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Molecular epidemiology of Rabbit Haemorrhagic Disease Virus (RHDV) in Australia: when one became many

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Abstract

Rabbit Haemorrhagic Disease Virus (RHDV) was introduced into Australia in 1995 as a biological control agent against the wild European rabbit (*Oryctolagus cuniculus*). We evaluated its evolution over a 16 year period (1995–2011) by examining 50 isolates collected throughout Australia, as well as the original inoculum strains. Phylogenetic analysis of capsid protein VP60 sequences of the Australian isolates, compared to those sampled globally, revealed that they form a monophyletic group with the inoculum strains (CAPM V-351 and RHDV351INOC). Strikingly, despite more than 3000 re-releases of RHDV351INOC since 1995, only a single viral lineage has sustained its transmission in the long-term, indicative of a major competitive advantage. In addition, we find evidence for widespread viral gene flow, in which multiple lineages entered individual geographic locations, resulting in a marked turnover of viral lineages with time, as well as a continual increase in viral genetic diversity. The rate of RHDV evolution recorded in Australia – $4.0 (3.3 - 4.7) \times 10^{-3}$ nucleotide substitutions per site per year – was higher than previously observed in RHDV, and evidence for adaptive evolution was obtained at two VP60

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Data Accessibility

All RHDV sequences generated here have been submitted to GenBank and assigned accession numbers (see Table 1).

Author Contributions

Designed research: JK, TS, JA, PJE. Performed research: JK, RS, GM, DP, TS, JA, ECH. Analyzed data: JA, ECH. Wrote the paper: JK, RS, GM, DP, TS, JA, PJE, ECH.

residues. Finally, more intensive study of a single rabbit population (Turretfield) in South Australia provided no evidence for viral persistence between outbreaks, with genetic diversity instead generated by continual strain importation.

Keywords

Rabbit Haemorrhagic Disease Virus; evolution; epidemiology; phylogeny; biocontrol; European rabbit

Introduction

The wild European rabbit (*Oryctolagus cuniculus*) was successfully introduced into Australia in 1859 at Barwon Park, a sheep station in southern Victoria (Ratcliffe & Calaby 1958). While small domestic breeding populations were recorded around settlement houses in 1825, they did not spread as their behaviour rendered them susceptible to predators (Stodart & Myers 1964; Myers *et al.* 1994). By the early 1900s the rabbits derived from the Barwon Park had combined with many other major releases (Peacock & Abbott 2013) and had crossed the borders of all mainland states and territories, spreading at a rate of about 70 km a year (McClusky *et al.* 1974; Parer 1985), the fastest of any colonising mammal anywhere in the world (Caughley 1977). Native animal species such as the greater bilby *Macrotis lagotis*, burrowing bettong *Bettongia lesueur*, hairy-nosed wombat *Lasiorninus spp.* and the common wombat *Vombatus ursinus* facilitated the rapid colonization of some habitats by providing existing burrow systems, while their numbers greatly declined due to competition for food and shelter (Ryan *et al.* 2003; Cooke 2012).

The introduction of the rabbit has irrevocably changed the Australian landscape. The environmental and ecological impacts have been well documented, with rabbits significantly affecting the recruitment and re-generation of both native flora and fauna (Lange & Graham 1983; Cooke 1988; Cooke 2012). In Australia, rabbits are major environmental and agricultural pests (Gong *et al.* 2009; Cooke 2012), and have been vigorously targeted to suppress their numbers through mechanical/chemical control methods (Cooke 2008) and the deliberate introduction of four biological control agents. Myxoma virus (MYXV) was successfully released in Australia in the 1950s, followed by the European rabbit flea *Spilopsilus cuniculi* in the mid-1960s and the Spanish rabbit flea *Xenopsylla cunicularis* in the early 1990s to enhance MYXV transmission (Kerr *et al.* 2012). In 1990 a single strain (CAPM V-351) of Rabbit Haemorrhagic Disease Virus (RHDV) – a positive-sense single stranded RNA virus that belongs to the genus *Lagovirus* within the *Caliciviridae* family (Green *et al.* 2000) – was imported into Australia and held in quarantine for testing as a biological control agent for the wild European rabbit. The CAPM V-351 strain was used (via passage) to manufacture RHDV351INOC, a suspension for use in laboratory trials, and later used to inoculate field rabbits. The virus escaped from quarantine field trials in late 1995, and was subsequently approved for release in 1996 and registered with the Australian Pesticides and Veterinary Medicines Authority. RHDV quickly established itself in mainland Australia (Kovaliski 1998) and has since become endemic in many rabbit populations (e.g. Mutze *et al.* 2010a). Naturally recurring outbreaks of RHD have kept

