there was a high infection rate of *H.parasuis* in Sichuan province from

201 January 2014 to May 2016. The present research revealed that *H. parasuis* incidence was the

202 highest in nursery pigs and infection rates in winter and spring were much higher than during the

203 other two seasons. In addition, it is also demonstrated serovars 4 and 5 were the most prevalent

204 in the Sichuan province, and mPCR combined with AGD was dependable for *H. parasuis*

205 serotyping.

206 **ACKNOWLEDGEMENTS**

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211 **REFERENCES**

212 **Brockmeier SL, Loving CL, Mullins MA, Register KB, Nicholson TL, Wiseman BS, Baker RB, Kehrli**

213 **ME. 2013.** Virulence, transmission, and heterologous protection of four isolates of *Haemophilus parasuis*.

214 *Clinical and Vaccine Immunology* **20(9):**1466–1472. DOI 10.1128/CVI.00168-13.

215 **Cai X, Chen H, Blackall PJ, Yin Z, Wang L, Liu Z, Jin M. 2005.** Serologicalcharacterization of

216 *Haemophilus parasuis* isolates from China. *Veterinary Microbiology* **111(3-4):**231–236 DOI

217 10.1016/j.vetmic.2005.07.007.

218 **Chen S, Chu Y, Zhao P, He Y, Jian Y, Liu Y, Lu Z. 2015.** Development of arecombinant OppA-based

219 indirect hemagglutination test for the detection ofantibodies against *Haemophilus parasuis*. *Acta Tropica*

220 **148:**8–12 DOI 10.1016/j.actatropica.2015.04.009.

221 **Del Río ML, Martín CB, Navas J, Gutiérrez-Muñiz B, Rodríguez-Barbosa JI, Rodríguez Ferri EF. 2006.**

222 aroA gene PCR-RFLP diversity patterns in *Haemophilus parasuis* and Actinobacillus species. *Research in*

223 *Veterinary Science* **80(1):**55-61 DOI 10.1016/j.rvsc.2005.03.004.

224 **Del Río ML, Gutiérrez CB, Rodríguez Ferri EF**. 2003. Value of Indirect Hemagglutination and

225 Coagglutination Tests for Serotyping *Haemophilus parasuis*. *Journal of Clinical Microbiology* **41(2):** 880-

226 882.

227 **Del Río ML, Gutiérrez B, Gutiérrez CB, Monter JL, Rodríguez Ferri EF. 2003.** Evaluation of survival of

228 *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* in four liquid media and two swab specimen

229 transport systems. *American Journal Veterinary Research* **64(9):**1176-1180

230 **Fablet C, Marois C, Dorenlor V, Eono E, Eveno E, Jolly JP, Le Devendec L, Kobisch M, Madec F, Rose**

231 **N. 2012.** Bacterial pathogens associated with lung lesions in slaughter pigs from 125 herds. *Research in*

232 *Veterinary Science* **93(2):**627-630 DOI 10.1016/j.rvsc.2011.11.002.

233 **Gentle A, Ashton PM, Dallman TJ, Jenkins C. 2016.** Evaluation of molecular methods for serotyping

234 *Shigella flexneri. Journal of Clinical Microbiology* **54(6):**1456-1461 DOI 10.1128/JCM.03386-15.

235 **Howell KJ, Peters SE, Wang J, Hernandez-Garcia J, Weinert LA, Luan SL, Chaudhuri RR, Angen Ø,**

236 **Aragon V, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Maskell DJ, Tucker**

237 **AW, BRaDP1T Consortium. 2015.** Development of a multiplex PCR for rapid molecular serotyping of

238 *Haemophilus parasuis. Journal of Clinical Microbiology* **53(12):**3812-3821 DOI 10.1128/JCM.01991-15.

