Differential serological diagnostics of different Rabbit Haemorrhagic Disease Viruses co-circulating in Australia

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Abstract:

The lagovirus Rabbit Haemorrhagic Disease Virus (RHDV) has been circulating in Australia since the mid-1990s when it was deliberately released to control overabundant rabbit populations. In recent years, the viral diversity of different RHDVs circulating in Australia has increased. An additional strain of an antigenic RHDV variant was deliberately released to boost rabbit control in 2017, and two exotic incursions of lagoviruses, including the novel calicivirus RHDV2, have also been reported in Australia. RHDV2 has since become the dominant strain in Australian wild rabbits. To allow for ongoing epidemiological studies and impact assessments of these viruses on rabbit populations, it is essential that serological tools are updated. Here we describe the adaptation of a competition enzyme linked immunosorbent assay (cELISA) for the specific detection of antibodies against the Australian RHDV2 virus, as well as modified isotype ELISAs to allow for the more sensitive detection

of RHDV2 specific IgM and IgA antibodies. We also assessed the patterns of cross-reactivity of sera raised against all virulent caliciviruses known to be present in Australia in a series of available competition ELISAs and isotype ELISAs originally developed for the prototype RHDV. The new serological assays provide valuable tools to study presence, prevalence and impact of RHDV2 on Australian rabbit populations; however the discrimination of different antigenic variants of RHDVs at the serological level remains challenging.

Introduction:

Rabbit Haemorrhagic Disease Virus (RHDV, sometimes also referred to as RHDV1 or GI.1c, according to a new proposed nomenclature [1]), belongs to the genus lagovirus within the family *caliciviridae*. RHDV was released in Australia as a biological control agent for introduced wild rabbits, a devastating agricultural and environmental pest species in this country [2]. While initially very effective in reducing rabbit populations across large parts of the continent [3, 4], RHDV was not effective in the more temperate areas of South Eastern Australia. This lack of effectiveness was likely due to the presence of endemic, non-pathogenic caliciviruses (Rabbit calicivirus Australia 1, also termed RCV-A1, or GI.4) that can provide transient and partial immunological cross protection to RHDV [5, 6], thereby reducing both case fatality and infection rates [7]. In addition to the impeding effects of RCV-A1, rabbit populations have been recovering in recent years [8], and developing genetic resistance to RHDV has been reported in some Australian rabbit populations [9, 10]. In an attempt to 'boost' rabbit biocontrol in Australia and to maintain the substantial economic and environmental gains made by the long term suppression of rabbit populations by RHDV [11, 12], an additional strain of RHDV was released nationwide in Australia in March 2017 [13]. This strain termed RHDVa-K5 is a naturally occurring antigenic variant of RHDV from Korea [14] which was shown experimentally to be more effective in infecting rabbits from known genetically resistant populations [9] and in overcoming partial protection conveyed by the benign RCV-A1 [15]. These antigenic variants of RHDV, referred to as RHDVa (GI.1a, or RHDV1a), were first reported in the late 1990s [16], and although they exhibit antigenic differences they are considered to be of the same serotype [17].

Prior to the release of RHDVa-K5, the incursions of two additional RHDV variants were reported in Australia. The first incursion was another variant RHDVa (GI.1a) strain in the greater Sydney area that most closely resembled a Chinese isolate [18] that appeared in early 2014. This virus, termed

RHDVa-Aus, caused a number of recorded outbreaks mostly in domestic rabbit farms, however, its distribution appeared geographically limited to the east and north east of New South Wales [19]. In May 2015, the incursion of a second exotic virus, the recently emerged RHDV2 (GI.2) was reported in Australia [20]. RHDV2 is a new lagovirus that was first reported in Europe in 2010 [21, 22]. It is not only genetically distinct from RHDV and RHDVa but, unlike RHDV and RHDVa, is also able to cause highly fatal disease in very young rabbits [23, 24] and capable of fatally infecting several species of hares, making it the only known lagovirus that does not exhibit strict species specificity [25-30]. Since its arrival in Australia, RHDV2 has become the dominant strain circulating in the field, seemingly replacing older RHDV1 strains and accounting for the majority of reported cases in wild and domestic rabbits [31].

With the increasing diversity of co-circulating RHDVs both in Australia and Europe it was essential to update the diagnostic tools available, to allow for specific identification of strains and to measure the impact they were having on wild rabbit (and hare) populations. Due to the genetic variability, the development of specific molecular diagnostics was comparably straightforward, and accordingly several tailored approaches have been described in both Europe and Australia [13, 29, 32, 33]. In contrast, the development of specific serological tools was more challenging. Although strain specific epitopes have been described for lagoviruses and specific monoclonal antibodies have been developed [16, 34-38], discriminatory serology remains difficult, in particular for closely related RHDVs, due to the large number of shared epitopes [17]. The infection history of populations can often only be inferred based on reactivity patterns [39, 40].

In Australia, the development of specific serological tools to discriminate between different lagoviruses was of particular importance. Here, RHDV2 was actively circulating at the time the new strain RHDVa-K5 was released nationwide [13] and specific serological tools were needed to investigate the respective impacts and potential interactions the two strains had on wild Australian rabbits at a population level. Secondly, in the years leading up to the national release of RHDVa-K5, extensive serological monitoring of several Australian rabbit populations was carried out. Screening these sample banks with a serological assay specific to RHDV2 might allow a more accurate determination of the exact time point this virus entered into Australia and started to circulate in wild rabbit populations before it was detected in May 2015 [20].

