Criticisms with evidence from the text or from other sources

Specific suggestions on how to improve the manuscript

Comment on language and grammar issues

Organize by importance of the issues and number your points

Provide constructive criticism and avoid personal opinions

Comment on strengths as well as weaknesses

Cover Page

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

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~~**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~~ can 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 were 5/12 (37.6%), 4 (27.8%) and 13 (15%) followed by ~~molecular serotyping~~ non-typable (MSNT) isolates (13.5%), which ~~are~~ were differentiated ~~on a genetic basis~~usig PCR of a polysaccharide biosynthesis locus. ~~Nevertheless, t~~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.

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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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Manuscript to be reviewed

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2 relationship between serovars and pathological patterns in Taiwan

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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 ~~intended as~~ an

29 indicator of virulence and pathotype and is also crucial for vaccination programs and vaccine

30 development. ~~According to a polysaccharide biosynthesis locus analysis, H. parasuis~~ *H. parasuis* isolates

31 ~~could~~ can be classified by a molecular serotyping assay (except for serovars 5 and 12). The aim of

32 this study was to identify ~~H. parasuis~~ *H. parasuis* isolates from diseased pigs in Taiwan by using a molecular

33 serotyping assay and to analyze the relationship between serovars and pathological patterns.

34 **Methods:** From August 2013 to February 2017, a total of 155 diseased animals from 124 infected herds were examined for infection with *H. parasuis.* 133 isolates of *H. parasuis* were recovered and serotyped~~from 277 lesions. on 155~~

35 ~~diseased animals from 124 infected herds serotyped~~ by multiplex PCR and ~~analyzed~~ correlated with

36 pathological data.

37 **Results:** The results ~~showed that~~identified the dominant ~~serovars of H. parasuis~~ *H. parasuis* ~~in Taiwan~~ were serovars

38 5/12 (37.6%), 4 (27.8%) and 13 (15%) followed by molecular serotyping non-typable (MSNT)

39 isolates (13.5%), ~~which are differentiated on a genetic basis~~. ~~Nevertheless, t~~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 ~~F~~M~~urthermore, m~~Most ~~H. parasuis~~ *H. parasuis* isolates were isolated from nursery pigs infected with porcine

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

44 *parasuis* infection was significantly higher than that of serosal lesions.

45 **Discussion**: Collectively, the distribution of serovars in Taiwan is similar ~~to that found in~~ other

46 countries, but MSNT isolates remain due to genetic variations. ~~Furthermore, p~~Our data suggests pulmonary lesions

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47 ~~may be~~are optimum sites for ~~H. parasuis~~ *H. parasuis* isolation~~, the diagnosis of Glässer's disease~~, and may also

48 serve as points of origin for systemic ~~H. parasuis~~ *H. parasuis* infections in hosts.

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49 Keywords: Haemophilus parasuis, Glässer's disease, polyserositis, serotyping

50

51 **Introduction**

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

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

54 meningitis and poor production performance, ~~causing~~ resulting in severe economic losses in the swine

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

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

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

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

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

60 *parasuis* is very important, not only for ~~subtyping for~~ epidemiological research but also for

61 choosing efficacious inactivated whole-cell bacterial vaccines.

62 Fifteen serovars~~, and~~ conventional serotyping cross-reactive (CSCR) and non-typable

63 (CSNT) isolates of ~~H. parasuis~~ *H. parasuis* have been described and demonstrated by gel immunodiffusion

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

65 non-reaction to antisera, there are still approximately 15% to 40% CSCR and CSNT isolates

66 reported in a variety of countries ~~of areas~~ by GID (Table S1) (Blackall et al. 1996; Cai et al.

67 2005; Castilla et al. 2012; Del Rio et al. 2003; Kielstein & Rapp-Gabrielson 1992; Luppi et al.

68 2013; Ma et al. 2016; Oliveira et al. 2003; Rapp-Gabrielson & Gabrielson 1992; Rubies et al.

69 1999; Tadjine et al. 2004). Despite using an indirect hemagglutination assay (IHA) designed to

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70 reduce the proportion of CSCR isolates, ~~there were still approximately~~ 7.5% to 18% of isolates are still untypable ~~CSCR and~~

71 ~~CSNT isolates~~ (Table S1) (Angen et al. 2004; Cai et al. 2005; Del Rio et al. 2003; Dijkman et al.

72 2012; Howell et al. 2015). This phenomenon makes it more difficult to ~~survey and design~~conduct an effective

73 vaccination program against *H. parasuis*.