239 **Kielstein P, Rapp-Gabrielson VJ. 1992.** Designation of 15 serovars of *Haemophilus parasuis* on the basis of

240 immunodiffusion using heat-stable antigen extracts. *Journal of Clinical Microbiology* **30(4):**862-865

241 **Luppi A, Bonilauri P, Dottori M, Iodice G, Gherpelli Y, Merialdi G, Maioli G, Martelli P. 2013.**

242 *Haemophilus parasuis* serovars isolated from pathological samples in Northern Italy. *Transboundary and*

243 *Emerging Disease* **60(2):**140-142 DOI 10.1111/j.1865-1682.2012.01326.x.

244 **Oliveira S, Pijoan C. 2004.** *Haemophilus parasuis* : new trends on diagnosis, epidemiology and control.

245 *Veterinary Microbiology* **99(1):**1-12 DOI 10.1016/j.vetmic.2003.12.001

246 **Olvera A, Calsamiglia M, Aragon V. 2006.** Genotypic diversity of *Haemophilus parasuis* field strains.

247 *Applied and Environmental Microbiology* **72(1):**3984-3992 DOI 10.1128/AEM.02834-05.

248 **Oliveira S, Galina L, Pijoan C. 2001.** Development of a PCR test to diagnose *Haemophilus parasuis*

249 infections. *Journal of Veterinary Diagnostic Investigation* **13(6):**495-501

250 **Oliveira S, Blackall PJ, Pijoan C. 2003.** Characterization of the diversity of *Haemophilus parasuis* field

251   isolates by use of serotyping and genotyping. *American Journal of Veterinary Research* **64(4):**435-442.

252   **Rapp-Gabrielson VJ, Gordon JK, Jeffrey TC, Stephen KM. 1997.** *Haemophilus parasuis*: Immunity in

253   swine after vaccination. *Veterinay Medicine* **92:**83–90.

254   **Turni C, Blackall PJ. 2005.** Comparison of the indirect haemagglutination and gel diffusion test for

255   serotyping *Haemophilus parasuis*. *Veterinary Microbiology* **106(1–2):**145-151 DOI

256   10.1016/j.vetmic.2004.12.019

257   **Turni C, Blackall PJ. 2007.** Comparison of sampling sites and detection methods for *Haemophilus parasuis*.

258   *Australian Veterinary Journal* **85(5):**177-184 DOI 10.1111/j.1751-0813.2007.00136.x

259   **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**

260   **J. 2014.** Identification of putative virulence-associated genes among *Haemophilus parasuis* strains and the

261   virulence difference of different serovars. *Microbial Pathogenesis* **77:**17–23 DOI

262   10.1016/j.micpath.2014.10.001

263   **Zhang J, Xu C, Guo L, Shen H, Deng X, Ke C, Ke B, Zhang B, Li A, Ren T, Liao M. 2012.** Prevalence

264   and characterization of genotypic diversity of *Haemophilus parasuis* isolates from southern China.

265   *Canadian Journal of Veterinary Research* **76(3):**224-229

266   **Zhang J, Xu C, Guo L, Ke B, Ke C, Zhang B, Deng XL, Liao M. 2011.** A rapid pulsed-field gel

267   electrophoresis method of genotyping *Haemophilus parasuis* isolates. *Letters in Applied Microbiology*

268   **52(6):**589-595 DOI 10.1111/j.1472-765X.2011.03048.x

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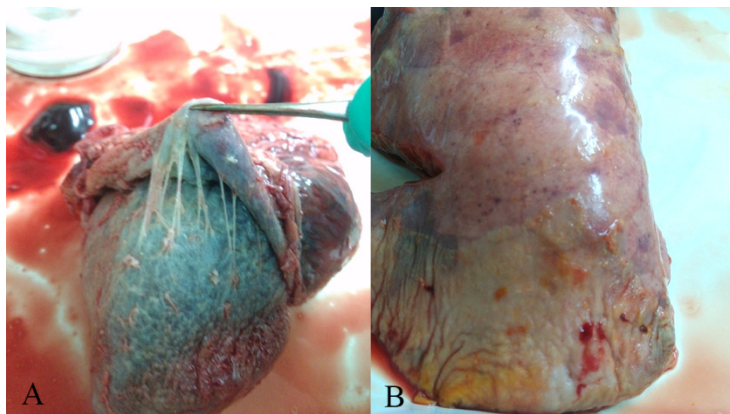
281   **Figure 1 Gross lesions in *H. parasuis* infected pigs.** (A) Fibrinous pericarditis. (B) Fibrinous pleuritis.