This study describes the adaptation of a European RHDV2 cELISA to the Australian strain of RHDV2, and the modification of IgM and IgA isotype ELISAs for the detection of RHDV2 antibodies. It also assesses the cross-reactivity patterns of reference sera raised against all virulent RHDV strains

94 known to be circulating in Australia in a panel of serological assays to infer disease dynamics of
95 RHDV in rabbit populations.

96

97 **Material and methods:**

98 *Production of antigen, virus inoculum and experimental vaccine*

99 Two five week old New Zealand white rabbits were infected with 0.5 ml of a 2% clarified liver
100 homogenate of the first RHDV2 field isolate found in Australia (BlMt-1, Gen Bank# KT280060) [20].
101 Rabbits were monitored twice daily for signs of terminal rabbit haemorrhagic disease (RHD), which
102 was defined as 10% weight loss within a 24 hour period, no resistance to handling or lateral
103 recumbency, or hypothermia following a fever episode, often in combination with lethargy. Both
104 rabbits experienced a peracute form of RHD, one was found displaying signs of terminal RHD at 66
105 hours post infection (h.p.i.) and was euthanized, the second was found dead 90 h.p.i. with no prior
106 signs of terminal RHD detected.

107 The liver of one of the RHDV2 infected rabbits was used for the production of an experimental
108 vaccine, according to methods published previously [17]. Briefly, a 20% w/v liver homogenate
109 (containing approximately 3 x 10^8 capsid gene copies/ml) was prepared in sterile PBS and clarified by
110 centrifugation for 20 min at 2,000 g. Chloroform was added to the supernatant to a final
111 concentration of 2% (v/v) and incubated at 4°C overnight, followed by a second clarification at
112 10,000g and 4°C. Part of the clarified supernatant was removed, mixed 1:1 with glycerol and stored
113 at -80°C as RHDV2 inoculum for subsequent infections (0.5 ml/rabbit). Formalin was added to the
114 remaining supernatant to a final concentration of 0.8% v/v and incubated at room temperature
115 overnight. A second inactivation step was performed by adding formalin to a total concentration of
116 1% v/v with overnight incubation at 4°C. This preparation was stored at 4°C until used. Immediately
117 prior to use, the vaccine was brought to room temperature, mixed 1:1 with Addavax (Invivogen, San
118 Diego, USA), and between 0.6 and 1.2 ml were injected subcutaneously in the scruff of the neck.

119 For the production of RHDV2 ELISA antigen, a 10% w/v homogenate of the liver of the second rabbit
120 was clarified using two centrifugation steps (20 min at 3000g, followed by 30 min at 6000g, at 4°C),
121 and then passed through a 0.8 µm filter. The resulting homogenate was mixed 1:1 with glycerol and
122 stored at -80°C.

123 A commercially produced preparation of RHDVa-K5 was used as inoculum to produce hyperimmune
124 sera, diluted to 10,000 RID_{50}/ml (Elizabeth Macarthur Agricultural Institute, Menangle, Australia).
125 RHDVa-Aus inoculum was prepared from a 2% of clarified liver homogenate of RHDVa-Aus (Ber-3,

126 GenBank # KY628310) [19]. The infectious dose of the RHDVa-Aus and the RHDV2 inoculum was not
127 titrated.

128 *Production of reference sera*

129 For the production of RHDVa hyperimmune sera, five-week old rabbits were infected with RHDVa-
130 Aus (n=4) and RHDVa-K5 (n=5). Due to the age related innate resistance to lethal RHDV infection [41,
131 42] it was expected that rabbits of this age group would not succumb to fatal RHDV infection, but
132 survive and mount a strong antibody response. Rabbits were orally infected with 0.5 ml of virus
133 inoculum using a 1 ml syringe. Rabbits were monitored twice daily for the first four days and then
134 daily afterwards. A small (0.1-0.2 ml) blood sample was collected from the marginal ear vein at day
135 0, 7 and 14. Two rabbits in each group were sacrificed and bled at 14 dpi the remaining rabbits at 20
136 dpi (RHDVa-K5) and 22 dpi (RHDVa-Aus) (Table 1).

137 Archival serum samples from five rabbits collected at various time points were used as RHDV
138 hyperimmune reference sera. In this previous study (T. Strive, unpublished), three 5 week old New
139 Zealand white rabbits were infected orally with 500 LD_{50} of a commercial RHDV preparation (RHDV
140 v351, Elizabeth Macarthur Agricultural Institute, Menangle, Australia), blood samples were collected
141 at 7 dpi and a final bleed was carried out at 14 dpi. Hyperimmune RHDV sera from an additional four
142 rabbits harvested at various time points were available from a previous study [41]. Seven RCV-A1
143 polyclonal sera were also included [38], as well as an additional seven negative control sera.

144

145 For the production of RHDV2 polyclonal sera, rabbits needed to be partially vaccinated prior to the
146 challenge, as young rabbits are not innately resistant to lethal RHDV2 infection [23]. Three five-week
147 old rabbits and two adult rabbits of approximately three years of age were injected with the
148 experimental vaccine preparation as described above. One adult animal was challenged 10 days
149 after vaccination (dpv) the remaining four rabbits in this group were challenged six dpv. Monitoring
150 and blood sampling of these animals was carried out as for the RHDVa infected rabbits (Table 1).

