

SCIENTIFIC SECTION

Editor
 COLIN WILKS

Associate Editor
 KL HUGHES,

Editorial Committee
 NORMAN ANDERSON, GLENN BROWNING,
 COLIN CHAPMAN, ROBIN CONDRON,
 JOHN DUFTY, TREVOR FARAGHER,
 KEITH HUGHES, ALAN LAWTHOR,
 CARL PETERSON, ANDREW TURNER,
 COLIN WILKS

Editorial ASSISTANT and
 Desktop Publishing
 ANNA GALLO

The Australian Veterinary Journal welcomes original contributions on all aspects of veterinary science.

Submission of a manuscript for publication will be held to imply that it is a record of unpublished original work of the author(s) and, if accepted will not be published elsewhere in whole or part without permission. The Journal reserves the right to reject any manuscript.

Send contributions to the Scientific Section of the AVJ to:

Editor,
 AVA House, 272 Brunswick Road,
 Brunswick, Victoria 3056.

Telephone: (03) 9387 2982, 9387 8808.
 International -61 3 9387 8808
 Telefax: (03) 9388 0112,
 International +61 3 9388 0112.
 Email avjsc@ava.com.au.

'Instructions for authors' were published in the February 1999 issue. They can be found in <http://www.farmwide.com.au/nff/vetasscn/aboutava/instruct.htm>.

'Statistical guidelines for authors' were published in December 1998. They can be found in <http://www.farmwide.com.au/nff/vetasscn/aboutava/statguid.htm>.

Establishment, validation and use of the Kielstein-Rapp-Gabrielson serotyping scheme for *Haemophilus parasuis*

M RAFIEE and PJ BLACKALL

Australasian Pig Institute, Queensland Department of Primary Industries, Animal Research Institute, Yeerongpilly, Queensland 4105

Objectives To produce antisera to the 15 recognised reference strains of the Kielstein-Rapp-Gabrielson (KRG) serotyping scheme for *Haemophilus parasuis*, validate those sera and use them to serotype 46 Australian field isolates of *H parasuis*.

Design Antisera were produced in rabbits and validated by cross-testing with the reference strains and re-testing 15 Australian field isolates of *H parasuis* that had been previously serotyped in the United States of America. The validated antisera were then used to determine the serovar of 46 Australian isolates.

Results Monospecific antisera were produced for 14 of the 15 KRG serovars of *H parasuis*. Two Australian field isolates, confirmed previously as serovars 1 and 7, were used to produce monospecific antisera for serovars 1 and 7 respectively. The antiserum for serovar 4 gave a one-way cross reaction with the antigen of serovar 14. The typing antisera correctly typed all 15 *H parasuis* that had been previously typed by antisera produced overseas. The 46 field isolates were shown to belong to serovars 2 (two isolates), 4 (one isolate), 5 (18 isolates), 12 (two isolates) and 13 (four isolates). The remaining 19 isolates were non-typable.

Conclusion Serotyping of *H parasuis* isolates is now available in Australia. *H parasuis* serovars 5 and 13 remain the predominant serovars present in Australian pigs.

Aust Vet J 2000;78:172-174

Key words: *Haemophilus parasuis*, serological characterisation, KRG scheme

Haemophilus parasuis, an organism dependent upon nicotinamide adenine dinucleotide (NAD) or V-factor for in-vitro growth, is the causative agent of porcine polyserositis and arthritis (Glässer's disease).¹ The main lesions associated with this disease are fibrinous or serofibrinous meningitis, serositis, pleuritis, pericarditis, peritonitis and arthritis that can occur in various combinations or occasionally singly.¹

In North America Glässer's disease has emerged as a significant problem in segregated early weaning (SEW) production systems.² The disease is now regarded as one of the remaining infectious diseases that can cause significant economic losses in these systems.² As there is now considerable interest in the use of SEW or modified SEW production systems in Australia, it is likely that Glässer's disease may emerge as an increasing problem in the Australian pig industry.

The Kielstein-Rapp-Gabrielson (KRG) serotyping scheme³ is now the internationally recognised scheme for serotyping *H parasuis*. Interest in the use of the KRG scheme arises from the fact that there is conflicting evidence on whether the protection provided by inactivated *H parasuis* vaccines is serovar-specific or, indeed, even strain specific.⁴⁻⁷ A previous study, performed by forwarding Australian isolates to the United States of America, has shown that KRG serovars 1, 2, 4, 5, 9, 10/7, 12 and 13 are present in Australia with serovars 5 and 13 being the most common.⁸ In this study, we report the production of antisera for the 15 KRG serovars, the validation of those antisera and then the use of those antisera to serotype a further 46 Australian field isolates.

