Comparison of the indirect haemagglutination and gel diffusion test for serotyping *Haemophilus parasuis*

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Running Headline

IHA and GD for serotyping *H. parasuis.*

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The aim of this study was to compare the use of indirect haemagglutination (IHA) and gel diffusion (GD) tests for serotyping *Haemophilus parasuis* by the Kielstein-Rapp-Gabrielson scheme. All 15 serovar reference strains, 72 Australian field isolates, nine Chinese field isolates, and seven isolates from seven experimentally infected pigs were evaluated with both tests. With the IHA test, 14 of the 15 reference strains were correctly serotyped – with serovar 10 failing to give a titre with serovar 10 antiserum. In the GD test, 13 reference strains were correctly serotyped – with antigen from serovars 7 and 8 failing to react with any antiserum. The IHA methodology serotyped a total of 45 of 81 field isolates while the GD methodology serotyped a total of 48 isolates. For 29 isolates, the GD and IHA methods gave discordant results. It was concluded that the IHA is a good additional test for the serotyping of *H. parasuis* by the KRG scheme if the GD methodology fails to provide a result or shows unusual cross-reactions.

Keywords:

Hemophilus parasuis, indirect haemagglutination, IHA, gel diffusion, GD, serotyping

38 # 1. Introduction

39 *H. parasuis* is the causative agent of Glässer's disease, porcine polyserositis and
40 arthritis, which is a significant disease in the pork industry worldwide (Hill et al., 2003;
41 Oliveira et al., 2003). It is believed that stress factors, such as transport, unfavourable
42 environment and management conditions, are involved in *H. parasuis* breaching naso-
43 pharyngeal barriers and infecting systemically (Nicolet, 1986). So far 15 serovars of *H.*
44 *parasuis* have been recognized (Rafiee and Blackall, 2000). The existence of non-typable
45 isolates (40% of isolates in some studies) indicates that there might be more than the 15
46 serovars (Rafiee and Blackall, 2000). Of the 15 known serovars, serovars 1, 5, 10, 12, 13, and
47 14 have been shown to be highly virulent causing death or moribundity within four days,
48 serovars 2, 4, and 15 were moderately virulent causing polyserositis, but not death, serovar 8
49 was considered to be of mild virulence resulting in mild symptoms and lesions, while serovars
50 3, 6, 7, 9, and 11 were avirulent causing no clinical symptoms or lesions (Kielstein and Rapp-
51 Gabrielson, 1992). However, research to establish virulence markers and link outer
52 membrane proteins and DNA profiles with virulence is still inconclusive (Ruiz et al., 2001;
53 Hill et al., 2003).

54

55 There are several schemes that have been proposed for serotyping *H. parasuis*, with the
56 Kielstein-Rapp-Gabrielson (KRG) (Kielstein and Rapp-Gabrielson, 1992) scheme (originally
57 based on heat stable antigens tested by agar gel precipitation) being the internationally
58 recognized standard scheme (del Rio et al., 2003). Recently two studies (del Rio et al., 2003;
59 Tadjine et al., 2004) have independently proposed the use of the Indirect Haemagglutination
60 (IHA) test in place of the gel diffusion (GD) test for the performance of KRG serotyping of *H.*
61 *parasuis*. To evaluate the performance of the IHA methodology, the results of serotyping by
62 the IHA test were compared with results obtained by the GD test for the 15 serovar reference

63 strains (against which the antisera were raised) and for 72 Australian, nine Chinese field
64 isolates of *H. parasuis* and 7 serovar 12 isolates from experimentally infected pigs.
65

66 **2. Materials and methods**

67 *2.1 Bacteria*

68 Reference strains for *H. parasuis* serovars 1 to 15 are stored at the Microbiology
69 Research Laboratory at the Animal Research Institute (ARI), Yeerongpilly, Queensland
70 (Table 1). A total of 72 Australian and nine Chinese field isolates of *H. parasuis* were also
71 used in this study. A further seven isolates, each obtained from a different pig, with all seven
72 pigs having been challenged with the serovar 12 reference strain, were also examined.
73