rabbit numbers low in semi-arid and arid areas (Mutze *et al.* 2010a), while it has had less impact in higher rainfall and some coastal areas in south-eastern Australia (Henzell *et al.* 2002). For example, in Cattai, New South Wales, RHDV was released on a number of occasions and spread only a few hundred metres before dying out (Richardson *et al.* 2007). The reduced capacity to control rabbits within the higher rainfall and coastal regions may in part be due to transient immunological cross-protection exerted by the related but non-pathogenic rabbit calicivirus, RCV-A1, circulating in those populations (Cooke *et al.* 2002; Henzell *et al.* 2002; Mutze *et al.* 2010b; Strive *et al.* 2009, Strive *et al.* 2010, Strive *et al.* 2013). While RCV-A1 isolates are clearly divergent from RHDV (Jahnke *et al.* 2010), all Australian RHDV field strains characterized to date are closely related to those of the released RHDV351INOC strains (Kinnear & Linde 2010). On-going field sampling has to date not conclusively identified RHDV strains of greatly reduced virulence, although this is an area of active research. In addition to the interference of RCV-A1, developing resistance to infection with low doses of CAPM V-351 RHDV has also been reported (Elsworth *et al.* 2012).

Australia is unique in its use of viral pathogens as agents of biocontrol and it is critical to measure the evolutionary capacity of these pathogens as an indicator of their long-term effectiveness and their co-evolutionary arms race with the rabbit host. Both MYXV and RHDV have significantly suppressed rabbit numbers for long periods of time and provided combined agricultural benefits estimated at \$AUD70 billion (Cooke *et al.* 2013). However, despite these combined control measures the annual surplus loss to Australia due to rabbits is calculated at \$AUS206 million (Gong *et al.* 2009). More generally, the detailed study of the spread of RHDV in the Australian environment provides valuable information on the rates, patterns and dynamics of pathogen evolution in a largely naïve environment, and represents a powerful analogy to cases of disease emergence following cross-species virus transmission to a novel host (Holmes 2009; Parrish *et al.* 2008). Importantly, because the founding viruses (i.e. inoculant strains) are known, the analysis of RHDV in Australia provides a uniquely accurate picture of viral evolutionary dynamics following emergence.

RHDV consists of two open reading frames (ORFs): ORF1, comprising nucleotides (nt) 10 to 7044, and ORF2 which is located between nt 7025 to 7378. ORF1 encodes a large polyprotein that is cleaved into mature non-structural proteins and a major structural protein, the capsid protein VP60, which protects viral RNA and in which the major immune-dominant epitopes are located (reviewed in Abrantes *et al.* 2012). In Europe, initial analysis of the RHDV capsid protein revealed a high level of sequence similarity (Milton *et al.* 1992), consistent with rapid spread in a naïve host population. Further analysis of RHDV isolates showed that they clustered into several genetic groups (denoted ‘genogroups’), which were better associated with the year of virus isolation than to geographic location (Le Gall *et al.* 1998; Muller *et al.* 2009; Alda *et al.* 2010). However, little is known about the pattern and dynamics of RHDV spread in Australia since its introduction, despite its potential impact on rabbit ecology and evolution.

Herein we report the molecular epidemiology of RHDV in Australia since its introduction as a biocontrol agent in 1995, using the VP60 region as a phylogenetic marker. Our study provides a unique opportunity to monitor genetic changes in an RNA virus introduced into a

naïve population, albeit one that also harbors a related (but benign) RNA virus, RCV-A1 (Strive *et al.* 2009). In particular, as there have been many reintroductions of the original released RHDV351INOC strain in an effort to initiate outbreaks and maximise the impact of the disease, we assess whether these later introductions have become established in the field. Finally, by analysing a set of samples spanning a period of 16 years from a single site in South Australia, we address whether the virus persists between the outbreaks that occur at varying intervals in most Australian rabbit populations.

Materials and methods

Samples

Field isolates of RHDV were collected from individual rabbits found freshly dead in the states/territories of Australian Capital Territory, New South Wales, South Australia, Tasmania and Western Australia (Fig. 1). Liver samples were collected in individual containers and frozen as soon as practicable. However, in some cases samples of bone marrow or larvae of carcase-feeding flies were collected from carcasses that were too decomposed to retrieve any viable organ tissue. All samples used in this study were obtained from rabbit carcasses found during naturally occurring RHDV outbreaks. No live animals were shot, trapped or otherwise handled to obtain tissues, and therefore no Animal Ethics permit was required.