74 Conventional serotyping ~~was developed~~is used extensively ~~by many researchers~~ (Kielstein et al. 1991;

75 Morozumi & Nicolet 1986; Rapp-Gabrielson & Gabrielson 1992). ~~Finally, t~~The Kielstein-Rapp

76 Gabrielson (KRG) scheme recognize~~d~~ 15 serovars of ~~H. parasuis~~ *H. parasuis* on the basis of a GID test with

77 specific rabbit antisera (Kielstein & Rapp-Gabrielson 1992). ~~In proposing, the KRG scheme, t~~The

78 authors noted a correlation between serovar and virulence (Kielstein & Rapp-Gabrielson 1992).

79 According to ~~field~~ serotyping results, serovar 4 tends to be found in pulmonary infections; CSNT

80 and CSCR isolates are mainly found in systemic infections (Angen et al. 2004). Unfortunately,

81 ~~there appears to be~~others report little correlation between serovar and virulence as isolates in the same serovar

82 often exhibit different virulence levels (Aragon et al. 2010; Olvera et al. 2007).

83 Previous studies ~~have~~ established ~~that~~ the serovar and pathotype of ~~H. parasuis~~ *H. parasuis* are based on

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

85 A multiplex PCR (mPCR) ~~designed~~ based on a polysaccharide biosynthesis locus analysis was

86 employed to ~~further define and characterized~~molecularly type ~~H. parasuis~~ *H. parasuis* serovars (Howell et al. 2015). As a

87 result, 14 of ~~the~~ 15 serovars of ~~H. parasuis~~ *H. parasuis* (serovars 5 and 12 could not be differentiated),

88 ~~including previous CSNT and CSCR isolates~~ were identified using this assay (Howell et al.

89 2015). Using the molecular typing assay many of the CSNT and CSCR isolates were successfully typed in a recognized serotype.

90 Although Glässer's disease is ~~very~~ common in Taiwan, ~~no published work has been~~

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~~91 performed to~~ serotyp~~inge~~of pathogenic ~~H. parasuis~~ *H. parasuis* isolates from Taiwanese pigs is not done. The principal aim of

92 this study was to use molecular serotyping to identify and characterize ~~H. parasuis~~ *H. parasuis* serovars ~~that~~

93 ~~had been~~ isolated from Taiwanese pigs infected with *H. parasuis*, and ~~analyze the relationship of~~correlate

94 serovars and pathological patterns.

95

96 **Materials & Methods**

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97 Bacterial isolate collection and identification

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

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

100 diseased pigs were located in the meninges, pleura, pericardia, peritonea, synovial cavities and

101 lungs. Lesions were swabbed; ~~samples were~~ plated on chocolate agar (at 37°C, 5% CO2, 18 to 72

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

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

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

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

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

107 Molecular serotyping mPCR

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

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

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

111 H2O, which was heated to 100°C for 30 min ~~and~~ then centrifuged at 4,000 x g for 1 min. The

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

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

114 Each PCR reaction was performed in a total volume of 30 µL containing ultrapure H2O, 1 x

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

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

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

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

119 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

120 molecular serotyping mPCR ~~products~~ amplicons were stained with ethidium bromide and analyzed using a

121 20-cm-long 2% agarose gel. A 50-bp DNA ladder RTU (GeneDireX, Las Vegas, NV, USA) and

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

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

124 and 3 hr. The results were confirmed by twice repeating tests.

125 Sequencing and analysis of unexpected PCR-amplified products

126 ~~The u~~Unexpected amplicons of the molecular serotyping mPCR products were cloned using a

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

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

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

130 Search Tool) database.

131 Pathological examination

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

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

134 primarily on ~~the organs of~~ meningeal, pleural, pericardial, peritoneal, and synovial cavities, and

135 lungs, which were fixed in 10% formalin.

136 Diagnoses of other diseases

137 *Pasteurella multocida* and *Streptococcus sp*. isolated from diseased pigs on blood agar were

138 initially determined by colony morphology. Pasteurella multocida was identified by catalase test

139 (positive), oxidase test (positive), Gram stain (Gram negative bacillus) and colony PCR modified

140 from previous study (Townsend et al. 1998). Streptococcus sp. was identified by catalase test

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141 (negative), Gram stain (Gram positive coccus) and colony PCR (Marois et al. 2004). Salmonella

142 sp. isolated from diseased pigs on blood agar and MacConkey agar was initially determined by

143 colony morphology and then identified by GNB-14 computer-coding system which can identify

144 most gram negative bacillus based on 14 biochemical characteristics including capable of using

145 lactose or sucrose, glucose, citrate, ornithine, arginine and lysine; producing carbon dioxide or

146 hydrogen and hydrogen sulfide; hydrolyzing urea; motility test; indole test; indole-3-propionic

147 acid test; Voges-Proskauer test; oxidase test (Tsai & Ho 2006). Nucleic acid extraction was

148 performed on a MagNA Pure LC 2.0 by using the MagNA Pure LC total nucleic acid isolation

149 kit (Roche Applied Science, Indianapolis, IN, USA). Following cDNA synthesis was using

Commented [H6]: Nucleic acid extraction of porcine tissue?