151 *RHDV2 specific monoclonal antibody*

152 A mouse monoclonal antibody (Mab) conjugated to horse radish peroxidase (4H12-HRP, lot 150416)
153 and raised against a European strain of RHDV2 was purchased from the Instituto Zooprofilatttico
154 Sperimentale (IZS, Brescia, Italy).

155 *ELISA*

156 The RHDV IgM, IgA and IgG isotype ELISA [38], the RHDV competition ELISA (cELISA) [43] and the
157 RCV-A1 blocking ELISA (bELISA) [44] were performed as described previously.

158 The RHDV2 cELISA workflow and volumes used were similar to the RHDV cELISA, with modifications.
159 Plates were coated overnight at 4°C with a polyclonal RHDV2 rabbit serum (A#Jul, 17 dpi) at a 1:2000
160 dilution in carbonate buffer (pH 9.6). The plates were washed with PBS –Tween (PBS-T) prior to the
161 addition of serial dilutions of rabbit sera starting at 1:40 dilution followed immediately with the
162 addition of RHDV2 crude antigen at a 1:20 dilution and incubated at 37°C for 1 hour. The plate was
163 washed and the MAb 4H12-HRP at a 1:300 dilution (0.8 µg/ml) was added and incubated at 37°C for
164 1 hr. The plate was washed and OPD substrate was added and incubated for 5 minutes before being
165 stopped by the addition of 1M sulphuric acid. The plate was read at 492nM. Optimal concentrations
166 of antigen and MAb were determined by checkerboard titrations using rabbit sera known to be
167 positive and negative to RHDV2. Concentrations were selected that resulted in an optical density
168 (OD) of between 1 and 1.2 for a panel of 15 negative reference sera as well as the highest signal to
169 noise ratio when compared to the positive control serum. A serum was scored positive when the OD
170 was reduced to 75% or less compared to the OD of the negative reference serum at the same
171 dilution.

172 RHDV2-adapted IgM and IgA ELISAs were carried out as described previously for RHDV [38],
173 substituting RHDV virus like particles with crude RHDV2 virus antigen at a 1:20 dilution, and using
174 MAb 4H12-HRP as the detecting antibody at a 1:300 dilution (0.8 µg/ml).

175 *RHDV2 cELISA analysis of field samples collected before and after RHDV2 arrival*

176 Sera from wild rabbits were obtained as described previously [45]. Two field sites located in the
177 central tablelands of New South Wales were visited to collect samples prior to the arrival of RHDV2
178 in October 2012 (n=20) and April 2014 (n=20), respectively, and again throughout 2017 and 2018
179 (n=48) after the arrival of RHDV2 in Australia but before the nationwide release of RHDVa-K5.

180

181 **Results:**

182 *Serum production*

183 As expected, none of the five week old rabbits infected with RHDVa-Aus or RHDVa-K5 developed
184 severe clinical signs or succumbed to RHD. A short fever episode of ≥40°C was observed in all four
185 kittens infected with RHDVa-Aus and three out of four kittens infected with RHDVa-K5 between 2

186 and 4 dpi. These fever episodes were not associated with any changes in behavior and all animals
187 continued to gain weight throughout the experiment.

188 In the group of rabbits used to produce RHDV2 polyclonal serum, no adverse reaction to the
189 experimental vaccine was observed in any of the animals at the injection sites. No behavioral
190 abnormalities or elevated temperatures were recorded in any of the rabbits in this group following
191 vaccination and subsequent challenge with RHDV2.

192 *Cross-reactivity of reference sera in RHDV isotype ELISAs*

193 High levels of cross-reactivity in the RHDV IgM, IgA and IgG isotype ELISAs were observed in sera
194 raised against RHDVa-Aus and RHDVa-K5 compared to the RHDV-Czech polyclonal sera (Table 1).
195 Sera raised against RHDV2 also showed varying levels of cross-reactivity in this assay, but with
196 different patterns. Cross-reactivity was highest in the IgG ELISA, with detectable titres in all animals
197 at 8 dpi, and in one case day 6 post vaccination and prior to RHDV2 challenge. IgM antibodies were
198 detected in all RHDV2 polyclonal sera although titres were lower compared to those of the RHDVa-
199 K5, RHDV-Aus and RHDV-Czech reference sera collected at similar time points post infection. IgA
200 antibodies are indicative of active replication of RHDV following a natural infection and are not seen
201 in vaccinated rabbits [17] and accordingly, no IgA responses were observed in this group following
202 vaccination. IgA cross-reactivity in the RHDV2 polyclonal sera was present but with overall lower
203 titres and with a later onset compared to rabbits infected with RHDV and RHDVa strains, indicating
204 an active but possibly attenuated infection with RHDV2 in these previously vaccinated animals (Table
205 1).

206 *Increased sensitivity and specificity in the RHDV2 adapted IgA and IgM ELISAs*

207 In the isotype ELISAs adapted for RHDV2, the titres of the RHDV2 reference sera were at least four
208 fold greater when compared to the respective original RHDV isotype assays, and where sufficient
209 amounts of serum were available for testing, both IgM and IgA antibodies were detected earlier
210 (Table 1). RHDV-Czech, RHDVa-K5, and RHDVa-Aus reference sera also cross-reacted in the RHDV2
211 IgM and IgA ELISA, but had lower titres compared to the RHDV2 reference sera (Table 1). Due to the
212 high level of cross reactivity of RHDV2 polyclonal sera in the in the IgG assay, the IgG isotype ELISA
213 was not adapted.

