Molecular serotyping of *Haemophilus parasuis* isolated from diseased pigs and the relationship between serovars and pathological patterns in Taiwan

Wei-Hao Lin [1,2] , **Hsing-Chun Shih** [2] , **Chuen-Fu Lin** [3] , **Cheng-Yao Yang** [4] , **Yung-Fu Chang** [5] , **Chao-Nan Lin** [Corresp., 1,2] , **Ming-Tang Chiou** [1,2,6]

[1] Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan

[2] Animal Disease Diagnostic Center, College of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan

[3] Department of Veterinary Medicine, College of Veterinary Medicine, National Chiayi University, Chiayi, Taiwan

[4] Graduate Institute of Veterinary Pathobiology, College of Veterinary Medicine, National Chung-Hsing University, Taichung, Taiwan

[5] Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, New York, United States

[6] Research Center for Animal Biologics, National Pingtung University of Science and Technology, Pingtung, Taiwan

Corresponding Author: Chao-Nan Lin
Email address: cnlin6@mail.npust.edu.tw

Background: *Haemophilus parasuis* (*H. parasuis*) is the etiological agent of Glässer's disease, and causes severe economic losses in the swine industry. Serovar classification is intended as an indicator of virulence and pathotype and is also crucial for vaccination programs and vaccine development. According to a polysaccharide biosynthesis locus analysis, *H. parasuis* isolates could be classified by a molecular serotyping assay (except for serovars 5 and 12). The aim of this study was to identify *H. parasuis* isolates from diseased pigs in Taiwan by using a molecular serotyping assay and to analyze the relationship between serovars and pathological patterns.

Methods: From August 2013 to February 2017, a total of 133 isolates from 277 lesions on 155 diseased animals from 124 infected herds serotyped by multiplex PCR and analyzed with pathological data.

Results: The results showed that the dominant serovars of *H. parasuis* in Taiwan were serovars 5/12 (37.6%), 4 (27.8%) and 13 (15%) followed by molecular serotyping non-typable (MSNT) isolates (13.5%), which are differentiated on a genetic basis. Nevertheless, the serovar-specific amplicons were not precisely the same sizes as previously indicated in the original publication, and MSNT isolates appeared with unexpected amplicons or lacked serovar-specific amplicons. Furthermore, most *H. parasuis* isolates were isolated from nursery pigs infected with porcine reproductive and respiratory syndrome virus. The percentage of lung lesions (30.4%) showing *H. parasuis* infection was significantly higher than that of serosal lesions.

Discussion: Collectively, the distribution of serovars in Taiwan is similar to that found in other countries, but MSNT i solates remain due to genetic variations. Furthermore, pulmonary lesions may be optimum sites for *H. parasuis* isolation, the diagnosis of Glässer's disease, and may also serve as points of origin for systemic *H. parasuis* infections in hosts.

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2   **relationship between serovars and pathological patterns in Taiwan**

3   Wei-Hao Lin[1,2], Hsing-Chun Shih[1], Chuen-Fu Lin[3], Cheng-Yao Yang[4], Yung-Fu Chang[5], Chao-

4   Nan Lin[1,2*], Ming-Tang Chiou[1,2,6*]

5   [1]Department of Veterinary Medicine, College of Veterinary Medicine, National

6   Pingtung University of Science and Technology, Pingtung 91201, Taiwan.

7   [2]Animal Disease Diagnostic Center, College of Veterinary Medicine, National Pingtung

8   University of Science and Technology, Pingtung 91201, Taiwan.

9   [3]Department of Veterinary Medicine, College of Veterinary Medicine, National Chiayi

10   University, Chiayi 60004, Taiwan.

11   [4]Graduate Institute of Veterinary Pathobiology, College of Veterinary Medicine, National

12   Chung-Hsing University, Taichung, Taiwan.

13   [5]Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine,

14   Cornell University, Ithaca, NY 14850, USA.

15   [6]Research Center for Animal Biologics, National Pingtung University of Science and

16   Technology, Pingtung, Taiwan.

18   *Corresponding Author:

19   Chao-Nan Lin[1,2]

20   VM208, 1, Shuefu Road, Neipu, Pingtung 91201, Taiwan

21   Email address: cnlin6@mail.npust.edu.tw

22   Ming-Tang Chiou[1,2,6]

23   VM212, 1, Shuefu Road, Neipu, Pingtung 91201, Taiwan

24   Email address: mtchiou@mail.npust.edu.tw

26 **Abstract**

27 **Background:** *Haemophilus parasuis* (*H. parasuis*) is the etiological agent of Glässer's disease,

28 and causes severe economic losses in the swine industry. Serovar classification is an indicator of

29 virulence and pathotype and is also crucial for vaccination programs and vaccine development.

30 *H. parasuis* isolates can be classified by a molecular serotyping assay except for serovars 5 and

31 12. The aim of this study was to serotype *H. parasuis* isolates from diseased pigs in Taiwan by

32 using a molecular serotyping assay and to analyze the relationship between serovars and

33 pathological patterns.

34 **Methods:** From August 2013 to February 2017, a total of 155 diseased animals from 124

35 infected herds were examined for infection with *H. parasuis*. One hundred thirty three isolates of

36 *H. parasuis* were recovered and serotyped by multiplex PCR and correlated with pathological

37 lesions.

38 **Results:** The dominant *H. parasuis* were serovars 5/12 (37.6%), 4 (27.8%) and 13 (15%)

39 followed by molecular serotyping non-typable (MSNT) isolates (13.5%). The serovar-specific

40 amplicons were not precisely the same sizes as previously indicated in the original publication,

41 and MSNT isolates appeared with unexpected amplicons or lacked serovar-specific amplicons.

42 Most *H. parasuis* isolates were isolated from nursery pigs infected with porcine reproductive and

43 respiratory syndrome virus. The percentage of lung lesions (30.4%) showing *H. parasuis*

44 infection was significantly higher than that of each serosal lesions.

45 **Discussion:** Collectively, the distribution of *H. parasuis* serovars in Taiwan is similar other

46 countries, but MSNT isolates remain due to genetic variations. Our data suggests those prevalent

47 serovar isolates prefer to cause both serosal and pulmonary lesions rather and only pulmonary

48 lesions. Pulmonary lesions are optimum sites for *H. parasuis* isolation and may also serve as

49   points of origin for systemic *H. parasuis* infections in hosts.

50   Keywords: *Haemophilus parasuis*, Glässer's disease, polyserositis, serotyping

51

52   **Introduction**

53   *Haemophilus parasuis* (*H. parasuis*), a part of normal upper respiratory microbiota, is the

54   etiological agent of Glässer's disease which induces sudden death, polyserositis, polyarthritis,

55   meningitis and poor production performance, resulting severe economic losses in the swine

56   industry (Amano et al. 1994; Moller & Kilian 1990; Vahle et al. 1997; Zhang et al. 2014).

57   Vaccination is an effective strategy for preventing increased mortality and economic losses

58   caused by virulent *H. parasuis* (Miniats et al. 1991a; Smart & Miniats 1989). However, only

59   partial protection is observed with heterologous *H. parasuis* strain challenges due to poor cross-

60   protection (Miniats et al. 1991b; Nielsen 1993; Takahashi et al. 2001). Thus, serotyping of *H.*

61   *parasuis* is very important, not only for epidemiological research but also for choosing

62   efficacious inactivated whole-cell bacterial vaccines.

63   Fifteen serovars, conventional serotyping cross-reactive (CSCR) and non-typable (CSNT)

64   isolates of *H. parasuis* have been described and demonstrated by gel immunodiffusion assay

65   (GID) (Kielstein & Rapp-Gabrielson 1992). Due to the persistence of cross-reactivity or non-

66   reaction to antisera, there are still approximately 15% to 40% CSCR and CSNT isolates reported

67   in a variety of countries by GID (Table S1) (Blackall et al. 1996; Cai et al. 2005; Castilla et al.

68   2012; Del Rio et al. 2003; Kielstein & Rapp-Gabrielson 1992; Luppi et al. 2013; Ma et al. 2016;

69   Oliveira et al. 2003; Rapp-Gabrielson & Gabrielson 1992; Rubies et al. 1999; Tadjine et al.

70   2004). Despite using an indirect hemagglutination assay (IHA) designed to reduce the proportion

71   of CSCR isolates, 7.5% to 18% of isolates are still untypable (Table S1) (Angen et al. 2004; Cai

72   et al. 2005; Del Rio et al. 2003; Dijkman et al. 2012; Howell et al. 2015). This phenomenon

73   makes it more difficult to conduct an effective vaccination program against *H. parasuis*.

