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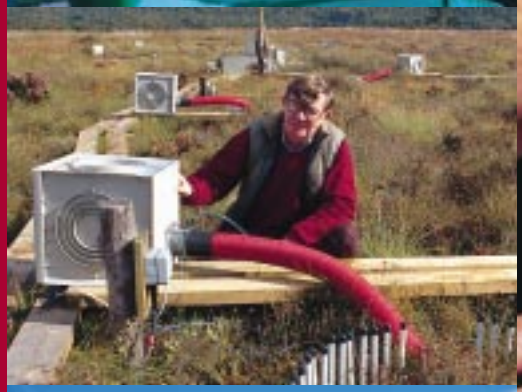

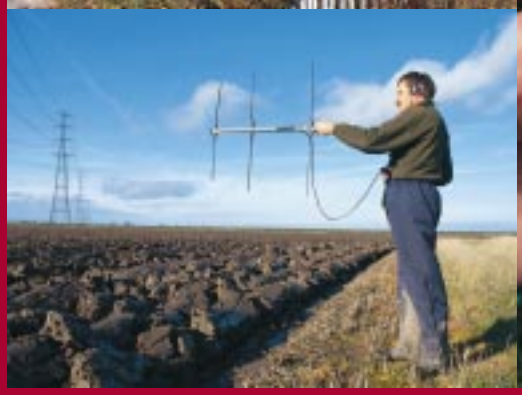

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Routes to the development of a parapoxvirus vaccine for red squirrels

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1. Background

The UK population of the native red squirrel *Sciurus vulgaris* has fallen dramatically over the past 150 years so much so that red squirrels are already locally extinct in many parts of England and Wales. This decline in the reds has in part been blamed on the concomitant increase in grey squirrels *Sciurus carolinensis* which were introduced to the UK from America in the mid-nineteenth century. There can be little doubt that the reason for the decline in red squirrel numbers is multi-factorial, but there is a growing body of evidence that a disease caused by a parapoxvirus (PPV) may play a major role in the local collapse of red squirrel populations.

Middleton (1930) reviewed the status of the reds in light of the introduction of the greys from America. He found that although there were natural fluctuations in red squirrel numbers there was a dramatic fall in the UK population in the first quarter of the 20th century. Many of the local population crashes he reviewed could be explained in terms of outbreaks of epidemic disease. Although most of the post-mortem reports examined suggested that coccidiosis was to blame, he noted that the occurrence of external symptoms was not always consistent with this and suggested that a disease of unknown aetiology was likely. Some of the disease epidemics appeared to have occurred soon after the first appearance of greys in the locality and this led Middleton to conclude that it was possible "…that the grey squirrel may act as a carrier of disease which is fatal to the red squirrel, but non-pathogenic to the grey species." A similar situation is found with another poxvirus, myxoma virus. In its natural hosts, the American rabbit (*Sylvilagus brasiliensis* and *Sylvilagus bachmani*), myxoma virus causes a benign cutaneous fibroma, but in the European rabbit *Oryctolagus ciniculus* it produces the systemic, usually fatal disease, myxomatosis (Fenner and Ross 1994).

In the 1960's attempts were made to isolate the agent responsible for outbreaks of a myxomatosis-like disease affecting reds in both Shropshire and Norfolk (Edwards 1962; Visozo and others 1964; Visozo 1968). Although viral agents, one tentatively identified as being an encephalomyocarditis virus and one with similarity to the myxoviruses, were identified, their specific role in the outbreaks of scab disease was questioned. It was not until 1981 that Scott and others identified a virus from an eyelid lesion on a red squirrel, and deemed it responsible for the outbreaks of scab disease. Using electron microscopy the virus resembled, but was clearly distinguishable from, orf virus, the type species of the genus parapoxvirus. The red squirrel from which the lesion had been taken had been found dead in Blickling Park, Norfolk. Three days earlier it had been spotted alive, but obviously unwell, apparently deaf and blind in one eye. This squirrel was thought to have had direct contact with greys.