214 *Cross-reactivity of reference sera in specific competition or blocking ELISAs for RCV-A1, RHDV and*
215 *RHDV2*

216 The RHDV2 cELISA developed here from Australian and European reagents proved to be highly
217 sensitive and specific. Low levels of reactivity were detected as early as 6 dpv with titres increasing

218 until the end of the trial (Table 1). Sera raised against RHDV-Czech, RHDVa-Aus and RHDVa-K5 only
219 showed low levels of cross-reactivity in this assay, only in one case exceeding titres of 1:40 (1:160,
220 K#315, 22 dpi, Table 1). None of the seven archival RCV-A1 control sera reacted in the RHDV2 cELISA.

221 In contrast, the RHDV cELISA showed low to moderate levels of cross-reactivity with RHDV2
222 polyclonal sera, and very high levels of cross-reactivity with the sera raised against the two RHDVa
223 strains (Table 1). Only one of the RHDV-Czech reference sera tested here showed a very low level of
224 cross-reactivity with the RCV-A1 bELISA (1:10, K#177, 14 dpi, Table 1). Only sera from the terminal
225 bleeds were tested in the RHDV cELISA and RCV-A1 bELISA as there was not sufficient serum left
226 from the previous sampling points.

227 *Evaluation of pre- and post RHDV2 field samples*

228 Of the 40 serum samples tested that were collected from wild rabbits in NSW prior to the detection
229 of RHDV2 in Australia, 5 samples (12.5%) were seronegative in all six serological assays used (Table
230 2). Seven samples (17.5%) tested positive for RHDV IgM and A, and 33 samples (82.5%) reacted in
231 the RHDV IgG ELISA. The more specific but less sensitive RHDV cELISA returned a positive result for
232 25 sera (62.5%), and 80% of rabbits (n=32) tested positive in the RCV-A1 bELISA. Seven samples
233 showed cross-reactions in the RHDV2 cELISA which were considered to be unspecific as RHDV2 was
234 thought to be absent from Australia in 2012. Of these, 4 samples showed low-level cross-reactivity at
235 a titre of 1:20, whereas three samples returned a higher titre of 1:80. This higher reading in all three
236 cases coincided with the presence of IgA or IgM and IgA responses, indicating a recent infection,
237 however, not all animals with IgM and/or IgA responses (n=11) cross-reacted in the RHDV2 cELISA
238 (Table 2). In all but one sample (2012, OC-OCT-19), sera returning a positive reading in the RHDV2
239 cELISA consistently had a higher titre in the RHDV cELISA (Table2).

240 In the 48 samples collected from wild rabbits throughout 2016 and 2017, after the known arrival of
241 RHDV2 in Australia, 7 (14.6%) tested negative in all six assays, 7 (14.6%) had detectable IgA
242 antibodies, 38 (79.2%) tested positive for IgG, 23 (47.9%) reacted in the RHDV cELISA, 26 (54.2%) in
243 the RCV-A1 bELISA, and none (0%) had detectable IgM antibodies (Table 2). Twenty three (47.9%)
244 sera tested positive in the RHDV2 cELISA, with 10 of these returning titres of >1:80 (Table 2). In 12 of
245 the 23 samples returning a positive test result in the RHDV2 cELISA, the corresponding titres in the
246 RHDV cELISA were much lower. In the remaining 11 samples the titres of the RHDV cELISA were
247 higher compared to the RHDV2 cELISA. In these samples, similar to the pre-RHDV2 samples from
248 2012 and 2014, none of the RHDV2 cELISA titres exceeded a titre of 1:80.

249 *The ratio of the two specific cELISAs for RHDV and RHDV2 can be used to infer the presence of RHDV2*
250 *specific antibodies*

251 We explored if the ratios between the RHDV and RHDV2 cELISAs could be used to infer the presence
252 of RHDV2 antibodies in the populations, similar to an approach used previously to discriminate
253 between RHDV2 and European Brown Hare Syndrome Virus (EBHSV) antibodies in wild hare
254 populations in Europe [25]. While there was some level of cross-reactivity between the respective
255 cELISAs in the reference sera produced for this study, the titres were always higher for the
256 respective specific viral antigen. To investigate this further, negative results were set to a titre of 1:5
257 for the purpose of forming ratios between titres, and only samples were included in the analysis that
258 had returned a positive reaction in either of the two assays [25]. The RHDV2 cELISA/RHDV cELISA
259 titre ratios were >1 in all reference sera raised against RHDV2, with ratios ranging from 1 to 100. In
260 all reference sera raised against RHDV or RHDVa the ratios were <1, ranging from 0.008 to 0.125 for
261 sera raised against RHDVa-K5 and RHDVa-Aus, and from 0.0002 to 0.0156 for sera raised against
262 RHDV-Czech (Figure 1).