74   Conventional serotyping is used extensively (Kielstein et al. 1991; Morozumi & Nicolet

75   1986; Rapp-Gabrielson & Gabrielson 1992). The Kielstein-Rapp-Gabrielson (KRG) scheme

76   recognizes 15 serovars of *H. parasuis* on the basis of a GID test with specific rabbit antisera and

77   the authors noted a correlation between serovar and virulence (Kielstein & Rapp-Gabrielson

78   1992). According to serotyping results, serovar 4 tends to be found in pulmonary infections;

79   CSNT and CSCR isolates are mainly found in systemic infections (Angen et al. 2004).

80   Unfortunately, others report little correlation between serovar and virulence as isolates in the

81   same serovar often exhibit different virulence levels (Aragon et al. 2010; Olvera et al. 2007).

82   Previous studies established the serovar and pathotype of *H. parasuis* are based on

83   differences at the genome level (Brockmeier et al. 2014; Howell et al. 2013; Howell et al. 2017).

84   A multiplex PCR (mPCR) based on a polysaccharide biosynthesis locus analysis was employed

85   to molecularly serotype *H. parasuis* serovars (Howell et al. 2015). As a result, 14 of 15 serovars

86   of *H. parasuis* (serovars 5 and 12 could not be differentiated) were identified using this assay

87   (Howell et al. 2015) Using the molecular typing assay many of the CSNT and CSCR isolates

88   were successfully typed in a recognized serovar.

89   Although Glässer's disease is common in Taiwan, serotyping of pathogenic *H. parasuis*

90   isolates from Taiwanese pigs is not clear. The principal aim of this study was to molecularly

91   serotype *H. parasuis* isolated from Taiwanese diseased pigs, and correlate serovars with

92   pathological patterns.

93

94   **Materials & Methods**

95   **Bacterial isolate collection and identification**

96   *H. parasuis* field isolates were collected from diseased pig herds between August 2013 and

97 February 2017 in Taiwan (Table S2). Lesions suspected of being caused by *H. parasuis* in

98 diseased pigs were located in the meninges, pleura, pericardia, peritonea, synovial cavities of

99 joints and lungs. Lesions were swabbed and plated on chocolate agar (at 37°C, 5% CO_2, 18 to 72

100 hours for growth rate variation for various isolates), blood agar (at 37°C, 16 to 24 hours) and

101 MacConkey agar (at 37°C, 16 to 24 hours). The bacterial isolates were identified by colony

102 morphology, Gram stain (Gram negative bacillus), nicotinamide adenine dinucleotide (NAD)

103 dependence (only growing on chocolate agar) and virulence-associated trimeric autotransporter

104 group 3 colony PCR (Pina et al. 2009).

Molecular serotyping mPCR

106   The molecular serotyping assay for *H. parasuis* isolates was modified from a previously

107 published method (Howell et al. 2015). The sp-sp amplicon was used as an internal control. A

108 loopful of bacteria from a passaged plate of pure culture was resuspended in 30 μL ultrapure

109 H_2O, which was heated to 100°C for 30 min then centrifuged at 4,000 x g for 1 min. The

110 supernatant was used in the mPCR reaction. Isolates from various lesions or pigs from the same

111 herd were serotyped. If they belonged to same serovar, they were considered one isolate.

112   Each PCR reaction was performed in a total volume of 30 μL containing ultrapure H_2O, 1 x

113 DreamTaq buffer, 250 μM dNTP, 0.2 μM concentrations of forward and reverse serovar-specific

114 primers, 0.04 μM concentrations of forward and reverse species-specific primers, 1.25 U of

115 DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 1 μL of

116 supernatant. The thermocycling conditions consisted of 5 min at 94°C, 30 cycles of 30 sec at

117 94°C, 30 sec at 58°C and 1 min at 72°C, and then a final extension at 72˚C for 5 min. The

118 molecular serotyping mPCR amplicons were stained with ethidium bromide and analyzed using

119 a 20-cm-long 2% agarose gel. A 50-bp DNA ladder RTU (GeneDireX, Las Vegas, NV, USA)

120 and Bio-1D software (Vilber Lourmat, Collégien, France) were used to estimate molecular size.

121   The electrophoresis conditions were an electric field 6 V/cm (300 V, 50-cm full-length electric

122   field) and 3 hr. The results were confirmed by twice repeating tests.

123   **Sequencing and analysis of unexpected PCR-amplified products**

124   Unexpected amplicons of the molecular serotyping mPCR products were cloned using a TA

125   cloning kit (Yeastern Biotech Co., Ltd. Taipei, Taiwan) and sequenced using an automated DNA

126   sequencer (ABI 3730XL, USA). Sequence data were analyzed using MEGA7 (Molecular

127   Evolutionary Genetics Analysis Version 7.0) software and BLAST (Basic Local Alignment

128   Search Tool) database.

129   **Pathological examination**

130   Cases of sick animals or fresh, complete carcasses were subjected to necropsy for gross

131   morphological examinations and H&E staining. Histopathological examination focused

132   primarily on meningeal, pleural, pericardial, peritoneal, and synovial cavities of joints, and lungs.

133   Typical meningeal gross lesions were characterized by yellow to white exudate accumulation in

134   the subarachnoid space, on pia mater and in the sulci (Fig. S1). Meningeal histopathological

135   lesions were principal neutrophils and few mononuclear inflammatory cells infiltration on pia

136   mater with fibrin and cellular debris deposit (Fig. S2). Serosal lesions were characterized by

137   yellow to white exudate accumulation in pleural, pericardial, abdominal and joint synovial

138   cavities and yellow to white fibrin covering the visceral and parietal serosa (Fig. S3; Fig. S4; Fig.

139   S5). The histopathological lesions of serositis were principal neutrophils and few mononuclear

140   inflammatory cells infiltration with fibrin deposit (Fig. S6). In typical cases, *H. parasuis* resulted

141   in bronchopneumonia with numerous neutrophils, mononuclear inflammatory cells, erythrocytes,

142   cellular debris and fibrinous exudate accumulation in alveoli (Fig. S7; Fig. S8). Due to disease

143   duration, lesions varied in field. Other lesions infected *H. parasuis* were also involved to

144   determine pathological patterns, including chronic fibrous serositis with angiogenesis and

145   mononuclear inflammatory cells infiltration (Fig. S9), and meningitis with principal

146   macrophages infiltration (Fig. S10).

147   **Detection of porcine reproductive and respiratory syndrome virus**

148   Nucleic acid extraction of pulmonary tissue was performed on a MagNA Pure LC 2.0 by

149   using the MagNA Pure LC total nucleic acid isolation kit (Roche Applied Science, Indianapolis,

150   IN, USA). Following cDNA synthesis was using PrimeScript™ RT reagent kits (Takara, Kyoto,

151   Japan). Porcine reproductive and respiratory syndrome virus (PRRSV) reverse transcription real-

152   time PCR was performed as previously described (Lin et al. 2013).

153   **Statistical analysis**

154   Fisher's exact test was used to compare the frequency of *H. parasuis* infected lesions and

155   the percentage of various lesion patterns using GraphPad Prism software (GraphPad Software,

156   La Jolla, CA, USA). Variables were considered significant at a 0.05 level (two-sided).

157

158   **Results**

159   ***H. parasuis* isolates, origins and pathological lesion patterns**

160   One hundred thirty three isolates of *H. parasuis* were isolated from August 2013 to

161   February 2017. The isolates were taken from 277 lesions on 155 diseased animals from 124

162   infected herds. Isolates from a herd serotyped as a single serovar were calculated as one isolate.

163   Of 155 *H. parasuis* cases, 12 cases (7.7%) belonged to suckling pigs ($\leq$ 3-week-old), 133 cases

164   (85.2%) belonged to nursery pigs (4- to 12-week-old), 7 cases (4.5%) belonged to growing pigs

165   (13- to 26-week-old) and one case belonged to a breeding boar. Age information for two cases

166   was unknown. Eighty-six cases (55.5%) had *H. parasuis* isolated from lung lesions with or

167   without serosal lesions.

168   One hundred eight animals were necropsied with complete pathological examination and

169   further correlated to pathological pattern and isolation proportion (Table S3). Of the *H. parasuis*

170   infected animals, 54.6 % had serositis and pulmonary tissue lesions, 41.7% had serosal lesions

171   only, and 3.7 % displayed only pulmonary lesions (Fig. 1).

172   One hundred six cases (98.1%) had bronchopneumonia, 64 cases (59.3%) displayed *H.*

173   *parasuis* positive lung lesions. Seventy-eight cases (72.2%) registered as positive for PRRSV via

174   reverse transcription real-time PCR screening. The proportion of 204 *H. parasuis* infected

175   lesions from 108 animals with complete pathological examination were meninges (10.3%),

176   pleura (20.1%), pericardium (16.2%), peritoneum (13.7%), joint synovial cavity (9.3%) and lung

177   (30.4%) (Fig. 2). The percentage of lung lesions showing *H. parasuis* infection was significantly

178   higher than the percentage of serosal lesions ($P < 0.05$).