Since the initial identification of PPV-associated disease in Norfolk, it has also been confirmed in red squirrels from Lancashire, Cumbria, Northumberland and North Wales (Sainsbury and Gurnell 1995; Sainsbury and Ward 1996; Sainsbury and others 1997; Jackson 1998). In contrast, it has been found only once in a single grey squirrel from Hampshire (Duff and others 1996). An enzyme-linked immunosorbent assay (ELISA) was developed in order to study the seroprevalence of antibodies to the virus in red and grey squirrels from across the UK (Sainsbury and others 2000).

Table 1. Distribution of squirrels tested for antibodies to the squirrel parapoxvirus (updated from Sainsbury and others 2000).

	Greys		Reds	
	Negative	**Positive**	**Negative**	**Positive**
Hampshire	0	20	36*	0
East Anglia	13	36	8	0
Staffordshire	1	10	0	0
Lancashire	2	19	1	3
Cumbria	108	99	92	2
Northumberland / Durham	30	25	18	4
Scotland	96	1[#]	32	0
Northern Ireland	149	39	0	0

The number of red and grey squirrels with antibodies to parapoxvirus. Serum samples have been collected over the past 6 years and tested by ELISA. *Samples are from the Isle of Wight. [#]This is the first seropositive grey squirrel found in Scotland.

The results (summarised in Table 1) indicated that a high percentage of apparently healthy greys had been exposed to the PPV, but only 4% of red squirrels had antibody to the virus. Of the nine seropositive red squirrels found, seven had died with PPV-associated symptoms. Furthermore, it appeared that in some areas where the red squirrel was now extinct and the greys had been established for a long time, the seroprevelance in greys was approaching 100 %, whereas in areas which supported both red and grey squirrels the seroprevelance in the greys was lower. The study also confirmed that PPV disease was found in reds only in areas where the greys were seropositive and that in Scotland which supports substantial numbers of reds only one grey squirrel, just over the border from England, has been shown to have antibody. In Northern England some populations of greys have antibodies whereas others do not. Similarly in Northern Ireland grey squirrels with antibody are restricted to some woods only.Thus further studies in this region could provide valuable insights into the epidemiology of the disease.

These findings support the suggestion that the greys could be carrying the virus asymptomatically and passing it to the reds which readily succumb to the PPV-associated disease. Reservoir species have been reported for other poxviruses and appear to be important for the maintenance of the virus in the wild. In addition to myxoma virus in the American rabbit, it has been demonstrated that two species of African squirrel, *Funisciurus anerythrus* and *Heliosciurus rufobrachium*, are likely to be important in sustaining monkeypox virus infections (Khodakevich and others 1987) and in Europe, bank voles *Clethrionmys glareolus*, field voles *Microtus agrestis* and wood mice *Apodemus sylvaticus* appear to be the reservoir for cowpox virus (Crouch and others 1995, Bennett and others 1997). Clinical signs of infection in each of these species are either minor or absent, although each of the viruses can cause serious disease in other species.

2. The disease

PPV disease in red squirrels is characterised by an exudative erythematous dermatitis (Figure 1), particularly around the eyes, nose, mouth and concha, although lesions are also found on other exposed skin surfaces such as between the digits and the genital region (Scott and others 1981; Sainsbury and Gurnell 1995; Sainsbury and Ward 1996). No signs of lesions

affecting internal organs are found. As with other parapoxviruses it is thought that the virus remains locally at the site of infection and that unlike the other poxvirus genera there is no systemic phase of infection. Although red squirrels experimentally inoculated with the virus on the right thigh also developed lesions at other sites it is thought that mechanical transfer of the virus to these other sites by grooming was likely. In addition and probably as a result of the development of scabby lesions, red squirrels infected with the PPV suffered a rapid loss of condition, appetite and weight and were generally lethargic in their behaviour (Tomkins and others 2002).