263 When applied to the pre- and post RHDV field samples, 13 of the 39 post RHDV2 samples that were
264 positive in either one or both cELISAs returned a ratio of >1. Only one serum in the pre-RHDV2
265 sample had a ratio of >1 (Figure 1). This particular sample was seronegative in all assays except for a
266 very low level (1:40) RCV-A1 titre, and a very low (1:20) titre for RHDV2. When only test results from
267 dilutions at 1:40 or greater were considered, none of the pre-RHDV2 samples had a ratio of >1 in this
268 analysis.

269

270 **Discussion/conclusions:**

271 Specific serology of different lagoviruses can be challenging, as high levels of cross-reactivity are
272 often observed even between antigenically very different members of this genus [38, 46, 47]. For the
273 more specific detection of different viruses, less sensitive and more specific cELISAs or bELISAs have
274 been described [44, 48, 49], however, isotype ELISAs detecting IgG antibodies are so cross-reactive
275 that they have historically been used to infer the presence of previously unknown and often
276 antigenically quite different lagoviruses [46, 47, 50, 51].

277 These difficulties notwithstanding, there is a need for improved serological tools to discriminate
278 between antibodies to the different lagoviruses circulating in Australia, to better understand their
279 role in naturally occurring disease dynamics as well as biological control operations of wild rabbits.
280 For over two decades, only RHDV and RCV-A1 were known to be present in Australia, and their

281 disease dynamics have been studied extensively, utilising specific cELISAs or bELISAs for RHDV and
282 RCV-A1, as well as isotype ELISAs for both viruses [38, 44, 50]. The recent arrival of two different
283 strains of RHDVa (RHDVa-Aus and RHDVa-K5) as well as RHDV2 has highlighted the need to update
284 and extend the panel of serological tools available on this continent to enable better studies of the
285 epidemiology and interactions of these viruses.

286 Disease dynamics of RHDV can be inferred by interpreting the reaction profiles in a different isotype
287 and cELISAs [43]. In this approach, IgM is interpreted as an indicator of a recent outbreak and first
288 time exposure of an individual to RHDV, a boost in IgA titres as a measure for re-exposure to RHDV,
289 and the highly sensitive but cross-reactive IgG is used to infer the presence of maternal antibodies in
290 very young rabbits. In our study, the reference sera raised against RHDV-Czech, RHDVa-Aus, RHDVa-
291 K5 and RHDV2 all reacted to varying degrees in these assays, indicating that they should be suitable
292 to detect broad patterns of disease activity caused by any of the virulent lagovirus strains in wild
293 rabbit populations in Australia, however they do not allow for the discrimination between the
294 various strains. Adaptation of the IgM and IgA ELISA to RHDV2 by using RHDV2-specific antigen and
295 Mab increased the sensitivity of these assays for RHDV2 IgM and IgA detection substantially. As
296 RHDV2 has rapidly become the dominant strain in the Australian landscape [31], switching to these
297 more sensitive assays for future large scale field epidemiology studies should be considered.

298 The RHDV2 cELISA described here proved to be highly specific for the detection of RHDV2
299 antibodies, with low levels of cross-reactivity to the experimentally produced RHDV and RHDVa
300 reference sera as well as the 40 sera from wild rabbits collected before the arrival of RHDV2 in
301 Australia. In contrast, the existing RHDV cELISA [48] showed low to medium levels of cross-reactivity
302 to RHDV2 and high levels of cross-reactivity to both RHDVa-Aus and RHDVa-K5. However, in the
303 RHDV and RHDV2 cELISAs, titres were higher in the sera raised against the respective strain in all but
304 one case, such that the ratios of the reciprocal titres of the two assays can potentially be used to
305 infer which strain the rabbit was most likely exposed to. There was only one exception in the pre-
306 RHDV2 sera analysed here where the animal tested negative in all RHDV assays but showed a low
307 titre of 1:20 recorded in the RHDV2 cELISA. The observed relatively high chance of cross-reactivity at
308 low serum dilutions (Table 1) indicates that these are not likely to be very informative, and broad
309 scale population level serological studies would benefit from only considering titres of ≥ 1:40, even if
310 this means a possible slight reduction in sensitivity.

311 The analysis of the reference sera as well as field sera collected pre- and post-RHDV2 arrival in
312 Australia indicates that the ratio between the RHDV2 and RHDV cELISA titres should allow for the
313 detection of the presence of RHDV2 specific antibodies with high levels of certainty, even in the

presence of RHDV or RHDVa antibodies, and thus allow for retrospective analysis of archival field sera for a more accurate estimate of the time of RHDV2 arrival in Australia. However, due to the high levels of cross-reactivity of RHDV2 reference sera in the RHDV cELISA, detecting RHDV or RHDVa antibodies in samples collected post RHDV2 arrival is challenging. In particular, inferring mixed infections of RHDV/RHDVa/RHDV2 or the absence of RHDV antibodies in RHDV2 positive populations will be difficult to discern with certainty. Similarly, the discrimination of antibodies to RHDV and RHDVa strains is not feasible with the serological tools currently available. Despite these remaining challenges in the differential serodiagnostics of Australian lagoviruses, the additional assays described here represent an important addition to the tool kit that will benefit ongoing continent-wide lagovirus epidemiology studies, and help inform the development of effective rabbit management strategies.

Declarations:

Ethics approval and consent to participate: All work involving animals was approved by the CSIRO Ecosystem Sciences Animal Ethics Committee (ESAEC #10-13, #11-01, #13-01, #13-10, #DOMRAB) and the Orange Animal Ethics Committee (ORA11/14/001, ORA14/17/001) and carried out in accordance with the Australian code for the care and use of animals for scientific purposes.

