Stoat zona pellucida genes with potential for immunocontraceptive biocontrol in New Zealand

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ABSTRACT

Prospective methods for control of stoats (*Mustela erminea*) and other vertebrate pests in New Zealand include use of virally-vectored immunocontraception (VVIC) as a tool for broad-scale suppression of their reproduction. In this report, we provide the complete stoat and ferret (*M. putorius furo*) zona pellucida C (ZPC) and stoat ZPB complementary DNA (cDNA) and protein sequences, together with partial coding sequence for putative stoat ZPA protein. Stoat ZPC protein expressed in a vaccinia-based system was specifically recognised by rabbit anti-porcine whole zona pellucida immune serum. We suggest that primer sequences used in this study could be used to isolate zona pellucida–coding sequences from other mustelid species. We also review prospective viral vectors for antigen delivery to stoats, and conclude that canine adenovirus-1 warrants further examination for its suitability for use.

Keywords: mustelid, stoat, *Mustela erminea*, immunocontraception, adenovirus, zona pellucida, New Zealand

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1. Introduction

With the benefit of hindsight, the establishment of several mustelid species—ferrets (*Mustela putorius furo*), stoats (*M. erminea)* and weasels (*M. nivalis)*—in New Zealand to control European rabbits (*Oryctolagus cuniculus*; see Long 2003) seems badly misdirected. While the intended prey of these predators remain abundant in New Zealand, stoats, weasels and ferrets now impact severely on the endemic biota of the country, especially on iconic birds such as the kiwi (*Apteryx* spp.) and kaka (*Nestor meridionalis*) (McLennan et al. 1996; Wilson et al. 1998).

Significant resources are now directed towards control of mustelids in New Zealand, with the primary focus on development and delivery of improved methods of 'traditional' control such as trapping and poisoning (Murphy & Fechney 2003).

1.1 BACKGROUND

A recurrent problem with broad-scale control of invasive pest animals is the inability of traditional 'one-by-one' methods—even where these eliminate large numbers of individuals—to reduce pest populations below thresholds from which they can rapidly recover by compensatory reproduction and/or survival.

Thus, it seems that long-term 'solutions' to invasive animal problems are likely to require both control of individuals by traditional methods, and implementation of one or more strategies that result in continuing suppression of the reproductive capabilities of remaining individuals in the population.

Many potential approaches to feral animal contraception have been investigated and described. In the current context it is of interest to consider these on the basis of two key parameters, namely, the mode of action of the proposed contraceptive agent, and the means by which the agent is delivered.

With regard to mode of action, a majority of agents (e.g. GnRH, cabergoline) can be categorised as operating through physiological disruption of the complex network of metabolic interactions associated with successful reproduction. In contrast, it has also been proposed that suppression of reproduction could be achieved through immunological means, e.g. by exposure of animals to antigens that elicit auto-immune reactions directed against proteins essential for successful reproduction. This approach—commonly known as immunocontraception—has been reviewed elsewhere (see Chambers et al. 1999).

The second factor—delivery of the contraceptive agent—is another critical element with respect to control of populations of feral animals. While it is realistic to treat domestic, performance and companion animals individually, it is impossible to access every individual in an invasive population. Under real-life conditions it is, therefore, necessary to disseminate the agent of interest throughout that population, either by 'passive' means, such as delivery by aerial baiting or provision in salt licks, or by 'active' means, such as use of a replicating disseminating organism.

1.2 VIRALLY-VECTORED IMMUNOCONTRACEPTION

Approaches to animal control that aim to combine an immunological response to a reproductive antigen with delivery by a viral infection are commonly known as 'virally-vectored immunocontraception' (VVIC). The rationale for this approach for biological control of mustelids, as well as data from various laboratory models, have been described elsewhere (Chambers et al. 1999; Hinds et al. 2000). In addition to the technical matters that are the focus of this paper, successful use of a VVIC-based approach for control of mustelids in New Zealand would also require resolution of a range of social and policy issues (Fitzgerald et al. 2005).