179   **Serovar distribution by molecular serotyping assay**

180   Of the 133 isolates, 91 (68.94%) isolates were typed using molecular serotyping mPCR.

181   The most common serovars were serovar 5/12 (38.2%) and serovar 4 (27.5%) followed by

182   serovar 14 (2.3%), serovar 1 (0.8%), serovar 2 (0.8%) and serovar 9 (0.8%) (Fig. 3). However,

183   the product sizes of the serovar-specific primers analyzed by Bio-1D software were varied from

184   the original publication (Howell et al. 2015). Furthermore, there were still 41 isolates (29.8%)

185   classified as MSNT; these were divided into four groups based on the appearance of unexpected

186   amplicons or the lack of serovar-specific amplicons. Eighteen isolates (13%) positive for a

187   species-specific (sp-sp) marker were categorized as MSNT group 1. Nineteen isolates (14.5%)

188   were placed in MSNT group 2; these displayed amplicons of 300, 830, and 1000 bp. Two

189   isolates (1.5%) which showed unexpected amplicons at 500 and 660 bp were categorized as

190   MSNT group 3. One isolate (0.8%), showing an amplicon of 300 bp was categorized as an

191   MSNT group 4 isolate (Fig. 3; Fig. S11; Fig. S12; Fig. S13; Fig. S14; Fig. S15).

192   **Identification of serovar-specific amplicons**

193   The amplicons generated from molecular serotyping mPCR analyzed using Bio-1D

194   software were not precisely the same sizes as previously indicated in the original description of

195   this assay (Howell et al. 2015). The product size of a specific amplicon found in serovar 4 was

196   mentioned at 320 bp in the original publication but the PCR run generated an amplicon of nearly

197   350 bp which might be confused with serovar 6. In serovar 5, the PCR results generated an

198   amplicon larger than 450 bp mentioned in the original publication which might be confused with

199   serovar 7. The product size of serovar 9 serovar-specific primers, mentioned at 710 bp in the

200   original publication, was smaller than the 700 bp ladder marker and might be confused with

201   serovar 8. In light of these conflicting results, the isolates were serotyped again to confirm the

202   sizes, and the amplicons were subsequently sequenced. Comparisons of the molecular serotyping

203   original publication described, Bio-1D software analyzed, and BLAST product sizes are shown

204   in Table 1 (Howell et al. 2015). The product sizes analyzed by Bio-1D software, BLAST and

205   sequence are more consistent.

206   The unexpected PCR products of the MSNT isolates were cloned for sequencing (Table 2).

207   The MSNT group 2 amplicon was 297 bp; this product was generated with a serovar 13 specific

208   forward and a serovar 14 specific reverse primer pair targeting *gltP* gene as a marker of serovar

209   13 in a polysaccharide biosynthesis locus. These primers were paired because the target

210   sequences in the respective serovars shared homologous segments. The other PCR generated an

211   amplicon product of the MSNT group 2 isolate determined to be 836 bp, and was identified as a

212   serovar 13 specific product. The Bio-1D software measured a 500 bp product of the MSNT

213   group 3 isolate as 499 bp; this amplicon was identified as a serovar 7 specific product. The 300

214   bp product found in the MSNT group 4 isolate, (sequencing results indicated it was 297 bp) was

215   generated by pairing a serovar 13 specific forward primer with a serovar 14 specific reverse

216   primer. This result was the same as that generated using the same primer pair of DNA isolated

217 from MSNT group 2.

Serovar distribution based on molecular serotyping assay and sequencing

219 The molecular serotyping assay combined with sequencing results reduced the percentage

220 of isolates classified as MSNT from 30.1% to 13.5% (Fig. 3). The dominant serovars were

221 serovar 5/12 (37.6%), serovar 4 (27.8%) and serovar 13 (15%) followed by serovar 14 (2.3%),

222 serovar 7 (1.5%), serovar 1 (0.8%), serovar 2 (0.8%) and serovar 9 (0.8%). Combining the

223 sequencing results showed that serovar 13 is a common serovar.

Relationship between pathological lesion patterns and serovars

225 The distribution of *H. parasuis* serovars in lesions from necropsied animals were serovar 5

226 (42.6%), serovar 4 (21.3%), serovar 13 (20.4%), and MSNT group 1 (13%). These categories

227 were further subdivided into animals displaying both serosal and pulmonary lesions, those with

228 only pulmonary lesions, and those with lesions found only in serosa. The respective percentages

229 of lesions vs. serovars, and the pattern of lesions in infected animals were showed in Fig. 1 and

230 Table S4. Necropsied animals with both serosal and pulmonary lesions were the most frequent;

231 animals with pulmonary lesions alone were the least frequent ($p < 0.0001$). Serovars 4 and 5/12

232 showed similar results, the MSNT group 1 both serosal and pulmonary lesions were more

233 frequent than serosal lesions alone. Serovar 13 had more serosal lesions than the combination of

234 serosal and pulmonary lesions.

235 Nine herds (7.3%) had populations infected with two *H. parasuis* serovars. One herd

236 contained a population with lesions infected with serovars 1 and 4. Three herds were infected

237 with serovars 4 and 5. Serovar 5, 13 and 5, 14 co-infections were seen in single herds. The

238 infected lesions were located in animals displaying a variety of tissue lesion patterns. Other 4

239 herds contained individuals co-infected with two *H. parasuis* serovars. One clinical case showed

240 pleural and pulmonary lesions coinfected with *H. parasuis* serovars 4 and 7, respectively. A

241 separate herd contained one case of pulmonary lesions with serovar 5, as well as pleural,

242 pericardial, and peritoneal lesions infected with *H. parasuis* serovar 13. One case was co-

243 infected with serovars 5 (pulmonary) and 13 (pleura and pericardium). A fourth case showed

244 coinfection with serovar 4 and an MSNT group 1 isolate taken from separate pulmonary lesions.

245

246 **Discussion**

247   This is the first study describing serovars of *H. parasuis* defined by molecular serotyping in

248 Taiwan. The most common serovars are serovar 5/12, 4 and 13, followed by MSNT isolates.

249 Even though serotyping assays vary, the serovar population profile of *H. parasuis* in Taiwan is

250 similar to profiles described in several other studies (Table S1) (Angen et al. 2004; Blackall et al.

251 1996; Cai et al. 2005; Castilla et al. 2012; Del Rio et al. 2003; Dijkman et al. 2012; Howell et al.

252 2015; Kielstein & Rapp-Gabrielson 1992; Luppi et al. 2013; Ma et al. 2016; Oliveira et al. 2003;

253 Rapp-Gabrielson & Gabrielson 1992; Rubies et al. 1999; Tadjine et al. 2004). Most commercial

254 *H. parasuis* vaccines are inactivated vaccines, which provide protection against the same serovar

255 but are unable to provide protection from challenge using different serovars (Miniats et al.

256 1991b; Nielsen 1993; Smart & Miniats 1989; Takahashi et al. 2001). Candidate serovar

257 composition in *H. parasuis* vaccine determines the success of a vaccine strategy against *H.*

258 *parasuis* (Takahashi et al. 2001). Therefore, the distribution of serovars in herds is an important

259 factor in outlining vaccination strategies and vaccine developments aimed at the prevention and

260 control of Glässer's disease.

261   IHA was applied to *H. parasuis* serovar differentiation to decrease the proportion of *H.*

262 *parasuis* isolates classified as CSCR (Cai et al. 2005; Del Rio et al. 2003). De-encapsulation due

263 to multiple passages results in non-reaction with antisera and cross reactivity of isolate antigens

264 to diagnostic (immune-based) test reagents are the primary factors behind CSNT and CSCR *H.*

265   *parasuis* isolates, respectively (Cai et al. 2005; Kielstein & Rapp-Gabrielson 1992; Oliveira et al.