Materials and Methods

Bacteria

Reference strains for *H parasuis* serovars 1 to 15 were obtained from Dr Vicki Rapp-Gabrielson of Solvay (now Fort Dodge) (Minnesota, USA). A total of 17 Australian field isolates of *H parasuis*, all previously serotyped by the KRG scheme⁸ were also used. Full details of the reference strains and the previously serotyped field isolates are given in Table 1. A further 46 Australian field isolates of *H parasuis*, all identified by traditional phenotypic methods⁹ were also used in this study.

Media

The agar medium TM/SN¹⁰ was used for the general growth of the *H parasuis* strains and isolates. Chocolate agar (CA) was used for the production of antigens to be inoculated into rabbits. TM/SN was used for the production of antigen to be used in the serotyping tests. Sheep blood agar (SBA), containing 5% sheep red blood cells, was used for confirming the purity of bacterial suspensions. All incubation was at 37°C in air.

Production of antisera

Fresh overnight growth of the reference strain of *H parasuis* on TM/SN was harvested into 1 mL of nutrient broth (NB). This suspension was then used to spread inoculate 15 CA plates, which then were incubated overnight. After incubation, each plate was individually harvested into 1.5 mL of phosphate buffered saline (PBS, pH 7.2). This suspension was checked for purity by inoculation onto SBA. Those suspensions shown to be pure were then pooled together, adjusted to an optical density equivalent to a MacFarland No 9 tube (approximately 2 to 4 X 10⁹ colony forming units/mL) and inactivated with 0.3% (v/v) formaldehyde solution. The resultant antigens were kept at 4°C.

Hyperimmune antisera to reference strains and selected field isolates were produced in rabbits as described previously.¹¹ For each strain or isolate, two rabbits (12 weeks of age) were used. For some strains or isolates, a second or third round of three intravenous (IV) injections at 2 d intervals at 10 to 17 d after the initial series was required to elicit a serovar specific antibody response.

Serum samples were obtained by ear vein bleeding the rabbits 9 to 12 d after the last IV inoculation. If the serum sample showed acceptable levels of antibody, a maximum blood volume was collected from the anaesthetised rabbit by cardiac puncture.

Serotyping procedure

Heat stable cell extracts of the reference strains and field isolates were prepared using autoclaving at 121°C for 2 h as described previously¹² except that TM/SN was used as the growth medium.

Serotyping was performed by immunodiffusion in a 1% agar gel prepared in PBS containing 1% sodium azide. An 8 µL volume of each antiserum and antigen was added to the wells and allowed to react for up to 72 h at room temperature in a moist environment. The resulting precipitin bands were recorded at 24, 48 and 72 h.

Results

We have achieved the production of *H parasuis* antisera to serovars 1 to 15. However we had some considerable difficulty in producing specific antisera for some of the serovars. In some cases the production of serovar-specific antisera required the use of repeated rounds of IV inoculations of inactivated cells. For serovars 4 and 8 even this approach was not sufficient and fresh sets of rabbits were used. For KRG serovar 7, we were unable to produce specific antibodies using strain 174, the reference strain. Hence for serovar 7, we produced antisera against Australian field isolate HS197, which had been shown to be serovar 7 in a study using antisera produced in the United States of America.⁸

Similarly, we were unable to produce a specific antiserum using the reference strain for KRG serovar 1, strain 4. We then produced antisera to Australian field isolate HS145, which had been previously⁸ shown to be serovar 1.

All the typing antisera were checked against the reference strains for all 15 serovars of *H parasuis* in repeated gel immuno-

diffusion tests. We have been able to produce monospecific antisera for 14 of the 15 serovars. There was a one way cross-reaction between the serovar 4 antiserum and the type strain of serovar 14, which was observed as a pale band near the centre well containing the antiserum. There was no such reaction between the serovar 14 antiserum and the serovar 4 antigen.

To further validate the typing antisera, we used the sera to retest 15 Australian field isolates of *H parasuis* that had been previously serotyped.⁸ These isolates were selected to represent the dominant serovars found in our previous study and consisted of serovar 2 (one isolate), serovar 4 (one isolate), serovar 5 (five isolates), serovar 7 (one isolate), serovar 9 (two isolates) and serovar 13 (five isolates). Using the typing antisera produced in the current study, all 15 Australian isolates were allocated to the same serovars as that established using the typing antisera produced in the United States of America.⁸

The typing antisera were then used to serotype 46 Australian field isolates of *H parasuis* that had not been previously serotyped. Of the 46 isolates tested, 27 could be confidently assigned to a serovar with serovars 5 and 13 being the dominant serovars (Table 2). The remaining 19 isolates could not be serotyped.

