Bayesian evaluation of temporal changes in sensitivity and specificity of three serological tests for multiple circulating strains of rabbit haemorrhagic disease virus

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Abstract

Competition and indirect ELISAs are currently being used to monitor rabbit hemorrhagic disease viruses (RHDV1 and RHDV2) in rabbits worldwide. Temporal changes in the sensitivity (Se) and specificity (Sp) of assays were investigated using Bayesian Latent Class models (BCLM) in the Australian wild rabbit population where both viruses circulate simultaneously and a long-term serological dataset exists. When cELISA1 was compared to IgG1 ELISA, the Se of cELISA1 improved while the Sp of IgG1 ELISA declined over the 2011-2021. This corresponded with a decline in the true RHDV1 prevalence in 2018-21, suggesting that a large proportion of RHDV1 exposed rabbits survived the introduction and dominance of RHDV2 up to approximately 2017/2018, after which they died and were not replaced. The Se and Sp estimates for 2014-15 for both cELISA1 and IgG1 ELISA, and the true prevalence when analysing all three tests together were similar to those obtained from the analysis of cELISA1/IgG1. The same was also true for the Se and Sp of cELISA2 and IgG1 estimates from 2018 onwards. This suggests that RHDV1 was the dominant infection status in 2014-15, but RHDV2 was the dominant infection status in 2018-2021. Further, the increase in Se of cELISA2 and the low Sp of IgG1 ELISA in the cELISA2/IgG1 ELISA analysis, compared to the Se of cELISA2 and Sp of IgG1 ELISA when analysing all three tests together suggests that the underlying infection status was more influenced by RHDV2 and that the higher Se of IgG1 ELISA is due to cross-reaction of RHDV2 antibodies on IgG1 ELISA. The true prevalence data suggests that RHDV2 exposure peaked in 2017. Our findings show that test characteristics changed in response to the changing virus prevalences over time. IgG1 ELISA currently has a high Se, should be used to monitor both viruses and will perform better than both cELISAs.

Introduction:

Wild rabbits have immense environmental and economic impacts within their invasive range. For example, rabbits are among the most environmentally significant pest animal in Australia (Kearney et al., 2019). Their impacts are wide and varied, and include direct herbivory and the associated reduction in the regeneration of vegetation; competition for food resources; land degradation through reduced soil porosity, increased compaction, and erosion; weed infestation; and supporting large populations of introduced predators (Finlayson et al., 2021). The distribution of rabbits in Australia is extensive, spanning greater than half the entire continent. The impacts of rabbits combined with their extensive distribution results in them

having significant economic impacts to the Australian agricultural industry which are currently estimated at approximately $200M AUD annually (Gong et al., 2009, Cooke et al., 2013, Bradshaw et al., 2021).

Rabbit biocontrol viruses have proven to be highly effective in suppressing rabbit populations and reducing their impacts in Australia. Two viruses have been used as biocontrol agents; 1) myxoma virus, which was introduced in 1950 and causes the disease myxomatosis, and 2) rabbit hemorrhagic disease virus (RHDV1), which was introduced in 1995 and causes rabbit hemorrhagic disease (Cooke and Fenner, 2002, Fenner and Ratcliffe, 1965, Ratcliffe et al., 1952). Both viruses were highly effective at reducing rabbit populations following their introduction and they both now circulate naturally in wild rabbit populations (Ratcliffe et al., 1952, Cooke and Fenner, 2002, Cooke et al., 2013, Cooke, 1996, Mutze et al., 1998). However, RHDV1 is the only virus that continues to be released intentionally by land managers and its transmission in wild rabbit populations is consequently more closely monitored and researched relative to that of myxoma virus (Taggart et al., 2022). In addition to the deliberate introduction of both myxoma virus and RHDV1 into Australia for rabbit management, a third virus, rabbit hemorrhagic disease virus 2 (RHDV2), emerged naturally in Australian rabbit populations in approximately 2014 (Ramsey et al., 2020, Taggart et al., 2021, Hall et al., 2015). RHDV2 also substantially reduced rabbit populations in Australia following its emergence and it now circulates widely among rabbit populations across Australia, replacing previously dominant RHDV1 strains (Ramsey et al., 2020, Mahar et al., 2018). As a result, the transmission of RHDV2 is now also closely monitored and researched in wild rabbit populations.