74 *2.2 Antisera*

75 The antisera used in this work have been produced and described previously (Rafiee
76 and Blackall, 2000).
77

78 For the IHA test, the antisera were adsorbed with 3% sheep red blood cells (SRBCs).
79 SRBCs were prepared by centrifugation of sheep blood at 250 x g for 10 min. The serum and
80 white blood cell layer were removed and the SRBCs were re-suspended in phosphate buffered
81 saline (PBS, pH 7.2) and centrifuged again. After a further two washes, the SRBCs were
82 resuspended to a final concentration of 3% (600 μl cells in 19.4 ml PBS). The antisera were
83 incubated in a 56°C water bath for 30 minutes. After incubation, 800 μl of serum was added
84 to 3.2 ml of 3% SRBCs, followed by 30 min incubation at 37°C. The adsorbed serum was
85 then centrifuged at 500 x g for 10 minutes and the resulting supernatant used in the IHA test.
86

87 *2.3 Antigen*

88 For the GD method, antigen was produced as previously described (Rafiee and
89 Blackall, 2000). The *H. parasuis* strain was grown on a TM/SN plate (Reid and Blackall,
90 1984) overnight at 37°C and harvested into 1 ml of nutrient broth. This suspension was then
91 used to spread inoculate two TM/SN plates and a blood agar plate. After overnight incubation
92 the growth from each TM/SN plate was harvested into 1.5 ml PBS, after checking purity on
93 the blood agar plate. The suspensions were pooled and autoclaved for 2 hr at 121°C. After
94 autoclaving, the suspension was centrifuged (17570 x g, 15 min) and the supernatant retained
95 as the antigen to be used in the GD test. The antigen was kept at 4°C until used.
96
97 For the IHA method, *H. parasuis* grown on a TM/SN plates overnight at 37°C was
98 harvested into 1 ml nutrient broth and spread inoculated onto one TM/SN plate. The growth
99 was harvested into 1 ml saline. After centrifuging (17570 x g, 2 min) the pellet was
100 resuspended in 9 volumes of saline. The suspension was kept at room temperature overnight,
101 before spinning at 8,000x g for 10 min. The collected supernatant was stored at -20°C until
102 used.
103
104 *2.4 GD test*
105 This test was performed as described by Rafiee and Blackall (2000). Briefly, wells
106 were punched into a 1% agar gel prepared in PBS containing 1% sodium azide. Volumes of 8
107 μl of the respective antisera and antigen were added to the wells and incubated in a moist,
108 dark environment for a maximum of 72 hr at room temperature. Gels were checked for
109 precipitating bands at 24, 48 and 72 hr.
110
111 *2.5 Indirect Haemagglutination Test*

112 A mixture of 300 μl of antigen, 2.7 ml of PBS and 3,000 μl of 3% SRBC was
113 incubated for 90 min at 37°C with gentle inversion of the tubes every 15 min. The sensitized
114 SRBCs were washed twice with PBS, centrifuging at 500 g for 10 min. The cell pellet was
115 then resuspended into a final volume of 13 ml using PBS. A 50 μl aliquot of PBS was added
116 to all wells of a round-bottomed microtitre plate. Next, 50 μl of absorbed antisera was added
117 to the first column of the plates and then serially diluted across the wells. Then 50 μl of
118 antigen absorbed SRBC was added to each well. The controls were unsensitized SRBC and
119 sensitized SRBC only. After 2 hr incubation at room temperature the results were read. The
120 titre for each antiserum was expressed as the reciprocal of the highest dilution of serum
121 displaying a positive reaction. If an isolate showed reactions to more than one antiserum, the
122 isolate was assigned to the serovar with the highest titre, provided there was three dilutions or
123 more difference in the titres. Where reactions were within two dilutions or less, then the
124 isolate was recorded as cross-reacting with those antisera.
125
126
127 **3. Results**
128 With the GD testing of the reference strains, no band was detected for the homologous
129 reaction for serovars 7 and 8. All other serovar reference strains gave a single band with the
130 corresponding antiserum. Serovar 1, 10 and 15 resulted in weak bands, while serovar 3, 4, 5,
131 6, 12, 13 and 14 gave strong bands. The bands for serovar 2, 9 and 11 were even stronger.
132
133 The IHA testing of the reference strains resulted in high titres for homologous
134 antigens and antisera. Serovar 1 and 11 showed a two-way cross-reaction, with the cross-
135 reacting serovar being at a lower titre (Table 1). The level of cross-reaction with the serovar 1