150 PrimeScript™ RT reagent kits (Takara, Kyoto, Japan). Porcine reproductive and respiratory

151 syndrome virus (PRRSV) reverse transcription real-time PCR was performed as previously

152 described (Lin et al. 2013). Porcine epidemic diarrhea virus reverse transcription real-time PCR

153 was performed by using PEDV-133F (5'-TTG-GCT-GCT-GGG-CTA-TGG-3') and PEDV

154 133R (5'-TGA-AAA-GGT-ACT-GCG-TTC-CC-3') following the thermocycling conditions

155 consisted of 10 min at 95°C and 45 cycles of 10 sec at 95°C and 60 sec at 60°C. Each 10 µL 156 reactionmixture contained 0.2 µM concentrations of the forward and reverse primers and 3 µL of

157 the cDNA. All diseases were diagnosed by both pathogen detection and histopathological lesions

158 to avoid subclinical infection.

159 Statistical analysis

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

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

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

163

164 Results

165 ~~H. parasuis~~ *H. parasuis* isolates, origins and pathological lesion patterns

166 One hundred thiry three isolates of~~A total of 133 isolates of H. parasuis~~ *H. parasuis* were isolated from August 2013 to February 2017.

~~The isolates were taken from 277 lesions on 155 diseased animals from 124 infected herds. Nine herds (7.3%) contained isolates representing different serovars.~~

Of 155 ~~H. parasuis~~ *H. parasuis* cases, 12 (7.7%) cases belonged to suckling pigs (≤ 3-week-old), 133 (85.2%) cases belonged to nursery pigs (4- to 12-week-old), 7 (4.5%) cases belonged to growing pigs (13- to 26-week-old) and one case belonged to a breeding boar. Age information for two cases was unknown. Eighty-six cases (55.5%) had ~~H. parasuis~~ *H. parasuis* isolated from lung lesions with or without serosal lesions.

~~There were 108 cases belonging to live animals or fresh, complete carcasses that~~One hundred eight animals were necropsied. In our study, 54.6 % of the ~~H. parasuis~~ *H. parasuis* positive animals had serositis and pulmonary tissue lesions, 41.7% of the ~~H. parasuis~~ *H. parasuis* infected animals had serosal lesions only, and 3.7 % ~~of~~ ~~the infected animals~~ displayed only pulmonary tissue lesions ~~(H. parasuis was only isolated from 178 lung lesions)~~ (Fig. 1).

~~Of the 106~~One hundred six cases (98.1%) ~~diagnosed with~~had bronchopneumonia, 64 cases (59.3%) displayed ~~H. parasuis~~ *H. parasuis* positive lung lesions. Thirteen cases (12%) had Streptococcus sp. infected serosal lesions. Seventy-eight cases (72.2%) registered as positive for PRRSV via reverse transcription real-time PCR screening. Seven bronchopneumonia cases were lung lesions positive for Pasteurella multocida. Twenty-six cases (24.1%) were diagnosed with salmonellosis. One case (0.9%) was diagnosed with porcine epidemic diarrhea.

~~The 277 H. parasuis~~ *H. parasuis* infected lesions included meninges (9.4%), pleura (21.3%), pericardium (16.6%), peritoneum (13.4%), synovial cavity (7.6%) and lung (31.8%). The proportion of 204 ~~H. parasuis~~ *H. parasuis* infected lesions ~~of~~ from 108 animals diagnosed with Glasser's disease ~~diagnosed cases~~ were 10.3%, 20.1%, 16.2%, 13.7%, 9.3% and 30.4%, respectively, for the tissues listed above (Fig. 2). The

Commented [H7]: 155 cases but only 133 isolates. Were 22 cases identified by histopathology only?

Commented [H8]: These are all based on histopathology? Were the 64 lung lesions included in the 106 bronchopneumonia lesions or were they deeper lesions with alveolar involvement? How many of these samples were also culture positive. Can this be consolidated into a table to show relationship of culture positive to histopathology lesions

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Commented [H9]: Can this section be reduced or if co-infection is a significant component expand discussion

Commented [H10]: The information in lines 185-190 is very confusing. The number of infected lesions varies and the proportion varies. Settle on one (277 or 204) Figure 2 seems to use 204

189 percentage of lung lesions showing ~~H. parasuis~~ *H. parasuis* infection was significantly higher than the

190 percentage of serosal lesions ($P < 0.05$).