The RHDV1 and RHDV2 strains are genetically distinct and have readily available molecular diagnostics for strain differentiation (Hall et al., 2018). However, a large portion of the ongoing monitoring of RHDV1 and RHDV2 relies on the collection of rabbit blood samples and inferring virus dynamics from serological assays. Differentiating seropositivity to RHDV1 Vs RHDV2 is challenging as both viruses are closely related and share similar antigenic epitopes (World Organisation for Animal Health (OIE), 2016). Several ELISAs have been used to detect RHDV1 and RHDV2 antibodies in wild rabbit populations. A competition ELISA (cELISA) developed in the early 1990s is used to detect all antibodies raised against RHDV1 (Capucci et al., 1991), whereas isotype ELISAs are used to detect specific antibodies (IgA, IgM, and IgG) developed against the virus (Capucci et al., 1997, Liu et al., 2012, Cooke et al., 2000). However, RHDV1 cELISA and isotype assays cannot differentiate between exposures from different RHDV1 variants and acts as a consensus test to detect antibodies against RHDV1 strains generally. RHDV2 assays have also been developed by adapting the methodology from the equivalent RHDV1 assays; this include the development of an RHDV2 cELISA, and RHDV2 IgA and IgM isotype assays (Strive et al., 2019). However, since the arrival of RHDV2 the antibodies produced in response to RHDV1 and RHDV2 infections cross-react on the opposing cELISA due to the viruses sharing common epitopes in their antigens. Therefore, now that both viruses, and their variants, circulate within rabbit populations, exposure to one specific virus can be difficult to differentiate via serology. Consequently, rabbits are commonly classified as being sero-positive to RHDV1 or RHDV2 based on the ratio of the reciprocal titres from both cELISAs (Strive et al., 2019).

The performance of a diagnostic test is characterized by its sensitivity (Se, the probability of a true positive test) and specificity (Sp, the probability of a true negative test). Test evaluation is commonly carried out by comparing the test of interest to a reference test, which is assumed to have a perfect ability to classify subjects as diseased or not. However, reference tests are not always available and assuming a reference test (with perfect Se and Sp) is often not reasonable; this is especially the case when working with wild animals or data from ongoing surveillance programs. Bayesian Latent class models (BLCM) enable estimation of Se and Sp of the tests under evaluation without the assumption of a perfect reference test. Hence, samples obtained directly from ongoing surveillance programs can be used to establish the Se and Sp of the tests within the population in which they are intended to be used, and the inherent bias associated with an imperfect reference test can be avoided. In a BLCM, the true status of an animal is treated as an existing, but unknown (latent), variable and the test characteristics and true disease or infection prevalence can be estimated according to this latent variable. This enables the continuous assessment of test performance in the population in which it is intended to be used, and in populations with multiple circulating virus strains (Greiner and Gardner, 2000).

The objective of our present study was to quantitatively evaluate the performance of three serological assays for detecting antibodies against RHDV1 & RHDV2 through the use of BCLM. We used a long-term RHDV1 and RHDV2 surveillance dataset from a wild rabbit population where the true underlying serological status of rabbits was unknown. RHDV1 and RHDV2 both co-circulate in this population and have changed drastically in their prevalence through time as a consequence of competition between the two viruses for the available susceptible host population (Ramsey et al 2020; Taggart et al 2021).

