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Riemerella anatipestifer diagnostics

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Executive Summary

Riemerella anatipestifer infection is a contagious disease affecting mainly ducks, geese and turkeys. It has been reported in other waterfowl, chickens and pheasants. The affected birds display symptoms of respiratory disease (such as ocular and nasal discharges, mild coughing and sneezing), greenish diarrhoea, reduced growth rate, nervous clinical signs (such as tremors of head and neck and incoordination) and exudative septicaemia causing mortalities as high as 80%.

In recent years the genus *Riemerella* has been subjected to phylogenetic analysis. These studies clearly showed that identification by classical conventional bacteriology alone was not accurate enough to distinguish *Riemerella anatipestifer* from other potential pathogens such as *Riemerella columbina*. Two polymerase chain reaction (PCR) assays claimed to be specific for *R. anatipestifer* have been published and appear to offer considerable advantages over the classic phenotypic methods for identification of suspect isolates..

There are 21 serovars recognised for *R. anatipestifer,* with the possibility of more serovars also existing. This presents a problem if using killed autogenous vaccines as the serovars are not cross-protective. So it is vital to know which serovars are on the farm to produce effective autogenous vaccines. There is little knowledge of the serovars present in Australia, as only limited Australian isolates have been sent overseas for typing (with this happening a number of years ago). These existing results are now clouded by recent developments. These developments include the recognition that some of the serovar reference strains used in the past are actually not *R. anatipestifer.* As well, there has been confusion caused by the fact that different research groups have used different strains to raise antisera for the same serovar.

Pulsed field gel electrophoresis (PFGE) and enterobacterial repetitive intergenic consensus (ERIC) PCR are two typing techniques that have both been used to sub-type *R. anatipestifer*. However, both these methods have disadvantages. PFGE is a very time consuming method. While ERIC PCR is much quicker than PFGE, this method does not allow comparisons between laboratories.

This project took on board all these challenges and set out to:

1) Compare and validate both published *R. anatipestifer* specific PCR assays.
2) Sort through the confusion about the serovars and raise antisera against all of the established type strains that could confidently be aligned to one serovar.
3) Set up a genotyping method that was not time consuming but could be compared between laboratories to give the power to trace the origin of strains.
4) Set up a data base of genotypes for *R. anatipestifer*
5) Identify, serotype and genotype field isolates from Australia
6) Set up identification, serotyping and genotyping methods for *R. anatipestifer* and offer the assays (via a user pays system) as a service for the industry.

Both PCRs produced the expected positive reactions with all *R. anatipestifer* reference strains. One of the PCRs had a digestion step which only worked for 10 of the 20 reference strains and this additional step was not used for the analysis of the field isolates. Of the 24 field isolates only 12 produced a positive reaction (with all 12 giving a positive reaction in both PCR assays). The isolates that had positive reactions were seven isolates from ducks (one being a vaccine strain) and five from unidentified origins. The 12 isolates that gave a negative reaction in the PCR came from chickens bar one, which came from the eye of a duck.

After sorting through the literature 17 type strains representing serovar 1-3, 5, 6 and 8 – 19 were picked to raise antisera. A further two strains that were not typable and a field strain

described as serovar 4 were also used to raise antisera. Formalin killed antigen produced from these strains was injected into the marginal ear vein of New Zealand White rabbits. Each antisera produced was specific to the antigen raised with no cross-reactions observed. The specific reactions obtained with the two non-typeable strains means that there are now two new serovars of *R. anatipestifer* (yet to be formalised via the scientific publication process). The 12 Australian isolates of *R. anatipestifer* were serotyped as serovar 1, 6, 8 and 13, while three isolates were non-typable.

A total of nine overseas field isolates were also serotyped. Of these overseas isolates, one was serovar 1 and two were not typable. Six isolates (one from Denmark and five from Germany) reacted specifically with the antiserum from one of the two new serovars.

The genotyping method used in this project was the repetitive extragenic palindromic PCR (rep-PCR). This PCR was standardised by bioMerieux and produced as kits. BioMerieux have developed the DiversiLab system, which allowed loading of samples onto a matrix readable by the machine and software allowed for the creation of a public data base. This system is used throughout veterinary laboratories worldwide and hence allows for the comparison of isolates worldwide. Using the DiversiLab system, the 12 Australian isolates were quite diverse, with all isolates having a unique profile. A cluster of four isolates of serovars 1 and 6 are closely related. When the Australian isolates are compared to the type strains, only one serovar 13 Australian isolate grouped with the respective serovar reference strain (in this case the serovar 13 strain). At this stage there are not enough strains in this data base to make any further analysis, but once more strains are added this data base will help understand the epidemiology of *R. anatipestifer*.

All methods have now been established successfully in the laboratory and are offered as a user pay system to the industry. This now gives the industry the tools necessary to not only identify *R. anatipestifer* but also to serotype and genotype this organism. These are vital tools for the selection of vaccine strains, treatment and implementation of biosecurity.

Contents

Introduction

Riemerellla anatipestifer causes disease worldwide, mainly in domestic ducklings characterised by diarrhoea, lethargy, respiratory and nervous clinical signs and exudative septicaemia. Turkeys, geese, chicken and other birds are also affected. Mortalities can be as high as 80% on infected duck farms. Reduced growth rate, poor feed conversion, increased condemnations and high treatment cost together with high mortality rate causing large economic losses (Kiss et al. 2007).

R. anatipestifer is recognised as causing disease problems in Australian poultry, having been isolated from diseased ducks, swans and chickens (Munday et al. 1970; Jackson 1972; Grimes and Rosenfeld 1972; Rosenfeld 1973). Other than these early studies, there have been no other systematic studies of Australian isolates of *R. anatipestifer*.

A total of 21 serovars have been established with the possibility of more being present (Loh et al. 1992). Effective vaccination programs are complicated by the presence of this multitude of serovars which do not cross-protect (Sandhu 1979). Research has found that the main isolates of *R. anatipestifer* of three countries belong to a limited number of serovars. In the USA the majority of isolates were serovars 1, 2 and 5 with slight increase in incidence of 3, 7 and 11 over a period of 8 years. A total of 25 isolates from Singapore belonged to serovars 1, 3, 4, 8, 14 and 15. Finally seven isolates from England belonged to serovars 1, 2, 6 and 10 (Sandhu and Leister 1991). There is little knowledge about the serovars of *R. anatipestifer* present in Australia. The little public knowledge that exists is based on the few isolates examined in the early 1970s by a now irrelevant serotyping scheme that showed an Australian duck isolate and one chicken isolates belonged to serovar A (Jackson 1972; Rosenfeld 1973). Preliminary work in our laboratory conducted in the 1990s involved sending strains to America to be serotyped. This work has shown that we have *Riemerella anatipestifer* serovars 1, 6 and 10 in Australia (unpublished data).