266   2003; Rapp-Gabrielson & Gabrielson 1992; Turni & Blackall 2005). The presence of CSNT and

267   CSCR isolates confounds epidemiological surveys used to assess *H. parasuis* isolate population

268   profiles, and impairs efforts to generate effective vaccines against this pathogen. The correlation

269   between the capsule and serovar of *H. parasuis* is well established; a multiplex serotyping PCR

270   was developed with this in mind (Howell et al. 2015; Howell et al. 2013). This protocol can be

271   employed to type isolates previously classified as CSNT and CSCR via traditional

272   (immunological) methods. The mPCR serotyping reduced the incidence (percentage) of CSNT

273   and CSCR *H. parasuis.* Molecular serotyping has not completely eliminated the issue of CSNT

274   and CSCR isolates. One reason may be the sequence similarity of different serovar-specific

275   primers and serovar-specific products. Another factor may be deletions and/or unknown

276   sequences within certain antigenic markers (Ma et al. 2016). This underscores the importance of

277   MSNT isolate whole-genome sequencing for *in silico* serotyping and improving the molecular

278   serotyping assay. Emergence of MSNT isolates by the molecular serotyping assay may be due to

279   insufficient or incomplete sequence data for *H. parasuis* from Asia. When this assay was

280   developed, there were only nine Asian *H. parasuis* isolates in a 212-isolate database (7 from

281   Japan, 2 from China). Investigating the sequences and gene composition of Asian *H. parasuis*

282   isolate capsule loci may be key for assaying and serotyping MSNT isolates. Besides, absence of

283   serovar-specific markers in polysaccharide biosynthesis loci in MSNT isolates may create

284   antigenic variation impairing vaccine strategies. Therefore, it is also important to study the

285   antigenic variation due to gene mutation and/or absence in polysaccharide biosynthesis loci in

286   the future.

287   Thus far, molecular serotyping has been challenging as there are 15 serovars, making it

288   difficult to design serovar-specific primers yielding differential results. Some primer pairs

289   produce amplicons from different *H. parasuis* serovars that vary by less than 20 bp-a difference

290   that is hardly detected especially when the amplicon size is larger than 600 bp. In our study,

291   electrophoresis using longer agarose gels was performed to enhance the ability of the procedure

292   to discriminate DNA fragment sizes. Bio-1D software was applied to more accurately measure

293   product size based on the intensity of the bands and decrease human operation error. In the case

294   of molecular serotyping tests resulting in ambiguities, serovar-specific primer pairs may be used

295   (in simplex PCR format) to confirm or classify hard-to-identify serovars. According to sequence

296   analysis, the product sizes described in the original publication were not accurate (Howell et al.

297   2015). The corrected product sizes are important to avoid mis-serotyping.

298   According to a previous study, pigs were infected with *H. parasuis* serovars 1, 5, 10, 12, 13,

299   and 14 showed high mortality. Pigs challenged with serovars 2, 4, 8, and 15 showed

300   polyserositis. Pigs inoculated with serovars 3, 6, 7, 9, and 11 resulted in no clinical symptoms or

301   lesions (Kielstein & Rapp-Gabrielson 1992). Serovars 5/12, 4, 13 and 7 are the most common

302   serovars in most countries worldwide (Table S1). Previous study has showed serovar 4 and 13

303   have a higher prevalence in systemic infection than in respiratory disease only (Luppi et al.

304   2013). Our data also showed similar results of serovar 4, 5/12, 13 and MSNT isolates. There may

305   be some correlation between serovar and virulence because serovars are defined by capsule

306   which can directly interact with host cells and has been proven to be a key virulence factor

307   relating to phagocytosis resistance (Olvera et al. 2009). Besides, it should be considered if the

308   impact of some isolates resulting in only pulmonary lesions are underestimate in field due to

309   absence of serositis and typical pulmonary lesions. The role and economic impact of *H. parasuis*

310   in pulmonary infection animals related to porcine respiratory disease complex in field is also

311   worthy of investigation in the future. In our study, serovars 7 and 9 caused serositis with or

312   without respiratory lesions. Serovar 5 isolate was isolated from an animal with only

313 bronchopneumonia lesions and another with lesions in both the serosa and lung tissues in the

314 same herd. Therefore, the results show clinical manifestations of Glässer's disease are influenced

315 by multiple factors, including host, stress, environment, co-infection with different serovars or

316 other pathogens, and gene differences between infecting *H. parasuis* isolates (Boerlin et al. 2013;

317 Howell et al. 2014; Li et al. 2009). In general, most Glässer's disease cases in nursery pigs were

318 co-infected with PRRSV in our data. This may be because PRRSV can cause

319 immunosuppression by reducing non-specific bactericidal activity of pig alveolar macrophages

320 and stimulating interleukin-10 production, which down-regulates inflammatory cytokines (Drew

321 2000; Flores-Mendoza et al. 2008; Suradhat & Thanawongnuwech 2003). The previous studies

322 have showed PRRSV does not result in an increased Glässer's disease by experimental challenge

323 (Segales et al. 1999; Solano et al. 1997). However, significant association between *H. parasuis*

324 and PRRSV in field was reported (Palzer et al. 2015). Recently studies also showed PRRSV can

325 induce bronchopneumonia with *Bordetella bronchiseptica* which is a part of normal upper

326 respiratory microbiota and predispose to colonization with *H. parasuis* (Brockmeier 2004;

327 Brockmeier et al. 2001). Co-infection of pig alveolar macrophages with PRRSV and *H. parasuis*

328 leads to pro-inflammatory mediated immunopathology by synergistic effect (Kavanova et al.

329 2015; Li et al. 2017). In the future, the synergistic effect between PRRSV and *H. parasuis*

330 resulting in economic losses in field is worthy of further investigation. Other factors also interact

331 with *H. parasuis* including the stress of weaning and maternal antibody reduction. However,

332 highly virulent *H. parasuis* isolates might be considered primary pathogens (Aragon et al. 2012).

333 In our study, some *H. parasuis* isolates caused serositis and sudden death without co-infection,

334 even in growing pigs and breeding boars.

335   Previous studies showed *H. parasuis* can access the blood stream through invasion of the

336 mucosal surface in the nasal cavity (Vahle et al. 1997). In our study, pulmonary lesions showed

337   higher pathogenic *H. parasuis* infection rates than serosal lesions. These results are in

338   accordance with a previous study from the Netherlands (Dijkman et al. 2012). *H. parasuis*

339   invasion and survival in lung tissue is likely a key feature for the onset of disease (Olvera et al.

340   2009; Vahle et al. 1995). Our results show *H. parasuis* infected animals with lesions found in

341   dual anatomical locations (pulmonary and serosal) occur at a higher rate than infected animals

342   with lesions located in only one tissue type. Previous studies also mentioned lung is one of the

343   most successful sites for acute (serovar 12) and subacute (serovar 4) isolate recovery (Turni &

344   Blackall 2007). Therefore, lung is an important origin for *H. parasuis* isolation and a target organ

345   for Glässer's disease diagnosis. Pulmonary infections may be an important step for *H. parasuis*

346   systemic infections.

347   Others have reported isolation of multiple isolates from single pig farms (Cerda-Cuellar et

348   al. 2010; Oliveira et al. 2003; Olvera et al. 2006a; Olvera et al. 2006b). Our results also show

349   different serovars cause disease in a single herd, or even in a single animal, although the latter

350   scenario is fairly uncommon. In most situations, Glässer's disease is caused by one isolate

351   (Rafiee et al. 2000), but several isolates may be present at a given farm (Turni & Blackall 2010).

352   Therefore, it would be useful to develop a universal vaccine against multiple serovars. The

353   possibility of cross talk between different pathogenic *H. parasuis* isolates at a given site may be

354   worthy of investigation.

355

356   **Conclusions**

357   Our study shows the dominant serovars of *H. parasuis* in Taiwan are serovars 5/12, 4 and

358   13, followed by MSNT isolates. Proportions of isolates in those serovars resulting in both serosal

359   and pulmonary lesions are significantly higher than pulmonary lesion. Pulmonary lesions may be

360 most important for *H. parasuis* isolation, and may serve as points of origin for systemic *H.*

361 *parasuis* infections in hosts.

362

363 **References**

364 Amano H, Shibata M, Kajio N, and Morozumi T. 1994. Pathologic observations of pigs
365   intranasally inoculated with serovar 1, 4 and 5 of *Haemophilus parasuis* using
366   immunoperoxidase method. *Journal of Veterinary Medical Science* 56:639-644.
367   10.1292/jvms.56.639
368 Angen O, Svensmark B, and Mittal KR. 2004. Serological characterization of Danish
369   *Haemophilus parasuis* isolates. *Veterinary Microbiology* 103:255-258.
370   10.1016/j.vetmic.2004.07.013
371 Aragon V, Cerda-Cuellar M, Fraile L, Mombarg M, Nofrarias M, Olvera A, Sibila M, Solanes D,
372   and Segales J. 2010. Correlation between clinico-pathological outcome and typing of
373   *Haemophilus parasuis* field strains. *Veterinary Microbiology* 142:387-393.
374   10.1016/j.vetmic.2009.10.025
375 Aragon V, Segalés J, and Oliveira S. 2012. Glässer's Disease. In: Zimmerman JJ, Karriker LA,
376   Ramirez A, Schwartz KJ, and Stevenson GW, eds. *Diseases of Swine*. 10 ed. Chichester:
377   Wiley-Blackwell, 760-769.
378 Blackall PJ, Rapp-Gabrielson VJ, and Hampson DJ. 1996. Serological characterisation of
379   *Haemophilus parasuis* isolates from Australian pigs. *Australian Veterinary Journal*
380   73:93-95.
381 Boerlin P, Poljak Z, Gallant J, Chalmers G, Nicholson V, Soltes GA, and MacInnes JI. 2013.
382   Genetic diversity of *Haemophilus parasuis* from sick and healthy pigs. *Veterinary*
383   *Microbiology* 167:459-467. 10.1016/j.vetmic.2013.07.028
384 Brockmeier SL. 2004. Prior infection with *Bordetella bronchiseptica* increases nasal
385   colonization by *Haemophilus parasuis* in swine. *Veterinary Microbiology* 99:75-78.
386   10.1016/j.vetmic.2003.08.013
387 Brockmeier SL, Palmer MV, Bolin SR, and Rimler RB. 2001. Effects of intranasal inoculation
388   with Bordetella bronchiseptica, porcine reproductive and respiratory syndrome virus, or a
389   combination of both organisms on subsequent infection with Pasteurella multocida in
390   pigs. *American Journal of Veterinary Research* 62:521-525.
391 Brockmeier SL, Register KB, Kuehn JS, Nicholson TL, Loving CL, Bayles DO, Shore SM, and
392   Phillips GJ. 2014. Virulence and draft genome sequence overview of multiple strains of
393   the swine pathogen *Haemophilus parasuis*. *PloS One* 9:e103787.

