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Serological evidence for the presence of non-pathogenic rabbit haemorrhagic disease virus-like strains in rabbits (*Oryctolagus cuniculus*) of the Kerguelen archipelago

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Abstract Antibodies raised against a Lagovirus were found in healthy rabbits *Oryctolagus cuniculus* sampled in 2003 and 2004 in the Kerguelen archipelago. The serological test we used enabled the detection of antibodies due to both pathogenic and non-pathogenic viruses related to the rabbit haemorrhagic disease virus (RHDV). The overall proportion of seropositive rabbits was 35% and differed between sites. Since previous studies have failed to detect antibodies raised against pathogenic RHDV strains, the antibodies detected in the present study were likely due to non-pathogenic strains. The interest of these strains

circulating in the Kerguelen archipelago is that they have evolved independently of those detected in the other parts of the world and should derive from an ancestral RHDV precursor. Their characterization may help understanding the evolution of the virus and the emergence of the disease.

Keywords Rabbit haemorrhagic disease virus · Antibodies · *Oryctolagus cuniculus* · Kerguelen archipelago

Introduction

Rabbit haemorrhagic disease (RHD) is a viral disease specific to the wild rabbit (*Oryctolagus cuniculus*). Its causative agent is a Lagovirus, belonging to the *Caliciviridae* family. It was first described in China in 1984 and was then recorded in different parts of the world where it is now endemic. However, recent studies have shown that the rabbit haemorrhagic disease virus (RHDV) may have emerged long before the first recorded occurrence of the disease, early in the twentieth century (Kerr et al. 2009). When it emerged, RHD caused severe mortalities in wild rabbits, up to 90%, which generally resulted in long-term severe decrease in population size (Marchandeau et al. 2000; Moreno et al. 2007; Mutze et al. 2008).

Different works have demonstrated the existence of non-pathogenic strains related to the RHDV. Most of them were serologically evidenced in Europe (Marchandeau et al. 2005; Marchandeau et al. 1998; Rodak et al. 1990b; Trout et al. 1997) but also in Australia and New Zealand where RHDV was introduced as a biological control agent (Cooke et al. 2002; Nagesha et al. 2000; O'Keefe et al. 1999; Robinson et al. 2002). Following the isolation and the characterization of a RHDV-like virus genetically distinct

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to RHDV and called 'RCV' in a rabbitry in Italy (Capucci et al. 1996), other RHDV-like RNA sequences were described in the sera of live rabbits (Forrester et al. 2009; 2007; Moss et al. 2002). More recently, two different strains have been isolated and characterized in France and in Australia (Le Gall-Reculé et al. 2007; Strive et al. 2009). The last one called 'RCV-A1' represents an evolutionarily earlier lineage when compared to RCV and RHDV lineages.

The possible role of these non-pathogenic strains in reducing the impact of RHD has been pointed out and discussed by several authors (Boots et al. 2004; Cooke et al. 2002; White et al. 2001). Recent works have demonstrated the existence of recombination between strains and have therefore shown another role of the non-pathogenic strains as a genetic reservoir for RHD evolution (Abrantes et al. 2008; Forrester et al. 2008).

The wild rabbit was introduced in 1874 into the Kerguelen archipelago (Kidder 1876), and no subsequent introduction has been recorded (Boussès 1991). Introduced rabbits likely originated from Robben Island in South Africa (Boussès 1991) where domestic rabbits were introduced in 1656 from the Netherlands (Cooper and Brooke 1982). The rabbit population rapidly established, and the species was reported as a common prey of the skua (*Catharacta antarctica lonnbergi*) in 1875. To control rabbit populations, the myxoma virus was introduced in 1955–1956 but RHD has never been recorded in the Kerguelen and a study carried out in 2001–2002 failed to detect antibodies raised against RHDV pathogenic strains in 81 rabbits sampled in 11 areas of the archipelago (Cooke et al. 2004).