191 Serovar distribution by molecular serotyping assay

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

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

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

195 the product sizes of the serovar-specific primers analyzed by Bio-1D software ~~were not accurate~~varied

196 ~~compared to that described~~from ~~in~~ the original publication (Howell et al. 2015). Furthermore, there

197 were still 41 isolates (29.8%) ~~that were~~ classified as MSNT; these were divided into four groups

198 based on the appearance of unexpected amplicons or the lack of serovar-specific amplicons.

199 Eighteen isolates (13%) positive for a species-specific (sp-sp) marker were categorized as MSNT

200 group 1. Nineteen isolates (14.5%) were placed in MSNT group 2; these displayed amplicons of

201 300, 830, and 1000 bp. Two isolates (1.5%) which showed unexpected amplicons at 500 and 660

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

203 categorized as an MSNT group 4 isolate (Fig. 3; Fig. S1; Fig. S2; Fig. S3; Fig. S4; Fig. S5).

204 Identification of serovar-specific amplicons

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

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

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

208 predicted at 320 bp but the PCR run generated an amplicon of nearly 350 bp. In serovar 5, the

209 PCR results generated an amplicon of just under 500 bp, larger than the expected 450 bp result.

210 The product size of serovar 9 serovar-specific primers, predicted at 710 bp, was smaller than the

211 700 bp ladder marker. In light of these conflicting results, the isolates were serotyped again to

212 confirm the sizes, and the amplicons were subsequently sequenced. Comparisons of the

213 molecular serotyping original publication described, Bio-1D software analyzed, and BLAST

214 product sizes are shown in Table 1 (Howell et al. 2015). The product size analyzed by Bio-1D software

215 and BLAST are ~~similar but are quite different to that described in the original publication~~plus or minus a bp.

216 (Howell et al. 2015).

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

218 The Bio-1D software showed ~~that a 300 bp product from~~ the MSNT group 2 ~~isolate~~amplicon was ~~exactly~~

219 297 bp; this product was generated with a serovar 13 specific forward and a serovar 14 specific

220 reverse primer pair. These primers were paired because the target sequences in the respective

221 serovars shared homologous segments. The other PCR generated an amplicon product of the

222 MSNT group 2 isolate determined to be 836 bp, and was identified as a serovar 13 specific

223 product. The Bio-1D software measured a 500 bp product of the MSNT group 3 isolate as 499

224 bp; this amplicon was identified as a serovar 7 specific product. The 300 bp product found in the

225 MSNT group 4 isolate, (sequencing results indicated it was 297 bp) was generated by pairing a

226 serovar 13 specific forward primer with a serovar 14 specific reverse primer. This result was the

227 same as that generated using the same primer pair of DNA isolated from MSNT group 2.

228 Serovar distribution based on molecular serotyping assay and sequencing results

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

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

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

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

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

234 Relationship between pathological lesion patterns and serovars

235 ~~Of the total number of lesions~~The distribution of serotypes in lesions from ~~in~~ necropsied animals ~~found to be infected with~~ *~~H. parasuis~~*were~~,~~

236 ~~42.6% contained H. parasuis~~ *H. parasuis* serovar 5 (42.6%), ~~21.3% with~~ serovar 4 (21.3%), ~~20.4% with~~ serovar 13 (20.4%), and 13%

Commented [H11]: Was this amplicon located in the polysaccharide operon? Using a unmatched pair can result in amplicons from sites outside the gene being interrogated

Commented [H12]: Previously stated this was not typable using PCR. How was this serogroup determined?

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237 were infected with MSNT group 1 isolates. These categories were further subdivided into

238 animals displaying both serosal and pulmonary lesions, those with only pulmonary lesions, and

239 those with lesions found only in serosa (Fig. S6; Fig. S7; Fig. S8; Fig. S9; Fig. S10; Fig. S11;

240 Fig. S12; Fig. S13). The respective percentages of lesions vs. serovars, and the pattern of lesions

241 in infected animals were tabulated in Table 3 and Fig. 1. In necropsied animals, those with both

242 serosal and pulmonary lesions were the most frequent; animals with pulmonary lesions alone

243 were the least frequent ~~in terms of percent of infected hosts~~ ($p < 0.0001$). Serovars 4 and 5/12

244 ~~infected categories~~ showed similar results, ~~except for~~ the MSNT group 1, ~~in which the~~

~~245 proportions of~~ both serosal and pulmonary lesions were ~~significantly higher~~were more frequent than ~~that of~~ serosal

246 lesions alone~~.,~~ ~~and s~~Serovar 13 ~~in which proportion of~~had more serosal lesions ~~were higher~~ than for ~~both~~ the combination of

247 serosal and pulmonary lesions.

