CSIRO PUBLISHING
www.publish.csiro.au/journals/wr *Wildlife Research*, 2007, **34**, 567–577

Towards a unique and transmissible vaccine against myxomatosis and rabbit haemorrhagic disease for rabbit populations

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Abstract. Currently available vaccines against myxomatosis and rabbit hemorrhagic disease virus (RHDV) are not suited to immunise wild rabbit populations, as vaccines need to be delivered individually by conventional veterinary practices. As an alternative approach, research in Spain has focused on the development of a transmissible vaccine. A recombinant virus has been constructed based on a naturally attenuated myxoma virus (MV) field strain, expressing the RHDV capsid protein (VP60). Following inoculation of rabbits, the recombinant virus (MV-VP60) induced specific antibody responses against MV and RHDV, conferring protection against lethal challenges with both viruses. Furthermore, the recombinant MV-VP60 virus showed a limited horizontal transmission capacity, either by direct contact or in a flea-mediated process, promoting immunisation of contact uninoculated animals. Efficacy and safety of the vaccine have been extensively evaluated under laboratory conditions and in a limited field trial. The development of the transmissible vaccine strategy and the steps being taken to obtain the marketing authorisation for the vaccine in the European Union are presented in this review.

Myxomatosis and rabbit hemorrhagic disease

Myxomatosis and rabbit hemorrhagic disease (RHD) are considered the major viral diseases affecting European rabbit (*Oryctolagus cuniculus*) populations. Myxoma virus (MV), the causative agent of myxomatosis, is a large virus with a double-stranded DNA genome of 163 kb, which replicates in the cytoplasm of infected cells. MV belongs to the genus *Leporipoxvirus* of the family Poxviridae. The virus induces a benign disease in its natural host, *Sylvilagus* rabbits in the Americas. In European rabbits, however, MV causes myxomatosis, a systemic and usually fatal disease. The virus is spread by blood-feeding arthropod vectors such as mosquitoes or fleas. Epidemics occur annually or less frequently, depending on the emergence of large numbers of susceptible young rabbits and the availability of arthropod vectors (for reviews on myxomatosis see: Fenner and Ross 1994; Kerr and Best 1998; Fenner and Fantini 1999*b*). MV was deliberately released as a biological control agent for the European rabbit initially in Australia (1950) and soon after in France (1952), whence it rapidly spread across the entire rabbit range in Europe, and has become endemic since then. Following initial release, devastating epizootics occurred in both continents, with a mortality rate of ~99.5% of the infected rabbits. Rabbit populations were reduced by more than 90% as a whole and in some areas rabbits completely disappeared. The evolution of attenuated viral strains along with the development of host resistance led to a diminished incidence of the disease (Fenner and Ratcliffe 1965; Fenner and Ross 1994). Nevertheless, studies carried out in Europe and Australia indicate that myxomatosis still regulates rabbit population numbers (Trout *et al*. 1992; Williams *et al*. 1995). Control of myxomatosis among domestic rabbits is currently achieved by vaccination using heterologous vaccines based on Shope fibroma virus, a less virulent leporipoxvirus (Fenner and Woodroofe 1954; Jacotot *et al*. 1955), or homologous vaccines based on cell culture-attenuated strains of MV (McKercher and Saito 1964; Saito *et al*. 1964; Maral 1970; Saurat *et al*. 1978; Argüello 1986).

RHD, an acute and highly contagious disease in wild and domestic rabbits (Ohlinger and Thiel 1993; Chasey 1997), was first reported in the People's Republic of China (Liu *et al*. 1984). The disease spread throughout Europe between 1987 and 1989, transmitted largely through trade in domestic rabbits (Morise *et al*. 1991). It quickly expanded into wild rabbit populations and appeared in Britain in 1992 (Fuller *et. al*. 1993). In 1995 it was accidentally released in Australia, where the virus escaped from an experimental trial on a quarantined island (Mutze *et al*. 1998), and in 1997 it was illegally introduced in New Zealand (O'Keefe *et al*. 1999). RHD has also been reported in countries from Africa, Asia and America (for reviews on RHD see: Fenner and Fantini 1999*a*; Cooke 2002). Infected rabbits usually die within 48–72 h of necrotising hepatitis. The disease is responsible for high economic losses in rabbitries as well as high mortality rates in wild rabbit populations (Villafuerte *et al*. 1995; Marchandeau *et al*. 1998; Mutze *et al*. 1998). The etiological agent, RHD virus (RHDV), is the prototype species of the genus *Lagovirus* within the family Caliciviridae (Ohlinger *et al*. 1990), which has been designated as the type species of the genus *Lagovirus*. The virions (30–40 nm in diameter) have a 7.4-kb single-stranded positive-sense RNA genome and are non-enveloped and icosa-