Virus Amplification and Sequencing

Samples were analysed for the presence of RHDV by PCR at the laboratory of Biosecurity South Australia, Urrbrae, South Australia, or at CSIRO Ecosystem Sciences, Canberra. Viral RNA was extracted and purified from 20–30mg tissue (liver, bone marrow or fly larvae using the RNeasy mini-kit (QIAGEN) according to the manufacturer's protocol. The purified RNA was eluted in 50 µl RNase free water. Amplification was carried out using the QIAGEN OneStep RT-PCR Kit, (QIAGEN).

Reverse transcriptase Superscript III (Invitrogen) was used to produce cDNA templates for amplification according to the protocol suggested by the supplier. The capsid sequences of strains ACT/GUN-HS/1998, ACT/GUN-RP/1998, ACT/PARKWOOD/1998, NSW/OC-M2/2007, NSW/OC-M8/2007, ACT/AINSLE-2/2009, ACT/PI-1/2009, ACT/GUN-01/2009 and ACT/MTP-2/2010 were amplified in three overlapping fragments using primers RHDV-11 fw (5'-CACCCATGACYATACTTGACGCCATG-3')/Rab2b (5'-GGARTGYTGRGCRGTGTACAGTATGC-3'); RHDV-capsid-fw hindIII (5'-AAAAGCTTATGGAGGGCAAAGCCCGTGCAGC-3')/RHDV-12 rev (5'-ARCCTAACTCATARGCCTGCACAGTCG-3') and RHDV-f2(5'-GTTTTGGTAYGCTAATGCTGGGTCTGC-3')/RHDV-13rev (5'-TTTTTTATAGCTTACTTTAACTATAAACC-3'). The Platinum Taq Hot start polymerase (Invitrogen) was used with nucleotide, primer and Magnesium Chloride concentrations according to the manufacturer's recommendations. The cycle protocol was 95°C for 5 min, followed by 20 touchdown cycles of 95°C for 15 sec, 60°C for 30 sec (minus 0.5°C /cycle) and 72 for 90 sec, followed by another 25 cycles of 95°C for 15 sec, 50°C for 30 sec and 72 for 90 sec.

For the remaining samples viral RNA was extracted and purified from 20–30mg tissue (liver, bone marrow or fly larvae using the RNeasy mini-kit (QIAGEN) according to the manufacturer's protocol. The purified RNA was eluted in 50 µl RNase free water. Amplification was carried out using the QIAGEN OneStep RT-PCR Kit, (QIAGEN) and four overlapping primers; RHDVf3937 (5'-GACAGTGRCAAGTCACTCATGAACATYGC-3')/RHDVr4985 (5'-TTGACACCCACCCRATGTCCGTGA-3'); RHDVf4846 (5'-CCGATGGTGAGYCTYTTRCCTGC-3')/RHDVr6059 (5'-TGRCCGTTCCACCTGTTGTCATTGC-3'); RHDVf5926 (5'-GCAATYCAGGTRACAGTGGAAACAAGGC-3')/RHDVr 6986 (5'-CCAGGTTGAACACGAGYGTGCTYTTGG-3'); RHDVf 6793 (5'-GGACTTTCGCTCAACAACACTACTCGTCAGC-3')/RHDVr7411 (5'-ATAGCTTACTTTAAACTATAAACCCAA-3').

The PCR conditions were as follows: 50°C for 30 min, 94°C for 15 min, then 40 cycles at 94°C for 15 sec, 55°C for 30 sec, 72°C for a minute. After the 40 cycles the samples were held at 72°C for 10 min and held at 4°C until removed and stored at minus4°C overnight. Amplified fragments were cut and purified using QIAquick Gel Extraction Kit (QIAGEN) protocol and sequenced directly using the BIG DYE sequencing kit (supplied by the Institute of Medical and Veterinary Science (IMVS), Adelaide) according to the manufacturer's instructions. The PCR protocol was as follows: 96°C for 5min, then 24 cycles at 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min. Samples were removed after the 24 cycles and stored at minus 4°C until required. The samples were then sent to the IMVS or the Australian Genome Research Facility, Brisbane for sequencing. In total, we sequenced the VP60 genes of 46 RHDV strains sampled from Australia as well as the manufactured strain RHDV351INOC (the sequence of the CAPM V-351strain was taken from GenBank). All RHDV sequences generated here have been submitted to GenBank with accession numbers given in Table 1.