Consent for publication: All authors have read and approved the final version of the manuscript.

Availability of data and material: All data generated or analysed during this study are included in this published article.

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505 Tables

506 Table 1:

rabbit ID	dpv	dpi	RHDV IgM	RHDV IgA	RHDV IgG	RHDV cELISA	RHDV-2 IgM	RHDV-2 IgA	RHDV-2 cELISA	RCV-A1 bELISA
RHDV-2 polyclonal sera										
A#Zor	0		0	0	0	–	–	–	0	–
A#Zor	6		640	0	160	–	40960	0	160	–
A#Zor	10	0	640	0	160	–	40960	0	320	–
A#Zor	14	4	640	0	640	–	40960	0	160	–
A#Zor	16	6	2560	0	2560	–	40960	10240	1280	–
A#Zor	24	14	640	80	2560	–	10240	10240	2560	–
A#Zor	26	16	640	80	10240	–	10240	10240	2560	–
A#Zor	34	24	640	640	10240	320	2560	40960	5120	0
A#Jul	0		0	0	0	–	–	–	0	–
A#Jul	5		0	0	0	–	640	0	10	–
A#Jul	6	0	0	0	0	–	2560	0	80	–
A#Jul	14	8	0	20	2560	–	10240	640	320	–
A#Jul	16	10	0	0	10240	–	10240	640	1280	–
A#Jul	24	17	80	20	10240	20	10240	10240	1280	0
K#316	0		0	0	0	–	–	–	0	–
K#316	5		0	0	0	–	–	–	0	–
K#316	6	0	0	0	0	–	640	–	10	–
K#316	14	8	640	160	2560	–	40960	10240	1280	–
K#316	16	10	640	160	10240	–	40960	10240	160	–
K#316	24	17	640	40	10240	20	10240	2560	2560	0
K#317	0		0	0	0	–	–	–	0	–
K#317	5		0	0	0	–	–	–	0	–
K#317	6	0	160	0	0	–	–	–	10	–
K#317	14	8	640	80	2560	–	10240	10240	160	–
K#317	16	10	160	80	2560	–	10240	10240	160	–
K#317	24	17	320	320	2560	20	10240	2560	1280	0
K#318	0		0	0	0	–	–	–	0	–
K#318	5		0	0	0	–	–	–	10	–
K#318	6	0	0	0	0	–	–	–	20	–
K#318	14	8	160	80	2560	–	–	–	320	–
K#318	16	10	160	80	10240	–	10240	10240	320	–
K#318	24	17	640	80	10240	20	10240	2560	1280	0
RHDVa-K5 polyclonal sera										
K#307		0	0	0	0	–	–	–	0	–
K#307		7	40960	2560	2560	–	10240	2560	10	–
K#307		14	10240	2560	2560	–	2560	640	0	–
K#307		20	10240	10240	40960	320	640	2560	10	0
K#308		0	0	0	0	–	–	–	0	–
K#308		7	10240	1280	2560	–	10240	640	0	–
K#308		14	10240	1280	2560	–	2560	640	10	–
K#308		20	5120	2560	10240	320	640	2560	40	0

K#309	0	0	0	0	–	–	–	0	–
K#309	7	40960	2560	2560	–	10240	2560	10	–
K#309	14	10240	1280	2560	–	640	160	10	–
K#309	20	5120	1280	10240	640	640	640	40	0
K#310	0	0	0	0	–	–	–	0	–
K#310	7	40960	2560	2560	–	2560	160	10	–
K#310	14	10240	640	2560	320	640	640	10	0
K#311	0	0	0	0	–	–	–	0	–
K#311	7	40960	2560	2560	–	10240	160	20	–
K#311	14	2560	5120	2560	640	2560	2560	10	0
RHDVa-Aus polyclonal sera									
K#312	0	0	0	0	–	–	–	0	–
K#312	7	40960	640	10240	–	–	–	0	–
K#312	14	10240	2560	10240	640	10240	2560	10	0
K#313	0	0	0	0	–	–	–	0	–
K#313	7	40960	640	2560	–	2560	40	10	–
K#313	14	10240	1280	2560	320	2560	640	0	0
K#314	0	0	0	0	–	–	–	0	–
K#314	7	10240	2560	10240	–	640	40	0	–
K#314	14		2560	40960	–	–	–	0	–
K#314	22	10240	5120	40960	1280	2560	640	10	0
K#315	0	0	0	0	–	–	–	0	–
K#315	7	40960	10240	40960	–	10240	160	0	–
K#315	14	10240	2560	40960	–	2560	160	10	–
K#315	22	10240	1280	10240	640	2560	640	160	0
RHDV-Czech polyclonal sera									
K#175	4	640	0	0	–	–	–	0	0
K#175	7	40960	2560	40	–	640	0	0	0
K#175	14	40960	10240	40960	5120	160	1280	0	0
K#176	4	160	0	0	–	–	0	0	0
K#176	7	10240	1280	640	–	640	160	0	0
K#176	14	20480	10240	10240	320	40	160	0	0
K#177	4	2560	0	0	–	–	–	0	0
K#177	7	10240	160	2560	–	2560	160	0	0
K#177	14	40960	5120	10240	5120	2560	640	10	10
K#184	4	2560	0	160	–	0	0	0	0
K#185	4	80	0	0	–	0	0	0	0
K#135	0	0	0	0	–	–	–	0	–
K#135	15	40960	10240	40960	10240	640	160	0	0
K#136	0	0	0	0	–	–	–	0	–
K#136	15	40960	10240	40960	10240	640	640	10	0
K#137	0	0	0	0	–	–	–	D	–
K#137	4	40960	5120	0	–	2560	–	10	0
K#138	0	0	0	0	–	–	–	0	–
K#138	15	40960	40960	40960	20480	2560	640	0	0
K#139	0	0	0	0	–	–	–	D	–
K#139	15	10240	5120	40960	5120	0	0	0	0