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298 **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

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

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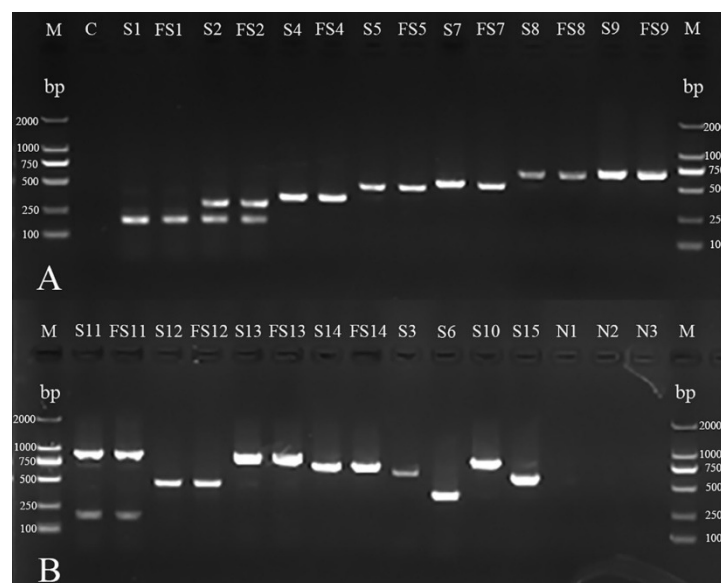
Figure 2 mPCR of serotyping for *H. parasuis* field isolates and standard strains. (A) M: DL 2000 DNA

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,

318   LS14031, GA15013, NC14041 (There were no field isolates of serovars 3, 6, 10 or 15 obtained in the

319   investigation). (B) M: DL 2000 DNA Marker (Takara); S11, S12, S13, S14, S3, S6, S10 and S15: standard

320   strains of serovars 11, 12, 13, 14, 3,6,10 and 15; FS11, FS12, FS13 and FS14: field isolates CD14021,

321   LS15044, NJ14021, MY14021; N1: non-typeable strain; N2: *A. pleuropneumoniae*; N3: *Streptococcus suis.*

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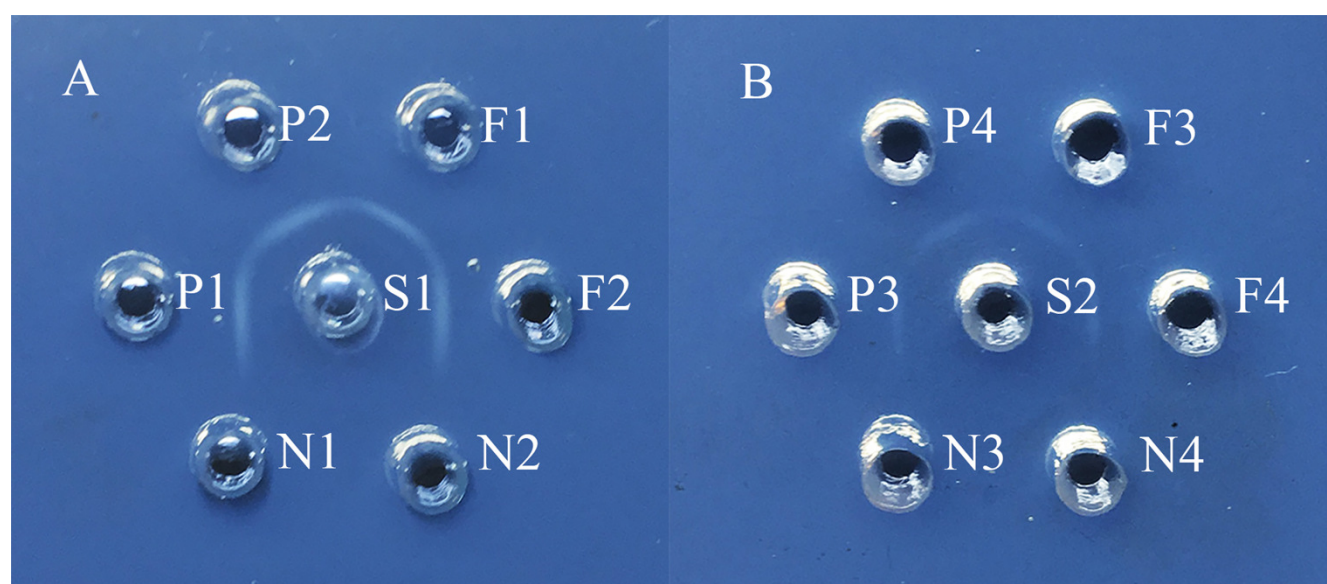
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330   **Figure 3 Agar gel diffusion (AGD) of serotyping for *H. parasuis* field isolates.** (A). S1: hyperimmune