136 antigen was such that, using the three dilution or greater rule, it was not possible to assign the
137 serovar 1 strain to serovar 1. The lower level cross-reaction with the serovar 11 antigen
138 meant that the serovar 11 reference strain could be assigned to serovar 11. The serovar 12
139 antiserum cross-reacted with the serovar 5 antigen, but this cross-reaction was one-way and
140 sufficiently low enough to confidently assign the serovar 5 strain to serovar 5. The antigen of
141 the reference strain for serovar 10 did not give a titre with the serovar 10 antiserum.
142
143 With the GD test, 44 field isolates showed only one band and four isolates gave
144 multiple bands (Table 2). Three of the four isolates that yielded a strong band with the
145 serovar 1 antiserum also showed a cross-reaction with serovar 11, with the band for serovar
146 11 being very weak. The only other isolate that showed a cross-reaction in the GD test was an
147 isolate that gave a strong band to the serovar 4 antiserum as well as weaker bands for the
148 antisera to serovars12, 14, and 15. A total of 33 of the 81 isolates were non-typable in the GD
149 – giving no band with any of the 15 antisera.
150
151 With the IHA test, 36 isolates could be confidently assigned to a serovar (Table 2). A
152 further 9 isolates gave cross-reactions that prevented assignment to a single serovar – four
153 being cross-reactive with antisera to serovars 1 and 11, four being cross-reactive with antisera
154 to serovars 8 and 9, and one being cross-reactive with antisera to serovars 5 and 12. A total of
155 36 isolates were nontypable in the IHA – failing to give any reaction with any of the 15
156 antisera.
157
158 By combining both the GD and the IHA results, only 21 isolates remained non-typable
159 (ie failed to react with any antiserum in either test). The combined approach resulted in 49
160 isolates being confidently assigned to a serovar with eleven isolates being unable to be

161 assigned to a serovar due to cross-reactions in either the IHA (less than three dilutions
162 difference in the titres) or GD tests. These eleven isolates consisted of four isolates that were
163 strongly cross-reactive with serovars 1 and 11 in the IHA (two also being cross-reactive in
164 GD), four that were strongly cross-reactive with serovars 8 and 9 in the IHA (and non-typable
165 in the GD), one that was strongly cross-reactive with serovars 5 and 12 in the IHA (identified
166 as serovar 12 in the GD test), one that was cross-reactive with serovar 1 and 11 in the GD
167 (identified as serovar 1 in IHA), and one that was cross-reactive with serovar 4, 12, 14, and 15
168 in the GD (identified as serovar 4 in the IHA) (Table 2).

169

170 Marked differences between the IHA and GD results were recorded for field isolates
171 assigned to serovars 4, 5, 12, and 13 by the GD test. Two of the 21 serovar 4 isolates
172 identified by GD were non-typable in the IHA (Table 2). Similarly, nine of the 14 serovar 5,
173 three of the serovar 13 and the only serovar 14 isolate were non-typable in the IHA test (Table
174 2).