Discussion

The production of the typing sera for the KRG scheme for the serotyping of *H parasuis* proved a difficult and demanding undertaking.

Table 1. Reference strains and previously serotyped Australian field isolates of *Haemophilus parasuis* used in this study

Strain/Isolate Code	KRG Serovar
4 ^a , HS145 ^b	1
SW140, HS172	2
SW114	3
SW124, HS236	4
Nagasaki, HS11, HS68, HS319, HS357, HS446	5
131	6
174, HS197, HS226	7
C5	8
D74, HS72, HS167	9
H367	10
H465	11
H425	12
IA-84-17975, HS330, HS331, HS356, HS435, HS436	13
IA-84-22113	14
SD-84-15995	15

^aThe strains shown with underlining are the reference strains supplied by Dr VJ Rapp-Gabrielson

^bThe Australian field isolates have been serotyped in a previous study⁸

Table 2. Geographical origin and serovar of Australian isolates of *Haemophilus parasuis* serotyped in this study

State	Serovar						Total
	2	4	5	12	13	NT	
New South Wales	-	1	2	-	2	1	6
Victoria	1	-	3	-	-	3	7
Queensland	1	-	13	1	2	15	32
South Australia	-	-	-	1	-	-	1
Total	2	1	18	2	4	19	46

NT = Non typable

We were unable to produce specific antisera using strain 174, the type strain for KRG serovar 7. This problem has been also reported by Rapp-Gabrielson and Gabrielson.¹¹ Our inability to produce specific antisera using strain 174 supports the suggestion of Rapp-Gabrielson and Gabrielson¹¹ that strain 174 produces only a poor or unstable level of serovar-specific antigens. It would appear to be sensible for a replacement serovar reference strain to be formally nominated in place of strain 174. To produce high-titre specific antisera for serovar 7, we were forced to use isolate HS197, an Australian field isolate of this serovar. Rapp-Gabrielson and Gabrielson¹¹ reached a similar conclusion and they used the American field isolate 512 in place of strain 174 to produce their serovar 7 antisera.

We also failed to produce a specific antiserum using the reference strain for KRG serovar 1. This problem has not been reported before for this strain. Similar to the situation with serovar 7, we were able to produce high-titre and specific antisera for KRG serovar 1 by using HS145, an Australian field isolate of this serovar.

Hence, to produce the full set of 15 KRG typing antisera, we used 13 recognised international reference strains and 2 previously characterised Australian field isolates. The results of the heterologous testing of these antisera clearly validate these typing antisera. All 15 antisera are monospecific with the exception of serovar 4 where we detected a weak cross-reaction with the serovar 14 reference strain. As the cross-reaction is one way only (ie. the serovar 4 reference strain did not cross-react with the serovar 14 antiserum), the reaction will not prevent confident identification of either serovar 4 or 14.

An interesting finding of our study was that we were able to produce specific antisera for KRG serovar 7 using Australian field isolate HS197. When this strain was examined using the antisera of Rapp-Gabrielson and Gabrielson,¹¹ it was found to give reactions to both KRG serovar 7 and 10.⁸ However, the antiserum we produced using this strain did not react with strain H367, the KRG serovar 10 reference strain. Hence, our finding that Australian field isolate HS197 can produce a specific antiserum that does not cross-react with serovar 10 indicates that the four Australian isolates reported as serovar 7/10 previously⁸ should be regarded as serovar 7. Further studies on KRG serovars 7 and 10 are required to understand fully the cross-reactions between these serovars detected when using the antisera of Rapp-Gabrielson and Gabrielson¹¹ on Australian field isolates of serovar 7.

The validity of the typing sera we have produced was further demonstrated by the fact that we were able to correctly serotype all 15 Australian field isolates that we had previously serotyped⁸ using the antisera produced by Rapp-Gabrielson and Gabrielson.¹¹ In the light of the one way cross-reaction we found between serovar 4 and 14, it is noteworthy that the Australian serovar 4 field isolate gave a specific reaction to the serovar 4 antiserum. Similarly the Australian serovar 7 isolate gave a specific reaction with serovar 7 and no reaction was seen to serovar 10 antiserum.