Contrary to other bacterial respiratory pathogens affecting poultry in Australia, the Australian diagnostic laboratories do not provide services such as identification, serotyping or genotyping for this bacterium. There is no advice available for the producer on the type of strain or the serovar and therefore, no advice can be provided on what strains need to be included in an autogenous vaccine.

Overcoming the shortfalls in the diagnostic tests available for *Riemerella anatipestifer* for the Australian poultry industry is a challenge. The confident identification of isolates as *R. anatipestifer* has long been a challenge for diagnostic laboratories. Indeed, even the correct taxonomic allocation of the organism has been a challenge – the organism has been allocated to at least three different genera – *Pasteurella, Moraxella* and now *Riemerella,* always with the same species name (Sandhu 2008). The organism is relatively inert in conventional phenotypic identification tests, adding to the difficulty of confident identification (Sandhu 2008). In addition, a range of organisms that resemble *R. anatipestifer* (in colonial morphology and in disease pathology) have been recognised by sophisticated phylogenetic analysis. The potential pathogens that resemble *R. anatipestifer* include – *Riemerella columbina*, *Coenonia anatina* and *Pelistega europaea* (Christensen and Bisgaard 2009), In addition, there are a number of isolates of organisms that are clearly members of the genus *Riemerella* but which appear to possibly be a new species (Christensen and Bisgaard 2009). Overall, there are a number of very similar organisms that make confident identification of *R. anatipestifer* difficult.

There have been at least two PCR assays claimed to provide species-specific identification of *R. anatipestifer*. In a recent study, both PCR assays have been shown to have some problems but one PCR, the assay developed by Kardos et al (2007) was shown to be the better option – giving the required negative reactions with the key non-target organisms *Riemerella columbina* and *Coenonia anatina* (Christensen and Bisgaard 2009). The only problem in the Kardos et al (2007) assay was a weak reaction with isolates of the genus

Riemerella that have not yet been confidently assigned to a species (Christensen and Bisgaard 2009). As these problem isolates have only been associated with chickens, the Kardos et al (2007) PCR is an appropriate PCR to use when working with duck isolates.

Inactivated vaccines are regarded as effective control measures that can prevent mortalities (Sandhu 2008). These inactivated vaccines are serovar-specific – they provide protection only against those serovars in the vaccine (Sandhu 2008). However, the serological classification of *R. anatipestifer* has been a confusing area. In part, this has been caused by multiple different serological classification systems. In recent times, there has been a general acceptance that there are 21 serovars with those serovars capable of being detected by rapid plate agglutination (Sandhu 2008).

A key support mechanism for serotyping has been the development of DNA-based typing systems. For *R. anatipestifer* both PFGE and ERIC-PCR have been shown to be capable of generating results that match the known field epidemiology (Kiss et al 2007). In similar work dealing with *Haemophilus parasuis,* we have found genotyping to be a very useful support tool for serotyping (Turni and Blackall 2010). In this work with *H. parasuis*, multiple isolates from a farm are first screened by genotyping to establish the number of genotypes present. Representative isolates of each genotype are then serotyped (based on the assumption that isolates from a farm with an identical genotype must have the same serotype BUT isolates that have a different genotype may or may not have the same serovar). This approach conserves the use of antisera – allowing many isolates to be initially grouped by the genotyping method and only selected representative isolates subjected to serotyping (Turni and Blackall 2010). This combined use of genotyping and follow serotyping is a powerful and sustainable method for understanding disease outbreaks and guiding vaccination programs.

As PFGE is a very time consuming method and as ERIC PCR patterns cannot be compared between laboratories, a more standardised method is needed. In particular, a method that has a library accessible from anywhere in the world would be attractive. The DiversiLab system is based on a technique known as repetitive sequence-based PCR (rep PCR). This system is a standardized, repeatable, genotyping system that allows reproducible genotyping and the comparison of genotypes using a publically available data base for all users of DiversiLab. This system is extensively used by diagnostic laboratories throughout the world. This will allow the comparison of the Australian isolates to other isolates in the world and allows other laboratories within Australia to compare their isolates.

The aim of this project is to establish key diagnostic methods in Australia and make them available to the Poultry Industry. The outcome will be a laboratory-based service that can provide confident species level identification and a serotyping/genotyping service that can be used to guide vaccination programs. In the absence of these assays, vaccination programs are essentially blind guesses in which it is hoped that the vaccine contains the right strains to ensure protection.

Objectives

The objective of this project is to establish the relevant diagnostic methods for *Riemerella anatipestifer* to provide the industry with the diagnostic tools to identify, serotype and genotype *Riemerella anatipestifer*.

The outcomes of this research will be:
A) rapid diagnosis of *Riemerella anatipestifer* Australia wide for the industry and
B) an ability to ensure that vaccines contain the appropriate serovar necessary to ensure protection.
The benefit of this project is that the end–user will be provided with all the relevant information about the strains of *Riemerella anatipestifer* on the farm. This knowledge is needed to make informed decisions about autogenous vaccines, treatment options and biosecurity measures to be put in place.
All of the diagnostic assays developed in this proposal will be provided on an on-going basis at the completion of the project to industry on a user pays basis. This model has been successful since the development of our first diagnostic assays back in the 1980s. As we propose a user pays model, the benefits of this project will be immediately available to industry.

Methodology

Bacteria

After sorting through the literature and evaluating all strains by PCR seventeen type strains representing serovars 1 to 3, 5 and 6, and 8 to 19 were chosen for antisera production. Three other strains which included two non-typable isolates, strains 4237/2 sv (Ryll et al 2001) and 18470/13 (Christensen and Bisgaard 2009), and one field isolate previously identified as serotype 4, strain 4280 (Ryll et al 2001), were also used to raise antisera in rabbits (Table1).

A total of 33 other isolates were used in this study. Of these 33 isolates, 24 were collected between 1964 and 2011 in Australia from ducks and chicken. The Australian isolates were originally confirmed as *Riemerella anatipestifer* by phenotypic characterizations. Six of the 24 isolates from Australia had been sent overseas for identification and serotyping.

The remaining nine isolates came from overseas, six from Denmark isolated from the upper respiratory tract of Pekin ducks in 1996 (Ryll et al 2001) and three single strains from the USA, Morocco and Singapore (Table 2).

Identification

Two published PCR methods (Kardos et al 2007; Rubbenstroth et al. 2013) were used in this work.

To produce a DNA template, a loopful of cells from a culture on sheep blood agar (SBA), incubated overnight in 5% CO_2 at 37ºC, was suspended in 200 µl H_2O and heated for 15 min at 98ºC. This template was used for both PCRs and the published methods were adhered to, except that for the Kardos et al (2007) BSA was not added and the annealing temperature was at 56.7ºC as suggested by Rubbenstroth et al (2013). As described in the original publication, restriction digestion with *Mob*II was used to confirm the identity of the amplicons generated using the PCR of Kardos et al (2007).