175

176 The results of testing the seven serovar 12 isolates obtained from the experimentally
177 infected pigs are shown in Table 3. In the GD test, all seven isolates reacted specifically with
178 the antiserum for serovar 12. In the IHA test, all seven isolates showed reactions to both
179 serovars 5 and 12. However, all seven isolates could be confidently assigned to serovar 12 on
180 the basis of a difference of at least three dilutions in the IHA titres (Table 3). There was
181 evidence of some variation – with the serovar 12 IHA titres varying between 1/1,280 and
182 1/640 while the serovar 5 titres varied between 1/40 and 1/160 (Table 3).

183

184

185

186 ## 4. Discussion

187 The current study produced different results to the study by Rafiee and Blackall
188 (2000), despite both studies being done in the same laboratory and having used the same
189 serovar reference strains and antiserum. Contrary to Rafiee and Blackall (2000), the current
190 study could not detect specific reactions between the serovar 7 and 8 antiserum and their
191 respective antigen. Difficulties in producing acceptable antiserum levels for serovar 7 and 8
192 had been reported by Rafiee and Blackall (2000) and Tadjine et al. (2004). These difficulties
193 in both production and lack of homologous reaction in the GD test may reflect a lack, or a low
194 level expression of the serovar-specific antigens, which in turn might be attributed to storage
195 and *in vitro* passage (Kielstein and Rapp-Gabrielson, 1992; Prakash et al., 2003). On the
196 other hand, in the IHA test both antisera 7 and 8 gave a homologous reaction in the current
197 study. Serovar 7 isolates from the field also showed no reaction in the GD test, while the
198 IHA showed a specific reaction, possible due to the IHA tests greater sensitivity (Tizard,
199 2000). Cross-reactions in GD between serovar 14 and 4 (Rafiee and Blackall, 2000) and
200 serovar 5 and 1 (Tadjine et al., 2004), were not observed in the current study. Tadjine et al.
201 (2004) used boiled instead of autoclaved extracts.

202

203 In the current study serovar 5 showed a one-way cross-reaction with serovar 12, while
204 serovars 1 and 11 showed a two-way cross-reaction in the IHA test. However, only serovar 1
205 had a true cross-reaction, as the homologous antibody titres in the other cross-reactions were 3
206 or more twofold dilutions apart. Contrary to del Rio et al. (2003) one-way cross-reactions in
207 the IHA for serovar 10 and 13 were not observed in the current study. The homologous
208 reaction for serovar 10, as found by del Rio et al. (2003) and Tadjine et al. (2004), was also
209 not detected in the IHA. However, neither del Rio et al. (2003) nor Tadjine et al. (2004)
210 provided sufficient information to identify the actual serovar reference strains used.

211

212 Tadjine et al. (2004) reported extensive cross reactions in their use of the GD test with
213 field isolates. Other studies using autoclaved antigen in the GD test have not reported
214 extensive cross-reactions (Blackall et al., 1996; Rafiee and Blackall, 2000). In the current
215 study some cross reactions in the GD test with a few field isolates were detected. Cross-
216 reactions with serovars 1 and 11 were detected in both GD and IHA. The isolate of serovar 4
217 that cross-reacted with three other serovars (12, 14, and 15) in the GD test obviously had a
218 different profile for those antigens detected in the IHA, as no cross-reaction was detected in
219 this assay.

220

221 The current study found only minimal cross-reactions when the IHA test was used on
222 field isolates, a finding also reported by the previous IHA evaluation studies (del Rio et al.,
223 2003; Tadjine et al., 2004). Cross-reactions that were predicted by the reference strain results
224 were detected (4 isolates showed 1 and 11 and 1 isolate 5 and 12 cross-reactions). These
225 cross-reactions were not reported by del Rio et al. (2003) and Tadjine et al. (2004). The other
226 cross-reactions found amongst the field isolates in the current study involved 4 isolates that
227 were cross-reactive with serovars 8 and 9 in the IHA and non-typable in the GD. As the titres
228 of these four isolates with both the serovar 8 and 9 antisera were low, it is possible that these
229 isolates actually represent a new serovar that has some low level cross-reaction with serovars
230 8 and 9.