When we examined the 46 Australian field isolates that had not been serotyped previously, we found serovars 2, 4, 5, 12 and 13, all of which had been recognised previously in our earlier study.⁸ As with our earlier study,⁸ the two most common serovars were 5 and 13. The current study found a higher level of non-typable isolates, 19 out of 46, compared with our previous study which reported 5 out of 31 as non-typable.⁸ The level of non-typable isolates we have found (41%) is higher than

that reported in USA (15%) and Germany (26%).^{3,11} However, it should be noted that we have shown that 12 of the 19 non-typable isolates reported here, while sourced from three farms, all had an identical ERIC-PCR fingerprint, suggesting that they are simply multiple isolates of the same untypable strain.¹³

There is confusion in the literature on whether protection from inactivated vaccines is serovar specific or indeed even strain specific.⁴⁻⁷ In the light of this uncertainty in the literature, the newly established capacity to serotype *H parasuis* combined with our confirmation of earlier findings that a wide variety of serovars are present in Australian pigs⁸ has important implications for the development and use of vaccines against diseases due to *H parasuis* in Australian pigs. At the very least, inactivated vaccines should contain the serovars prevalent in the target pig population, or be produced from isolates of *H parasuis* obtained from the target herd. As more than one serovar of *H parasuis* can be present in a herd at the same time, the choice of strains and serovars to include in a vaccine is of prime importance. As a consequence of the successful completion of this study, the Australian pig industry now has access to an Australian based *H parasuis* serotyping service that can support attempts to develop effective prevention and control programs for the diseases associated with this organism.

Acknowledgments

The assistance of our colleagues at the Yeerongpilly Veterinary Laboratory, the Toowoomba Veterinary Laboratory, the Rockhampton Veterinary Laboratory, the Bendigo Regional Veterinary Laboratory, the Victorian Institute for Animal Science (Attwood), the Elizabeth MacArthur Agricultural Institute (Camden), the Orange Veterinary Laboratory and Veterinary Pathology Services in providing the field isolates used in this study is acknowledged. This work was funded, in part, by the Pig Research and Development Corporation.

References

- Nicolet J. *Haemophilus parasuis*. In: Leman AD, Straw BE, Mengeling WL, D'Allaire S, Taylor DJ, editors. *Diseases of Swine*. 6th edn. Iowa State University Press, Ames, 1992:526-528.
- Pi Joan C. Pathogenesis of disease in SEW pigs. *Pig News Inform* 1997;18:65N-66N.
- Kielstein P, Rapp-Gabrielson VJ. Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. *J Clin Microbiol* 1992;30:862-865.
- Riising H-J. Prevention of Glässer's disease through immunity to *Haemophilus parasuis*. *Zbl Vet Med B* 1981;28:630-638.
- Smart NL, Miniats OP. Preliminary assessment of a *Haemophilus parasuis* bacterin for use in specific pathogen free swine. *Can J Vet Res* 1989;53:390-393.
- Kielstein P, Rassbach A. Serologische Typisierung und Nachweis immunogener Kreuzreaktionen von *Haemophilus parasuis* (Glässersche Krankheit). *Mh Vet-Med* 1991;46:586-589.
- Miniats OP, Smart NL, Rosendal S. Cross protection among *Haemophilus parasuis* strains in immunized gnotobiotic pigs. *Can J Vet Res* 1991;55:37-41.
- Blackall PJ, Rapp-Gabrielson VJ, Hampson DJ. Serological characterisation of *Haemophilus parasuis* isolates from Australian pigs. *Aust Vet J* 1996;73:93-95.
- Blackall PJ, Pahoff J. Characterisation of porcine haemophilus isolated from Australian pigs, 1988-1992. *Aust Vet J* 1995;72:18-21.
- Reid GG, Blackall PJ. Comparison of adjuvants for an inactivated infectious coryza vaccine. *Avian Dis* 1987;31:59-63.
- Rapp-Gabrielson VJ, Gabrielson DA. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. *Am J Vet Res* 1992;53:659-664.
- Morozumi T, Nicolet J. Some antigenic properties of *Haemophilus parasuis* and a proposal for serological classification. *J Clin Microbiol* 1986;23:1022-1025.
- Rafiee M, Bará MR, Stephens CP, Blackall PJ. Application of ERIC-PCR for the fingerprinting of *Haemophilus parasuis*. *Aust Vet J* 2000; In press.

(Accepted for publication 10 January 2000)