Materials and Methods

Study population and sample collection

We monitored the RHDV1 and RHDV2 viruses in wild rabbit populations at 41 sites in Queensland, New South Wales, Australian Capital Territory, Victoria, South Australia and Western Australia (Cox et al., 2019). Monitoring occurred at approximate three-monthly intervals in Summer (January), Autumn (April), Winter (July) and Spring (October) between 2011 and 2021. However, not all sites were monitored at every time point. During each monitoring period we collected samples from up to 20 shot rabbits using a small caliber rifle (ethics permit number: ORA 19/22/020; CWLA-AEC 2016-02; CES-AEC 12-15). We immediately collected blood from the open pleural cavity of shot rabbits. Serum was subsequently separated and stored at -20°C. The rabbit shooting operations were approved by the animal ethics committee of their respective jurisdictions.

Serology assays

Serum samples were tested for general detection of antibody response to RHDV1 variants using a cELISA (cELISA1) assay developed by Capucci et al. (1991). Sera were also tested on an RHDV1 IgG isotype ELISA (IgG1 ELISA), similar to that described by Capucci et al. (1997) but with minor modifications. An RHDV2 cELISA (cELISA2), modified from the cELISA1, was used to detect general antibody response to RHDV2 (Strive et al., 2019). Sera were serially diluted from 1:40 dilution to 1:2560 dilution on cELISA1 and cELISA2, and from 1:40 to 1:40,960 dilution on RHDV1 IgG1 ELISA assay. A titre [?]1:40 was considered positive on all assays. All sera were tested on cELISA1 and IgG1 ELISA. Sera from all sites were tested retrospectively on cELISA2 until the RHDV2 sero-prevalence dropped below 5%. This resulted in the majority of sera prior to 2014 not being tested on cELISA2.

Bayesian Latent Class model (BLCM)

To evaluate the impact of the underlying mix of RHDV1 and RHDV2 strains on the performance of the above-described tests, data were stratified by time period ([?]2013, 2014-2015, 2016-2017, 2018-2021) and sampling season to account for anticipated differences in the true prevalence of each strain. These time periods were choses to obtain sufficiently large samples for the analyses (Table 1).

For the analyses, two different BLCMs were used: 1) a model with two tests included at a time, where it was assumed that the tests were conditionally independent given disease status (Branscum et al., 2005); and 2) a model with three tests, allowing for pairwise conditional covariance between tests. All models were fit using non-informative prior (i.e. Beta(1,1)) distributions for all parameters, except for conditional covariance parameters, that were modelled as uniform across their possible range (Gardner et al., 2000). The approach of analyzing the tests combined and pairwise, was inspired by Toft et al. (2007) and allows for comparing estimates across models to assess the underlying assumptions of the BLCM. Furthermore, an analysis where the results from cELISA1 and cELISA2 were interpreted in parallel against the IgG test was used to assess the potential of combining cELISA1 and cELISA2 results.

The posterior distribution of the Se, Sp and true prevalence were reported as the median and corresponding 95% posterior probability interval. The Markov Chain Monte Carlo (MCMC) chains were run for 20,000 iterations (with three different sets of starting values) and the first 10,000 iterations were discarded as a burn-in phase. Convergence of the MCMC chains after the initial burn-in was assessed by visual inspection of the time-series plots of Se, Sp and the true prevalence as well as the Gelman–Rubin statistic as suggested by Toft et al. (2007). All MCMC analyses were carried out using JAGS (Plummer, 2003) from R (R Core Team, 2021), with the runjags-package (Denwood, 2016). JAGS models were constructed using the function "template_huiwalter()" to generate generic JAGS code and were subsequently modified to accommodate the specific features of the individual models. All data management and post-processing of MCMC estimates were done in R.

Results

A total of 5,310 sera from our RHDV1 and RHDV2 surveillance program were available for analysis (Table 1). Data for all three assays was available for 4,320 sera whereas no cELISA2 data was available for 990 sera sampled before 2014.

Table 1: Cross tabulation of the RHDV1 cELISA (cELISA1), RHDV1 IgG (IgG1 ELISA), RHDV2 cELISA (cELISA2) and test results (positive test: +; negative test: -) stratified by time period and sampling season. Prior to 2014, rabbits were not tested using cELISA2, so data from before 2014 were only cross tabulated with respect to cELISA1 and IgG1 ELISA test status.