If any of the Australian strains did not produce the expected amplicon size in the PCR, the amplicons were sequenced using PCR with universal primer pairs 27F and 1410R as described previously (Turni and Blackall 2011). Sequences were aligned to known sequences.

Production of antisera

Formalin killed antigen of each reference strain was injected into the marginal ear vein of two New Zealand White rabbits (animal ethics approval number QAAFI/399/12/POULTRY CRC) according to Sandhu et al (1991) with some modifications (see Figure 1). Cells were harvested in 0.85% NaCl containing 0.3% formalin from 5% sheep blood agar plates after 24 to 48 hour incubation. The cells were washed twice in 0.85% NaCl containing 0.3% formalin and adjusting to McFarland 5. Rabbits were immunised by successive doses of 0.1, 0.1, 0.2, 0.5, 0.5, 1.0, 1.5, and 2.0 ml at 3 to 4 days interval and were bled out two days after the last injection.

Figure 1. Preparing to inject the marginal ear vein of a rabbit.

Serotyping

A loopful of growth from a sheep blood agar plate incubated for 24 h was suspended in 1 ml nutrient broth. From this suspension, 200µl were spread on two dextrose starch agar plates (100 µl/plate). After incubation of these plates for 24 h incubation under 5% CO_2 at 37ºC cells were harvested in 0.85% NaCl containing 0.3% formalin. The density was adjusted to McFarland 10. After suspension 1 ml was taken off and centrifuged (16,000 *g*, 2 min). The pelleted cells were resuspended in 180 µl of 0.85% NaCl containing 0.3% formalin. This suspension was boiled for one hour and the supernatant used in gel diffusion precipitation test as described for serotyping of *Pasteurella multocida* (Brogden 1982; Heddleston et al 1972).

rep-PCR and DiversiLab

The DNA extraction was performed on an overnight culture using the UltraClean™ Microbial DNA Isolation Kit (MO Bio Laboratories) according to the manufacturer's instructions. The concentration of the DNA was adjusted to 35 ng/µl and 2 µl of the DNA preparation was used as template in the 25 µl rep-PCR reaction. The rep-PCR was set up and run with the

Pseudomonas fingerprinting kit (bioMérieux) according to the manufacturer's instructions. The fingerprints were compared with DiversiLab® software version 3.4.

Results

Identification

All the type strains gave positive results in both PCRs. However, the *Mob*II restriction digestion, a second stage in the Kardos et al (2007) assay, was only successful for 9 out of 17 type strains (Table 1). Therefore this extra step was not used on the 12 Australian field strains.

The examination of the 24 Australian field isolates, which had been identified phenotypically as *Riemerella anatipestifer*, resulted in only 12 isolates giving a positive reaction in both PCR assays (Table 2). Of the 12 isolates that were confirmed *as R. anatipestifer,* seven isolates were from ducks and five isolates were of unknown origin. Of the 12 isolates that did not produce positive results in either of the species specific PCRs, 11 isolates came from chicken and one isolate from the eye of a duckling. Further sequence analysis of the 12 isolates that yielded negative results in the two species specific PCRs revealed that most of them were similar to *Riemerella columbia* and *Riemerella*-like taxon II. The isolate from the eye of the duckling was *Moraxella lacunata*.

The isolates from Denmark were all identified as *R. anatipestifer,* with positive results in both PCRs (Table 2). The isolates from the USA and Singapore were both confirmed as *R. anatipestifer*. The isolate from Morocco was a *Riemerella*-like taxon II.

Table 1. List of the *R. anatipestifer* strains used to raise antisera and the results of the PCR analysis of those strains.

Strain	Serovar	Reference	Kardos et al (2007) PCR	*Mob*II digestion	PCR
HPRS 1795	1	Bisgaard (1982)	+	+	+
HPRS 2591	2	Bisgaard (1982)	+	-	+
HPRS 2212	3	Bisgaard (1982)	+	+	+
HPRS 2514	5	Bisgaard (1982)	+	+	+
HPRS 2336	6	Bisgaard (1982)	+	+	+
HPRS 2174	8	Bisgaard (1982)	+	-	+
HPRS 2528	9	Bisgaard (1982)	+	+	+
HPRS 2564	10	Bisgaard (1982)	+	+	+
HPRS 2560	11	Bisgaard (1982)	+	+	+
8755/9	12	Bisgaard (1982)	+	-	+
11693/2	13	Bisgaard (1982)	+	+	+
CVLS D664/63	14	Ryll and Hinz (2000)	+	-	+
CVLS D743/85	15	Ryll and Hinz (2000)	+	-	+
DRL S4801	16	Ryll and Hinz (2000)	+	-	+
CVLS 977/83	17	Ryll and Hinz (2000)	+	-	+
CVLS 540/86	18	Ryll and Hinz (2000)	+	+	+
CVLS 30/90	19	Ryll and Hinz (2000)	+	-	+
4237/2 sv		Ryll and Hinz (2000)	+	+	+
18470/13		Christensen and Bisgaard (2009)	+	-	+
4280	4	Ryll et al (2001)	+	-	+

Table 2. List of *R. anatipestifer* and *R. anatipestifer*-like isolates used in this study and the results of the identification work.*

Isolate	Year	Country	Final Identification	Animal	Tissue	Kadros PCR	Rubbenstroth PCR
BR 138	1999	Australia	*R. anatipestifer*	?	?	+	+
BR 139	1999	Australia	*R. anatipestifer*	?	?	+	+
BR 140	1999	Australia	*R. anatipestifer*	?	?	+	+
BR 141	1999	Australia	*R. anatipestifer*	?	?	+	+
BR 142	2000	Australia	*R. anatipestifer*	?	?	+	+
BR 143	NA	Australia	*R. anatipestifer*	Duck	V	+	+
BR 144	1977	Australia	Not RA	Chicken	Sinus	(-)	(-)
BR 145	1981	Australia	Not RA	Chicken	Lung	(-)	(-)
BR 146	1981	Australia	*R. anatipestifer*	Duck	Pharynx	+	+
BR 147	1981	Australia	Not RA	Chicken	Trachea	-	-
BR 148	1981	Australia	Not RA	Chicken	Trachea	-	-
BR 149	1981	Australia	Not RA	Chicken	Trachea	-	-
BR 150	1981	Australia	Not RA	Chicken	Sinus	-	-
BR 987	2011	Australia	*R. anatipestifer*	Duck	Heart and blood	+	+
BR 988	2011	Australia	*R. anatipestifer*	Duck	Brain	+	+
BR 1205	1990	Australia	Not RA	Chicken		(-)	-
BR1206	1990	Australia	Not RA	Chicken		-	(-)
BR 1207	1972	Australia	Not RA	Fowl	Ex trachea	-	-
BR 1208	1979	Australia	Not RA	Fowl	Trachea	-	-
BR 1209	1964	Australia	*Moraxella lacunta*	Duckling	Eye	(-)	(-)
BR 1210	1964	Australia	*R. anatipestifer*	Duck	Liver	+	+
BR 1211	1971	Australia	*R. anatipestifer*	Duck	Ex liver and pericardial sac	+	+
BR 1212	1964	Australia	*R. anatipestifer*	Duckling	Eye	+	+
BR 1213	1990	Australia	Not RA	Chicken	?	(-)	-
IPDH 180/88	1988	Morocco	Not RA	?	?	(-)	(-)
4159/1 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	+	+
4159/2 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	+	+
4159/4 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	+	+
4237/1 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	+	+
4005/2 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	+	+
4340/1 N	1996	Denmark	*R. anatipestifer*	Duck	Nasal cavity or Pharynx	+	+
S 80	?	Singapore	*R. anatipestifer*	Duck	?	+	+
P 1767	?	USA	*R. anatipestifer*	?	?	+	+