231

232 It was found that passaging the reference strain serovar 12 *in vivo* did not alter the
233 results of the IHA. Two of the passaged serovar 12 isolates had a lower titre than the other
234 isolates, indicating that there is slight variability in resulting titres.

235

236 A major difference between the current study and the previous studies were the
237 number of isolates that could not be serotyped by IHA. The current study found 36 (44%)
238 field isolates could not be serotyped by IHA, while 33 (41%) field isolates were non-typable
239 by GD. In contrast, del Rio et al. (2003) reported that 37% of the field isolates from Spain
240 were non-typable by GD, while only 7% were non-typable by IHA. Tadjine et al. (2004)
241 reported less than 10% of the field strains from North America could not be typed with the
242 IHA, while more than 30% could not be typed with the GD. An important finding of the
243 current study was that some field isolates of serovars 4 and 5, and all field isolates of serovar
244 13 and 14, could not be identified by IHA but were identified by GD. The finding of the
245 current study that some field isolates of serovars 4 and 5 could be identified by the IHA test
246 while others could not, suggests that there are differences in the antigenic profiles of these
247 serovars. Previous studies have found that genetic variation occurs between isolates of the
248 same serovar (Blackall et al., 1997; Oliveira and Pijoan, 2004).

249

250 Overall conclusions from this study differ from those reached by the previous
251 evaluations of the IHA methodology for serotyping *H. parasuis* (del Rio et al., 2003; Tadjine
252 et al., 2004). These previous studies have both recommended the use of the IHA
253 methodology above the GD methodology. Results of the current study demonstrate that the
254 GD method can be used as the primary serotyping method with the IHA being used as a
255 secondary test that can be used when the GD test gives either a non-typable result or cross-
256 reactions.

257

258

259

260

261 ## 5. Acknowledgements

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264

265

266 ## 6. References

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301

TABLE 1. Results of the IHA with soluble antigen from the reference strains representing serovars 1 to 15 and rabbit hyperimmune sera produced against whole-cell antigens of these reference strains. Titres are expressed as the reciprocals of the final dilutions of serum giving positive reactions.

	Antisera raised against														
antigen	HS 145	SW 140	SW 114	SW 124	Nagasaki	131	HS 197	C5	D74	H367	H465	H425	IA84-17975	IA84-22113	SD84-15995
serovar (Reference strain)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 (HS 145)	1280										320				
2 (SW 140)		2560													
3 (SW 114)			5120												
4 (SW 124)				1280											
5 (Nagasaki)					10240							320			
6 131						1280									
7 (197)							5120								
8 (C5)								1280							
9 (D74)									10240						
10 (H367)															
11 (H465)	160										2560				
12 (H425)												2560			
13 (IA84-17975)													2560		
14 (IA84-22113)														2560	
15 (SD84-15995)															2560

308

309 TABLE 2. Results of GD compared to IHA typing for field isolates from Australia and China

		IHA results											
GD results	number of samples	non-typable	1	1 + 11	2	3	4	5	7	8 + 9	12	12 + 5	13
non-typable	33	21		1			3		3	4			1
1	1			1									
1 + 11	3		1	2									
2	1				1								
3	1					1							
4	21	2					19						
5	14	9						5					
12	2										1	1	
4+12+14+15	1						1						
13	3	3											
14	1	1											
Total	81	36	1	4	1	1	23	5	3	4	1	1	1

310

311

312 TABLE 3. Results from the 7 pigs challenged with serovar 12. Titres are expressed as the

313 reciprocals of the final dilutions of serum giving positive reactions.

314

Isolates	GD result	IHA		
		Serovar 5 titre	Serovar 12 titre	result
HS 1928	12	80	1280	12
HS 1929	12	40	640	12
HS 1931	12	80	1280	12
HS 1932	12	160	1280	12
HS 1933	12	40	640	12
HS 1934	12	80	1280	12
HS 1935	12	160	1280	12

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