10.1071/WR06160 1035-3712/07/070567

hedral. The RHDV capsids are made of a major protein component of 60 kDa (VP60) and a minor polypeptide of 12.9 kDa (VP10) (Wirblich *et al*. 1996). Commercial vaccines against RHD are prepared from the livers of experimentally infected rabbits (Argüello 1991), since *in vitro* systems are not available for efficient virus propagation. This approach involves the need to handle large amounts of highly infectious material. The RHDV capsid protein gene has been successfully expressed in several heterologous systems. VP60 protein has been produced in *Escherichia coli* (Boga *et al*. 1994), *Saccharomyces cerevisiae* (Boga *et al*. 1997), *Pichia pastoris* (Farnos *et al*. 2005), recombinant virus-based systems such as baculovirus (Laurent *et al*. 1994; Marín *et al*. 1995; Nagesha *et. al*. 1995), poxvirus (Bertagnoli *et al*. 1996*a*, 1996*b*; Fischer *et al*. 1997), potyvirus (Fernandez-Fernandez *et al*. 2001) and plants (Castañón *et al*. 1999). The recombinant VP60 obtained in all these systems has been shown to induce protection of rabbits against a lethal challenge with RHDV. In some cases the recombinant VP60 protein self-assembled to form virus-like particles (VLPs) which were highly immunogenic (Laurent *et al*. 1994; Nagesha *et al*. 1995; Plana-Durán *et al*. 1996). Nevertheless, high levels of protection have also been obtained with recombinant VP60 protein constructions that do not form VLPs (Marín *et al*. 1995; Castañón *et al*. 1999; Farnos *et al*. 2005), or do not even reproduce the native protein conformation (Boga *et al*. 1994).

The need to control myxomatosis and RHD in wild rabbit populations in Spain

While the above-mentioned results offer promising opportunities for the protection of domestic rabbits against myxomatosis and RHD, control of both diseases in wild rabbit populations remains an unsolved problem of great concern. In this regard, it should be noted that the European rabbit plays a key ecological role in Mediterranean ecosystems. In Spain, the rabbit is the staple prey of a wide variety of predators (Delibes and Hiraldo 1981; Delibes-Mateos *et al*. 2007). These include carnivores such as the Iberian lynx (*Lynx pardina*), which is considered by the IUCN (World Conservation Union) to be the most critically endangered cat species worldwide, and several birds of prey such as the Spanish imperial eagle (*Aquila adalberti*), the most endangered raptor in Europe. Both endangered predators are specialist rabbit predators (Ferrer and Negro 2004). In addition, rabbits are among the most important small game species in several European countries.

Following the introduction to Spain of myxomatosis, and especially RHD, wild rabbit abundances have sharply declined, both diseases having become endemic. Recently, some have called for the rabbit to be reclassified under the IUCN Red List of threatened species in the Iberian Peninsula (Ward 2005). Iberian lynxes, Spanish imperial eagles and other predators have been reported to suffer from this decline (Fernández 1993; Real and Mañosa 1997; Moreno *et al*. 2004). In Spain, where more than 30 000 hunting areas cover more than 70% of the country, rabbits are an important economic resource and their decline is reflected in the fact that the number of rabbits shot is half the number two decades before (Delibes-Mateos *et al*. 2007). Thus, an effective way of vaccinating wild rabbit populations could offer a good solution from the point of view of the local hunting socio-economy and nature conservation.

Approaches for a wildlife vaccination

Immunisation of wildlife is difficult to achieve because the animals are free ranging, thus precluding the use of vaccines that require individual administration by conventional veterinary practices, when dealing with large populations.

The oral route is considered a feasible way of vaccine administration to widespread wildlife populations. A good example is the recombinant vaccine that has been succesfully used for the control of sylvatic rabies in Europe and North America (Brochier *et al*. 1991; Pastoret and Brochier 1998, 1999). The vaccine is based on a recombinant vaccinia virus (VRG) that expresses the immunising glycoprotein of rabies virus (Blancou *et al*. 1986; Brochier *et al*. 1990). The VRG is delivered in baits that are dropped by helicopter or plane according to a grid. From 1989 to 1995, ~8.5 million VRG vaccine doses were dispersed in Western Europe to vaccinate red foxes, and in North America to vaccinate raccoons and coyotes. In Europe, the use of VRG has led to the elimination of sylvatic rabies form large areas, which have consequently been freed from the need for vaccination (Brochier *et al*. 1996; Pastoret and Brochier 1999; Pastoret 2001; Cliquet and Aubert 2004).

Another approach could be the use of vaccines based on viral vectors capable of spreading within an animal population. This is a potentially useful approach for delivering antigens to wild animals, especially when the distribution, size, and turnover rate of a population preclude capture or baiting techniques as the only means for antigen delivery. The European rabbit is an example of such a population. With this in mind, research in Spain has explored the possibility of vaccinating wild rabbits against both myxomatosis and RHD by using an MV-VP60 recombinant virus capable of spreading through rabbit populations by horizontal transmission. The principle of this control method consists on the direct administration of the recombinant vaccine to a small number of rabbits, which would eventually lead to the immunisation of a fraction of animals within the population sufficient to reduce the spread of both diseases.

Interestingly, in parallel to the development of the recombinant MV-VP60 vaccine in Spain, research in Australia focused on the novel concept of virally vectored immunocontraception (VVIC), using MV as a transmissible vector to immunosterilise wild rabbits, by expressing reproductive proteins to cause autoimmune infertility (Robinson and Holland 1995; Robinson *et al*. 1997). Basically, the same technical approach to reach the opposite result: a reduction in wild rabbit population numbers.