Previous studies conducted at the Pest Animal Control Cooperative Research Centre (Canberra, Australia) have indicated that the most promising candidate antigen for use in other VVIC contexts (mouse, rabbit) is the zona pellucida C (ZPC) protein, which is a subunit component of the outer glycoprotein matrix of the mammalian oocyte (Wassarman et al. 1999).

The ZPC glycoprotein has been successfully delivered using viral vectors, and shown to be able to immunise and reduce fecundity in mice (Jackson et al. 1998; Hardy et al. 2003; Lloyd et al. 2003) and rabbits (Mackenzie et al. 2006). While other zona pellucida subunits (ZPA, ZPB) are also capable of reducing fecundity when delivered in this manner, their effects in both rabbits and mice appear to be more variable (Kerr et al. 1999; Mackenzie et al. 2006; Ronald Jackson, unpubl. data). On this basis, it is considered that the mustelid ZPC subunit has greatest potential for use as an immunocontraceptive antigen in a reproductive vaccine for mustelids.

1.3 OBJECTIVES

The study reported here aimed to further investigate two of the major technical objectives whose achievement would be essential for use of VVIC in mustelids in New Zealand. We provide a description of several potentially effective infertility-inducing antigens identified from stoats and ferrets. We then present the results of a literature review of viral vectors potentially suitable for delivery of antigen-encoding genes to mustelids.

2. Methods

2.1 ANIMALS AND RNA EXTRACTION

Ovaries were isolated from stoats trapped in New Zealand (kindly supplied by Dr Janine Duckworth, Landcare Research New Zealand), or obtained from domestic ferrets by surgical removal during routine veterinary de-sexing procedures (kindly supplied by Dr Andrew Braid, CSIRO Sustainable Ecosystems, Canberra Australia). Ovaries were stored in RNAlater™ (Qiagen

Pty Ltd, Doncaster, Victoria, Australia) at –80°C. Total RNA was isolated from ovaries using the RNeasy® Mini Protect kit (Qiagen Pty Ltd, Doncaster, Victoria, Australia) as recommended by the manufacturer.

2.2 PCR AMPLIFICATION OF ZONA PELLUCIDA CDNAS

Zona pellucida complementary DNA (cDNA) was synthesised using the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA USA) with the proofreading Advantage™ 2 polymerase mix. All procedures were performed using methods recommended by the manufacturer. First strand syntheses for 5′-RACE and 3′-RACE used 1 µg of total RNA in each reaction; first strand reaction products were stored at –80°C until required.

Gene-specific 5′-RACE oligonucleotides for PCR amplification of stoat ZPA, and stoat and ferret ZPC encoding sequences were designed on the basis of DNA sequences conserved between corresponding ZP cDNA sequences from three carnivores: domestic cat (*Felis catus*) (ZPA: GenBank accession number U05776; ZPC: U05778); domestic dog (*Canis familiaris*) (ZPA: U05779; ZPC: U05780) (Harris et al. 1994); and red fox (*Vulpes vulpes*) (ZPA: AY598031; ZPC: AY598032) (Reubel et al. 2005). The following oligonucleotides were used in the 5′-RACE reactions: ZPA gene specific primer GSP1: GGGTAGGTTTGCAGGATTAAGGCAAGTGGACC and nested primer GSP2: GCTAAGTAGAACTGAGGTAGGCATTTTTCAGAG; ZPC primer GSP1: CCTTTCCCAGGAAGATCAGAGGCCCCACGGTG and nested primer GSP2: TGCTTCTCGGAGCCCCAGTCCTCCTCCAT.

Because neither fox nor dog ZPB homologues were available at the time of the study, we chose to base primer sequence choice on a short region of DNA sequence conserved between the cat (U05777; Harris et al. 1994), rabbit (L12167; Lee et al. 1993) and pig (*Sus scrofa*) (L11000; Yurewicz et al. 1993) ZPB cDNAs. This strategy was based on the assumption that the relatively high DNA sequence conservation between the known zona pellucida genes meant this region was also likely to be conserved among other members of the Carnivora. For PCR amplification of stoat ZPB cDNA, the overlapping nested 5′-RACE oligonucleotides used were: GSP1: CTTGGGTACAGCGTGAGGTCACTGT and nested primer GSP2: AGGTCACTGTGTTTCCATAGTAACA.