Evolutionary Analysis

The 47 VP60 sequences obtained were combined with those of four Australian viruses already available on GenBank (NSW/EU650679NYNGAN/2005; NSW/EU650680NARRAWA/2006; ACT/AINSLE-2/2009; ACT/PI-1/2009; Table 1), along with the original CAPM V-351strain, and aligned using BioEdit 7.0.9.0 (Hall 1999). This produced a total data set of 52 VP60 sequences, length of 1737 nt. This sequence alignment was first screened for recombination using the RDP, GENECONV and BootScan methods implemented in the RDP3 Alpha 44 program (Martin *et al.* 2010). Only recombination events detected by all three methods were considered strongly supported.

To place the evolution of the Australian RHDV isolates in the context of those viruses sampled in other geographical localities they were combined with a data set of 82 RHDV sequences sampled from a variety of countries, including three from New Zealand (Table S1, supporting information). This phylogenetic tree was rooted using the sequences of non-pathogenic rabbit caliciviruses from Australia (RCV-A1, the most basal lineage), Europe, and North America. This produced a final data set of 134 VP60 sequences, 1737 nt in

length. A phylogenetic tree of these data was estimated using the maximum likelihood (ML) method available within the PhyML package (Guindon *et al.* 2010). This analysis employed the General Time Reversible model (GTR) of nucleotide substitution with a proportion of invariant (I) sites and a gamma distribution of among-site rate variation with four rate categories (Γ_4) (i.e. the GTR+I+ Γ_4 substitution model). To search among trees we employed both Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) branch-swapping. A bootstrap resampling procedure involving 1000 replicate ML trees (utilizing NNI branch-swapping) was used to estimate the support for individual nodes.

To infer aspects of the evolutionary dynamics of RHDV in Australia – rates of nucleotide substitution per site and the Time to the Most Recent Common Ancestor (TMRCA) – we analysed the 50 Australian field RHDV sequences (as well as CAPM V-351 and RHDV351INOC) using the Bayesian Markov chain Monte Carlo (MCMC) approach available within the BEAST package (version 1.7.5; Drummond *et al.*, 2012). A variety of analyses were undertaken, utilizing (i) GTR+I+ Γ_4 and GTR+CP nucleotide substitution models, with the latter specifying a different substitution rate for each codon position (CP), and (ii) constant population size, exponential population growth and time-aware Gaussian Markov random field (GMRF) Bayesian skyride coalescent models. A relaxed (uncorrelated lognormal) molecular clock was used in all cases with relatively uninformative priors. Sequences were coded by month of sampling, and in cases where this information was not known we assumed that they were sampled in the middle of their assigned year. The MCMC analysis was run until all parameters converged (100 million generations), and statistical uncertainty was reflected in values of the 95% Highest Posterior Density (HPD). All BEAST runs produced highly congruent results (see Results and Discussion). The BEAST analysis also enabled us to estimate the Maximum Clade Credibility (MCC) tree from the distribution of posterior trees, in which support for individual nodes is reflected in posterior probability values. Prior to the BEAST analysis we tested the extent of temporal structure in the Australian RHDV sequence data through a regression of the root-to-tip genetic distance of each sequence on the ML tree against its time of sampling using the Path-O-Gen program (kindly provided by Dr. Andrew Rambaut, University of Edinburgh; <http://tree.bio.ed.ac.uk/software/pathogen>).

Finally, we performed an analysis of site-specific selection pressures acting on VP60 using the SLAC (Single Likelihood Ancestor Counting), FEL (Fixed Effects Likelihood), REL (Random Effects Likelihood), MEME (Mixed Effects Model of Evolution), FUBAR (a Fast, Unconstrained Bayesian AppRoximation for inferring selection) and Branch-Site REL methods implemented at the Datamonkey interface of the HyPhy package (<http://www.datamonkey.org>) (Kosakovsky Pond & Frost 2005; Kosakovsky Pond *et al.* 2006; Murrell *et al.* 2012). All these methods consider the relative numbers of synonymous (d_S) and nonsynonymous (d_N) nucleotide substitutions per site. In each case, the best-fit model of nucleotide substitution was determined using the automated tool in Datamonkey. Only sites with p -values <0.05 or with posterior probability values greater than 0.95 were considered as providing significant evidence for positive selection under these analytical criteria.

Results and Discussion

Molecular Epidemiology of RHDV in Australia

Our phylogenetic analysis of the 50 field RHDV VP60 (capsid) sequences sampled from rabbits collected across five Australian states and territories (Fig. 1), in the context of those sampled globally, revealed that the Australian viruses form a monophyletic group, with the Czech CAPM V-351 and RHDV351INOC sequences falling near the root of this cluster (Fig. 2; the phylogenetic position of RHDV351INOC is shown in Fig. 3). Such a phylogenetic pattern is consistent with a single introduction of RHDV into Australia from these two inoculum strains, followed by *in situ* evolution over the next 16 years. Similarly, the close evolutionary relationship between the Australian viruses and those sequences sampled in New Zealand is indicative of a common source in both countries. Indeed, previous characterisation of RHDV samples from New Zealand revealed a high similarity with the CAPM V-351 and RHDV351INOC strains indicative of a common source (O'Keefe *et al.* 1998). Hence, these results are compatible with the notion that RHDV was illegally imported into New Zealand from Australia in 1998 (Zheng *et al.* 2002).