The rationale basis of the transmissible MV-VP60 vaccine

Efficacy

Several facts led to anticipate that the proposed transmissible recombinant vaccine could be effective for the control of myxomatosis and RHD among rabbit populations, both in terms of efficacy and safety. First, recombinant poxvirus systems had been successfully developed as vectors for delivering a wide range of vaccine antigens to humans and animals (reviewed in Pastoret and Vanderplasschen 2003). Viruses used include vaccinia virus (Moss 1996), avipoxviruses (Fischer *et al*. 1997), raccoon poxvirus (Hu *et al*. 1996), capripoxvirus (Romero *et al*. 1994), swinepox virus (Van der Leek *et al*. 1994), and myxoma virus (Bertagnoli *et al*. 1996*a*). It is important to note that

recombinant vaccines based on poxvirus vectors (ALVAC strain of canarypox virus) have been authorised and are currently used in the European Union (Moulin 2005). Second, as mentioned above, the expression of RHDV VP60 capsid protein in several heterologous systems had been shown to induce protective immunity. Remarkably, no indications of toxicity or side effects associated with the expression of VP60 have so far been reported. In addition, molecular epidemiology studies have revealed a low genomic variation within isolates collected from different geographic areas and over a period of several years (Nowotny *et al*. 1997; Le Gall *et al*. 1998; Moss *et al*. 2002; Le Gall-Recule *et al*. 2003). Although some authors have reported the emergence of a new antigenic variant termed RHDVa (Capucci *et al*. 1998; Schirrmeier *et. al*. 1999), which can be differentiated from precedent strains by reactivity with certain monoclonal antibodies, these strains still share over 93% amino acid identity in the VP60 protein sequence with typical European isolates of RHDV. Importantly, current commercial vaccines induce full protection against challenge infections with these RHDVa isolates (Capucci *et al*. 1998; Schirrmeier *et al*. 1999). The latest phylogenetic studies further confirm the low genetic variability among RHDV isolates observed so far (Matiz *et al*. 2006; van de Bildt *et al*. 2006; Forrester *et al*. 2006*a*, 2006*b*, 2007). These results suggest that, to date, a single RHDV serotype exists. Since this is also the case for MV, vaccination with a recombinant MV-VP60 is expected to provide effective protection against all currently circulating strains of MV and RHDV. Third, MV presented some features that made it a good choice for the development of transmissible recombinant vaccines for rabbits. Suitable insertion sites for heterologous genes had been previously described (Jackson and Bults 1992; Jackson *et al*. 1996), and the ability of MV-based recombinants to induce a strong immune response, including mucosal immunity, had been established (Kerr and Jackson 1995). Doses as low as 5 PFU of a recombinant MV induced high titres of specific antibodies against the recombinant antigen in inoculated rabbits (Kerr and Jackson 1995), suggesting that the viral doses delivered by arthropod vectors in nature, which are in the range of 1–100 PFU (Fenner and Ratcliffe 1965), could be sufficient to elicit adequate antibody responses. In addition, field experiments carried out in Australia had addressed issues related to the introduction, spread, and monitoring of MVs released into wild rabbit populations in areas where MV is already endemic (Parer *et al*. 1985; Robinson *et al*. 1997).

Safety

Since the transmissible immunisation strategy involves the environmental release of a recombinant virus, considerations regarding safety issues were considered as important as the potential efficacy of the candidate vaccine. It is for this reason that safety concerns have been at the core of its rational design.

The biological characteristics of MV make it a good candidate as a vaccine vector in terms of safety considerations. MV exhibits a very narrow host range, as it infects only lagomorphs (*Sylvilagus* spp., *Oryctolagus* spp. and *Lepus* spp. under certain conditions). The virus has been widely distributed throughout Australia and Europe for more than 50 years (and even for centuries in the Americas) with no evidence of infection of other species. Thus, the host-restricted nature of MV minimises the risk of recombinant vaccine spreading to non-target species in nature. Moreover, given the current widespread geographic distribution of MV, which is similar to the distribution of RHDV, the field use of a recombinant MV-VP60 vaccine would not involve the introduction of a virus species that was not already present in a particular area.

Transmissible protection

A critical step in the development of the transmissible vaccine was the choice of an appropriate parental MV strain for the construction of the MV-VP60 recombinant. In the case of the VVic. strategy in progress in Australia, this step does not represent a major challenge. Since the final goal of this strategy is to reduce rabbit numbers, field strains of intermediate level of virulence (i.e. with mortality rates of ~70%), which are reportedly highly transmissible (Fenner and Ratcliffe 1965; Fenner and Ross 1994; Kerr and Best 1998), are in principle suitable as parental strains for the construction of immunocontraceptive recombinant viruses (Kerr *et al*. 2003). The fact that the viral vector causes significant mortality rates in the target population is not regarded as a problem. However, in the case of a recombinant virus intended as a protective vaccine, the parental virus should ideally be an MV strain with virtually no pathogenic potential and at the same time capable of horizontal transmission among rabbits.

Previous results obtained by several authors indicated that it is unlikely such an MV strain can be obtained by cell culture passage attenuation of virulent field strains, as these attenuated viruses usually lack the ability to spread through horizontal transmission (Saito *et al*. 1964; Saurat *et al*. 1978). Presumably, these attenuated MV variants have lost gene functions that are not essential for virus replication in cell cultures but are necessary for virus dissemination *in vivo*. This seems to be the case of MV strain SG33, a non-transmissible cell culture attenuated strain used in France to vaccinate rabbits against myxomatosis (Saurat *et al*. 1978). SG33 has a deletion of ~13 kb (Petit *et al*. 1996), which includes the Serp2 gene, which has been shown to be important in the pathobiology of MV (Messud-Petit *et al*. 1998).