* Kardos PCR = PCR published by Kardos et al (2007); Rubbenstroth PCR = PCR published by Rubbenstroth et al (2013); Not RA = not *R. anatipestifer*; V = tissue origin not known but isolate used as a vaccine strain; ? = not known; + = fragment of expected size; - = no fragment; (-) = fragment but not of expected size.

Serotyping

Antisera was produced for all the 17 typing strains, as well as the two strains which had no serovar assigned and the one that had serovar 4 assigned to it. Each antisera raised reacted with the strain it was raised against and no cross-reactions were observed (Table 3).

The specificity of the sera raised against strains 4237/2 sv and 18470/13 is evidence that these strains represent two new serovars. For the purpose of the current report, these new serovars are termed candidate serovar 20 (c20 as represented by strains 4237/2 sv) and candidate serovar 21 (c12 as represented by strain 18470/13). The specificity of the antiserum against strain 4280 indicates that this strain is a suitable serovar 4 reference strain.

Table 3. Gel diffusion results for all the antisera against all antigens.

antisera	1	2	3	5	6	8	9	10	11	12	13	14	15	16	17	18	19	(4237/2 SV)	(18470/13)	4
antigen serotype (strain)																				
1 (HPRS 1795)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 (HPRS 2591)	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3 (HPRS 2212)	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 (HPRS 2514)	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6 (HPRS 2336)	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8 (HPRS 2174)	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9 (HPRS 2528)	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
10 (HPRS 2564)	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
11 (HPRS 2560)	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
12 (8755/9 Salp)	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
13 (11693/2 Hjerne)	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
14 (CVLS D664/83)	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
15 (CVLS D743-85)	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
16 (DRL S4801)	-	-	-	-	-	-	-	-	-	-	-		-	+	-	-	-	-	-	-
17 (CVLS 977/83)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
18 (CVLS 540/86)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
19 (CVLS 30/90)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
(4237/2 SV)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
(18470/13)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
4 (4280)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Testing the Australian field isolates confirmed as *R. anatipestifer* revealed that they belonged to different serovars with serovar 1 being the most prevalent (5 isolates). Two isolates belonged to serovar 6, one to serovar 8 and one to serovar 13, while three isolates were non-typable (Table 4).

The *R. anatipestifer* isolates from Denmark belonged to serovar 1 (one isolate) and serovar represented C20 (five isolates). Both isolates from the USA and Singapore were non-typable.

Table 4 Serotyping of the 12 Australian field isolates and the six isolates from Denmark and one isolate each from Singapore and the USA.

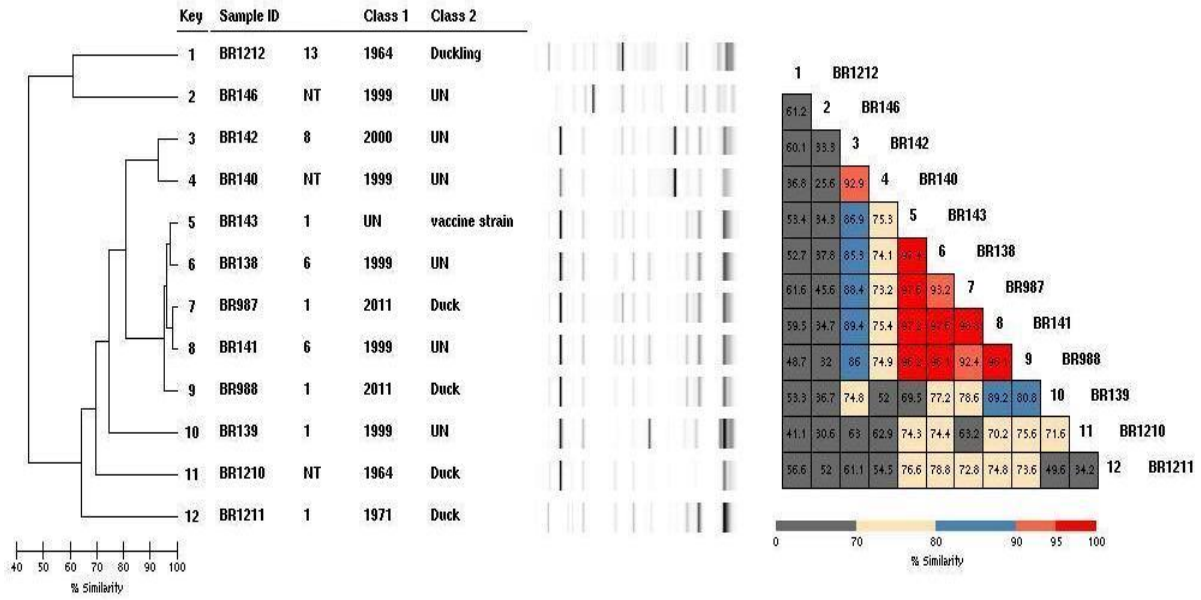
Isolate	Year	Country	Final Identification	Animal	Tissue	Serovar (This study)	Previous Serotyping Result*
BR 138	1999	Australia	*R. anatipestifer*	?	?	6	NA
BR 139	1999	Australia	*R. anatipestifer*	?	?	1	NA
BR 140	1999	Australia	*R. anatipestifer*	?	?	NT	NA
BR 141	1999	Australia	*R. anatipestifer*	?	?	6	NA
BR 142	2000	Australia	*R. anatipestifer*	?	?	8	NA
BR 143	NA	Australia	*R. anatipestifer*	Duck	?	1	NA
BR 146	1981	Australia	*R. anatipestifer*	Duck	Pharynx	NT	NA
BR 987	2011	Australia	*R. anatipestifer*	Duck	Heart and blood	1	NA
BR 988	2011	Australia	*R. anatipestifer*	Duck	Brain	1	NA
BR 1210	1964	Australia	*R. anatipestifer*	Duck	Liver	NT	1+6
BR 1211	1971	Australia	*R. anatipestifer*	Duck	Ex liver and pericardial sac	1	1
BR 1212	1964	Australia	*R. anatipestifer*	Duckling	Eye	13	1
4159/1 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	C20	NA
4159/2 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	C20	NA
4159/4 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	C20	NA
4237/1 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	C20	NA
4005/2 SV	1996	Denmark	*R. anatipestifer*	Duck	Pharynx	C20	NA
4340/1 N	1996	Denmark	*R. anatipestifer*	Duck	Nasal cavity or Pharynx	1	NA
S 80	?	Singapore	*R. anatipestifer*	Duck	?	NT	NA
P 1767	?	USA	*R. anatipestifer*	?	?	NT	NA