Test	Test	cELISA1/IgG1 ELISA	cELISA1/IgG1 ELISA	cELISA1/IgG1 ELI
Year	**Season**	+/+	+/+	+/-
2013	Spring	154	154	8
	Summer	88	88	11
	Autumn	111	111	4
	Winter	103	103	6
Test	Test	**cELISA1/IgG1 ELISA/cELISA2**	**cELISA1/IgG1 ELISA/cELISA2**	**cELISA1/IgG1 E**
Year	**Season**	+/+/+	+/+/-	+/-/+
2014-2015	Autumn	12	136	1
	Winter	69	157	0
	Spring	62	132	1
	Summer	13	71	0
2016-2017	Autumn	153	94	1
	Winter	127	113	0
	Spring	223	125	1
	Summer	144	155	4
2018	Autumn	17	9	3
	Winter	6	2	0
	Spring	12	7	4
	Summer	38	28	2

The posterior estimates for Se, Sp and true prevalence were obtained when analyzing data from Table 1 using a model with two or three tests (Table 2).

The analysis of cELISA1 against IgG1 ELISA, without cELISA2, was the only analysis done for data prior to 2014 (Table 2). The performance of cELISA1 improved over the 2011-2021 period, particularly with respect to Se. In contrast, the Sp of IgG1 ELISA decreased over this same period. Before 2014, there was a remarkably high RHDV1 seroprevalence, consistent with the long period of time that RHDV1 had

been circulating in the Australian rabbit population. However, the emergence of the new and more virulent RHDV2 reduced the occurrence of RHDV1 seropositive rabbits markedly, especially from 2018 onwards.

When we assume the underlying infection status is primarily driven by RHDV1, the Sp for cELISA1, when comparing cELISA1 and IgG1 ELISA, remained relatively consistent over time (Table 2). Comparatively, when we assume the underlying infection status is driven by both RHDV1 and RHDV2, by comparing cELISA1 and cELISA2, the Sp of cELISA1 increased over time from 2014-15 to 2018-21. This may be due to the gradual decrease in the RHDV1 dominance and corresponding increase in RHDV2 dominance to the mixture of the assumed underlying infection status.

Table 2: Posterior estimates (median and 95% posterior credible interval) of the sensitivity (Se) and specificity (Sp) obtained from Bayesian latent class models for the tests under evaluation, as well as the true prevalence estimates resulting from these test evaluations. Missing estimates for a test indicate that the analysis was done without this test.

	cELISA1	cELISA1	cELISA2	cELISA2	IgG1 ELISA	IgG1 ELISA	Preva
Year	Se	Sp	Se	Sp	Se	Sp	Autu
<2014	75.3 [70.6;80.8]	93.9 [89.8;98.4]			98.4 [94.9;100]	96.4 [87.4;100]	71.5 [
2014-2015	79.1 [69.6;92.1]	97.6 [95.5;100]			99 [97.4;100]	81 [65.7;98.6]	54.6 [
2016-2017	90.8 [83.3;99.2]	98.5 [97.1;100]			99.4 [98.5;100]	72.3 [64.2;81.9]	46.4 [
2018-2021	95.6 [82.7;100]	97.1 [94.2;100]			92.5 [84.6;100]	56.3 [52.2;60.4]	8.2 [4
2014-2015	80.5 [67;93.4]	97 [92.6;100]	22.9 [19.5;26.6]	92.1 [89.1;94.9]	98.1 [94.3;100]	76.8 [63.1;95]	53 [41
2016-2017	77.5 [71.3;84.8]	98.6 [95.4;100]	58.8 [55.4;62.2]	86.6 [77;95.3]	90.6 [85.2;99]	76.6 [70.3;83.1]	55.5 [
2018-2021	19.6 [16.2;24.2]	82.7 [79.4;86.7]	71.6 [61.5;86.4]	81.1 [74.8;89.1]	92.5 [82.6;99.4]	82.5 [70.1;92.3]	54.8 [
2014-2015	94.2 [84.2;100]	60.5 [55.8;65.5]	53.5 [38;73.2]	91.2 [88.7;93.7]			2.2 [0
2016-2017	78.2 [69.9;87.4]	79.2 [67.2;94.7]	65.1 [56.3;75]	82.2 [74.2;91.9]			42.6 [
2018-2021	81.9 [54.4;100]	97.3 [92.2;100]	61.1 [50.6;73.2]	63.5 [59;68.1]			9.5 [2
2014-2015			42.5 [30.5;59]	92.5 [89.9;95.1]	97.8 [92.4;100]	43 [37.3;49.7]	8.8 [0
2016-2017			67.9 [57.7;78.4]	87.6 [79.4;97.3]	90.1 [84.3;96.5]	59.6 [48.7;73.7]	47.3 [
2018-2021			71.4 [58.2;89.7]	83.5 [75.8;93.9]	91.1 [79.5;100]	80.6 [66.2;97.2]	52.3 [