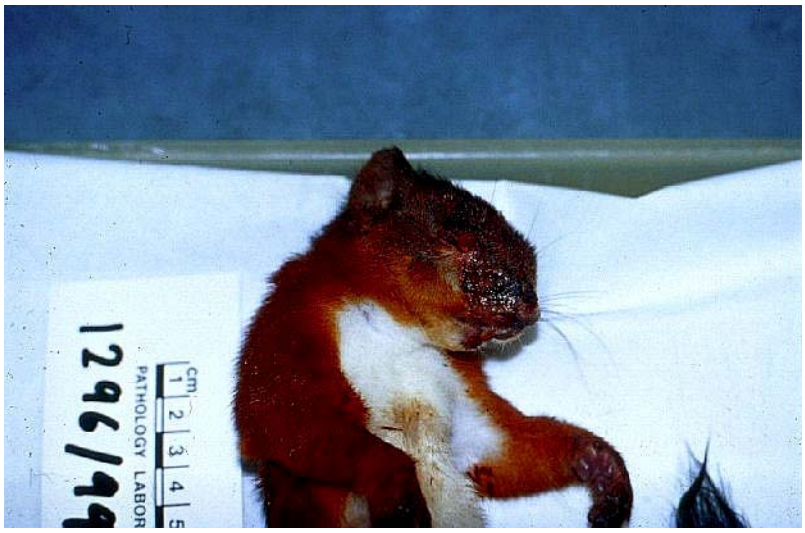

Figure 1. A confirmed case of PPV disease showing typical lesions around the face and footpads of a red squirrel. This squirrel was found in Gateshead in 1999.

PPV disease is not normally found in grey squirrels. The only grey squirrel found with confirmed PPV disease had facial skin lesions similar to that found in reds (Duff and others 1996). Greys experimentally infected with the same innoculum as the reds showed no obvious signs of disease either externally or internally (Tomkins and others 2002), although they did produce an antibody response specific to the virus. It has been suggested that the disease is manifested in greys only when the immune system is compromised by stress or another underlying infection.

3. The virus

The *poxviridae* are a family of large double-stranded DNA viruses which infect both vertebrate *Chordopoxviridae* and invertebrate *Entomopoxviridae* hosts. The *chordopoxviridae* can be further subdivided into 8 genera which are related genetically, antigenically, and share a similar morphology and host range (Table 2). The poxviruses are unique amongst viruses in that transcription, replication and assembly of the virus all occur within the host cell cytoplasm.

Table 2. The classification of Poxviruses showing the genera affecting vertebrates

Family	Genus	Species
Chordopoxvirinae (Poxviruses of vertebrates)	Orthopoxvirus	Vaccina
		Variola
		Cowpox
		Monkeypox
		Ectromelia
		Camelpox
	Avipoxvirus	Fowlpox
		Canarypox
		Pigeonpox
		Turkeypox
		Mynapox
	Capripoxvirus	Sheeppox
		Goatpox
		Lumpy skin disease
	Leporipoxvirus	Myxoma
		Shope Fibroma
		Squirrel Fibroma
	Suipoxvirus	Swinepox
	Molluscipoxvirus	Molluscum contagiosum virus
	Yatapoxvirus	Tanapox virus
		Yaba monkey tumour virus
	Parapoxvirus	Orf virus
		Bovine papular stomatitis
		Psuedocowpox
		Parapox of red deer
		Parapox of red squirrels
		Parapox of seals

Note that not every member of each genera is listed

All poxviruses are related genetically, illustrated by the co-linearity of their genomes. Although the individual genome size varies slightly within a genus and more widely between the genera (~140kb – 300kb) there is a central core of genes, conserved across the genera, which are essential for survival and replication of the viruses (Fenner 1996; Moss 1996). These include the genes which encode the machinery for transcribing the viral genes into mRNA, the machinery for replicating the virus genome and the genes for the major structural elements of the virion. The remaining genes, found at either end of the linear genome, usually dictate which host species the virus can infect, and also confer a replicative advantage to the virus in the face of the immune response of that host. Thus they are considered to determine the virulence of the virus (Smith and others 1991; Johnson and others 1993; Massung and

others 1994). These genes also tend to be specific to the different genera and sometimes to individual species of virus, probably as a result of the co-evolution between the virus and the host it infects.