The alternative approach adopted was to select a viral strain among circulating field viruses. The rationale was that if a suitable non-pathogenic strain could be isolated from the field, it should be capable of horizontal spread among rabbits, since the virus was circulating in nature. For this purpose, 20 field isolates collected from infected wild rabbits from 12 regions throughout Spain, over the period 1992–95, were analysed in terms of virulence and transmissibility (Bárcena *et al*. 2000*b*). From this study an MV strain arose, Isolate 6918, which potentially fulfilled the desired characteristics of low pathogenicity along with horizontal transmissibility.

Since preservation of the biological properties of the original MV strain was of major importance, the effect of inserting foreign DNA into the MV genome was carefully considered. The conventional procedure for the isolation of recombinant poxviruses based on thymidine kinase (TK) gene disruption results in a severe attenuation of the virus (Buller *et al*. 1985). Provided that Strain 6918 was already non-virulent, further attenuation was not desirable since this could result in a loss of vaccine efficacy and affect the virus transmissibility. Thus, it was decided not to disrupt any viral gene. The RHDV VP60 gene was inserted in the intergenic site between the open

reading frames M061R (TK gene) and M062R, as recombinant MVs with a foreign gene inserted at this site had been previously shown to retain overall wild-type biological properties (Jackson *et al*. 1996).

Construction and characterisation of MV-VP60 recombinant virus

The VP60 construct inserted in the MV genome (VP60-T2) was a gene fusion of RHDV VP60 gene with a DNA fragment coding for a linear epitope tag from the swine transmissible gastroenteritis virus (TGEV) nucleoprotein, recognised by monoclonal antibody DA3 (Martín-Alonso *et al*. 1992). This peptide tag was included in the construction to enable monitoring the spread of the recombinant virus in the field. The presence of antibodies against the TGEV tag in rabbits should be indicative of infection with the recombinant MV-VP60, as TGEV is a porcine virus that does not circulate in rabbit populations.

The transient dominant selection approach (Falkner and Moss 1990) was used for the isolation of the recombinant virus. This procedure enabled the construction of the recombinant MV-VP60 without any marker genes inserted in the final recombinant viral genome. This is a very desirable feature for recombinant viruses intended for field release, since the health and environmental risks associated with the wide spread of selectable marker genes (i.e. antibiotic resistance genes or genes encoding metabolic enzymes such as β-galactosidase) is currently regarded as a problem of major concern.

The recombinant MV-VP60 virus obtained expressed the VP60 construct to high levels in infected cells (Bárcena *et al*. 2000*a*). The VP60 construct expressed was antigenically similar to the viral capsid, as it was recognised by hyperimmune antisera against RHDV and also by conformational (RHDV-specific) monoclonal antibodies. As expected, the recombinant construction was also recognised by monoclonal antibody DA3, reflecting the presence of the TGEV peptide tag. The recombinant VP60 construct did not self-assemble into VLPs, since it contained a point mutation at amino acid position 296 (D296G) that abrogates VLP formation (Bárcena *et al*. 2004).

Transmissible protection induced by the recombinant MV-VP60

After injection of domestic or wild rabbits with the recombinant MV-VP60, only a small transient lesion at the inoculation site (usually ~0.5 cm) and, in some cases, localised discrete secondary nodules were observed. The presence of the virus could be detected by PCR 2–10 days post-infection (dpi). The virus was detected at the inoculation site, the draining lymph node, and samples of skin and in secondary nodules when these were present. No virus was detected in internal organs (blood, spleen, liver, and lungs). No febrile response or loss of bodyweight was detected in the inoculated rabbits, and the overall health of the rabbits was largely unaffected. The clinical symptoms appeared 5–7 dpi and completely resolved in all inoculated rabbits normally by 15 dpi. Thus, the symptomatology induced by the recombinant virus in the inoculated rabbits (Bárcena *et al*. 2000*a*) was similar to that previously described for the parental 6918 MV strain (Bárcena *et al*. 2000*b*).

The immune response elicited by rabbits inoculated with the recombinant MV-VP60 was monitored by ELISA for the presence of anti-MV, anti-RHDV, and anti-TGEV (anti-peptide tag) antibodies (Bárcena *et al*. 2000*a*). Rabbits inoculated with MV Strain 6918 or the recombinant MV-VP60 virus progressively developed similar anti-MV antibody titres. Anti-VP60 and anti-TGEV antibodies were detected in rabbits inoculated with the recombinant virus, while animals immunised with the parental virus remained seronegative. Antibodies against MV and RHDV were still detectable at least 8 months after inoculation with the recombinant virus.