248 Nine herds had populations infected with two ~~H. parasuis~~ *H. parasuis* serovars. One herd contained a

249 population with lesions infected with serovars 1 and 4. Three herds were infected with serovars 4

250 and 5. Serovar 5, 13 and 5, 14 co-infections were seen in single herds. The infected lesions were

251 located in animals displaying a variety of tissue lesion patterns~~. Four different herds contained~~

~~252 individuals co-infected with two H. parasuis serovars.~~ One clinical case showed pleural and

253 pulmonary lesions coinfected with ~~H. parasuis~~ *H. parasuis* serovars 4 and 7, respectively. A separate herd

254 contained one case of pulmonary lesions with serovar 5, as well as pleural, pericardial, and

255 peritoneal lesions infected with ~~H. parasuis~~ *H. parasuis* serovar 13. One case was co-infected with serovars 5

256 (pulmonary) and 13(pleura and pericardium). A fourth case showed coinfection with serovar 4

257 and an MSNT group 1 isolate, ~~which were each~~ taken from separate pulmonary lesions.

258

259 Discussion

Commented [H13]: Don't need both. The data in table 3 doesn't seem to correlate with the data in figure 1.

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This is the first study describing ~~the presence of the~~serotypes of ~~H. parasuis~~ *H. parasuis* ~~serovars~~ defined by molecular serotyping in Taiwan. The most common serovars are serovar 5/12, 4 and 13, followed by MSNT isolates. Even though serotyping assays vary, the serovar population profile of *H. parasuis* in Taiwan is similar to ~~that~~ profiles described in several other studies (Table S1) (Angen et al. 2004; Blackall et al. 1996; Cai et al. 2005; Castilla et al. 2012; Del Rio et al. 2003; Dijkman et al. 2012; Howell et al. 2015; Kielstein & Rapp-Gabrielson 1992; Luppi et al. 2013; Ma et al. 2016; Oliveira et al. 2003; Rapp-Gabrielson & Gabrielson 1992; Rubies et al. 1999; Tadjine et al. 2004). Most commercial ~~H. parasuis~~ *H. parasuis* vaccines are inactivated vaccines, which provide protection against the same serovar ~~H. parasuis challenge~~ but are unable to provide protection from challenge using different serovars (Miniats et al. 1991b; Nielsen 1993; Smart & Miniats 1989; Takahashi et al. 2001). Candidate serovar composition in ~~H. parasuis~~ *H. parasuis* vaccine determine~~d~~s the success of a vaccine strategy against ~~H. parasuis~~ *H. parasuis* (Takahashi et al. 2001). Therefore, the distribution of serovars in herds is an important factor in outlining vaccination strategies and vaccine developments aimed at the prevention and control of Glässer's disease.

IHA was applied to ~~H. parasuis~~ *H. parasuis* serovar differentiation to decrease the proportion of *H. parasuis* isolates classified as CSCR (Cai et al. 2005; Del Rio et al. 2003). De-encapsulation due to multiple passages ~~causing~~ results in non-reaction ~~to~~ with antisera and/or cross reactivity of isolate antigens to diagnostic (immune-based) test reagents are the primary factors behind CSNT and CSCR H. parasuis isolates (Cai et al. 2005; Kielstein & Rapp-Gabrielson 1992; Oliveira et al. 2003; Rapp Gabrielson & Gabrielson 1992; Turni & Blackall 2005). The presence of CSNT and CSCR isolates ~~can~~ confounds epidemiological surveys used to assess ~~H. parasuis~~ *H. parasuis* isolate population profiles, and ~~may~~ impairs efforts to generate effective vaccin~~es~~ation ~~programs~~ against this pathogen.