*Previous serotyping result = unpublished data, serotyping performed at National Animal Disease Center, Ames, Iowa, USA; ? = Not known; NT = Non-typeable (no reaction to any of the 20 antisera tested in the current study); NA = not previously tested

Rep-PCR and DiversiLab

The analysis by rep-PCR revealed that the Australian field isolates were very diverse (Figure 2). A cluster of isolates did occur – consisting of isolates of serovars 1 and 6 and included the vaccine strain (Key numbers 5 to 9 in Figure 2). However, not all serovar 1 isolates were in this cluster e.g. BR139 and BR1211 were serovar 1 but quite separate from the cluster.

Figure 2. Rep-PCR pattern of the 12 Australian isolates. UN stands for unknown.



When the Australian isolates were compared to the overseas isolates and to the type strains, the cluster of isolates of serovars 1 and 6 remained as cluster (Figures 3 and 3 a).

Figure 3 Rep-PCR patterns of all isolates and strains in the study.

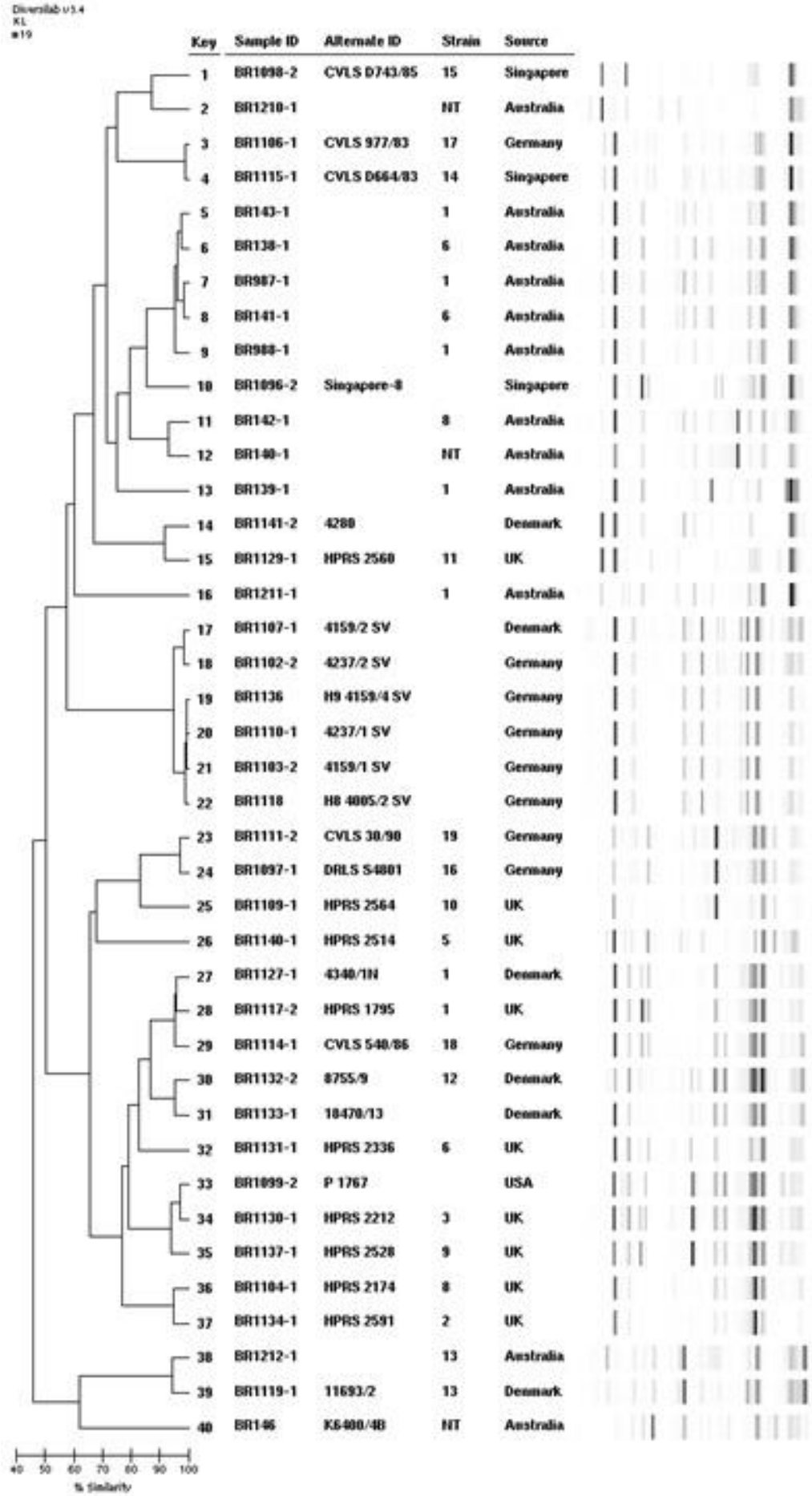


Figure 3a. The top part of the total Rep PCR patterns seen in this study (Australian isolates circled).