To determine whether administration of recombinant MV-VP60 could protect wild rabbits from lethal challenges with MV or RHDV, and if this protection could be transmitted by direct contact to uninoculated rabbits, challenge experiments were conducted (Bárcena *et al*. 2000*a*). In these experiments groups of six rabbits were injected with 10^4 PFU of recombinant MV-VP60. After 3 days the inoculated rabbits were placed in a cage together with a group of six uninoculated rabbits (first-passage groups) for 6 days. Subsequently, the first-passage rabbits were placed in the same cage with a new group of six uninoculated rabbits (second-passage groups) for another 6 days. The different groups of rabbits were then separated, and 35 days after the initial immunisation with recombinant MV-VP60, rabbits were challenged with RHDV or virulent MV. A similar approach was used to evaluate the flea-mediated transmission of the recombinant virus and the concomitant induction of protection against myxomatosis and RHD. For this, special cages divided into two contiguous compartments separated by two metallic nets 25 cm apart were used. Under these conditions there was no direct contact between rabbits placed in different compartments, but the fleas readily spread through the whole cage. Finally, induction of oral protection with the recombinant virus was also analysed. In this case groups of rabbits were immunised by oral administration of 10^7 PFU of recombinant MV-VP60, and then the contact transmission experiment proceeded as described above.

The results obtained in the different challenge experiments can be summarised as follows:

- Direct immunisation of rabbits with recombinant MV-VP60 either by a single injection or by oral administration induced nearly 100% protection against a lethal challenge with MV or RHDV.
- Protection could be transmitted to unvaccinated rabbits by contact transmission or by flea-mediated transmission. On the whole, 50% of the rabbits in a first passage had detectable antibody levels against MV and RHDV and were protected from a lethal challenge with either of these viruses.
- Around 10% of the rabbits in a second passage had detectable antibody levels against MV and RHDV and were protected from a lethal challenge with either of these viruses.

The serologic analysis showed that infection of previously immunised rabbits with virulent MV or RHDV induced a high increase in the corresponding antibody titres. This result indicates that the immunity evoked by the recombinant MV-VP60 is readily reinforced by exposure to virulent virus. In areas where myxomatosis or RHD is endemic, vaccinated rabbits would be readily re-exposed to the viruses. Therefore, a high level of immunity is likely to be maintained in vaccinated rabbits over a prolonged period.

Interestingly, oral administration of a high dose of the recombinant virus was able to induce transmissible protection against a lethal challenge with MV and RHDV. This result opens the possibility of combining both characteristics of the proposed vaccine, i.e. oral administration and horizontal transmissibility among rabbits, in field immunisation of wild rabbit populations to control myxomatosis and RHD.

Safety evaluation of the recombinant MV-VP60 under laboratory conditions

The promising results obtained under laboratory conditions suggested that the recombinant MV-VP60 might be effective as a transmissible vaccine in large-scale immunisation schemes for the control of myxomatosis and RHD in wild rabbit populations. However, before considering its environmental release, vaccine safety considerations had to be extensively evaluated.

As indicated above, administration of either the parental MV Strain 6918, or the recombinant MV-VP60 to healthy rabbits under laboratory conditions by standardised procedures was safe, as all rabbits exhibited only mild clinical symptoms and rapidly recovered (Bárcena *et al*. 2000*a*, 2000*b*). The safety assesment of the vaccine was further extended (Torres *et al*. 2000) by analysing the potential risks of vaccine administration under a varied range of situations that might occur if the recombinant virus is used for large-scale field immunisation of rabbits: risks associated with vaccine dose, age or physiological condition.

Concerning vaccine dosage and the possibility of accidental administration of an overdose, the safety experiments demonstrated that administration of the recombinant virus to rabbits was safe even when a 100-fold overdose (10^6 PFU) was inoculated. Assessment of vaccine effects in immunosuppressed rabbits was considered relevant, given the incidence in nature of immunocompromised individuals due to infections, or to environmental or genetic causes. For this reason the effect of vaccine administration in rabbits treated with prednisolone, a potent immunosuppressor (Oehling *et al*. 1976) commonly used for the safety evaluation of veterinary vaccines (Argüello 1986; Ciuchini *et al*. 1986; Pedersen 1993) was analysed. Results showed that prednisolone-treated rabbits exhibited similar symptoms to those observed in control rabbits. Another important aspect addressed was the effect of recombinant MV-VP60 infection in rabbit reproduction. Results showed that recombinant virus inoculation did not alter the reproduction parameters analysed and none of the rabbits born from vaccinated does showed myxomatosis-associated clinical signs. In conclusion, the overall results obtained demonstrated a notable lack of adverse effects attributable to the recombinant virus (Torres *et al*. 2000), regardless of dose, route or life-history stage of individuals. Finally, the biological stability of the recombinant virus was analysed. The environmental release of the recombinant virus would involve a certain number of serial passages in its host, even when this capability seemed to be limited to only two serial passages under laboratory conditions (Bárcena *et al*. 2000*a*). Should there be a tendency for the virus to evolve to a virulent state, serial passage in rabbits would cause it to do so. Accordingly, the biological stability of the recombinant MV-VP60 was studied by subjecting the virus to 10 serial passages in rabbits. The results obtained indicated that the recombinant virus maintained grossly the same biological characteristics through the passages, and no evident increase in virulence was detected (Torres *et al*. 2000). Thus, the attenuated nature of the recombinant MV-VP60 seemed to be a stable trait. On the other hand, the genetic analysis indicated that the VP60 gene remained stably integrated in the MV genome after serial passage in rabbits.

Field trial

Despite all the safety data obtained under laboratory conditions, the response of rabbits to the recombinant MV-VP60 might be expected to be different in the field, given the greater variability in health or immune status among individuals, due to biological and environmental conditions, as well as varying levels of pressure from parasites, pathogens, competitors and predators. In order to address these safety concerns, a field trial under controlled conditions was performed (Torres *et al*. 2001).