The aim of the present work was to enlarge the knowledge of caliciviruses by looking for the presence of both RHDV and RHDV-like viruses in the Kerguelen archipelago. The interest of studying RHDV here is that this rabbit population has been isolated twice, in Robben Island and in Kerguelen, for a long time, well before the estimated emergence of virulent strains of RHDV, which may enable the detection of viral strains that directly evolved from putative ancestors of the current pathogenic strains. This particular situation provides a unique opportunity for studying virus evolution. We thus sampled rabbits and took blood samples to detect antibodies raised against the VP60 (major viral protein) of the RHDV group.

Materials and methods

Study area

The Kerguelen archipelago (48°18'–50°00'S, 68°28'–70°35'E) is situated in the south of the Indian Ocean. It is

constituted of a large island, called "Grande Terre" (6,600 km²), surrounded by over 300 secondary islands and islets. The climate is characterized by low temperatures (mean annual temperature 4.6°C, Météo-France, 1951–1999 record), strong winds and a mean annual rainfall of 695 mm.

Rabbit sampling

Rabbit samples were collected in November and December 2003 in four sites on Grande Terre: Ratmanoff, Pointe Morne, Port-aux-Français and Pointe Molloy; and in December 2004 in two other sites: Port-Jeanne d'Arc and Armor (Fig. 1). Rabbits were sampled by shooting and a blood sample was taken on a strip of blotting paper.