This phylogenetic analysis also tentatively suggests that rabbits which died from RHDV in the initial 1995 mainland outbreak may have derived from two different genetic lines. Cooke (1996) reported that there were two occasions where the virus had spread beyond the quarantined rabbit pens, in July and early September 1995. SA/Curnamona/1995 and SA/PLUM/1995 were collected four days and 50 km apart, just two weeks after the virus was first reported on the mainland, yet they appear in two separate clades with SA/Curnamona/1995 closely related to RHDV351INOC.

To explore the evolution of the Australian viruses in more detail, and because they form a distinct monophyletic group, we performed an analysis of these viruses in isolation, examining the diversification of the virus in both time and space across Australia (Fig. 3). This analysis clearly revealed some geographical clustering by state of sampling, as reflected in the color-coding by Australian state/territory shown in Fig. 3. However, more striking is that despite this geographic clustering, multiple lineages have entered the viral populations of Australian Capital Territory, New South Wales and South Australia, such that the structure of RHDV genetic diversity has changed with time, with few apparent constraints to viral spread. For example, those viruses sampled in the Australian Capital Territory from 2009 onwards fall into two lineages, both of which are distinct from a more basal lineage sampled in 1998, such that there has been a clear lineage replacement. South Australia appears particularly diverse in this respect with multiple lineages circulating through time (Fig. 3), although whether this is due to more intensive sampling in this region is unclear. This phylogenetic analysis also provides clear evidence for viral gene flow, and sometimes between spatially disjunct localities, although we have likely not sampled intermediate localities. For example, strains from Tasmania, which is separated from the closest mainland state, Victoria, by the 200 km wide Bass Strait, cluster with those from South Australia, which is >500 km distant. Similarly, strains from South Australia cluster with those from Western Australia located thousands of kilometres away.

Perhaps the most striking outcome of this analysis was the strong temporal structure in the data such that different time-points are characterized by the presence of different viral lineages (Fig. 3). This pattern has a number of important implications. First, despite the many reintroductions of RHDV (RHDV351INOC) into Australia (Table 2), only one viral lineage (i.e. that derived from the initial release) has managed to become established and sustain its transmission in the long-term, indicative of a major competitive advantage. For example, although there have been 196 releases of RHDV351INOC in Western Australia, all those viruses sampled from Western Australia form a single clade that is distinct from RHDV351INOC (and CAPM V-351). It is therefore possible that the fitness of the founding lineage may have been enhanced because it encountered a largely susceptible rabbit population. Secondly, this main lineage has also experienced a major ‘turnover’ through time, such that viral lineages replace each other in specific geographical localities, which is particularly notable in the cases of New South Wales and South Australia (Fig. 3). In the specific example of Turretfield (TF) in South Australia, viral lineages fall into at least six phylogenetically distinct locations, and viruses from different years never share common ancestry (Fig. 3). Although two viral lineages co-circulate at Turretfield during 2008 (TF3, TF4), single lineages with different origins are detected there in 2009 (TF5) and 2011 (TF6).

This pattern of lineage turnover is compatible with the idea that viruses do not persist between disease outbreaks at individual geographic localities. Indeed, it is striking that viruses sampled from successive years in individual locations never cluster as sister groups. Rather, it is feasible that insect vectors such as blowflies (e.g. *Calliphora* sp.) import viral strains into rabbit populations each year (Asgari *et al.* 1998). Flies feeding on rabbit carcasses can carry RHDV on their legs and their regurgita, and anal excretions can also be a potential source of virus (Asgari *et al.* 1998). Indeed, flies were identified as the likely vector facilitating the escape of the virus from quarantine in 1995 (McCull *et al.* 2002). The importance of flies as the main means of long distance virus transmission is also highlighted by the large geographic expansion of common viral lineages observed here.