The recombinant virus was subjected to the mandatory risk-assessment process relative to the release of genetically modified organisms (GMOs) in Spain. On the basis of the efficacy and safety data previously reported (Bárcena *et al*. 2000*a*, 2000*b*; Torres *et al*. 2000) the Spanish competent authority (the Biosafety National Commission) authorised a limited field trial.

The first environmental release of a vaccine based on a GMO should necessarily be conducted in relatively biocontainment conditions, such as on an island. Therefore, the experiment took place in Isla del Aire, an island of 34 ha located 1 km to the east of Menorca (Balearic Islands). The island holds a population of ~300 rabbits and has no natural predators. There is no human activity on the island.

To evaluate the effects of delivering the recombinant MV-VP60 to rabbits under field conditions, a total of 147 adult rabbits were captured and tagged with a microchip. A blood sample was extracted from each rabbit, and 76 rabbits (Group A) were inoculated with the recombinant virus. The other 71 rabbits (Group B) were used as contact non-vaccinated rabbits. All the rabbits were released near their point of capture. On Days 8, 24 and 32 post-vaccination (dpv), rabbits were captured and thoroughly examined for clinical signs. In addition, a blood sample was extracted from the rabbits captured 32 dpv.

Around 65% of the inoculated rabbits (Group A) captured 8 and 24 dpv showed clinical signs associated with the vaccine. These consisted of a localised primary nodule at the inoculation site and, in some rabbits, scanty secondary skin lesions in the form of small discrete nodules. No clinical signs were observed in inoculated rabbits (Group A) captured 32 dpv. None of the inoculated rabbits captured exhibited classical severe myxomatosis symptoms. None of the contact uninoculated rabbits captured, either marked with a microchip (Group B) or not marked (NM), showed any apparent clinical symptomatology associated with myxomatosis. Furthermore, the lack of statistically significant differences in the total rabbit population of the island during the first 32 days following vaccination indicated that there was no detectable increase in mortality that could be associated with the vaccine release. In conclusion, the results obtained showed a remarkable lack of undesirable effects in the rabbit population attributable to the recombinant virus release, and no adverse phenomena were observed in wildlife of the island throughout the observation period. Thus, the data obtained added to the extensive body of knowledge regarding

the safety of the recombinant MV-VP60 transmissible vaccine, and extended it to include evaluation in field conditions, in a relatively simple ecosystem (Torres *et al*. 2001).

Analysis of vaccine efficacy was another objective of the field evaluation. The substantial live trapping conducted during the study generated extensive serological data suitable to address this question (Table 1).

To evaluate the immune response elicited by the recombinant virus in directly immunised rabbits, sera samples from inoculated rabbits (Group A) obtained 32 dpv were analysed for the presence of anti-MV and anti-RHDV antibodies by ELISA, and compared with the sera samples obtained before vaccine administration (0 dpv). At 0 dpv, 82.7% of Group A rabbits were seronegative (titre <1/10) for both MV and RHDV. The rest of the animals (17.3%) exhibited low anti-MV or anti-RHDV antibody titres (1/10–1/100). At 32 dpv, 100% of Group A rabbits showed anti-MV antibody titres ranging from 1/1000 to 1/100 000, and anti-RHDV antibody titres ranging from 1/100 to 1/100 000. It is important to note that all the rabbits from Group A were doubly seropositive (MV+ RHDV+).

In order to analyse the ability of the vaccinal virus to disseminate among the rabbit population and induce an immune response in contact rabbits, the antibody response against both MV and RHDV was evaluated in microchip-marked uninoculated rabbits (Group B). At 0 dpv, 80% of Group B rabbits were seronegative (titre <1/10) for both MV and RHDV. The rest of the animals (20%) exhibited low anti-MV or anti-RHDV antibody titres (1/10–1/100). The number of double seropositive animals (MV+ RHDV+) in Group B increased from 8% (0 dpv) to 64% (32 dpv), while the number of single seropositive animals to MV (MV+, RHDV–) increased from 0% (0 dpv) to 12% (32 dpv). According to this, 56% of the contact non-vaccinated rabbits from Group B developed significant antibody titres against both MV and RHDV upon vaccinal virus release (although the titres observed in this group of animals were consistently lower than those of directly vaccinated rabbits from Group A), and 12% of the animals developed antibodies against MV but not against RHDV.

Similar results were obtained when the serological study was extended to all of the contact non-vaccinated rabbits captured 32 dpv, including microchip-marked (Group B) and NM rabbits. The number of non-vaccinated rabbits captured 32 dpv (71) was large enough to be considered representative of the total non-vaccinated rabbit population in the experimental area, which was estimated at 230 rabbits. At 32 dpv, 58.6% of the contact rabbit population (Group B + NM) was seropositive for both MV and RHDV, and 11.4% were single-positive for MV. Since only 8.8% of the total rabbit population was seropositive for MV at 0 dpv, it was concluded that the vaccinal virus disseminated among rabbits by horizontal transmission, inducing an antibody response against both MV and RHDV in ~50% of the contact rabbits, and 11.4% of the animals developed antibodies against MV but not against RHDV. Considering directly vaccinated rabbits (Group A) and contact rabbits (Group B + NM) together, the serological situation of the total rabbit population in the experimental area 32 days after vaccination was as follows: 68.1% of the rabbits were seropositive to both MV and RHDV, 8.8% were seropositive only for MV, 19.8% were seropositive only for RHDV, and only 3.3% of the rabbits were seronegative for both MV and RHDV.