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The correlation between the capsule and serovar ~~of H. parasuis has been~~is well established; a multiplex serotyping PCR was developed with this in mind (Howell et al. 2015; Howell et al. 2013). This protocol can be employed to type isolates ~~that have been~~previously classified as CSNT and CSCR via traditional (immunological) methods. CSNT and CSCR isolates ~~have~~ were recently ~~been~~ serotyped using molecular serotyping in the UK (Howell et al. 2015). ~~However, even though~~ mPCR serotyping ~~has~~ reduced the incidence (percentage) of CSNT and CSCR ~~H. parasuis~~ *H. parasuis* ~~isolate~~, ~~some isolates still yielded ambiguous PCR results~~. ~~As a result, m~~Molecular serotyping has not completely eliminated the issue of CSNT and CSCR isolates. One reason may be the sequence similarity of different serovar-specific primers and serovar-specific products. Another factor may be deletions and/or unknown sequences within certain antigenic markers (Ma et al. 2016). This underscores the importance of MSNT isolate~~s~~ whole-genome sequencing for in silico serotyping and improving the molecular serotyping assay. Gaps in the molecular serotyping assay may be the result of insufficient or incomplete sequence data for ~~H. parasuis~~ *H. parasuis* from Asia. When this assay was developed, there were only nine Asian ~~H. parasuis~~ *H. parasuis* isolates in a 212-isolate database (7 from Japan, 2 from China). Investigating the sequences and gene composition of Asian ~~H. parasuis~~ *H. parasuis* isolate capsule loci may be key for assaying and serotyping MSNT isolates.

Thus far, molecular serotyping has been challenging as there are 14 serovars, making it difficult to design serovar-specific primers ~~that~~ yielding differential results detectable using agarose gel. Some primer pairs produce amplicons from different ~~H. parasuis~~ *H. parasuis* serovars that vary by less than 20 bp-a difference that most agarose gels cannot detect with accuracy. In this study, electrophoresis using longer agarose gels was performed to enhance the ability of the procedure to discriminate DNA fragment sizes. Bio-1D software was applied to more accurately measure

product size based on the intensity of the bands and decrease human operation error. In the case of molecular serotyping tests ~~that~~ resulting in ambiguities, serovar-specific primer pairs may be used (in simplex PCR format) to confirm or classify hard-to-identify serovars. According to sequence analysis, the product sizes ~~that~~ described in the original publication were not accurate (Howell et al. 2015). The corrected product sizes are important to avoid mis-serotyping.

According to a previous study, pigs were infected with ~~H. parasuis~~ *H. parasuis* serovars 1, 5, 10, 12, 13, and 14 showed high mortality(ref). Pigs ~~that were~~ challenged with serovars 2, 4, 8, and 15 showed polyserositis. Pigs inoculated with serovars 3, 6, 7, 9, and 11 resulted in no clinical symptoms or lesions (Kielstein & Rapp-Gabrielson 1992). Serovars 5/12, 4, 13 and 7 are the most common serovars in most countries or regions worldwide (Table S1). There may be some correlation between serovar and virulence because serovars are defined by capsule which can directly interact with host cells and has been proven to be a key virulence factor relating to phagocytosis resistance (Olvera et al. 2009). However, in this study, serovars 7 and 9 caused serositis with or without respiratory lesions. Serovar 5 isolate was isolated from an animal with only bronchopneumonia lesions and another with lesions in both the serosa and lung tissues in the same herd. Therefore, the results show ~~that~~ clinical manifestations of Glässer's disease are influenced by multiple factors, including host, stress, environment, co-infection with different serovars or other pathogens, and gene differences between infecting ~~H. parasuis~~ *H. parasuis* isolates (Boerlin et al. 2013; Howell et al. 2014; Li et al. 2009). In general, the results showed ~~that~~ most Glässer's disease cases were in nursery pigs co-infected with PRRSV. This may be because PRRSV can cause immunosuppression by reducing non-specific bactericidal activity of pig alveolar macrophages and stimulating interleukin-10 production, which down-regulates inflammatory cytokines (Drew 2000; Flores-Mendoza et al. 2008; Suradhat & Thanawongnuwech 2003). Other pathogens may also indirectly promote Glässer's disease, like porcine epidemic diarrhea causing malnutrition. Other factors include the stress of weaning and maternal antibody reduction.

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330 However, highly virulent ~~H. parasuis~~ *H. parasuis* isolates might be considered primary pathogens (Aragon et

331 al. 2012). ~~According to this~~In our study, some ~~H. parasuis~~ *H. parasuis* isolates ~~could~~ caused serositis and sudden

332 death without co-infection, even in growing pigs and breeding boars.