For 3′-RACE syntheses, nested mustelid-specific oligonucleotides were deduced from the 5′-untranslated regions of the ZPA, ZPB and ZPC 5′-RACE sequences and used to generate full-length 3′-RACE products. The oligonucleotides used for 3′-RACE reactions were:
ZPA GSP1: GACCTACCTGGCTGATTTGATGATA and nested primer GSP2: GCTGATTTGATGATACATTTGGTCA;
ZPB GSP1: GGTTCTTGGGAGTTTAGGAGGGTCT and nested primer GSP2: GAGTTTAGGAGGGTCTTACGGCCAG;
ZPC GSP1:CCCGGGCGTTACCAGGGGGGTGATGGGAGC and nested primer GSP2: GTGATGGGAGCAGCCATGGACCTGAGCTGT.

PCR products were separated on 1% agarose gel in Tris/Acetate/EDTA electrophoresis buffer and stained with ethidium bromide. DNA fragments were visualised on a UV trans-illuminator and the desired bands excised using a sterile scalpel blade. PCR products were recovered from the agarose slices using the Nucleotrap® Gel Extraction Kit (Clontech Laboratories Inc., Mountain View, CA USA), ligated into pGem®-T Easy (Promega Corporation, Madison, WI USA), and used to transform JM109 competent cells. Colonies containing cloned PCR fragments were grown on LB agar plates containing 100 µg/mL ampicillin, IPTG (0.5 mM) and X-gal (80 µg/mL). White colonies were picked and grown, plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen Pty Ltd, Doncaster, Victoria, Australia), and sequenced using an ABI PRISM® BigDye™ Terminator V3.1 Cycle Sequencing Kit and capillary DNA sequencer (Applied Biosystems, Foster City, CA USA).

2.3 EXPRESSION OF STOAT ZPC

Stoat ZPC cDNA was ligated between the NcoI and EcoRI restriction sites of the vaccinia T7 promoter expression vector pTM1 (Wyatt et al. 1995), such that the start codon of ZPC was in-frame with the translation initiation site of the vector. A transient expression assay was conducted by infecting confluent CV1 cells (ATCC CCL-70) grown in six well plates with vaccinia virus vTF7-3 (which constitutively expresses the T7 polymerase; Fuerst et al. 1986) at a multiplicity of infection of 1 PFU/cell. Infected cells were then transfected with pTM1-stoatZPC using the Effectene® Transfection reagent (Qiagen Pty Ltd, Doncaster, Victoria, Australia) and incubated in Minimal Essential Media at 37°C and 0.5% CO_2 for 24 h. After incubation, the transfected monolayer was recovered and lysed with SDS-PAGE loading buffer. Immuno-blots were performed using procedures described by Hardy et al. (2003), then reacted with polyclonal rabbit anti-whole porcine zona pellucida serum known to cross-react with authentic zona pellucida glycoproteins of many mammalian species. Bound antibodies were detected using horseradish peroxidise labelled goat anti-rabbit IgG (Silenus Laboratories, Melbourne, Australia) and developed using SigmaFast™ DAB with metal enhancer (Sigma-Aldrich, St. Louis, Missouri, USA).

2.4 REVIEW OF POTENTIAL VIRAL VECTORS

Two relatively recent reviews of viral infections of mustelidae are already available (Hinds et al. 2000; McDonald & Larivière 2001). Since the publication of those reviews, two further viruses have been recorded from naturally-occurring infections of mustelids, namely, an astrovirus from mink and a herpesvirus from badgers (*Meles meles*; Banks et al. 2002; Mittelholzer et al. 2003).