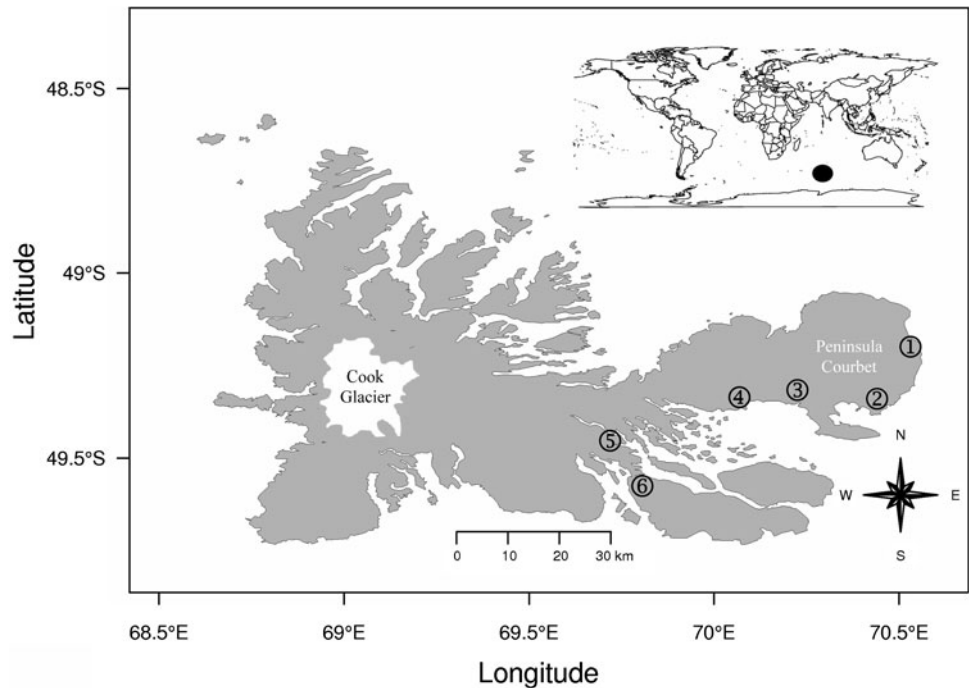
Antibody detection

Blood samples were screened by ELISA to detect antibodies raised against the VP60, which is the major RHDV capsid protein (Laurent et al. 1994). Briefly, two 6-mm-diameter discs of blotting paper were rehydrated in 100 µl of phosphate-buffered saline (PBS) on a 96-well microplate and stored at 4°C overnight. The eluate was then used directly in serological tests as an equivalent of a 1/20 dilution of fresh serum. ELISA plate wells (Becton–Dickinson, Falcon Probind, Meylan, France) were coated with 1 µg of recombinant baculovirus-purified VP60. RHDV antibody binding was visualized by incubating the plate wells with alkaline phosphatase antibody conjugate (Sigma Chemical, Saint Louis, Missouri, USA) at 37°C for 60 min. The absorbance of each sample was measured at a wavelength of 405 nm with a spectrophotometer 15 min after addition of the substrate solution (pNPP in 10% diethanolamine, pH 9.8) (Sigma Chemical, Saint Louis, Missouri, USA). The eluate sample titre was expressed as the inverse of the highest dilution for which the optical density was greater than three times the optical density of the negative serum standard. Titres ≥ 100 were considered to be positive.

Results

Seventy-seven rabbits were sampled in 2003 and 46 in 2004, all were adults. Antibodies were detected in 5 out of 6 sites (Table 1). On the whole, 35% (43/123) rabbits were seropositive (Table 1). The proportion of rabbits with antibodies significantly differed between sites ($\chi^2 = 52.7$, $df = 5$, $P < 0.005$). A high proportion was seropositive in Pointe Morne (74%, 14/19) in 2003 and Armor (88%, 14/16) in 2004. In contrast, a low proportion of rabbits carried antibodies in Pointe Molloy (27%, 4/15), and

Fig. 1 Location of the Kerguelen archipelago (WGS84) and of rabbit sampling sites on Grande Terre: ① Ratmanoff, ② Pointe Morne, ③ Port-aux-Français, ④ Pointe Molloy, ⑤ Armor, ⑥ Port Jeanne d'Arc



Port-aux-Français (4%, 1/23), whereas all rabbits sampled in Ratmanoff were seronegative (0%, 0/20).

Discussion

These results show the presence of a Lagovirus in different sites of the Kerguelen archipelago. Three convergent reasons suggest that this Lagovirus may be non-pathogenic. A previous study carried out in 2001–2002 failed to characterize RHDV antibodies raised against pathogenic strains (Cooke et al. 2004). No occurrence of the disease has ever been recorded on the archipelago. Moreover, the Lagoviruses detected in Kerguelen have evolved in a rabbit population that was isolated before the emergence of virulent RHDV. We previously took advantage of the difference of specificity between the test we used here and the test based

on monoclonal antibodies used by Cooke et al. (2004) to detect the circulation of a non-pathogenic RHDV-like strain in France (Marchandeu et al. 2005). The existence of such a non-pathogenic RHDV strain in the Kerguelen has been suggested by Cooke et al. (2004) who detected IgG in two rabbits out of 81. Indeed, the immunoglobulin profile of the rabbits they tested led them to conclude that no RHDV strain circulated in the population but they assumed that the presence of a RHDV-like strain may explain this result they considered atypical. Note that they found one of their seropositive rabbits in Ratmanoff where we failed to detect antibodies among the 20 rabbits that we sampled. This may indicate either that these viruses do not permanently circulate in this population, or that they have a low prevalence, which makes them difficult to detect with such small sample sizes. The other seropositive rabbit they found was sampled in Val Studer where we did not sample rabbits during the present study.

The overall prevalence of RHDV-like antibodies of 35% suggests that the virus efficiently circulates in rabbits of Grande Terre. However, large differences in prevalence occur between sites, even between very close sites (e.g., Pointe Morne and Port-aux-Français), which suggests that the subpopulations are not widely connected and/or that different circulation patterns of the virus occur between subpopulations.