RCV-A1 polyclonal sera									
RCV-3	7	0*	0*	0*	0*	–	–	0	160**
RCV-4	7	2560*	320*	320*	80*	–	–	0	320**
RCV-8	11	0*	0*	40*	0*	–	–	0	40**
RCV-11	28	0*	160*	2560*	0*	–	–	0	160**
RCV-12	28	0*	0*	640*	0*	–	–	0	160**
RCV-24	9	0*	0*	0*	0*	–	–	0	320**
RCV-25	9	160*	0*	320*	0*	–	–	0	160**

dpv = Days post vaccination
dpi = Days post infection
* Data from Liu et al., 2012a
**Data from Liu et al., 2012b
D= doubtful

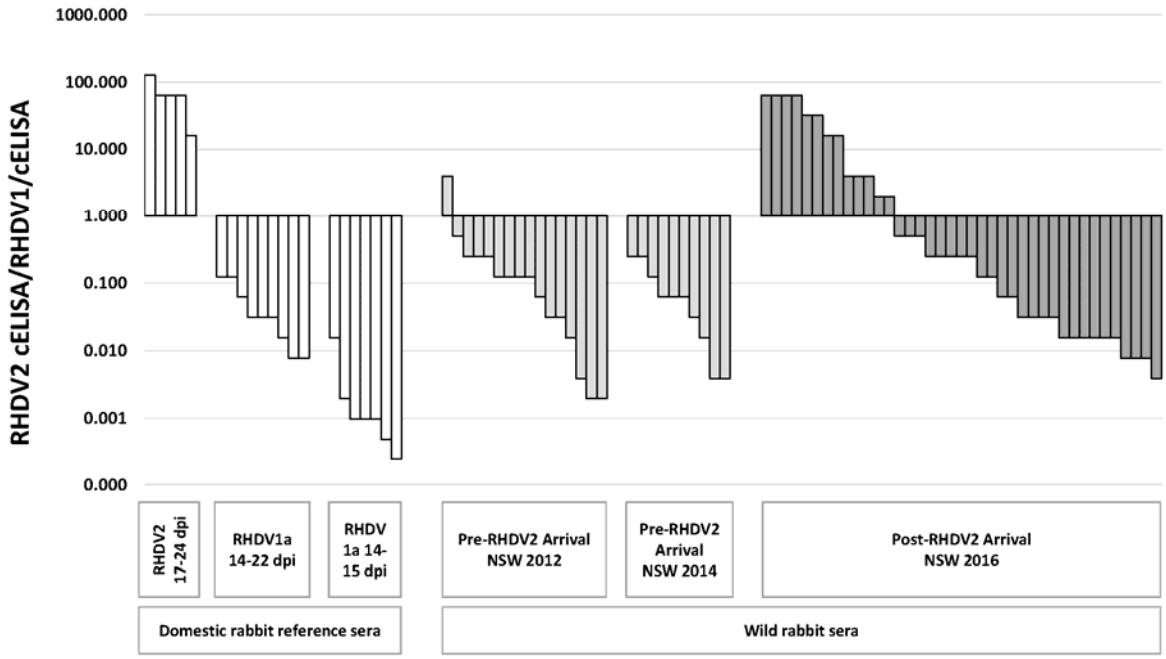
Table 2:

Year	rabbit ID	RHDV IgM	RHDV IgA	RHDV IgG	RHDV cELISA	RHDV-2 cELISA	RCV-A1 bELISA
Wild rabbit sera Pre-RHDV							
2012	OC-OCT-1	0	0	1280	0	*0*	160
2012	OC-OCT-2	0	POS	10240	160	*20*	>160
2012	OC-OCT-3	0	0	640	40	*0*	160
2012	OC-OCT-4	0	0	>40960	>>>640	*20*	160
2012	OC-OCT-5	0	0	10240	<80	*20*	>160
2012	OC-OCT-6	0	0	20480	0	*0*	>160
2012	OC-OCT-7	0	0	>40960	320	*0*	>160
2012	OC-OCT-8	0	0	20480	>>640	*0*	>160
2012	OC-OCT-9	0	0	2560	0	*0*	40
2012	OC-OCT-10	0	POS	10240	320	*80*	160
2012	OC-OCT-11	0	0	2560	160	*0*	10
2012	OC-OCT-12	0	0	>40960	>>640	*0*	>160
2012	OC-OCT-13	0	0	20480	80	*0*	>160
2012	OC-OCT-14	0	0	20480	40	*0*	>160
2012	OC-OCT-15	0	0	40960	20	*0*	>160
2012	OC-OCT-16	0	0	40960	160	*0*	>160
2012	OC-OCT-17	0	0	5120	20	*0*	>160
2012	OC-OCT-18	0	POS	2560	0	*0*	160
2012	OC-OCT-19	0	0	0	0	20	40
2012	OC-OCT-20	0	0	2560	40	*0*	>160
2014	MIR-APR-1	0	0	40960	320	*0*	0
2014	MIR-APR-2	POS	POS	>>40960	>640	*80*	40
2014	MIR-APR-3	0	0	0	0	*0*	0
2014	MIR-APR-4	0	0	0	0	*0*	0
2014	MIR-APR-5	0	0	2560	0	*0*	160
2014	MIR-APR-6	POS	0	5120	40	*0*	0
2014	MIR-APR-7	0	0	5120	0	*0*	160
2014	MIR-APR-8	POS	0	0	>640	*0*	80
2014	MIR-APR-9	POS	POS	>40960	>640	*0*	80
2014	MIR-APR-10	POS	0	20480	80	*0*	160
2014	MIR-APR-11	0	POS	2560	20	*0*	40
2014	MIR-APR-12	POS	POS	>40960	>640	*80*	80
2014	MIR-APR-13	0	0	0	0	*0*	0
2014	MIR-APR-14	0	0	640	0	*0*	10
2014	MIR-APR-15	0	0	10240	20	*0*	80
2014	MIR-APR-16	0	0	0	0	*0*	0
2014	MIR-APR-17	0	0	5120	160	*0*	0
2014	MIR-APR-18	POS	0	1280	0	*0*	160
2014	MIR-APR-19	0	0	0	0	*0*	0
2014	MIR-APR-20	0	0	2560	0	*0*	>160
Wild rabbit sera Post-RHDV							
2016	MIR-APR-1	0	0	320	20	*0*	40
2016	MIR-APR-2	0	0	5120	320	*1280*	10
2016	MIR-APR-3	0	0	0	0	*0*	0
2016	MIR-APR-4	0	POS	10240	>640	*80*	160

2016	MIR-APR-5	0	POS	320	10	*0*	0
2016	MIR-APR-6	0	POS	2560	40	*20*	40
2016	MIR-APR-7	0	0	2560	20	*640*	>160
2016	MIR-APR-8	0	POS	2560	40	*0*	10
2016	MIR-APR-9	0	0	0	0	*0*	10
2016	MIR-APR-10	0	0	0	0	*0*	0
2016	MIR-APR-11	0	0	0	0	*0*	0
2016	MIR-APR-12	0	0	2580	160	*0*	10
2016	MIR-APR-13	0	POS	1280	20	*1280*	0
2016	MIR-APR-14	0	POS	640	20	*0*	10
2016	MIR-APR-15	0	POS	1280	320	*0*	40
2016	MIR-APR-16	0	0	80	0	*0*	0
2016	MIR-APR-17	0	0	5120	>640	*20*	80
2016	MIR-APR-18	0	0	320	0	*80*	160
2016	MIR-APR-19	0	0	320	80	*0*	10
2016	MIR-APR-20	0	0	0	0	*0*	0
2016	MIR-JUL-1	0	0	320	40	*1280*	0
2016	MIR-JUL-2	0	0	640	20	*80*	0
2016	MIR-JUL-3	0	0	1280	160	*0*	40
2016	MIR-JUL-4	0	0	5120	>>>640	*80*	80
2016	MIR-JUL-5	0	0	0	0	*0*	0
2016	MIR-JUL-6	0	0	5120	>640	*20*	0
2016	MIR-JUL-7	0	0	0	0	*0*	0
2016	MIR-JUL-8	0	0	5120	>>640	*20*	80
2016	MIR-JUL-9	0	0	5120	640	*1280*	160
2016	MIR-JUL-10	0	0	640	160	*320*	20
2016	MIR-JUL-11	0	0	5120	>>640	*20*	0
2016	MIR-JUL-12	0	0	5120	160	*0*	40
2016	MIR-JUL-13	0	0	320	40	*160*	0
2016	MIR-JUL-14	0	0	0	0	*0*	0
2016	MIR-JUL-15	0	0	5120	>640	*0*	10
2016	MIR-OCT-1	0	0	2560	>160	*80*	10
2016	MIR-OCT-2	0	0	160	0	*320*	10
2016	MIR-OCT-3	0	0	5120	>640	*40*	160
2017	MIR-JAN-1	0	0	1280	320	*0*	0
2017	MIR-JAN-2	0	0	0	0	*320*	160
2017	MIR-JAN-3	0	0	10240	640	*0*	0
2017	MIR-JAN-4	0	0	640	320	*80*	0
2017	MIR-JAN-5	0	0	2580	20	*0*	0
2017	MIR-JAN-6	0	0	320	10	*0*	0
2017	MIR-JAN-7	0	0	160	320	*0*	0
2017	MIR-JAN-8	0	0	0	0	*80*	0
2017	MIR-JAN-9	0	0	320	0	*320*	160
2017	MIR-JAN-10	0	0	1280	40	*0*	10

POS = Positive at 1:40 dilution. Only one dilution was tested.

531 **Figures:**



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534 **Figure 1.** RHDV2/RHDV cELISA ratios of experimentally produced reference sera and selected field
535 caught rabbits.

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