3. Results

3.1 ISOLATION AND ANALYSIS OF STOAT ZONA PELLUCIDA CDNA

A partial stoat ZPC cDNA was isolated using the 5′ rapid amplification of cDNA ends (RACE) technique using total RNA from a stoat ovary and nested PCR primers deduced from the carnivore species cat, dog and red fox. Due to the nature of the SMART™ RACE technology, cDNAs are preferentially generated that contain 5′ ends that correspond to the authentic capped end of the mRNA. The 5′ RACE clones analysed contained a short 5′-untranslated region of 16 bases upstream of the first in-frame ATG codon. However, a second ATG codon was observed to lie within a consensus Kozak translation initiation signal (CCATGG) that corresponds to the ZPC start codons of other carnivore species. Therefore, this second in-frame ATG codon was considered most likely to be the authentic start codon, giving a 5′-untranslated sequence of 28 bases.

Based on the 5′ RACE-derived ZPC clone DNA sequence, a further set of mustelid-specific nested primers were designed for use with 3′-RACE. Using these primers, full-length 3′ RACE cDNA clones containing the entire ZPC coding region and polyadenylated tail were generated. The cDNA included the TAA stop codon overlapping the consensus polyadenylation signal and a short 3′-noncoding region upstream of the polyadenylation tract. A complete stoat ZPC cDNA sequence of 1348 bp, including the 5′ and 3′ untranslated regions has been deposited in the GenBank database, where it has been assigned accession number AY648050.

The deduced coding sequence of the stoat ZPC gene was 1281 bp in length, encoding a 426 amino acid residue protein with a calculated molecular mass of 46.9 kDa. Like other mammalian ZP proteins (Wassarman et al. 1999), the stoat ZPC contains a potential hydrophobic N-terminal signal peptide and a C-terminal transmembrane anchor domain, a ‘ZP domain’ (amino acids 196–237), N-linked glycosylation sites (amino acids 123, 145, 270) and an arginine-rich domain (amino acids 330–352) containing a putative furin cleavage site (amino acids 349–352).

Alignment of the stoat ZPC cDNA nucleotide sequence with others from the GenBank database showed that it is most closely related to sequences from other members of the Order Carnivora, and that it has relatively high sequence similarity to ZPC cDNAs from dog (87%), red fox (87%) and cat (85%). A comparison of deduced amino acid sequences (Fig. 1) showed that the stoat ZPC protein is closely related to ZPC proteins of other carnivore species with lower levels of relationship to those of pig, human (*Homo sapiens*) and mouse (*Mus musculus*) (Table 1).

TABLE 1. PERCENTAGE AMINO ACID IDENTITY/SIMILARITY (ALLOWING FOR FUNCTIONALLY CONSERVATIVE SUBSTITUTIONS) BETWEEN THE STOAT ZONA PELLUCIDA SUBUNITS AND SELECTED REPRESENTATIVE SPECIES.

STOAT	DOMESTIC FERRET	DOMESTIC DOG	RED FOX	DOMESTIC CAT	PIG	HUMAN	MOUSE
ZPA*	NA	69/80	66/78	67/77	56/68	55/69	44/63
ZPB	NA	80/84*	NA	72/80	67/76	68/77	44/61
ZPC	98/98	84/90	83/89	78/85	75/83	71/80	64/75

* Denotes 'strongly conserved amino acid groups'.

Using the same primers and procedures used with the stoat-derived materials, a ZPC-encoding cDNA was also isolated from domestic ferret ovarian tissue. A complete domestic ferret ZPC cDNA sequence of 1339 bp, including the 5′ and 3′ untranslated regions, has been deposited in the GenBank database, where it has been assigned accession number AY702973. The ferret sequence is 1281 bp in length, and encodes a 426 amino acid product (Fig. 1). The encoded stoat and ferret sequences show 98% amino acid identity.