In our study, the indirect ELISA test is based on purified VLP (viral like particle), formed by recombinant VP60 RHDV proteins expressed in Sf9-baculovirus system. It has been previously shown that the majority of the VLPs have

Table 1 Proportion of rabbits with RHDV antibodies by site

Site	Proportion of rabbits with antibodies
Ratmanoff	0% (0/20)
Pointe Morne	74% (14/19)
Port-aux-Français	4% (1/23)
Pointe Molloy	27% (4/15)
Armor	88% (14/16)
Port-Jeanne d'Arc	33% (10/30)

The number of positive rabbits over the number of rabbits sampled is given in parentheses

a similar morphology to that of RHDV (Laurent et al. 1994). This test enables the detection of VP60-specific antibodies, which are the main antibodies produced after RHDV and Lagovirus infections. Since the alignments of different Lagovirus VP60 sequences showed a relatively high amino acid identity and similarity, serological cross reactions could be observed (Capucci et al. 1995). Nevertheless, very early epidemiological field studies on farm rabbits, comparing indirect ELISA test, HIT and Western blot, have confirmed the specificity of ELISA, and experimental studies have shown the non-reactivity of convalescent rabbit sera with feline calicivirus (FCV) antigens, FCV belonging to the Vesivirus genus, another genus of the Caliciviridae family (Rodak et al. 1990a, b). Recently, a Rabbit Vesivirus (RaV), isolated from young rabbits showing signs of diarrhoea, has been characterized as a putative novel member of the genus *Vesivirus* (Martin-Alonso et al. 2005). Phylogenetic analyses clearly place this virus in the marine-related calicivirus branch, which is separated from the branch including FCV, distant from Lagoviruses, and thus there is no antigenic relationship between these two viruses.

Twenty-five years after its emergence, the studies carried out on RHD have changed and are not only focused on the description of the impact of the disease. Most current studies aim at describing the diversity in RHDV strains (Le Gall-Reculé et al. 2003; Moss et al. 2002), including non-pathogenic ones (Forrester et al. 2007, 2009; Le Gall-Reculé et al. 2007; Strive et al. 2009), to understand the evolution of the virus as well as the impact of the disease on the free-living rabbit populations. These strains may act as genetic reservoirs for RHDV since recombination between RHDV strains has been evidenced (Abrantes et al. 2008; Forrester et al. 2008). They may also modulate the impact of RHD due to more or less partial cross protection between strains. Low titres of antibodies raised against RCV protect against RHDV infection (Capucci et al. 1996), whereas higher titres are required with antibodies due to some RHDV-like viruses serologically detected in Australia (Robinson et al. 2002). In contrast, some works carried out in France evidenced the existence of RHDV-like viruses, which induce antibodies that do not protect against a RHDV infection (Marchandeu et al. 2005) (Le Gall-Reculé, pers. comm.).

Within the framework of these studies on RHDV diversity, the study of RHDV-like strains circulating in the Kerguelen archipelago is of major interest because the rabbit population has been isolated for 110 years before the first occurrence of the disease and before the estimated emergence of RHDV. Until now, Lagovirus antibodies have been detected in rabbit sera collected between 1975 and 1983 in the Czech Republic (Rodak et al. 1990b). The interest of the strains circulating in the Kerguelen

archipelago is that they have evolved independently of those detected in other parts of the world and should derive from an ancestral RHDV precursor. In this way, Strive et al. (2009) suggested that the ancestor of RCV-A1 may have arrived in Australia together with the first introduced European rabbits, 150 years ago. Further works are now required to isolate and characterize these RHDV-like viruses that may help understanding the evolution of the virus and the emergence of the disease. In particular, the description of a virus that has directly evolved from a potential ancestor of the RHDV could help identifying the parts of the genome that are involved in virus pathogenicity. Indeed, the sequence located between amino acids 300 and 311 in the VP60 protein gene has been suspected to influence the degree of pathogenicity of the Lagoviruses (Lavazza and Capucci 2008). However, the deletions observed in this area for the RCV do not occur in the RCV-A1 recently described in Australia (Strive et al. 2009), which suggests either that these deletions are not responsible for non-pathogenicity or that they are not involved alone in non-pathogenicity.

More generally, the long-lasting isolation of these detected viruses has the potential to provide new information for the study of the phylodynamics of caliciviruses.

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