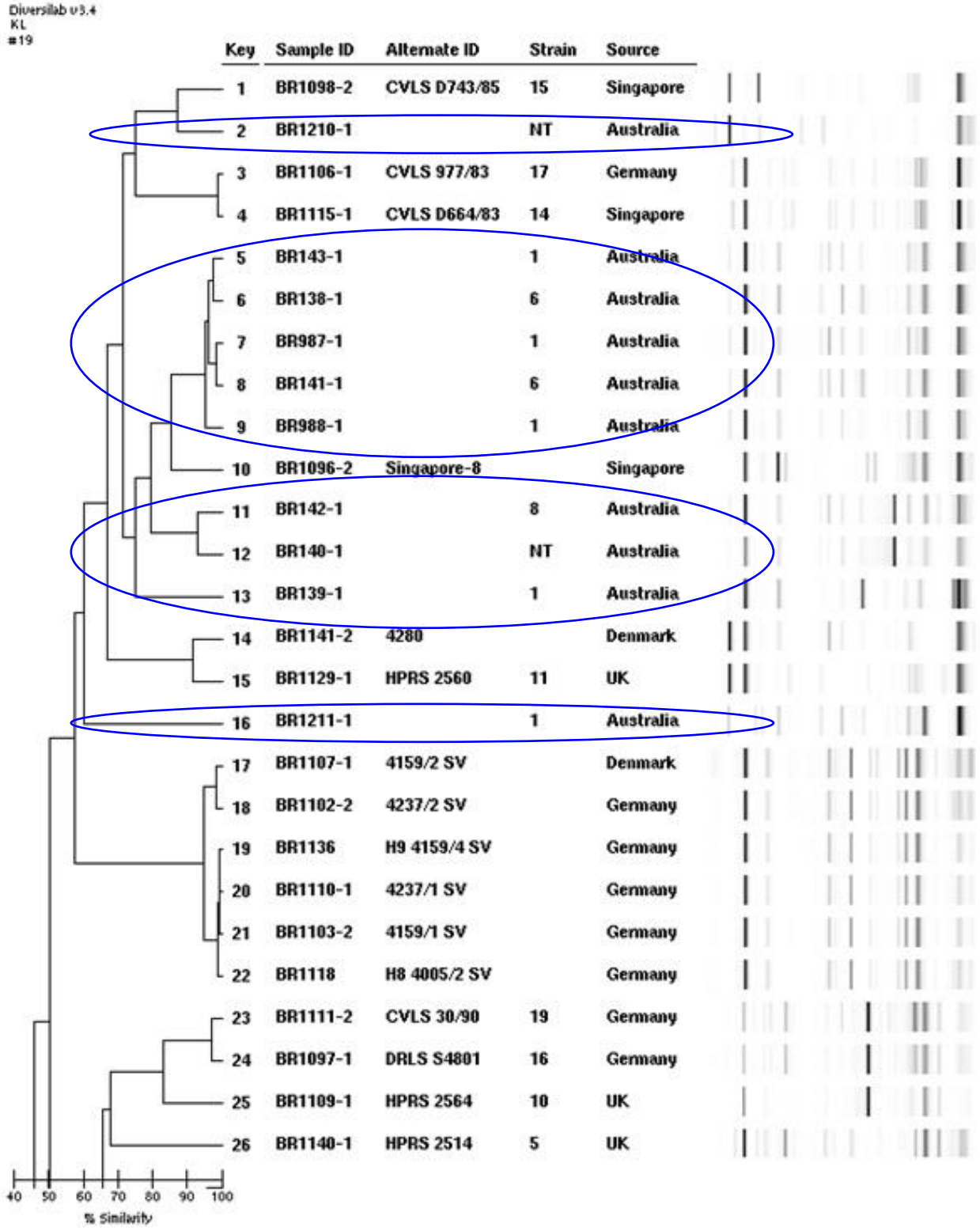
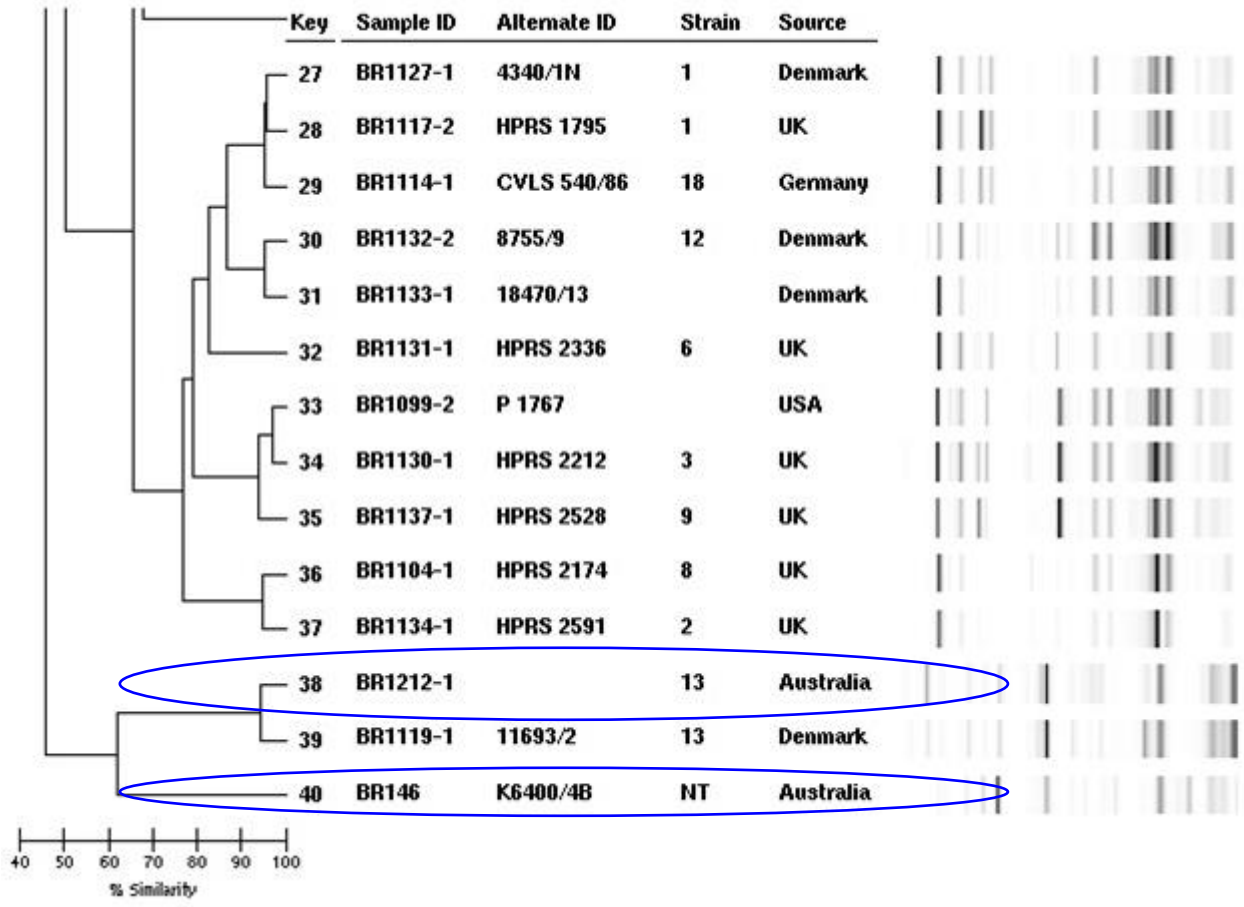


Figure 3b. The bottom part of the Rep PCR patterns seen in this study (Australian isolates circled).