394   10.1371/journal.pone.0103787

395   Cai X, Chen H, Blackall PJ, Yin Z, Wang L, Liu Z, and Jin M. 2005. Serological

396   characterization of *Haemophilus parasuis* isolates from China. *Veterinary Microbiology*

397   111:231-236. 10.1016/j.vetmic.2005.07.007

398   Castilla KS, de Gobbi DD, Moreno LZ, Paixao R, Coutinho TA, dos Santos JL, and Moreno

399   AM. 2012. Characterization of *Haemophilus parasuis* isolated from Brazilian swine

400   through serotyping, AFLP and PFGE. *Research in Veterinary Science* 92:366-371.

401   10.1016/j.rvsc.2011.04.006

402   Cerda-Cuellar M, Naranjo JF, Verge A, Nofrarias M, Cortey M, Olvera A, Segales J, and Aragon

403   V. 2010. Sow vaccination modulates the colonization of piglets by *Haemophilus*

404   *parasuis*. *Veterinary Microbiology* 145:315-320. 10.1016/j.vetmic.2010.04.002

405   Del Rio ML, Gutierrez CB, and Rodriguez Ferri EF. 2003. Value of indirect hemagglutination

406   and coagglutination tests for serotyping *Haemophilus parasuis*. *Journal of Clinical*

407   *Microbiology* 41:880-882. 10.1128/JCM.41.2.880-882.2003

408   Dijkman R, Wellenberg GJ, van der Heijden HM, Peerboom R, Olvera A, Rothkamp A,

409   Peperkamp K, and van Esch EJ. 2012. Analyses of Dutch *Haemophilus parasuis* isolates

410   by serotyping, genotyping by ERIC-PCR and Hsp60 sequences and the presence of the

411   virulence associated trimeric autotransporters marker. *Research in Veterinary Science*

412   93:589-595. 10.1016/j.rvsc.2011.10.013

413   Drew TW. 2000. A review of evidence for immunosuppression due to porcine reproductive and

414   respiratory syndrome virus. *Veterinary Research* 31:27-39. 10.1051/vetres:2000106

415   Flores-Mendoza L, Silva-Campa E, Resendiz M, Osorio FA, and Hernandez J. 2008. Porcine

416   reproductive and respiratory syndrome virus infects mature porcine dendritic cells and

417   up-regulates interleukin-10 production. *Clinical and Vaccine Immunology* 15:720-725.

418   10.1128/CVI.00224-07

419   Howell KJ, Peters SE, Wang J, Hernandez-Garcia J, Weinert LA, Luan SL, Chaudhuri RR,

420   Angen O, Aragon V, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW,

421   Maskell DJ, Tucker AW, and Consortium BRT. 2015. Development of a multiplex PCR

422   assay for rapid molecular serotyping of *Haemophilus parasuis*. *Journal of Clinical*

423   *Microbiology* 53:3812-3821. 10.1128/JCM.01991-15

424   Howell KJ, Weinert LA, Chaudhuri RR, Luan SL, Peters SE, Corander J, Harris D, Angen O,

425   Aragon V, Bensaid A, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW,

426   Holden MT, Tucker AW, Maskell DJ, and Consortium BT. 2014. The use of genome

427   wide association methods to investigate pathogenicity, population structure and serovar

428   in *Haemophilus parasuis*. *BMC Genomics* 15:1179. 10.1186/1471-2164-15-1179

429   Howell KJ, Weinert LA, Luan SL, Peters SE, Chaudhuri RR, Harris D, Angen O, Aragon V,

430   Parkhill J, Langford PR, Rycroft AN, Wren BW, Tucker AW, Maskell DJ, and
431   Consortium BRT. 2013. Gene content and diversity of the loci encoding biosynthesis of
432   capsular polysaccharides of the 15 serovar reference strains of *Haemophilus parasuis*.
433   *Journal of Bacteriology* 195:4264-4273. 10.1128/JB.00471-13
434 Howell KJ, Weinert LA, Peters SE, Wang J, Hernandez-Garcia J, Chaudhuri RR, Luan SL,
435   Angen O, Aragon V, Williamson SM, Langford PR, Rycroft AN, Wren BW, Maskell DJ,
436   and Tucker AW. 2017. "Pathotyping" multiplex PCR assay for *Haemophilus parasuis*: a
437   tool for prediction of virulence. *Journal of Clinical Microbiology* 55:2617-2628.
438   10.1128/JCM.02464-16
439 Kavanova L, Prodelalova J, Nedbalcova K, Matiasovic J, Volf J, Faldyna M, and Salat J. 2015.
440   Immune response of porcine alveolar macrophages to a concurrent infection with porcine
441   reproductive and respiratory syndrome virus and *Haemophilus parasuis* in vitro.
442   *Veterinary Microbiology* 180:28-35. 10.1016/j.vetmic.2015.08.026
443 Kielstein P, and Rapp-Gabrielson VJ. 1992. Designation of 15 serovars of *Haemophilus parasuis*
444   on the basis of immunodiffusion using heat-stable antigen extracts. *Journal of Clinical*
445   *Microbiology* 30:862-865.
446 Kielstein P, Rosner H, and Mueller W. 1991. Typing of heat-stable soluble *Haemophilus*
447   *parasuis* antigen by means of agar gel precipitation and the dot-blot procedure. *Journal of*
448   *Veterinary Medicine B: Infectious Diseases and Veterinary Public Health* 38.
449 Li J, Wang S, Li C, Wang C, Liu Y, Wang G, He X, Hu L, Liu Y, Cui M, Bi C, Shao Z, Wang
450   X, Xiong T, Cai X, Huang L, and Weng C. 2017. Secondary *Haemophilus parasuis*
451   infection enhances highly pathogenic porcine reproductive and respiratory syndrome
452   virus (HP-PRRSV) infection-mediated inflammatory responses. *Veterinary Microbiology*
453   204:35-42. 10.1016/j.vetmic.2017.03.035
454 Li JX, Jiang P, Wang Y, Li YF, Chen W, Wang XW, and Li P. 2009. Genotyping of
455   *Haemophilus parasuis* from diseased pigs in China and prevalence of two coexisting
456   virus pathogens. *Preventive Veterinary Medicine* 91:274-279.
457   10.1016/j.prevetmed.2009.06.004
458 Lin CN, Lin WH, Hung LN, Wang SY, and Chiou MT. 2013. Comparison of viremia of type II
459   porcine reproductive and respiratory syndrome virus in naturally infected pigs by zip
460   nucleic acid probe-based real-time PCR. *BMC Veterinary Research* 9:181. 10.1186/1746-
461   6148-9-181
462 Luppi A, Bonilauri P, Dottori M, Iodice G, Gherpelli Y, Merialdi G, Maioli G, and Martelli P.
463   2013. *Haemophilus parasuis* serovars isolated from pathological samples in Northern
464   Italy. *Transboundary and Emerging Diseases* 60:140-142. 10.1111/j.1865-
465   1682.2012.01326.x