Towards the commercialisation and release of a recombinant vaccine

Regulations and competent authorities at national and European levels

Since the purpose of the MV-VP60 recombinant virus is to be commercialised as a veterinary vaccine and released into the environment to immunise wild rabbit populations in Spain, the marketing and field use of this GMO-based vaccine must first be authorised by the competent national and European Union (EU) authorities, both as a veterinary medicine product (VMP) and as a GMO intended for field release.

National authorities in Spain for the environmental release of GMOs rely on two different commissions: the GMO Inter-Ministry Council, which evaluates proposals and makes decisions, and the Biosafety National Commission, which is an advisory commission. The GMO Inter-Ministry Council is responsible for coordinating and communicating with the European Commission (EC). In the case of GMO-based medicinal products, national authorities are principally responsible for the assessment of clinical trials and experiments in contained

Table 1. Seroprevalence of specific anti-myxoma virus (MV) and anti-rabbit hemorrhagic disease virus (RHDV) antibodies
dpv, days post- vaccination

Group of rabbits[A]	MV– RHDV–[B]	MV– RHDV+[B]	MV+ RHDV–[B]	MV+ RHDV+[B]
Group A (0 dpv)	82.7	7.7	0.0	9.6
Group A (32 dpv)	0.0	0.0	0.0	100.0
Group B (0 dpv)	80.0	12.0	0.0	8.0
Group B (32 dpv)	0.0	24.0	12.0	64.0
Total population (A + B) (0 dpv)	81.3	9.8	0.0	8.8
Contact (B + NM) (32 dpv)	4.3	25.7	11.4	58.6
Total population (A + B + NM) (32 dpv)	3.3	19.8	8.8	68.1

[A]On Day 0 dpv, sera samples were taken from 147 rabbits: 76 Group A, 71 Group B. On Day 32 dpv, sera samples were taken from 92 rabbits: 21 Group A, 25 Group B, 46 non-marked (NM).

[B]Percentage of rabbits seronegative for both MV and RHDV (MV– RHDV–), single positive for RHDV (MV– RHDV+), single positive for MV (MV+ RHDV–) and seropositive for both MV and RHDV (MV+ RHDV+).

areas before an application for marketing authorisation is submitted to European authorities (Moulin 2005).

At the European level, the Regulation EC No. 726/2004 (EC 2004) created the European Medicines Agency (EMEA), which regulates the authorisation and use of VMPs. Its Committee for medical products of veterinary use (CVMP) adopted in December 2004 guidelines for live recombinant vector vaccines (EMEA 2004) that were completed with a description of the exact procedure in March 2007 (EMEA 2007). The EMEA guideline and procedure complies with the European Directive 2001/18/EC on the deliberate release into the environment of GMOs (EC 2001) and with the pharmaceutical rules governing medicinal products in the EU (EC 1998). Following these regulations, the EMEA proceeds to authorise VMP, containing or consisting of GMOs, allowing direct access to the market at the European level.

The EMEA requirements for the marketing authorisation of a recombinant veterinary vaccine rely on three main points: quality, safety and efficacy of the recombinant vaccine. Additionally, the EMEA asks for an environmental risk-assessment report. The proposal must include a dossier on the product characteristics and the details of compliance with the requirements of Directive 2001/18/EC. The EMEA designates a *Rapporteur* and *Co-rapporteur* who lead the proposal evaluation, but all members of the CVMP and competent authorities under Directive 2001/18/EC are informed and can comment on the proposal. The final decision, however, remains within the CVMP, which discusses the risk versus the benefit of the use of the vaccine and recommends for or against the marketing authorisation (EMEA 2007).

The likely authorisation of the transmissible MV-VP60 vaccine

The transmissible MV-VP60 vaccine was first presented to the EMEA to obtain marketing authorisation in 2002 under the name Lapinvac-F1. The Spanish Hunting Federation and a private Spanish company devoted to VMP were the applicants of this first proposal. The EMEA reported technical problems in some of the evaluation experiments on the quality and safety of the recombinant vaccine, which did not fully comply with the European pharmacopeia, and asked for additional information, such as the complete genome sequence of the recombinant virus. The proposal was then withdrawn from the evaluation process. Two years later, the Spanish Hunting Federation together with a new private Spanish vaccine company resumed the process. Currently, the technical problems raised by the EMEA have been resolved, and new evaluation experiments to further assess the safety and efficacy of the recombinant vaccine are being undertaken. These include the evaluation of the effects of ingesting vaccine-inoculated rabbits in different predator species, and the evaluation of the duration of immunity in inoculated rabbits, up to one year. In addition, the complete genome sequence of the recombinant virus, which provides insights to the molecular basis of its attenuation, is available. A new proposal to the EMEA is expected to be presented before the end of 2007.