333 Previous studies showed ~~that H. parasuis~~ *H. parasuis* can access the blood stream through invasion of

334 the mucosal surface in the nasal cavity (Vahle et al. 1997). In this study, pulmonary lesions

335 showed higher pathogenic ~~H. parasuis~~ *H. parasuis* infection rates than serosal lesions. These results are in

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

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

338 2009; Vahle et al. 1995). The ~~general~~ results show ~~that H. parasuis~~ *H. parasuis* infected animals with lesions

339 found in dual anatomical locations (pulmonary and serosal) occur at a higher rate than infected

340 animals with lesions located in only one tissue type. Previous studies~~y~~ also mentioned ~~that~~ lung is

341 one of the most successful sites for acute (serovar 12) and subacute (serovar 4) ~~infections culture~~isolate recovery

342 (Turni & Blackall 2007). Therefore, lung is an important origin for ~~H. parasuis~~ *H. parasuis* isolation and a

343 target organ ~~of~~ for Glässer's disease diagnosis. ~~It has been suggested that p~~Pulmonary infections may

344 be an important step for ~~H. parasuis~~ *H. parasuis* systemic infections.

345 ~~Previous studies~~Others have reported ~~that~~ isolation of multiple~~several~~ isolates ~~can be isolated~~ from single pig farms

346 (Cerda-Cuellar et al. 2010; Oliveira et al. 2003; Olvera et al. 2006a; Olvera et al. 2006b). ~~In this~~

347 ~~study, the~~Our results also show ~~that~~ different serovars ~~can~~ cause disease in a single herd, or even in a

348 single animal, although the latter scenario is fairly uncommon. In most situations, Glässer's

349 disease is caused by one serovar ~~which may be one isolate and it can be prove by genotyping~~

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

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

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

353 worthy of investigation.

354

355 Conclusions

356 In summary, this study shows ~~that~~ the dominant serovars of ~~H. parasuis~~ *H. parasuis* in Taiwan are

357 serovars 5/12, 4 and 13, followed by MSNT isolates. Pulmonary lesions may be most important

358 for ~~H. parasuis~~ *H. parasuis* isolation, ~~the diagnosis of Glässer's disease,~~ and may serve as points of origin for

359 systemic ~~H. parasuis~~ *H. parasuis* infections in hosts. Developing a universal vaccine would be a giant step in

360 an overall strategy aimed at defending porcine farms from infection by variety of pathogenic *H.*

361 *parasuis* isolates.

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531

532 Figure legends

533 Figure 1 Distribution of *Haemophilus parasuis* serovars according to lesion pattern. 534 Serositis only: animals were diagnosed with ~~H. parasuis~~ *H. parasuis* positive serosal lesions. Pulmonary 535 lesion only: animals were diagnosed with ~~H. parasuis~~ *H. parasuis* positive pulmonary lesions. Data were 536 analyzed by Fisher's exact test and variables were considered significant at a 0.05 level (two

537 sided). 538 539 Figure 2 *Haemophilus parasuis* isolation proportion of 204 lesions of 108 pathological 540 diagnosed cases. 541 Fisher's exact test was used to compare the frequency of ~~H. parasuis~~ *H. parasuis* isolation lesions. P value < 542 0.05 was considered a significant difference. 543 544 Figure 3 Molecular serotyping results with or without sequence results for 133 Haemophilus 545 parasuis isolates. 546

547 Supplementary file

548 Supplementary Figure 1 Band patterns of molecular serotyping mPCR for Haemophilus 549 parasuis. 550 Lane M: 50 bp DNA Ladder, lane S5: serovar 5 or 12, lane G2: molecular serotyping non551 typable group 2, lane S4: serovar 4, lane S9: serovar 9, lane G1: molecular serotyping non552 typable group 1, lane NC: negative control. 553 554 Supplementary Figure 2 Band patterns of molecular serotyping mPCR for Haemophilus 555 parasuis. 556 Lane M: 50 bp DNA Ladder, lane S4: serovar 4, lane S5: serovar 5 or 12, lane G2: molecular 557 serotyping non-typable group 2, lane S14: serovar 14, lane G1: molecular serotyping non-typable 558 group 1, lane NC: negative control. 559 560 Supplementary Figure 3 Band patterns of molecular serotyping mPCR for Haemophilus 561 parasuis. 562 Lane M: 50 bp DNA Ladder, lane S5: serovar 5 or 12, lane S4: serovar 4, lane G1: molecular 563 serotyping non-typable group 1, lane G2: molecular serotyping non-typable group 2, lane NC: 564 negative control. 565 566 Supplementary Figure 4 Band patterns of molecular serotyping mPCR for Haemophilus 567 parasuis. 568 Lane M: 50 bp DNA Ladder, lane G1: molecular serotyping non-typable group 1, lane S4: 569 serovar 4, lane S5: serovar 5 or 12, lane S14: serovar 14, lane G4: molecular serotyping non570 typable group 4, lane G3: molecular serotyping non-typable group 3, lane NC: negative control. 571