331   antiserum of serovar 5; F1: *H. parasuis* field isolates GA14032; F2: *H. parasuis* field isolates CD14041; P1

332   and P2: positive control of reference strain for serovar 5; N1: negative control of *A. pleuropneumoniae*; N2:

333   blank control. (B). S2: hyperimmune antiserum of serovar 12; F3: *H. parasuis* field isolates CD14051; F4: *H.*

334   *parasuis* field isolates LS15044; P3 and P4: positive control of reference strain for serovar 12; N3: negative

335   control of *A. pleuropneumoniae*; N4: blank control.

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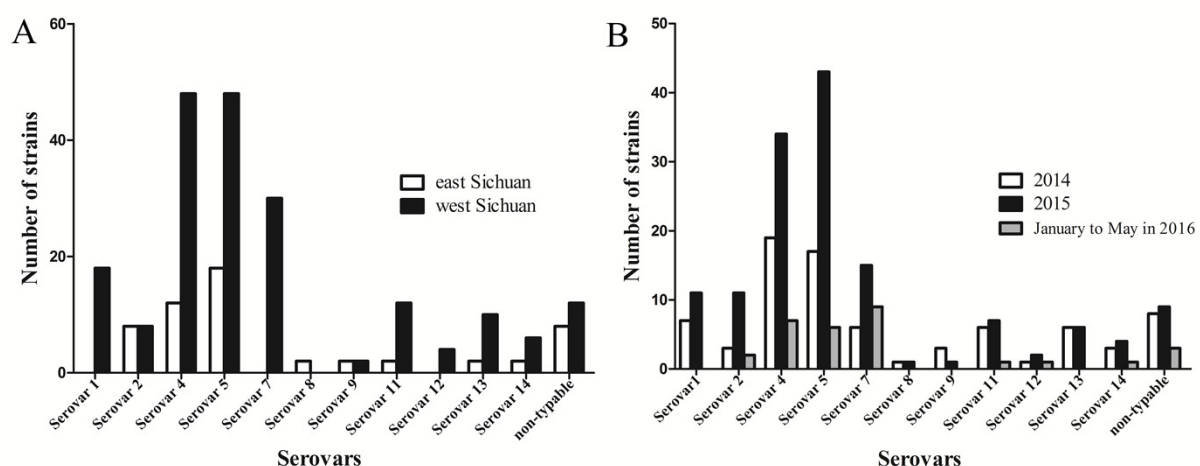
346   **Figure 4**: **Distribution and variation of different serovars of *H .parasuis* in Sichuan province, China.** (A)

347   Distribution of different serovars of *H. parasuis* in east and west Sichuan. (B) Variation of different serovars

348   of *H. parasuis* during a two year, five months survey period.