To further understand the factors shaping the spread of RHDV in Australia we analysed rates of viral evolution. Highly comparable rate estimates were obtained under a variety of nucleotide substitution and coalescent models. Mean values range from $4.0 - 4.7 \times 10^{-3}$ nucleotide substitutions per site per year (subs/site/year) across all analyses, with overlapping credible intervals irrespective of substitution and demographic model, suggesting that they are robust. Estimation of marginal likelihoods using path sampling (Baele *et al.* 2012) suggested that the Bayesian skyride model was the best-fit to the data. Under this model the evolutionary rate for RHDV in Australia was $4.0 (3.3 - 4.7) \times 10^{-3}$ subs/site/year, and hence substantially higher than all previous rate estimates for this virus (Kerr *et al.* 2009; Kinnear *et al.* 2010), including that accounting for a misdated GenBank sequence (at 1.9×10^{-3} subs/site/year; Hicks & Duffy 2012). This rate is also broadly equivalent to that estimated for the HA gene of human influenza virus (Rambaut *et al.* 2008), making it one of the highest observed in RNA viruses. An important internal control for the validity of this rate estimate is that the TMRCA for the Australian epidemic estimated from this analysis (1994.4 – 1995.4) accords with the date of the initial release of the virus in Australia. In addition, there was a clear association between genetic distance and

date of sampling (correlation coefficient = 0.85) indicating that there is strong temporal (i.e. molecular clock) structure in the data. Hence, RHDV has evolved with remarkable rapidity in Australia and which could be due to a selectively driven evolutionary process and/or increased rates of viral replication during epidemic expansion.

To determine the population dynamics of RHDV in Australia in more detail we reconstructed the changing pattern of viral genetic diversity through time using a Bayesian skyride analysis (Fig. 4). This revealed an exponential increase in the extent of RHDV genetic diversity through time, and can be thought of as a concomitant increase in viral effective population size under a neutral evolutionary model. Such population growth, at least in our sample of viruses, suggests that ecological, genetic, and immunological constraints, including the co-circulation of the benign RCV-A1 or rabbit resistance, did not impose a major selective challenge to RHDV as periodic selective sweeps would be estimated to periodically purge genetic diversity. RCV-A1 has been reported to interfere with effective RHDV biocontrol (Nagesha *et al.* 2000; Cooke *et al.* 2002; Robinson *et al.* 2002; McPhee *et al.* 2009; Mutze *et al.* 2010b). For example, RHDV351INOC releases produced localised disease outbreaks in areas with co-circulating RCV-A1, but failed to trigger wide scale epizootics (Richardson *et al.* 2007; Mutze *et al.* 2010b). Although interference by RCV-A1 might be expected to exert a selection pressure on RHDV, we found no clear signature for that selective process here. Intriguingly, previous reports have shown that genetic resistance to RHDV infection varied between rabbit populations from different localities (Elsworth *et al.* 2012), and did generally not overlap with areas where RCV-A1 was highly prevalent (Cooke *et al.* 2002, Jahnke *et al.* 2010). Hence, it has been suggested that RCV-A1, by providing partial protection from lethal RHDV infection, may actually relieve the selective pressure on rabbits to develop genetic resistance (Nystrom *et al.* 2011). The exponential population growth of RHDV may also have been assisted by the ability of the virus to travel considerable distances via insect vectors.

Finally, our analysis revealed that SA/TF-PT1350/2011 and SA/TF-PT1339/2011 were recombinant sequences, both with strong statistical support (p -values <0.001) under a variety of methods. For both isolates the first breakpoint was located in the Shell (S) domain and the second in the P1 subdomain of VP60, with SA/ROXDWNS/2011 and NSW/ROCKL-2/2011 acting as parental sequences in both cases (more detailed results are available from the authors on request). Although recombination has been described previously in RHDV (Abrantes *et al.* 2008; Forrester *et al.* 2008), its implications for RHDV evolution in Australia are unclear. Indeed, there is currently no evidence that these two recombinant viruses possess unusual phenotypic properties, and they had no substantive effect on our estimates of nucleotide substitution rates and divergence times; the estimated rate of nucleotide substitution under the Bayesian skyride model after removing the recombinant sequences was $3.9 (3.3 - 4.5) \times 10^{-3}$ subs/site/year.

Selection pressures on Australian VP60 sequences

The rapid evolution of RHDV necessitated that we perform a detailed analysis of site- and lineage-specific selection pressures acting on the Australian RHDV VP60 (capsid) sequences. Accordingly, multiple methods (SLAC, FEL, REL, MEME, FUBAR) suggested

that capsid residues 307 and 572 were evolving under positive selection ($p < 0.05$; posterior probability > 0.95), although this will need to be confirmed in precise fitness experiments. In contrast, the branch-site REL method revealed no compelling evidence for episodic diversifying selection on any individual viral lineage.