466   Ma L, Wang L, Chu Y, Li X, Cui Y, Chen S, Zhou J, Li C, Lu Z, Liu J, and Liu Y. 2016.
467   Characterization of Chinese *Haemophilus parasuis* isolates by traditional serotyping and
468   molecular serotyping methods. *PloS One* 11:e0168903. 10.1371/journal.pone.0168903
469   Miniats OP, Smart NL, and Ewert E. 1991a. Vaccination of gnotobiotic primary specific
470   pathogen-free pigs against *Haemophilus parasuis*. *Canadian Journal of Veterinary*
471   *Research* 55:33-36.
472   Miniats OP, Smart NL, and Rosendal S. 1991b. Cross protection among *Haemophilus parasuis*
473   strains in immunized gnotobiotic pigs. *Canadian Journal of Veterinary Research* 55:37-
474   41.
475   Moller K, and Kilian M. 1990. V factor-dependent members of the family *Pasteurellaceae* in the
476   porcine upper respiratory tract. *Journal of Clinical Microbiology* 28:2711-2716.
477   Morozumi T, and Nicolet J. 1986. Some antigenic properties of *Haemophilus parasuis* and a
478   proposal for serological classification. *Journal of Clinical Microbiology* 23:1022-1025.
479   Nielsen R. 1993. Pathogenicity and immunity studies of *Haemophilus parasuis* serotypes. *Acta*
480   *Veterinaria Scandinavica* 34:193-198.
481   Oliveira S, Blackall PJ, and Pijoan C. 2003. Characterization of the diversity of *Haemophilus*
482   *parasuis* field isolates by use of serotyping and genotyping. *American Journal of*
483   *Veterinary Research* 64:435-442.
484   Olvera A, Ballester M, Nofrarias M, Sibila M, and Aragon V. 2009. Differences in phagocytosis
485   susceptibility in *Haemophilus parasuis* strains. *Veterinary Research* 40:24.
486   10.1051/vetres/2009007
487   Olvera A, Calsamiglia M, and Aragon V. 2006a. Genotypic diversity of *Haemophilus parasuis*
488   field strains. *Applied and Environmental Microbiology* 72:3984-3992.
489   10.1128/AEM.02834-05
490   Olvera A, Cerda-Cuellar M, and Aragon V. 2006b. Study of the population structure of
491   *Haemophilus parasuis* by multilocus sequence typing. *Microbiology* 152:3683-3690.
492   10.1099/mic.0.29254-0
493   Olvera A, Segales J, and Aragon V. 2007. Update on the diagnosis of *Haemophilus parasuis*
494   infection in pigs and novel genotyping methods. *Veterinary Journal* 174:522-529.
495   10.1016/j.tvjl.2006.10.017
496   Palzer A, Haedke K, Heinritzi K, Zoels S, Ladinig A, and Ritzmann M. 2015. Associations
497   among *Haemophilus parasuis*, *Mycoplasma hyorhinis*, and porcine reproductive and
498   respiratory syndrome virus infections in pigs with polyserositis. *Canadian Veterinary*
499   *Journal* 56:285-287.
500   Pina S, Olvera A, Barcelo A, and Bensaid A. 2009. Trimeric autotransporters of *Haemophilus*
501   *parasuis*: generation of an extensive passenger domain repertoire specific for pathogenic

502   strains. *Journal of Bacteriology* 191:576-587. 10.1128/JB.00703-08

503   Rafiee M, Bara M, Stephens CP, and Blackall PJ. 2000. Application of ERIC-PCR for the

504   comparison of isolates of *Haemophilus parasuis*. *Australian Veterinary Journal* 78:846-

505   849.

506   Rapp-Gabrielson VJ, and Gabrielson DA. 1992. Prevalence of *Haemophilus parasuis* serovars

507   among isolates from swine. *American Journal of Veterinary Research* 53:659-664.

508   Rubies X, Kielstein P, Costa L, Riera P, Artigas C, and Espuna E. 1999. Prevalence of

509   *Haemophilus parasuis* serovars isolated in Spain from 1993 to 1997. *Veterinary*

510   *Microbiology* 66:245-248. 10.1016/S0378-1135(99)00007-3

511   Segales J, Domingo M, Solano GI, and Pijoan C. 1999. Porcine reproductive and respiratory

512   syndrome virus and *Haemophilus parasuis* antigen distribution in dually infected pigs.

513   *Veterinary Microbiology* 64:287-297.

514   Smart NL, and Miniats OP. 1989. Preliminary assessment of a *Haemophilus parasuis* bacterin

515   for use in specific pathogen free swine. *Canadian Journal of Veterinary Research*

516   53:390-393.

517   Solano GI, Segales J, Collins JE, Molitor TW, and Pijoan C. 1997. Porcine reproductive and

518   respiratory syndrome virus (PRRSv) interaction with Haemophilus parasuis. *Veterinary*

519   *Microbiology* 55:247-257.

520   Suradhat S, and Thanawongnuwech R. 2003. Upregulation of interleukin-10 gene expression in

521   the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus.

522   *Journal of General Virology* 84:2755-2760. 10.1099/vir.0.19230-0

523   Tadjine M, Mittal KR, Bourdon S, and Gottschalk M. 2004. Development of a new serological

524   test for serotyping *Haemophilus parasuis* isolates and determination of their prevalence

525   in North America. *Journal of Clinical Microbiology* 42:839-840.

526   Takahashi K, Naga S, Yagihashi T, Ikehata T, Nakano Y, Senna K, Maruyama T, and Murofushi

527   J. 2001. A cross-protection experiment in pigs vaccinated with *Haemophilus parasuis*

528   serovars 2 and 5 bacterins, and evaluation of a bivalent vaccine under laboratory and field

529   conditions. *Journal of Veterinary Medical Science* 63:487-491. 10.1292/jvms.63.487

530   Turni C, and Blackall P. 2007. Comparison of sampling sites and detection methods for

531   *Haemophilus parasuis*. *Australian Veterinary Journal* 85:177-184. 10.1111/j.1751-

532   0813.2007.00136.x

533   Turni C, and Blackall PJ. 2005. Comparison of the indirect haemagglutination and gel diffusion

534   test for serotyping *Haemophilus parasuis*. *Veterinary Microbiology* 106:145-151.

535   10.1016/j.vetmic.2004.12.019

536   Turni C, and Blackall PJ. 2010. Serovar profiling of *Haemophilus parasuis* on Australian farms

537   by sampling live pigs. *Australian Veterinary Journal* 88:255-259. 10.1111/j.1751-

538   0813.2010.00592.x

539   Vahle JL, Haynes JS, and Andrews JJ. 1995. Experimental reproduction of *Haemophilus*

540   *parasuis* infection in swine: clinical, bacteriological, and morphologic findings. *Journal*

541   *of Veterinary Diagnostic Investigation* 7:476-480. 10.1177/104063879500700409

542   Vahle JL, Haynes JS, and Andrews JJ. 1997. Interaction of *Haemophilus parasuis* with nasal and

543   tracheal mucosa following intranasal inoculation of cesarean derived colostrum deprived

544   (CDCD) swine. *Canadian Journal of Veterinary Research* 61:200-206.

545   Zhang B, Tang C, Liao M, and Yue H. 2014. Update on the pathogenesis of *Haemophilus*

546   *parasuis* infection and virulence factors. *Veterinary Microbiology* 168:1-7.

547   10.1016/j.vetmic.2013.07.027

548

549   **Figure legends**

550   **Figure 1** Distribution of *Haemophilus parasuis* serovars according to lesion pattern.

551   Serositis only: animals were diagnosed with *H. parasuis* positive serosal lesions. Pulmonary

552   lesion only: animals were diagnosed with *H. parasuis* positive pulmonary lesions. Data were

553   analyzed by Fisher's exact test and variables were considered significant at a 0.05 level (two-

554   sided).

555

556   **Figure 2** *Haemophilus parasuis* isolation proportion of 204 lesions of 108 pathological

557   diagnosed cases.

558   Fisher's exact test was used to compare the frequency of *H. parasuis* isolation lesions. *P* value <

559   0.05 was considered a significant difference.

560

561   **Figure 3** Molecular serotyping results with or without sequence results for 133 *Haemophilus*

562   *parasuis* isolates.

563

564   **Supplementary file**

565   **Supplementary Figure 1** Gross meningeal lesion in *H. parasuis* infected pigs.

566

567   **Supplementary Figure 2** Histopathological suppurative meningitis lesion in *H. parasuis*

568   infected pigs.

569

570   **Supplementary Figure 3** Gross pleural and peritoneal lesions in *H. parasuis* infected pigs.

571

572   **Supplementary Figure 4** Gross epicardial lesion in *H. parasuis* infected pigs.

573

574   **Supplementary Figure 5** Gross joint synovial cavity lesion in *H. parasuis* infected pigs.

575

576   **Supplementary Figure 6** Histopathological fibrinous serositis lesion in *H. parasuis* infected

577   pigs.

578

579   **Supplementary Figure 7** Gross lung lesion in *H. parasuis* infected pigs.

580

581   **Supplementary Figure 8** Histopathological pulmonary lesion in *H. parasuis* infected pigs.

582

583   **Supplementary Figure 9** Histopathological fibrous serositis lesion in *H. parasuis* infected pigs.