Comparison of the true prevalence estimates between the analysis of all three tests and those from any of the pairwise two-test analyses, suggests that in 2014-2015 a large proportion of rabbits were exposed to RHDV1 relative to RHDV2. Comparison of the same analyses also shows that in 2016-2017 a similar proportion of rabbits are exposed to both RHDV1 and RHDV2, and from 2018 onwards a much larger proportion of rabbits are exposed to RHDV2 compared to RHDV1.

The underlying latent infection status, when comparing all three tests, is a mixture of both viruses and this becomes evident when observing how the test characteristics change in response to the changes in the virus prevalence. When comparing all three tests, the Se of cELISA1 decreased from 80.5% in 2014-2015 to 19.6% from 2018 onwards, while the Se of cELISA2 increased from 22.9% to 71.6% over the same period. The Sp of both cELISAs decreased slightly over time, while in comparison, the Se and Sp of IgG1 ELISA were less affected by the change in the RHDV1 or RHDV2 dominance in the underlying infection status.

When the underlying latent infection status is a mixture of both RHDV1 and RHDV2, as inferred from the analysis using all 3 tests and the analysis using cELISA1/cELISA2 and cELISA2/IgG1 ELISA, the analyses suggests that RHDV2 was at peak prevalence in the 2016-17 period. After this, RHDV2 prevalence declined during the 2018-21 period. Further, the comparison of the true prevalence estimates from the analysis of cELISA1/IgG1 ELISA to those from all other analyses indicates that RHDV2 was the dominant strain in the 2018-21 period.

The Se and Sp estimates for cELISA1 and IgG1 ELISA during 2014-15, and the true prevalence estimates during this same period, are similar between the analysis of all three tests and the analysis of cELISA1/IgG1

ELISA. The same is also true for cELISA2 and IgG1 ELISA from 2018 onwards, that is, the Se and Sp estimates for cELISA2 and IgG1 ELISA from 2018 onwards, and the true prevalence estimates during this same period, are similar between the analysis of all three tests and the analysis of cELISA2/IgG1 ELISA. This suggests that a larger proportion of rabbits were exposed to RHDV1 compared to RHDV2 during the 2014-2015 period, but the reverse was the case from 2018 onwards, a larger proportion of rabbits were exposed to RHDV2. However, as expected, when cELISA2 and IgG1 ELISA are analyzed together, in isolation of ELISA1, the prevalence results for 2014-15 and 2016-17 change considerably compared to the analysis of cELISA1/IgG1 ELISA. The true prevalence estimates from analysis of cELISA2/IgG1 ELISA show that the proportion of rabbits exposed to RHDV2 increased over time; this is the reverse of the prevalence pattern observed from the analysis of cELISA1/IgG1 ELISA, where the proportion of rabbits exposed to RHDV1 decreases over time. The analysis of cELISA2/IgG1 ELISA shows a higher Se for cELISA2 and a lower Sp for IgG1 ELISA when compared to the analysis of all three tests. This is indicative of the underlying latent infection status being more strongly influenced by RHDV2 relative to RHDV1.