Discussion

A key outcome of this study is that none of the Australian field isolates from chicken were actually identified as *R. anatipestifer*. This would suggest that *R. anatipestifer* in Australia is strongly associated with ducks. Indeed, the report in the literature that *R. anatipestifer* has been isolated from diseased chickens (Rosenfeld 1973) was a case of mis-identification, as the isolates from Rosenfeld (1973) were included in the current study. This highlights the inadequacy of phenotypic identification for *Riemerella*-like organisms. The two PCR assays evaluated in the current study performed well and should be considered as front-line diagnostic tests in place of the conventional phenotypic methods.

Three of the Australian field isolates of *R. anatipestifer* were sent to the USA for serotyping in the 1990s. The serotyping results indicated that they were serovars 1 (two isolates) and 1 + 6. In the current study, the same result (serovar 1) was obtained for just one isolate. Of the other two isolates, one was non-typeable in the current study (serovar 1+6 in the American study) and the other was serovar 13 (serovar 1 in the American study). This contradiction in serotyping results highlights the difficulties observed when sorting through the literature and the strains. Different type strains have been used to raise antisera by different research groups. As an example such as strain HPRS1785 was used as the reference strain for

serovar 7 in Denmark (Bisgaard et al. 1982) and as the reference strain for serovar 9 by another group (Sandhu and Leister 1991). In addition, a close look at the phylogenetic relationships, phenotypic characteristics, whole cell fatty acid patterns and sequencing data identified some of the reference strains used for antisera production for serovars 4 and 20 as belonging to another species (Ryll et al 2001, Christensen and Bisgaard 2009). This explains why, in the current study, only 17 reference strains that could be identified as *R. anatipestifer* and were associated with a serovar.

On the basis of preliminary results provided by the Danish group that provided the reference strains (Bisgaard, pers comm.), we selected an additional three reference strains as representing potential new serovars. These additional strains were confirmed by the Danish laboratory (Bisgaard, pers comm.) and the results of the current study (see Table 1) as *R. anatipestifer*, but they had no serovar associated with them. One of these additional strains had been named in the published literature (Ryll et al 2001) as a serovar 4 strain (strain 4280).

As all three additional strains yielded a specific antiserum that reacted only with the homologous antiserum (Table 2), all three strains represent a new serovar. As strain 4280 has been named as serovar 4 (Ryll et al 2001), we have referred to the antiserum raised against strain 4280 as serovar 4 antiserum. For the other two strains, it is not yet possible to assign a serovar designation. For the purpose of the current study, we have adopted a provisional naming – candidate serovar 20 (C20) and candidate serovar 21 (C21). Formal recognition of the new serovars must await a formal publication in the peer review literature. It is worth noting that the C20 antiserum recognised a number of European field isolates.

Additional further new serovars appear to exist as three Australian isolates, one isolate from Singapore and one isolate from the US all failed to react with the 20 antisera used in the current study.

The rep-PCR results showed some similarities between Australian isolates of *R. anatipestifer* and reference strains (as well as field isolate) from Singapore. As an example, field isolate Singapore 8 was the first isolate or strain to join the cluster of Australian isolates that contained isolates BR143, BR 987 and BR 988 (serovar 1) as well as isolates BR138 and BR141 (both serovar 6) (see Figure 3a). Further isolates from both countries need to be examined by rep-PCR and serotyping to see if there is truly a link.

Overall, the rep-PCR patterns and serotyping results suggested that the Australian field isolates were quite diverse. While five of the isolates belonged to the same serovar (serovar 1), only three (BR143, BR 987 and BR 988) had a similar rep-PCR pattern, while the other two (BR139 and BR1211) showed quite different rep-PCR patterns. Only one of the Australian field isolates grouped (in terms of rep-PCR patterns) with the respective serovar reference strain (isolate BR1212 with the reference strain serovar 13, Figure 3b).

However, PCRs that are based on repetitive sequences in the genome do not necessarily produce clusters/groups that correspond with serovar groupings. This lack of correlation between this type of PCR typing and serotyping has been shown with ERIC PCR and serotyping for *Haemophilus parasuis* (Dijkman et al 2012). As well, similar findings to the current study have been reported rep-PCR patterns among different serovars of *Salmonella enterica* (Rasschaert et al 2005). Therefore, it seems a common observation that isolates with related/similar rep-PCR patterns can belong to different serovars.

This was confirmed by Huang et al. (1999) who used rep-PCR to look at field isolates of *R. anatipestifer* from Singapore and observed that some serovars (serovar 1, 7, 11, 13, 15 and 19) consisted of more than one rep-PCR pattern. However, they also found that strains from different serovars (e.g. serovar 2 and 19) had the same rep-PCR profile. In the current study isolates/strains with the same rep-PCR profile but different serovars were not found. However, some serovar 1 and 6 isolates were shown to have very similar rep-PCR patterns.

It is clear that further work needs to be done and more isolates need to be added to the database created in the current study in order to obtain more information on the diversity of *R. anatipestifer* in Australia. The current data suggests that there will be a reasonable degree of diversity, highlighting the importance of understanding that diversity. In the light of the diversity found in this work, the diagnostic tools developed in this work will be key support mechanisms to aid veterinarians to help the producer to establish effective management procedures, such as vaccine strategies, biosecurity strategies and adequate treatment options, for disease outbreaks associated with *R. anatipestifer.*

Implications

The outcomes of this research are rapid, accurate diagnostic tools for *Riemerella anatipestifer* and an ability to ensure that inactivated vaccines are based on the correct strains to ensure effective protection.

The benefit of this project is that the end – user will be provided with all the relevant information about the strains of *Riemerella anatipestifer* on the farm, providing the knowledge needed to make an informed decision about autogenous vaccines, treatment options and biosecurity measures to be put in place.

All of the diagnostic assays developed in this proposal will be provided on an on-going basis at the completion of the project to industry on a user pays basis. This model has been successful within the DAFF/QAAFI research group since the development of our first diagnostic assays back in the 1980s. The user pays model will ensure that the benefits of this project will be immediately available to industry.

Recommendations

The poultry industries need to be aware of the suite of diagnostic tests now available to both identify outbreaks associated with *R. anatipestifer* and the capacity of the typing assays (both genotyping and serotyping) to guide effective sustainable vaccination programs.

In the longer term, the capacities developed in this work are essential base tools that would support the development of new generation vaccines (e.g. rationally attenuated vaccines) for *R. anatipestifer*.

Acknowledgedments

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References

Bisgaard, M. (1982) Antigenic studies on *Pasteurella anatipestifer*, species *incertae sedis*, using slide and tube agglutination. *Avian Pathology* **11**, 341 – 350.

Brogden, K.A., Rhoades, K.R. and Rimler, R.B. (1982) Serologic types and physiologic characteristics of 46 avian *Pasteurella anatipestifer* cultures. *Avian Diseases* **26**, 891 – 896.