Poxviruses have been shown to be related antigenically, with the greatest degree of cross-reactivity being found within the individual genera. Using standard techniques such as direct and indirect fluorescent antibody staining, serum neutralisation tests and agar gel immunodiffusion tests it has been found that certain species of poxvirus are indistinguishable, whilst others can clearly be delineated from each other (Baxendale 1971; Baxby,1977; Baxby 1982; Carn 1993; Crouch and others 1995). The inability to distinguish between different poxvirus species may be as a result of the relative insensitivity of tests used, but also may reflect the conservation of particular epitopes on the major structural proteins. There are few reports of cross-reactive epitopes between the genera. One example, however, is that it has been shown that convalescent sera from sheep infected with orf virus (a parapoxvirus) cross-react with sheep pox and goat pox (capripoxviruses) antigens (Subba Rao and others 1984). This may be a reflection of the common host species of these viruses.

The squirrel poxvirus was initially classified as a parapoxvirus on the basis of its morphology as visualised by electron microscopy (Scott and others 1981) and, of the clinical pathology found in affected squirrels. Supporting evidence for its classification as a parapoxvirus comes from a recent study of the virus genome (K. Thomas PhD Thesis in preparation). The entire genome, obtained from a virus isolated from a particularly virulent outbreak of disease in Gateshead in 1999, has been cloned. The size of the genome (~150kb) and percentage content of G + C residues (~65%, in contrast to the orthopoxviruses which contain ~36% G + C residues) are both indicative of it being a parapoxvirus. A more detailed phylogenetic examination of genes should confirm this, but will require more information about the DNA sequence of individual genes to be obtained. Sands and others (1984), reported that the squirrel poxvirus was antigenically distinct from two of the known parapoxviruses: orf virus (OV) and bovine papular stomatitis virus (BPS). However, more recently it was reported that of a panel of twenty-seven monoclonal antibodies (Mabs) raised against OV, two were able to recognise the squirrel virus by immunofluorescence staining (Howsawi and others 1998). These two Mabs were thought to recognise separate antigens within OV. One of the Mabs recognised all the parapoxviruses tested, OV, BPS, pseudo-cowpox virus, seal PPV and the squirrelpox virus, whilst the other recognised every virus except the seal PPV. Of the 27 Mabs described, 18 reacted with all four OV isolates tested 19 reacted with both parapoxviruses of cattle (BPS and pseudo-cowpox virus) and 6 reacted with the seal PPV. Taken together, this supports the classification of the virus as a parapoxvirus, but suggests that it is more distantly related to the type species, OV, than the other parapoxviruses.

4. Poxvirus vaccines

Vaccines tend to fall into two categories; either the whole organism (alive or dead) is used, or a sub-unit (ie. a single, or group of, antigens) of the organism is used to produce a protective response in the vaccinates. With the poxviruses no effective sub-unit vaccines have been reported. Furthermore “live” poxviruses have always proved to be more effective than “killed” poxviruses at producing an effective protective immunity. Vaccination normally entails the topical application of the vaccine to broken skin. It would appear that immunity is primarily cell-mediated and that circulating antibody is not necessarily protective. This is presumably because the localised spread of infection is from cell to cell and therefore the viruses are protected from antibodies. Antibodies may help to limit the spread of virus via the

circulation (possibly not relevant for the parapoxviruses) but do not prevent the replication of the virus at the site of infection. The death of the PPV-infected red squirrels is not thought to be a result of a generalised viraemia or because of failure of any virally infected internal organs, but rather because the localised scabby lesions result in an inability to feed properly.

Immunity to infection varies between the poxviruses with some vaccines considered to give life-long protection whilst others require to be boosted. The differences here may be related to whether or not the virus causes a systemic disease as opposed to a localised infection.

The science of vaccinology stems from the work of Edward Jenner who was able to demonstrate that humans could be protected from smallpox by inoculating their skin with cowpox virus, naming the process vaccination. The disease smallpox was caused by the orthopoxvirus, variola, and was certified eradicated from the world by the World Health Organisation in 1980 following a carefully controlled worldwide vaccination program. It probably represents the most successful eradication of a disease by means of vaccination, but its success was possible because there are no wildlife reservoirs and no human carriers of variola virus. Other poxvirus-associated diseases have proved more difficult to eradicate, principally as a result of there being a constant source of infection other than the animals showing clinical signs of disease.