A model with conditional covariance (dependence assumed between tests) between cELISA1 and IgG1 ELISA, as well as cELISA1 and cELISA2 was used for the analyses of all three tests together (data on conditional covariance posterior estimates are not shown). Conditional independence given infection status was still assumed between cELISA2 and IgG1 ELISA in order to ensure identifiability of the model. The conditional covariance between tests is difficult to interpret and there are two primary concerns with the interpretation of the conditional covariance estimates: 1) whether they can be deemed different from zero; and 2) whether their inclusion changes the overall conclusions regarding test characteristics. For a few conditional covariance parameters in some of the analyses, the posterior 95% credibility interval did not include zero. However, comparisons between models with and without conditional covariance included suggested minimal, if any, influence on the overall Se and Sp estimates for all tests (data not shown). Nonetheless, the conditional covariances were included in the analysis of the three tests together for consistency and biological plausibility.

When the two cELISAs were compared to each other, in isolation of IgG1 ELISA, the true prevalence estimates were very different to the prevalence estimates using all three tests and they suggest that the underlying latent infection status becomes much more complicated to interpret in comparison to the underlying status using all three tests together. Consequently, the Se and Sp of both tests became difficult to interpret. There was no evidence that the latent infection status definition even remained the same for each time period when analyzing the two cELISAs in isolation of IgG1 ELISA.

Discussion

Our study highlights key challenges for serological surveillance programs in dynamic populations with competing and closely related infectious agents that cross-react on diagnostic assays. For such a surveillance program to yield meaningful information, the characteristics of the applied tests should be known and described quantitatively. This is especially the case if the objective of the surveillance program is to monitor the prevalence of a disease, rather than merely establish its presence or absence. For example, prior to 2014, IgG1 ELISA had a remarkably high Se and Sp and could have been considered a near perfect test. However, from 2018 onwards, the Sp of IgG1 ELISA became problematic if we considered the underlying lately infection status to be primarily driven by RHDV1, with Sp dropping from 96.4% prior to 2014 to 56.3% from 2018 onwards. This drop in the Sp of IgG1 ELISA when the latent infection status is considered to be primarily driven by RHDV1 was likely due to the increase in the prevalence and dominance of RHDV2 post 2015. However, if the underlying latent infection status was more broadly considered to represent exposure to either virus (RHDV1 or RHDV2), then the Sp of IgG1 ELISA would remain high, at 82.5%. This suggests that in the current rabbit population IgG1 ELISA is no longer a suitable diagnostic test for RHDV1 only, but rather better detects exposure to both viruses. These results highlight the importance of quantitively assessing test characteristics through time as their characteristics and the value of the results they provide

can change drastically.

The change in RHDV1 and RHDV2 prevalence over time may have guided the change in the test characteristics. The cELISA1 was originally designed to detect RHDV1 exposure in rabbit populations (Capucci et al., 1991) and served this purpose adequately until the arrival and dominance of RHDV2, post 2014. From 2018 onwards, cELISA1 remained a very good test with respect to detecting RHDV1 antibodies. However, when all three tests were analysed together and the latent serological status involved both viruses, the Se of cELISA1 dropped to 19.6% from 2018 onwards. This was as expected due to the decreasing proportion of RHDV1 exposed rabbits in the population when the latent infection status involved both viruses.

IgG1 ELISA was developed to detect RHDV1 IgG antibodies. However, the decrease in the Sp of IgG1 ELISA, when comparing cELISA1 and IgG1 ELISA, suggests that IgG1 ELISA detects a higher proportion of rabbits with RHDV2 antibodies compared to those detected with cELISA1. Consequently, the underlying latent infection status, when comparing cELISA1 and IgG1 ELISA, may not only include RHDV1 but rather be a mixture of RHDV1, and indirectly RHDV2. These findings support the results of previous laboratory studies (Strive et al., 2019), and show a high level of cross-reactivity, whereby RHDV2 antibodies in wild rabbits are frequently detected by IgG1 ELISA. Further, this cross-reactivity may have increased the Se of the ELISA1 over time.