584

585   **Supplementary Figure 10** Histopathological meningitis lesion in *H. parasuis* infected pigs.

586

587   **Supplementary Figure 11** Band patterns of molecular serotyping mPCR for *Haemophilus*

588   *parasuis.*

589   Lane M: 50 bp DNA Ladder, lane S5: serovar 5 or 12, lane G2: molecular serotyping non-

590   typable group 2, lane S4: serovar 4, lane S9: serovar 9, lane G1: molecular serotyping non-

591   typable group 1, lane NC: negative control.

592

593   **Supplementary Figure 12** Band patterns of molecular serotyping mPCR for *Haemophilus*

594   *parasuis.*

595   Lane M: 50 bp DNA Ladder, lane S4: serovar 4, lane S5: serovar 5 or 12, lane G2: molecular

596   serotyping non-typable group 2, lane S14: serovar 14, lane G1: molecular serotyping non-typable

597   group 1, lane NC: negative control.

598

599   **Supplementary Figure 13** Band patterns of molecular serotyping mPCR for *Haemophilus*

600   *parasuis.*

601   Lane M: 50 bp DNA Ladder, lane S5: serovar 5 or 12, lane S4: serovar 4, lane G1: molecular

602   serotyping non-typable group 1, lane G2: molecular serotyping non-typable group 2, lane NC:

603   negative control.Histopathological bronchopneumonia lesion in *H. parasuis* infected pigs.

604

605   **Supplementary Figure 14** Band patterns of molecular serotyping mPCR for *Haemophilus*

606   *parasuis.*

607 Lane M: 50 bp DNA Ladder, lane G1: molecular serotyping non-typable group 1, lane S4:
608 serovar 4, lane S5: serovar 5 or 12, lane S14: serovar 14, lane G4: molecular serotyping non-
609 typable group 4, lane G3: molecular serotyping non-typable group 3, lane NC: negative control.

610

611 **Supplementary Figure 15** Band patterns of molecular serotyping mPCR for *Haemophilus*
612 *parasuis.*

613 Lane M: 50 bp DNA Ladder RTU (GeneDireX), lane G2: molecular serotyping non-typable

614 group 2, lane S4: serovar 4, lane S5: serovar 5 or 12, lane G1: molecular serotyping non-typable

615 group 1, lane G3: molecular serotyping the non-typable group 3, lane NC: negative control.

616 **Ethics and consent to participate**

617 The study did not involve any animal experiment. The Institutional Animal Care and Use

618 Committee (IACUC) of National Pingtung University of Science and Technology did not deem

619 it necessary for this research group to obtain formal approval to conduct this study.

620

621 **Consent to publish**

622 Not applicable.

623

624 **Competing interest**

625 The authors declare that they have no competing interests.

626

627 **Funding**

628 No funding was obtained for this study.

629

630 **Authors' contributions**

631 Wei-Hao Lin, Chao-Nan Lin and Ming-Tang Chiou designed this study. Wei-Hao Lin performed

632 the laboratory experiments, analyzed data and wrote the manuscript. Hsing-Chun Shih assisted

633 the laboratory experiments. Chuen-Fu Lin, Cheng-Yao Yang, Yung-Fu Chang, Chao-Nan Lin

634 and Ming-Tang Chiou proofread and edited the manuscript.

635

636 **Availability of data and materials**

637 All the data supporting our findings is contained within the manuscript.

638

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646 Fang Wang, Guang-Ting Tsai, Ni-Jyun Ke, Ting-Han Lin, Sheng-Yuan Wang, Hong Liu, Jia-

647 Liang Hong, Joan Wang and Yu-Hsuan Chen.

Figure 1

Distribution of *Haemophilus parasuis* serovars according to lesion pattern.

Distribution of *Haemophilus parasuis* serovars according to lesion pattern. Serositis only: animals were diagnosed with *H. parasuis* positive serosal lesions. Pulmonary lesion only: animals were diagnosed with *H. parasuis* positive pulmonary lesions. Data were analyzed by Fisher's exact test and variables were considered significant at a 0.05 level (two-sided).

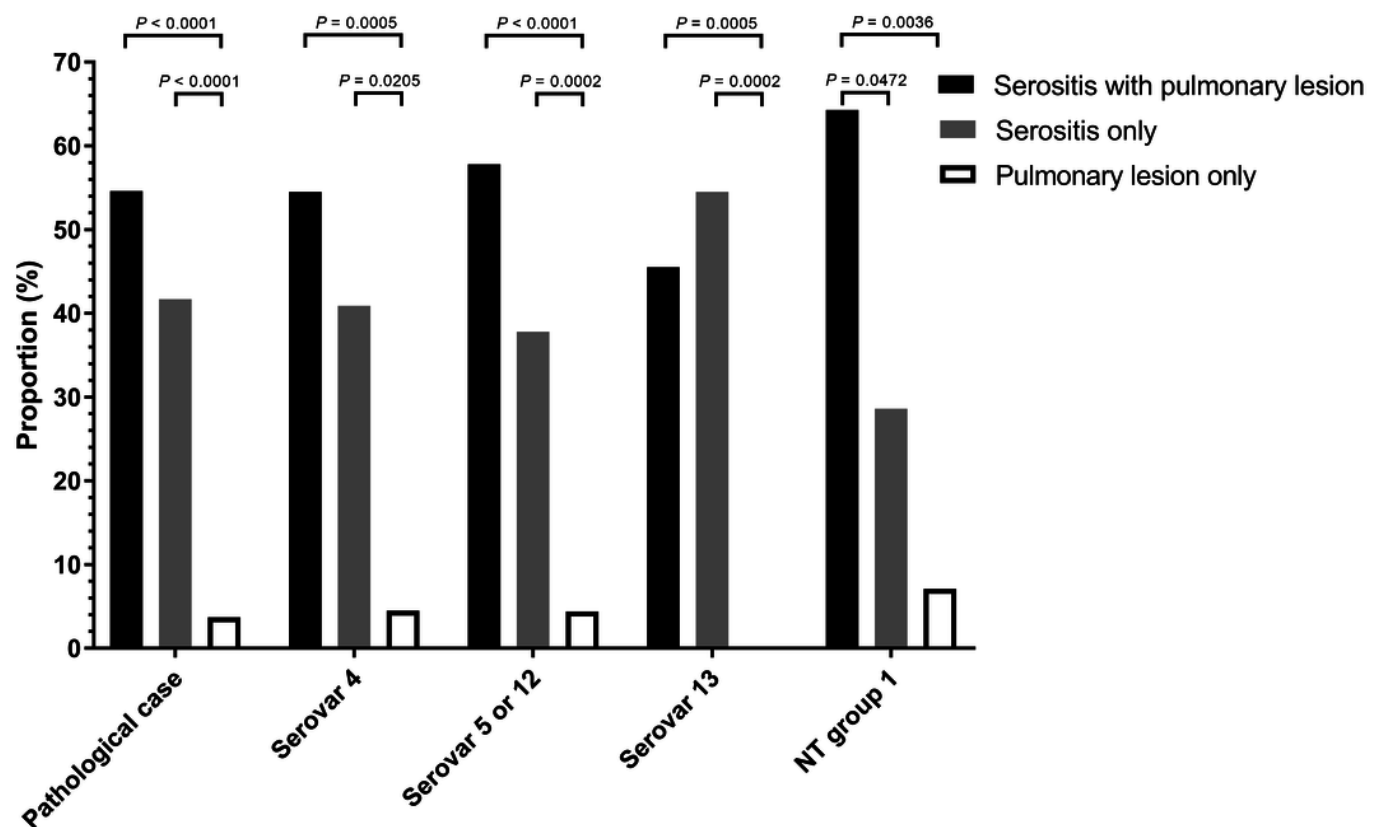



Figure 2

Haemophilus parasuis isolation proportion of 204 lesions of 108 pathological diagnosed cases.

Haemophilus parasuis isolation proportion of 204 lesions of 108 pathological diagnosed cases. Fisher's exact test was used to compare the frequency of *H. parasuis* isolation lesions. *P* value < 0.05 was considered a significant difference.