Generally vaccination with one member of a poxvirus genus has been effective at protecting against infection with another member of the same genus. The most obvious example of this is vaccinia virus (or cowpox virus) protecting against variola virus infection, and also against camelpox infection (Hafez and others 1992). Within the capripoxes, vaccination with any one member of the genus generally protects against the other species within the genus (Carn 1993). Traditionally pigeon pox virus was used as the vaccine to protect commercial chicken flocks against fowl pox virus (Tripathy and Hanson 1975), but turkey pox virus is equally, if not more, effective (Baxendale 1971). Protection against myxomatosis can be obtained by vaccinating with shope fibroma virus, another, less pathogenic member, of the leporipoxvirus genus (Barcena and others 2000).

The situation is less clear with the parapoxviruses. No studies have been reported where one member of the genus has been used to protect against infection with another member of the genus. In the examples given above the viruses either have a broad host range or at least are capable of infecting the host species where protection is being sought. In the parapoxviruses, apart from the two cattle viruses, it is thought that the different virus species cannot naturally infect the target hosts of the other virus species in the genus. Sands and others (1984) did inoculate lambs with the squirrel PPV, but at no time did the animals appear unwell and no lesions developed at the site of infection, although the animals did produce an antibody response to the virus. Nevertheless an orf virus vaccine, derived from an actual clinical case of orf in sheep, has been used successfully for over 70 years. This vaccine, however, may contribute to the environmental pool of the virus as it is based on a fully virulent isolate.

Cross-protection is generally not found between the genera. Inoculation with vaccinia virus does not protect lambs against subsequent infection with orf virus, the reciprocal test also being true (Robinson and Mercer 1988). Similarly the virus which causes contagious ecthyma in camels (a parapoxvirus) does not protect the camels from camelpox virus, an orthopoxvirus (Hafez and others 1992).

Heterologous vaccines (using one member of a genus to protect against another member) are effective, but generally not as effective as using the homologous virus as a vaccine. This is true for camel pox, fowl pox, lumpy skin disease and myxomatosis amongst others. It is certainly true for orf, where no heterologous vaccine has been found so far. However, the major drawback in using the homologous virus is the potential for producing pathology. One way round this would be to use inactivated virus, but studies have shown that such viruses do not offer good protection.

The alternative to using an inactivated virus is to use an avirulent or attenuated strain of virus. Such strains of virus can be obtained by adaptation to and subsequent growth in cell culture *in vitro*. During adaptation and growth *in vitro* the genes which dictate the virulence of the virus tend to be mutated or even deleted from the virus genome because their expression *in vitro* is non-essential for the growth of the virus. When such viruses are inoculated into the host, they tend to cause little or no pathology, but still offer some degree of protection against subsequent challenge with the wild-type virus. Such tissue culture grown viruses have been used as vaccines against camel pox (Hafez and others 1992), fowl pox (Baxendale 1971; Tripathy and Hanson 1975), the capripoxes (Carn 1993) and orf (Pye 1990), the degree of protection offered being related to the degree of attenuation of the virus. This, however, has been a problem for researchers trying to find an effective vaccine against myxomatosis. Cell culture passage-attenuated strains have been developed (McKercher and Saito 1964), but some show residual pathogenicity for young rabbits, have reverted to virulence or have lost the ability to disseminate amongst rabbits by contact or arthropod vectored transmission (Barcena and others 2000). As a result researchers were keen to find a naturally attenuated field isolate that could confer protection against myxomatosis. They also argued that since vaccination of a wild animal population was being sought a vaccine candidate with a limited capacity for horizontal transmission would be appropriate. The first field trial of such a vaccine has now been reported, with 100% of the vaccinated and 50% of the uninoculated in-contact rabbits producing antibodies to the vaccine (Torres and others 2001).

Another approach to obtaining an attenuated virus has been to remove certain, mainly virulence, genes from the viral genome using recombinant DNA techniques. This has arisen out of the desire to use the poxviruses as vectors for the expression of protective antigens from pathogens where sub-unit vaccines have been shown to be effective (Kaplan 1989; Baxby and Paoletti 1992). It had been recognised that different strains of vaccinia virus varied in their virulence and in their efficacy as a vaccine against smallpox. The variation between the strains stemming from the complement of intact virulence genes present in their genomes. Thus it was argued that the judicious removal of particular genes would result in a virus that could still be used as a vaccine, but would not be a problem in terms of pathogenicity (Tartaglia and others 1992). Although developed for heterologous vaccination the principle should also hold true for homologous vaccination.