The results obtained when analyzing each cELISA separately against the IgG1 ELISA provide insights into the ability of each cELISA to detect the correct strain. In the cELISA1/IgG1 ELISA analysis, the contrasting finding of higher Se for cELISA1 and lower true prevalence in the 2018-21 period, compared to the Se of cELISA1 and true prevalence estimates in the previous periods, indicates that RHDV2 antibodies are cross reacting on cELISA1 as reported by Strive et al. (2019). The 2016-17 and 2018-21 cELISA2 Se and Sp estimates from the cELISA2/IgG1 ELISA analysis, compared to the 2016-17 and 2018-21 cELISA1 Se and Sp estimates from the cELISA1/IgG1 ELISA analysis, suggest that the discriminatory ability of cELISA2 is not as good as cELISA1. However, this may very well be due to the implied latent infection status for either virus by comparing cELISA2 to the IgG1 test.

It appears that the best strategy for monitoring the serostatus of either virus is using the IgG1 ELISA test, as this assay overall performs better than the cELISA tests particularly for the 2018-2021 period, which represents the current scenario. However, the change in the true prevalence of the underlying status in all analyses, except cELISA1 and cELISA2, suggested that the change in dominance from RHDV1 to RHDV2 had little impact on Sp of cELISA1 and Sp of cELISA2 assays to truly detect RHDV1 and RHDV2 seronegative rabbits, respectively. This suggests that cELISA1 and cELISA2 may be used as a confirmatory (or discriminatory) test to supplement the IgG1 ELISA. When analyzed solely against IgG1 ELISA, the cELISA1 consistently showed a very high Se and Sp, between 80-92% percent. If the biology and the known introduction of RHDV2 was ignored, one might be tempted to conclude that RHDV prevalence was decreasing in Australia and that cELISA1 was very good tool for monitoring RHDV. However, consideration of the introduction of RHDV2 and the test results of cELISA2 presents a completely different picture of the RHDV epidemic, with a relatively constant prevalence and a marked decrease in the Se of the cELISA1. This emphasizes that a test developed for a specific purpose, such as monitoring an ongoing epidemic disease, needs to be continuously reassessed and reevaluated in order to account for changes in the underlying infection status.

The disadvantage of BLCM is that two or more tests are required for analysis and there are certain assumptions about the tests and populations, which must be fulfilled. These assumptions, known as the Hui-Walter paradigm, are: two or more populations with different true prevalences; two or more tests conditionally independent given disease status; each test must have constant Se and Sp across the populations. In the current study, the assumption of conditional independence given infection status could be relaxed due to explicitly accounting for this in our analysis, when considering all three tests simultaneously. We chose to model conditional dependence between cELISA1 and IgG1 ELISA and between cELISA1 and cELISA2. The rationale for this was that cELISA1 and IgG1 ELISA were both developed for RHDV1 and were developed to detect antibodies in response to RHDV1, whereas the two cELISAs, though based on the same method-

ology, were developed to detect antibodies in response to different viruses. We chose to assume conditional independence between IgG1 ELISA and cELISA2 to ensure that the model was still identifiable from our data. One challenge of BLCM is that the latent status is defined through the set of tests included in the comparison. Hence, it is advocated that when more than two tests are available, analyses on subsets of tests should be carried out to explore the impact on the latent status. A typical issue is that, if the prevalence of a disease in a population changes when the set of tests under evaluation changes, then this implies changes in the latent status. It is apparent that the disease definition is heavily influenced by the choice of tests in our analysis. However, given the dynamics of RHDV1 and RHDV2, this was expected. Still, there are some reassuring elements of the prevalence estimates.