572 Supplementary Figure 5 Band patterns of molecular serotyping mPCR for Haemophilus 573 parasuis. 574 Lane M: 50 bp DNA Ladder RTU (GeneDireX), lane G2: molecular serotyping non-typable 575 group 2, lane S4: serovar 4, lane S5: serovar 5 or 12, lane G1: molecular serotyping non-typable 576 group 1, lane G3: molecular serotyping the non-typable group 3, lane NC: negative control. 577 578 Supplementary Figure 6 Gross meningeal lesion in ~~H. parasuis~~ *H. parasuis* infected pigs. 579 580 Supplementary Figure 7 Gross epicardial lesion in ~~H. parasuis~~ *H. parasuis* infected pigs. 581 582 Supplementary Figure 8 Gross pleural and peritoneal lesions in ~~H. parasuis~~ *H. parasuis* infected pigs. 583 584 Supplementary Figure 9 Gross synovial cavity lesion in ~~H. parasuis~~ *H. parasuis* infected pigs. 585 586 Supplementary Figure 10 Gross lung lesion in ~~H. parasuis~~ *H. parasuis* infected pigs. 587 588 Supplementary Figure 11 Histopathological meningitis lesion in ~~H. parasuis~~ *H. parasuis* infected pigs. 589 590 Supplementary Figure 12 Histopathological fibrinous serositis lesion in ~~H. parasuis~~ *H. parasuis*

infected 591 pigs. 592 593 Supplementary Figure 13 Histopathological bronchopneumonia lesion in ~~H. parasuis~~ *H. parasuis* infected 594 pigs.

595

596 Ethics and consent to participate

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

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

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

600

601 Consent to publish

602 Not applicable.

603

604 Competing interest

605 The authors declare that they have no competing interests.

606

607 Funding

608 No funding was obtained for this study.

609

610 Authors' contributions

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

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

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

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

615

616 Availability of data and materials

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

618

619 Acknowledgements

620 This study was supported by the Animal Disease Diagnostic Center, College of Veterinary
621 Medicine, National Pingtung University of Science and Technology, Taiwan. We appreciate the
622 following people for their help with this study: swine veterinarians at the Animal Disease
623 Diagnostic Center for assistance in necropsy, Ying-Xiu Lian for assistance in viral nucleic acid
624 detection and Qiong-Yi Huang to demonstrate bacterial preservation. Swine veterinarians who
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625 assisted in this study: Guan-Shiuan Su, Chih-Chung Chang, Shu-Wei Chang, Kuan-Lin Li, Ling
626 Fang Wang, Guang-Ting Tsai, Ni-Jyun Ke, Ting-Han Lin, Sheng-Yuan Wang, Hong Liu, Jia
627 Liang Hong, Joan Wang and Yu-Hsuan Chen.
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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~~ *H. parasuis* positive serosal lesions. Pulmonary lesion only: animals were diagnosed with ~~H. parasuis~~ *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).
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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~~ *H. parasuis* isolation lesions. P value < 0.05 was considered a significant difference.
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Figure 3
Molecular serotyping results with or without sequence results for 133 *Haemophilus parasuis* isolates.

Table 1(on next page)

Product size by molecular serotyping assay

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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.

Table 2(on next page)

Unexpected products of serotyping multiplex PCR

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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

830 836 S13Group 2 Serovar 13

1000 N/A§ N/A

500 499 S7

Group 3 Serovar 7

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.

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Table 3(on next page)

Relationship between pathological diagnoses and serovars

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1 Table 3 Relationship between pathological diagnoses and serovars

Pathological lesion pattern

Serovar

Serositis with respiratory Serositis Respiratory

Total

(isolate)

2 1 (0.9%) 0 (0%) 0 (0%) 1

4 12 (11.1%) 8 (7.4%) 1 (0.9%) 21

5 or 12 26 (24.1%) 17 (15.7%) 2 (1.9%) 45

7 0 (0%) 1 (0.9%) 0 (0%) 1

9 1 (0.9%) 0 (0%) 0 (0%) 1

13 9 (8.3%) 12 (11.1%) 0 (0%) 21

14 1 (0.9%) 1 (0.9%) 0 (0%) 2

MSNT group 1 9 (8.3%) 3 (2.8%) 1 (0.9%) 13

4 and 7† 0 (0%) 1 (0.9%) 0 (0%) 1

4 and MSNT group

1† 0 (0%)

1 (0.9%) 0 (0%) 1

5 and 13† 0 (0%) 1 (0.9%) 0 (0%) 1

Total 59 (54.6%) 45 (41.7%) 4 (3.7%) 108

2 †co-infection two serovars in one case

3