5. Other relevant factors for squirrelpox virus vaccine development

Although the probable involvement of the PPV in the decline of the red squirrels has been hinted at for a number of years it is only in the last five years that an intensive study of the actual virus has been undertaken. There are still many unanswered questions which may become relevant when considering the development of a vaccine.

Little is known about the epidemiology or transmission of the virus. Is the grey squirrel the only reservoir host for the virus? A study to try and answer this is currently being undertaken. How is the virus transmitted amongst the greys and indeed transmitted to the reds? Is direct contact between the reds and greys required for transmission or can the virus survive on fomites for any length of time, or are biting arthropods important in transmission? Once infected is transmission amongst the reds more important than between greys and reds? Is there a period when reds are more susceptible to infection than at other times?

There are also a number of questions relating to a vaccine candidate. How avirulent must a potential vaccine candidate be to allow survival of vaccinated reds in the wild? There is some evidence that given the right conditions red squirrels can survive the PPV infection (Jackson 1998; Tomkins and others 2002). How would a potential vaccine be delivered given that poxvirus vaccines tend to be applied individually with mechanical insult of the skin? Would a vaccine give life long immunity or have to be boosted? Would it be appropriate to vaccinate reds, greys or both? Would a vaccine have any deleterious affect on any other wildlife species? This would obviously have to be answered before any license for general release of the vaccine would be granted. It may be that in the first instance it would be appropriate to vaccinate reds that were being reintroduced to localities where the red population was already extinct. Such translocation experiments have failed in the past because the reds released into the wild appeared to rapidly succumb to the PPV disease.

6. Summary

- It is possible to vaccinate against poxvirus disease. Therefore it should be possible to develop a candidate vaccine against the squirrel PPV.
- The candidate vaccine is likely to be a “live” poxvirus, as no inactivated poxvirus vaccines and no subunit vaccines have ever been used effectively against a poxvirus infection.
- The candidate vaccine is unlikely to be a heterologous virus as there is no evidence that poxviruses from the other genera will protect against infection with a parapoxvirus. Equally there is no evidence to support the view that vaccination with another parapoxvirus is likely to be effective in protecting against the squirrel PPV.
- The candidate vaccine is most likely to be an avirulent or partially attenuated strain of the squirrel PPV.
- Delivery of a vaccine in the first instance may be more effective if it is targeted to particular at risk populations.

7. Developmental stages in the production of a vaccine candidate

7.1 Heterologous vaccination

Although most of the available evidence would suggest that heterologous vaccination is unlikely to succeed it would seem appropriate in the first instance to confirm that this route of vaccination would not offer protection to the red squirrels. This could be tested by using another parapoxvirus, eg orf virus, and /or an orthopoxvirus, eg vaccina virus, in *in vivo* protection studies.

7.2 Homologous vaccination with a modified squirrelpox vaccine

This approach would concentrate on the production of an attenuated virus, either by adaptation and growth *in vitro* or by direct modification of the viral genome. Direct modification of the genome would require prior knowledge of the putative virulence genes contained therein and so would require specific sequence data to be obtained before virulence genes could be removed.

7.3 Production of an attenuated virus by *in vitro* passage

This approach is entirely dependent on chance in so far as the deletion of virulence determinants cannot be controlled. It is very likely that by serial passage of the virus in culture we could obtain an attenuated virus, but experience with both orf virus and vaccinia virus has shown that it is possible to obtain a virus that is so weakened by passage *in vitro* that it does not offer good protection in the field.

7.4 Production of an attenuated virus by recombinant DNA technology

By comparison with other poxvirus genomes it should be possible to identify those genes that are likely to encode virulence factors. Thus it should be possible to closely control the deletion of virulence determinants from the viral genome in comparison to that achieved by *in vitro* passage alone. The main drawback of this technique is the cost, not only in terms of the production of a vaccine, but also in terms of the downstream testing and registration of such a "recombinant" vaccine.