Comparing the analysis of all three tests against the analyses of cELISA1/IgG1 ELISA and cELISA2/IgG1 ELISA shows comparable results for true prevalence and test characteristics (Se and Sp) between the time periods when comparisons are made within the periods where either RHDV1 (2014-2015) or RHDV2 (2018-2021) was dominant. Also, the true prevalence estimates for the analysis with all three tests were generally higher than those for the two-test models, suggesting that the two-test models detected the latent infection status less accurately compared to the three-test model.

The model using the two cELISAs produced spurious prevalence estimates, with very low true prevalence for the period 2014-2015 as well as 2018-2021, but higher estimates for 2016-2017. This might be interpreted as evidence, that both strains had to be present in a test subject to be deemed as a "true" infection. Hence, the latent status implied by these two tests might not have a practical implication, and consequently the test characteristics from that particular evaluation should be disregarded.

The prevalence estimates from analysis of all three tests and the analyses of IgG1 ELISA against cELISA2 shows that the RHDV2 sero-prevalence may have peaked in 2016-17 period with a down fall in the 2018-21 period. Ramsey et al. (2020) also reported a peak in RHDV2 seroprevalence between 2016 and 2018 at some monitoring sites. The prevalence data from the analysis of cELISA2/IgG1 ELISA, in particular, resembles to that of an outbreak epidemic curve and provides more insights into the epidemiology of RHDV2. This is reflected in the increase in prevalence in the 2014-15 period, as observed at the start of a disease outbreak, peak prevalence in 2016-17 period, when the disease outbreak is at the peak level, and a decrease in prevalence in 2018-21 period, similar to a decrease in the disease outbreak post the peak levels. RHDV2 sero-prevalence peak in 2016-17 period suggests that the majority of rabbit populations and majority of rabbits within those populations may have been already exposed to RHDV2 at the end of 2017. Further, the high levels of RHDV2 exposure may have led to an increase in RHDV2 immunity being passed onto future generations which in turn may have led to fewer RHDV1 infections in rabbits from 2018 onwards. This is evident in the true prevalence estimates from the analysis of cELISA1/IgG1 ELISA.

The true prevalence estimates from analysis of cELISA1/IgG1 ELISA suggests that a large proportion of the RHDV1 exposed rabbits survived the incursion, spread and dominance of RHDV2 up until approximately 2017/2018. This is indicated by the estimated true prevalence remaining relatively constant in the analysis of cELISA1/IgG1 ELISA up to the 2018-2021 period when it shows a sharp decrease. Exposure to RHDV2, which is antigenically similar to RHDV1 may have resulted in a boost in RHDV1 IgA and /or IgG titres developed from previous RHDV1 exposures. Consequently, rabbits with RHDV1 antibodies may have been protected from RHDV2 infection following a boost in RHDV1 immunity following RHDV2 infection for the first few years after RHDV2 arrival. The sudden drop in true prevalence during the 2018-2021 period from the analysis of cELISA1/IgG1 ELISA may be due to natural mortality and a lack of replacement of RHDV1 exposed rabbits in a then RHDV2 dominant landscape.

Overall, this study found that the test characteristics (Se and Sp) for all 3 assays in this study changed in response to the dominance of RHDV1 and RHDV2 over the 2011-21 period. Accordingly, all 3 assays should be subject to periodic evaluation to detect exposures to both viruses in anticipation of change in the true prevalence of both viruses. Results from IgG1 ELISA assay should be used with both cELISAs when estimating sero-prevalence of either viruses due to IgG1 ELISA's high Se in the current scenario.

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Conflict of interest

The authors declare no conflict of interest in this study.

Author contributions

KKP conceived the study idea. BP, JK and RMJJEA led to the generation of the serology data. KKP, PLT, and RMJJEA collated the available serology data. NT, KKP, and PLT designed the study methods and analyzed the data. NT, KKP, and PLT interpreted the results. KKP, PLT, NT led to the writing of the manuscript. All authors contributed equally and critically to the study and gave their final approval to the publication of the manuscript.

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