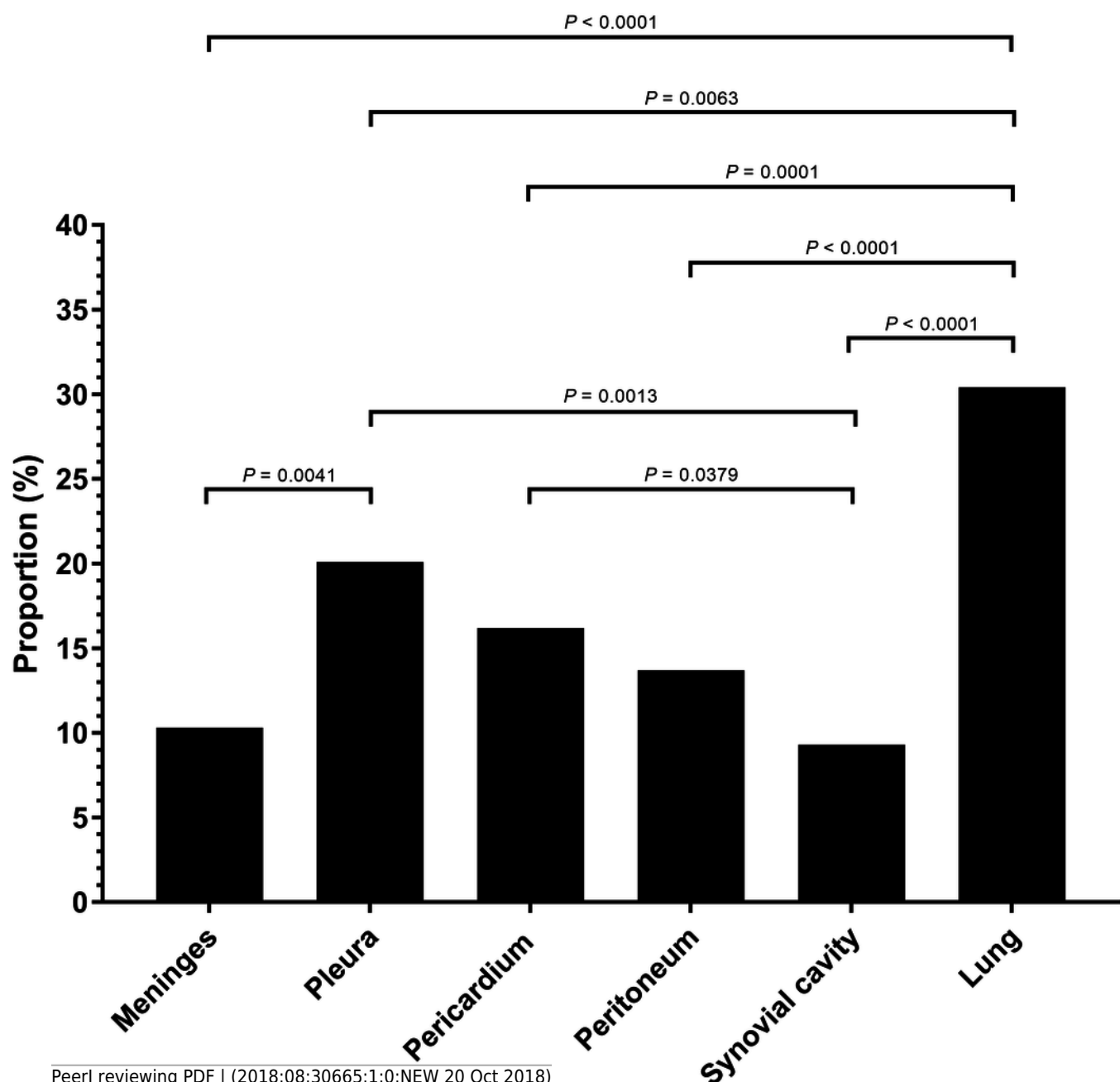
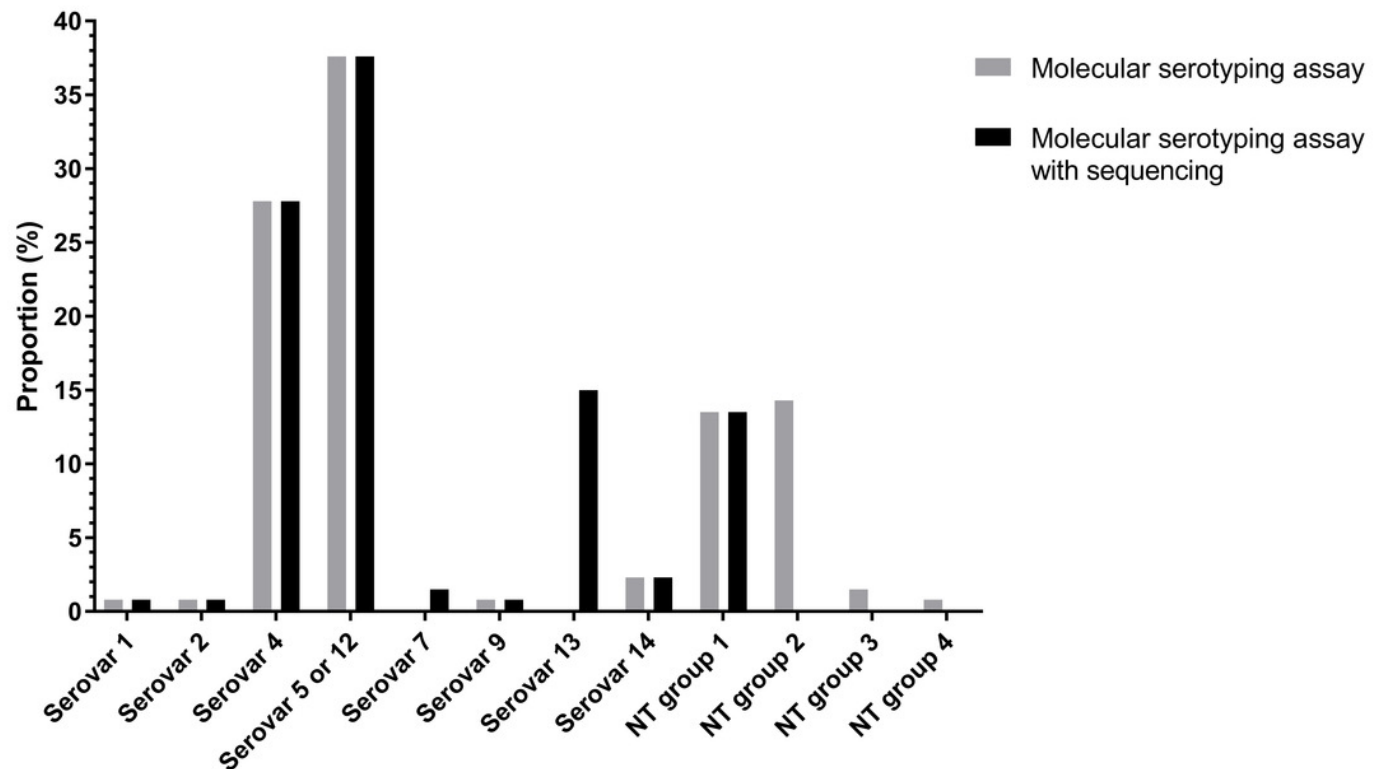



Figure 3

Molecular serotyping results with or without sequence results for 133 *Haemophilus parasuis* isolates.

Product size by molecular serotyping assay

1   Table 1 Product size by molecular serotyping assay (Howell et al. 2015)

Gene	Serovar	Product size in the original publication (bp)	Product size (bp) predicted by BLAST	Aligned sequence accession number	Product size (bp) measured by Bio-1D software	Product size (bp) according to sequence
funB	1	180	183	CL120103	184	183
wzx	2†	295	294	CL120103	N/A	N/A
glyC	3†	610	618	KC795327.1	N/A	N/A
wciP	4	320	349	KC795356.1	350	349
wcwK	5 or 12	450	468	KC795341.1	469	468
gltI	6†	360	378	KC795372.1	N/A	N/A
funQ	7†	490	499	CP009158.1	N/A	N/A
scdA	8†	650	634	KC795411.1	N/A	N/A
funV	9	710	676	KC795429.1	675	676
funX	10†	790	784	KC795448.1	N/A	N/A
amtA	11†	890	883	KC795474.1	N/A	N/A
gltP	13†	840	836	KF841370.1	N/A	N/A
funAB	14	730	710	KC795520.1	708	710
funI	15†	550	550	KC795537.1	N/A	N/A
HPS_219690793	All	275	276	CP020085.1	276	276

2   †This serotype was not detected in this study.

Unexpected products of serotyping multiplex PCR

1 Table 2 Unexpected products of serotyping multiplex PCR

Molecular serotyping non-typable isolate	Serovar according to sequence	Product size (bp) measured by Bio-1D software	Product size (bp) according to sequence	Amplified primer
Group 1	Unknown†	None‡	None	None
		300	297	S13F, S14R
Group 2	Serovar 13	830	836	S13
		1000	N/A§	N/A
Group 3	Serovar 7	500	499	S7
		660	N/A	N/A
Group 4	Serovar 13	300	297	S13F, S14R

2 †Serovar could not be defined without any serovar-specific product sequence result.

3 ‡There was no serovar-specific product.

4 §Cloning of serovar-specific product was failed.