Residue 307 has been previously identified as evolving under positive selection (Esteves *et al.* 2008; Kinnear & Linde 2010), and it is notable that while previous studies considered a global sample of viruses, we detected positive selection in a geographically restricted area (Australia), adding weight to it being a *bona fide* selected site. The complete atomic model of the RHDV capsid indicates that this residue is located within the most exposed loop (L1, amino acids 300–318) of the RHDV capsomer (Wang *et al.* 2013). This region plays a role in the interaction of RHDV with histo-blood group antigens (HBGAs) that were suggested as co-receptor structures on host mucosal surfaces (Nystrom *et al.* 2011). In addition, L1 conjugated to keyhole limpet hemocyanine was able to elicit cell and antibody-mediated immunity, suggesting that it is a major neutralization site (Wang *et al.* 2013). Thus, this region may be involved in both viral escape from immune recognition, and in overcoming genetic resistance based on changes in HBGA expression patterns in rabbits. Residue 572 has also been previously determined to be subject to adaptive evolution (Kinnear & Linde 2010); this residue is located in a less accessible region within subdomain P1 (or region F, according to the nomenclature defined by Neill 1992) and is not directly exposed to the host immune system. As a consequence, the factors driving the putative positive selection at this residue are unknown.

Evolutionary outcomes of RHDV351INOC reintroductions

Since 1996 a further 3508 releases of RHDV351INOC have been made in an effort to reduce localised rabbit populations as part of pest animal management programmes in Australia (Table 2). The majority of these (2451) were made in New South Wales. Our phylogenetic analysis (and particularly the strong temporal structure in the data) shows that these reintroductions were likely quickly outcompeted by circulating field strains. Although it is evident that the ongoing culture of CAPM-V351 and RHDV351INOC would mean that later reintroductions of these viruses would be genetically different from the original inoculants used in 1995, these viruses would still be phylogenetically distinct from those we sample in the field. In addition, Australian rabbit populations are now beginning to develop some level of resistance against CAPM-V351 (Elsworth *et al.* 2012). Reintroductions of the original RHDV351INOC isolate are therefore likely to provide little more than a one-off biocide tool that provides a short-term, localised reduction in rabbit populations, but which is unlikely to result in long-term effective rabbit control.

Overall, our molecular epidemiological analysis of RHDV in Australia provides an important snap-shot of the evolution and spread of a highly pathogenic virus in a largely naïve host environment. The resulting evolutionary pattern appears to be both rapid and unhindered, suggesting that any immunity or resistance in the rabbit has to date had relatively little effect and that there are a sufficient number of susceptible hosts to sustain exponential viral population growth. For the future it will clearly be important to monitor the evolutionary and epidemiological dynamics of RHDV in the face of mounting resistance.

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Fig. 1.

Map showing the sampling location of field RHDV isolates in Australia. Numbers on the map correspond to the isolate reference numbers in Table 1 and colours to the state/territory of origin as documented in Fig. 3. No map reference points are given for the two inoculum strains; CAPM V-351/1988 (sequence 1) and RHDV351INOC/1995 (sequence 2).

Fig. 2.

Phylogenetic relationships of RHDV sampled globally, including those from Australia. The major clusters of RHDV, as well as their geographic origins, are noted. Also shown are the phylogenetic positions of (i) the original Czech (CAPM V-351) strain, (ii) three viruses from New Zealand that fall within the phylogenetic diversity of the Australian viruses, and (iii) the background virus closest to the Australian cluster, Germany/M67473/1989. Bootstrap values (>70%) are shown for key nodes and all horizontal branches are drawn to a scale of

nucleotide substitutions per site. Background RHDV sequences are listed in Table S1 (supporting information).

Fig. 3.

Phylogenetic (Maximum Clade Credibility – MCC) tree depicting the evolutionary relationships among the Australian RHDV sequences. As the tree assumes a relaxed molecular clock, tip times reflect the date of sampling, and an estimated real-time (year) time-scale is given on the x-axis. Sequences are colour-coded according to their state/territory of sampling in Australia: Australian Capital Territory (ACT) = green, New South Wales (NSW) = blue, South Australia (SA) = red, Tasmania (TAS) = grey, Western Australia (WA) = pink, and those from outside Australia (i.e. inoculum strains) are

unshaded. Numbers after the decimal place in the virus names denote the fractional year (e.g. 2008.75 = September 2008), and the date of the common ancestor of the Australian viruses (1991.4–1995.4) inferred from this analysis is shown next to the relevant node. Viruses sampled from Turretfield (SA) are numbered according to date of sampling. All branches supported by posterior probability values >0.95 are marked by a * symbol.

Fig. 4. Bayesian skyline analysis of RHDV in Australia depicting changing estimates of relative genetic diversity (y-axis) plotted against time from the youngest sampled sequence (x-axis). The solid black line represents the median value while the shaded region of the plot depicts the 95% HPD values.