Christensen, H. and Bisgaard, M. (2009) Phylogenetic relationships of *Riemerella anatipestifer* serovars and related taxa and an evaluation of specific PCR tests reported for *R. anatipestifer*.. *Journal of Applied Microbiology* **108**, 1612 - 1619.

Dijkman, R., Wellenberg, G.J., van der Heijden, H.M.J.F., Peerboom, R., Olvera, A., Rothkamp, A., Peperkamp, K. and van Esch, E.J.B. (2012) Analysis of Dutch *Haemophilus parasuis* isolates by serotyping, genotyping by ERIC-PCR and Hsp60 sequences and the presence of the virulence associated trimeric autotransporters marker. *Research in Veterinary Science* **93**, 589 – 595.

Grimes, T.M. and Rosenfeld, L.E. (1972) *Pasteurella anatipestifer* infection in ducks in Australia. *Australian Veterinary Journal* **48**, 367-368.

Heddleston, K.L., Gallagher, J.E. and Rebers, P.A.(1972) Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. *Avian Disease* **16**, 925 – 936.

Huang, B., Subramaniam, S., Chua, K.-L., Kwang, J, Loh, H., Frey, J. and Tan, H.-M. (1999) Molecular fingerprinting of *Riemerella anatipestifer* by repetitive sequence PCR. Veterinary Microbiology **67**, 213 – 219.

Jackson, C.A. (1972) Suspected *Pasteurella anatipestifer* infection in ducklings in New South Wales. *Australian Veterinary Journal* **48**, 479.

Kardos, G., Nagy, J., Antal, M., Bistyak, A., Tenk, M. and Kiss, I. (2007) Development of a novel PCR assay specific for Riemerella anatipestifer. *Letters of Applied Microbiology* **44**, 145-148.

Kiss, L., Kardos, G,, Nagy, J., Tenk, M. and Ivanics, E. (2011). DNA fingerprinting of *Riemerella anatipestifer* isolates from ducks. *The Veterinary Record* **160**, 26 – 28.

Loh, H., Teo, T.R. and Tan, H.C. (1992). Serotypes of *Pasteurella anatipestifer* isolates from ducks in Singapore; a proposal of new serotypes. *Avian Pathology* **21**, 453 – 459.

Munday, B.L., Corbould, A., Heddleston, K.L. and Harry, E.G. (1970) Isolation of *Pasteurella anatipestifer* from black swan (*Cygnus atratus*). *Australian Veterinary Journal* **46**, 322-325.

Rasschaert, G., Houf, K., Imberechts, H., Grijspeerdt, K., De Zutter, L. and Heyndrickx, M. (2005) Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of *Salmonella enterica* isolates. *Journal of Clinical Microbiology* **43**, 3615 - 3623.

Rosenfeld, L.E. (1973) *Pasteurella anatipestifer* infection in fowls in Australia. *Australian Veterinary Journal* **49**, 55-56.

Rubbenstroth, D., Ryll, M., Knobloch, J.K.-M., Köhler, B. and Rautenschlein, S. (2013) Evaluation of different diagnostic tools for the detection and identification of *Riemerella anatipestifer*. *Avian Pathology* **42**, 17 - 26.

Ryll, M. and Hinz, K.H. (2000) Exclusion of strain 670/89 as type strain for serovar 20 of *Riemerella anatipestifer*. *Berl Munch Tierarztl Wochenschr* **113**, 65-66.

Ryll, M., Christensen, H., Bisgaard, M., Christensen, J.P., Hinz, K.H. and Köhler, B. (2001) Studies on the prevalence of *Riemerella anatipestifer* in the upper respiratory tract of clinically healthy ducklings and characterization of untypable strains. *Journal of Veterinary Medicine, Series B* **48**, 537 – 546.

Sandhu, T. S (1979). Immunization of white Pekin ducklings against *Pasteurella anatipestifer* infection. *Avian Diseases* **23**, 662 - 669.

Sandhu, T.S. (2008) *Riemerella anatipestifer* infection, P. 758 – 764. In Saif Y.M. (ed.), Disease of poultry, 12th ed. Blackwell Publishing Ltd., Oxford, United Kingdom.

Sandhu, T. S. and Leister, M. L. (1991). Serotypes of *'Pasteurella*" *anatipestifer* isolates from poultry in different countries. *Avian Pathology* **20**, 233 - 239.
Footnotes/References/Cross-references

Turni, C. and Blackall, PJ. (2010) Serovar profiling of *Haemophilus parasuis* on Australian farms by sampling live pigs. Australian Veterinary Journal **88**, 255 – 259.

Turni, C. and Blackall, PJ. (2011) An unusual strain of *Haemophilus parasuis* that fails to react in a species-specific polymerase chain reaction assay. *Journal of Veterinary Diagnostic Investigation* **23**, 355 – 358.

Plain English Compendium Summary

Sub-Project Title:	*Riemerella anatipestifer* diagnostics
Poultry CRC Sub-Project No.:	1.2.4
Researcher:	Conny Turni
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Sub-Project Overview	Delivery of improved and new diagnostis to Australian end-users
Background	*Riemerella anatipestifer* infection is a contagious disease primarily affecting ducks, geese and turkeys. However, it can also affect other waterfowl, chickens and pheasants. It displays respiratory symptoms, greenish diarrhoea, nervous symptoms and causes septicaemia with up to 80% mortality. Despite the significance of this infection, only phenotypic diagnostic tools have been available. Phenotypic identification has proven to be unreliable and this leaves the Australian poultry industry with no tools to either diagnose or serotype / genotype this bacterial species. This means that there are no tools available to give the industry advice on vaccine strain selection, biosecurity measurements or even treatment options. This research project set out to produce the necessary tools to get a handle on this disease.
Research	Tools for proper identification of *Riemerella anatipestifer* were established. Next, antisera against all serovar reference strains were produced and Australian field isolates successfully serotyped. Finally, a genotyping method was established and a data base created for *R. anatipestifer* isolates which at this stage includes Australian field isolates, as well as all the type strains for all the serovars and some overseas field strains.
Implications	The implications for the industry are that it is now possible to identify *R. anatipestifer* correctly and rapidly. Additionally, *R. anatipestifer* isolates can be serotyped, which is vital for autogenous vaccine production. An autogenous vaccine is only protective against the serovars that are in the vaccine. Therefore, it is now possible to collect the strains for the vaccine by serovar, making sure that all the serovars on the farm are in the vaccine. The final implication is that tools to genotype the isolates are now available. This will improve knowledge of the epidemiology of the disease, which is vital if proper biosecurity measures are to be implemented. All these new diagnostics tools are offered to the industry on a user-pay system. In summary the industry is now equipped to properly treat and prevent the disease.
Publications	The results are currently in the process of being written up for formal publication in the scientific literature.