Whichever approach is taken it is probable that more than one candidate vaccine would have to be developed as there is no way of predicting either the virulence or efficacy of protection of a modified virus prior to use. Thus any vaccine candidates would firstly be tested for virulence in red squirrels prior to challenge experiments. It is likely that not all the vaccine candidates would progress to the challenge experiment phase. As well as the production of a vaccine, information about the epidemiology and transmission of the wild type virus should be gathered if a successful strategy for vaccinating the reds is to be developed. This should inform us of how and when would be most appropriate for delivery of the vaccine.

Testing the safety and efficacy of a vaccine in red squirrels at an experimental level is only the first step in developing a vaccine that could be used in the field. Any vaccine that was developed would be subject to statutory control by the Veterinary Medicines Directorate. Thus a considerable amount of data, not only on the safety and efficacy of the vaccine in the target species, but also on the possible dissemination of the vaccine in the environment and possible effects on other wild life species, together with data on the actual production of the vaccine and the manufacturing process, would have to be collected before permission to test the vaccine in the field was granted. Testing in the field at two or three specific locations could be done under an Animal Test Certificate (ATC), but if the results from such field studies supported the wider use of the vaccine across the UK then a full marketing authorisation is likely to be required. Such an authorisation could have considerable cost implications depending on the type of vaccine developed for use. However there may be some flexibility within the system which would allow the vaccine to be used under a provisional marketing authorisation (a cheaper option) or even an extension of the ATC,

since the development of such a vaccine for red squirrels would obviously be financially non-profitable.

8. Timescales and costs

The following timescales and costs are based on taking the approach of developing a vaccine by recombinant DNA techniques and therefore represent the more expensive approach. We estimate that in order to make progress in as fast a timeframe as possible it would be appropriate to employ both a post-doctoral virologist and a post-doctoral biologist, together with appropriate technical support. We would envisage that the vaccine development work would run in parallel with the epidemiology / transmission studies for at least three years, after which time the efficacy of any vaccine candidates would be tested. This process is likely to take a further two to three years, but would require only one post-doctoral researcher with appropriate technical support. During this second phase of the work the data required for any subsequent application for an Animal Test Certificate should be collected. An indication of the estimated costs using the midpoint of the BBSRC salary scales for a Band 6 Post-doctoral researcher and a Band 7 Technical support position, is given below;

Post	Salary + NI / Year	Overheads (55%)	Consumable costs/year	Total/Year	Three year* total
Post-doctoral scientist (x2)	£53,900	£29,600	£20,000	£103,500	£316,800
Technical support	£20,825	£11,450	-	£32,275	£98,790
					£415,590

* Includes a 3% inflationary increase for years 2 and 3.

Thereafter the costs for each subsequent year, based on one post-doctoral researcher and a technical support position would be approximately **£90,000/year**.

Application to the Veterinary Medicines Directorate for an Animal Test Certificate would be approximately **£ 275** whilst application for a marketing authorisation will range from approximately **£ 4,000** to **£ 20,000**. At this stage it is not possible to give a properly costed estimate of any field trials which might be required.

The development of a vaccine based entirely on the adaptation of the wild-type virus to cell culture and its subsequent *in vitro* propagation would be a less expensive alternative to the recombinant approach costed above. The chances of success using this approach alone however may be less than that expected from the recombinant route. Nevertheless it should be possible to produce candidate vaccines for field trial by this approach, over the same rough timeframe as the recombinant approach if not sooner. Our estimate would be that in this instance it would be appropriate to employ one post-doctoral researcher with technical support. Since much of the data needed on the epidemiology and transmission of the virus in the wild will be the same, the timeframe required will be approximately the same as that outlined above. Thus using the same costings as above we estimate that this approach would cost approximately **£90,000** per year. Any subsequent testing and registration through the Veterinary Medicines Directorate should also be less costly due to the fact that the vaccine would not be considered as recombinant.

As with all scientific research no guarantees can be given as to the likelihood of success. We have undertaken this feasibility study because we believe it should be possible to develop a vaccine which would help prevent the decline of the red squirrel in the UK. What is certain is that there appears to be no quick or easy fix and that considerable time and resources will have to be spent if we are to protect the red squirrels from PPV-associated disease.

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