Table 1

The 50 Australian field isolates and two inoculum strains studied here, with sampling locations shown in the map in Fig. 1

Number (map reference)	Isolate	Collection Date	GenBank Accession
1 *	CAPM V-351/1988	Original imported (Czech) strain. Sampled between October 1987- July 1988.	U54983
2 *	RHDV351INOC/1995	Release strain manufactured from CAPM V- 351. Passaged >5 times	KF494921
3	SA/PLUM/1995	October 1995	KF494931
4	SA/CURNAMONA/1995	October 1995	KF494924
5	SA/MERNMORA/1995	November 1995	KF494929
6	SA/WILLOCHRACRK/1995	November 1995	KF494947
7	ACT/GUN-HS/1998	September 1998	KF494907
8	ACT/GUN-RP/1998	September 1998	KF494908
9	ACT/PARKWOOD/1998	September 1998	KF494910
10	SA/TF-07/1999	October 1999	KF494935
11	SA/TF-13/1999	October 1999	KF494936
12	SA/LOB-OLIVER08/2003	August 2003	KF494928
13	NSW/EU650679/NYNGAN/2005	Sept-Nov 2005	EU650679
14	TAS/COLLINS-1/2006	August 2006	KF494948
15	NSW/EU650680/NARRAWA/2006	Sept-Nov 2006	EU650680
16	SA/TF-19/2006	November 2006	KF494937
17	SA/TF-1907/2006	November 2006	KF494938
18	NSW/OC-M2/2007	September 2007	KF494912
19	NSW/OC-M8/2007	March 2007	KF494913
20	TAS/STHARM/2007	May 2007	KF494949
21	SA/FRNP-ORA/2008	June 2008	KF494926
22	SA/GRNW/2008	October 2008	KF494927
23	SA/TF-PT1731/2008	October 2008	KF494943
24	SA/TF-PT1743/2008	September 2008	KF494944
25	SA/TF-2186/2008	October 2008	KF494939
26	SA/BARM/2008	October 2008	KF494922
27	SA/ECH/2008	September 2008	KF494925
28	ACT/AINSLE-2/2009	2009	GU373617
29	ACT/PI-1/2009	2009	GU373618
30	ACT/GUN-01/2009	March 2009	KF494906
31	SA/BULG/2009	August 2009	KF494923
32	SA/TF-PT1839/2009	October 2009	KF494945

Number (map reference)	Isolate	Collection Date	GenBank Accession
33	ACT/MTP-2/2010	February 2010	KF494909
34	SA/ORR-1/2010	October 2010	KF494930
35	SA/PLUM-1/2010	August 2010	KF494931
36	SA/WILKA/2010	May 2010	KF494946
37	WA/MANMAN/2010	September 2010	KF494950
38	WA/MARCHA/2010	September 2010	KF494951
39	WA/NARRIN/2010	November 2010	KF494952
40	ACT/WESTCRK/2011	August 2011	KF494911
41	NSW/WOODSTK/2011	March 2011	KF494918
42	NSW/YOUNG/2011	July 2011	KF494919
43	NSW/VITTO/2011	February 2011	KF494920
44	NSW/ROCKF-1/2011	April 2011	KF494914
45	NSW/ROCKF-2/2011	April 2011	KF494915
46	NSW/ROCKL-1/2011	March 2011	KF494916
47	NSW/ROCKL-2/2011	March 2011	KF494917
48	SA/REMARK/2011	September 2011	KF494933
49	SA/ROXDWS/2011	August 2011	KF494934
50	SA/TF-2863/2011	August 2011	KF494940
51	SA/TF-PT1339/2011	August 2011	KF494941
52	SA/TF-PT1350/2011	September 2011	KF494942

*
No map reference shown

Table 2

Numbers of releases of RHDV in Australia from 1995 to 2011.

Year	New South Wales	Victoria	South Australia	Western Australia	Tasmania	Queensland	Australian Capital Territory	Northern Territory
1995						1 Original release site in South Australia		
1996	87	64	24	23		6	5	3
1997	336	120	1		20		17	6
1998	15		5			40	3	
1999	63			11	4			
2000	52				15			
2001	57					4		
2002	100					26		
2003	21		5			36		
2004	130		2		20	23		
2005	158					16		
2006	959		7	15	12	57	3	2
2007	188		1	10		20	10	
2008	39		4	30	18	32		
2009	130		9 (25)	50		70	8	4
2010	50			32	26	47	2	20
2011	66			35	2	39	3	
TOTAL	2451	184	58	196	117	416	51	35

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