Besides the demonstration of the quality, safety and efficacy of the vaccine, the likelihood of a positive authorisation of the recombinant vaccine by the EMEA will, in the end, depend on the final step in which all European countries consider the risks and benefits of the proposal. This consideration will be largely influenced by political and social aspects, such as the public perception of GMO releases into the environment across Europe, or the conflicting interests regarding the management of wild rabbits: conservation versus control of rabbits, within the EU context. To overcome this last step of the decision process, the applicant and Spanish competent authorities will have to show the interest and benefits of the recombinant vaccine, in terms of social and environmental implications, and should express a clear and unique opinion. The National Hunting Federation and, at different times, the Spanish Ministry of Environment have funded the recombinant vaccine. Thus, the interest of the recombinant vaccine relies mainly on small game hunters that want to recover rabbit populations but the implications for endangered predators that depend on rabbits should also be stated. An evaluation of the current situation of wild rabbit populations in Spain would certainly be helpful.

International issues in the use of the recombinant transmissible vaccine

Finally, a recombinant transmissible vaccine to be released in wild populations of a widely distributed species such the European rabbit, should take into account the countries outside Europe where rabbits are now distributed. Moreover, it should be noted that different countries have opposing goals in the management of wild rabbits: control of wild rabbits, considered as a pest species in most of their distribution range outside their native range, versus conservation within their native range.

Currently, there is a lack of any broad international agreement with the potential to mediate effectively in these conflicting cases, although both the World Organisation of Animal Health (OIE: http://www.oie.int/eng/en_index.htm) and the Convention of Biological Diversity (CBD, through the Cartagena Protocol of Biosafety: http://www.cbd.int/biosafety/default.shtml) are currently trying to aid in this field (Angulo and Gilma 2008). The EU signed the Cartagena Protocol of Biosafety, which includes among its obligations the need to inform the Protocol and the potential affected countries about any GMO release that may lead to an unintentional transboundary movement (EC 2003). However, the Protocol is already not very effective as it deals mostly with international transboundary movements of GM crops. New guidelines concerning more specific fields, such as GM viruses, have just begun.

Concluding remarks

Under laboratory conditions, direct administration to rabbits of recombinant MV-VP60 induced almost complete protection against myxomatosis and RHD. The recombinant virus (and the concomitant protection) could be quite efficiently transmitted in a first passage to contact uninoculated rabbits, whereas transmission was greatly reduced in a second passage. The results obtained in the field trial were in good agreement with those obtained at the laboratory. Direct immunisation of rabbits by inoculation induced a high antibody response against both MV and RHDV, and the recombinant virus disseminated among the rabbit population induced a lower but significant antibody response against both MV and RHD in ~50% of the contact uninoculated rabbits.

The limited horizontal transmission capacity so far exhibited by the recombinant MV-VP60, under laboratory conditions and in a field trial in a relatively simple ecosystem, raises questions about its potential efficacy as a transmissible vaccine intended to immunise wild rabbit populations. On the other hand, the limited transmissibility of the recombinant virus can be considered advantageous with respect to safety issues, as it would facilitate maintaining control over the recombinant virus dissemination in the environment, avoiding its spread and persistence into areas where the virus is unwanted. Upon introduction of the transmissible vaccine in a particular area, it is expected that the recombinant virus would undergo a limited number of passages among the target rabbit population, infecting fewer susceptible rabbits in each passage, until it would eventually disappear. It should be borne in mind that, in field conditions, there are several factors (i.e. previous immunity to MV, genetic resistance of rabbits, competing field strains of MV, etc.) that would limit the recombinant virus spread. In these conditions the success of the proposed strategy depends on the ability of the recombinant virus to reach a fraction of immunised animals within the rabbit population sufficient to reduce the spread of both myxomatosis and RHD. In order to be effective, the vaccinal virus would need to be reintroduced annually in each target population. Furthermore, key parameters of the epidemiology of MV and RHDV, including rabbit abundance, annual patterns of breeding, abundance of arthropod vectors (fleas or mosquitoes), abundance of predators, occurrence of myxomatosis and RHD, and serological status of rabbit populations, should be carefully analysed and taken into account so as to determine the optimal timing, intensity and frequency of the vaccinal virus release.

Recent studies performed in Australia aimed at the question of whether or not it is possible to impose a myxoma virus of choice on populations of rabbits in the field were MV is endemic (Kerr *et al*. 2003; Merchant *et al*. 2003*a*, 2003*b*). It was concluded that there is a lag between the appearance of susceptible animals in the population and the appearance of myxomatosis, indicating that there is a window of opportunity of one or two months for releasing the MV strain of choice (Merchant *et al*. 2003*b*). A selected MV field strain (Kerr *et al*. 2003) was introduced in four sites in order to monitor its spread and persistence in the field (Merchant *et al*. 2003*a*). On two of the sites the released virus was able to spread across the entire site, persisting for almost the entire observation period of 6 months. On the other two sites, however, the virus was detected for 62 and 78 days, and the subsequent inability to detect the introduced virus correlated with the appearance of unrelated MV field strains.

Similar studies performed in the environmental conditions of Spain have been designed and will be conducted on the event that the MV-VP60 vaccine is authorised by the EMEA, to define the best way to manage the transmissible immunisation strategy to control myxomatosis and RHD in wild rabbit populations.

Acknowledgements

Elena Angulo acknowledges funding by the ANR-Biodiversité project. The development of the MV-VP60 recombinant vaccine was supported by an agreement between the 'Fundación para el Estudio y Defensa de la Naturaleza y la Caza' (FEDENCA) and the 'Instituto Nacional de Investigación y Tecnologíia Agraria y Alimentaria' (INIA).

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Manuscript received 24 November 2006, accepted 18 July 2007