The same 5′ and 3′ RACE methodologies used to isolate ZPC cDNAs were used to isolate stoat ZPA and ZPB cDNAs. For stoat ZPA, a 1040 bp 5′-RACE clone was isolated and sequenced (accession AY779765), while for ZPB, a 680 bp 5′-RACE fragment was recovered and characterised. Full-length stoat ZPA and ZPB clones of approximately 2.1 kb and 1.8 kb, respectively, were subsequently isolated using mustelid-specific 3′-RACE primers (see section 2). The clones were ligated into pGem®-T Easy, and termini were sequenced to confirm that both the 5′ and 3′ ends of the coding sequence were present. Taken together with the observed lengths, we conclude that these represent full-length clones. The complete stoat ZPB cDNA (accession AY779766) contains an open reading frame of 1668 bp encoding a 555 amino acid product with a calculated molecular mass of 61.6 kDa. The stoat ZPA and ZPB cDNA sequences showed high nucleotide identity to other species from the Carnivora; stoat ZPA showed 85% identity to both of the corresponding dog and fox sequences and 87% to that of domestic cat, while the complete stoat ZPB cDNA sequence showed 85% identity with that of cat and 88% identity to the partial dog ZPB sequences. Amino acid conservation of deduced stoat zona pellucida proteins to selected mammalian ZPA and ZPB proteins (Table 1) are shown in Figs 2 and 3.

3.2 EXPRESSION OF STOAT ZPC IN CELL CULTURE

To confirm that the isolated stoat ZPC cDNA encoded an authentic zona pellucida protein, it was transiently expressed in mammalian cell culture and immunological cross-reactivity to known zona pellucida was confirmed. Immuno-blotting of an aliquot of the cell lysate using rabbit anti-whole porcine ZP sera demonstrated that the transfected cells expressed a highly immuno-reactive glycosylated ZP protein with a molecular mass of approximately 60–70 kDa (Fig. 4). During expression, the mammalian zona pellucida proteins are extensively modified and secreted as heterogeneously sized glycoforms

Figure 1. ClustalX (Jeanmougin et al. 1998) alignment of the deduced stoat and ferret ZPC amino acid sequences (bold) to the ZPC proteins of domestic dog, red fox, domestic cat, domestic pig, human and laboratory mouse obtained from the Genbank protein database. Shown within the alignment are gaps (-) introduced to maximise the alignment; and below are the positions of fully conserved amino acid residues (*), 'strongly' conserved amino acid groups (:) and 'weaker' conserved amino acid group (.) substitutions.

```
Stoat       MDLSCGVFICFLLLGGTELCYSQTIWSGE--TSSPLPSRPP-VVVECLEAQLVVTVSKDL
Ferret      MDLSCGVFICFLLLGGTELCYPQTIWSRE--TSSPLPSRPP-VVVECLEAQLVVTVSKDL
Dog         MGLSYGIFICFLLLGGMELCCPQTIWPTE--TYYPLTSRPP-VMVDCLESQLVVTVSKDL
Fox         MGLSYGIFIRFLLLGGMELCCPQTIWPTE--TYYPLTSRPP-VMVDCLESQLVVTVSKDL
Cat         MGLSYGLFICFLLWAGTGLCYPPTTTEDK--THPSLPSSPS-VVVECRHAWLVVNVSKNL
Pig         MAPSWRFFVCFLLWGGTELCSPQPVWQDE--GQRLRPSKPPTVMVECQEAQLVVIVSKDL
Human       MELSYRLFICLLLWGSTELCYPQPLWLLQGGASHPETSVQP-VLVECQEATLMVMVSKDL
Mouse       MASSYFLFLCLLLCGGPELCNSQTLWLLPGGTPTPVGSSSP-VKVECLEAELVVTVSRDL
            *  *  .*: :** ..  ** . .             *  . * *:* .: *:* **::*

Stoat       FGTGKLIRPADLILGPENCEPLVSADMEDVVRFEVGLHECGNGVQVTDSALVYTTFLLHS
Ferret      FGTGKLVRPADLTLGPENCEPLVSADMEDVVRFEVGLHECGNRVQVTDSALVYTTFLLHS
Dog         FGTGKLIRPADLTLGPENCEPLVSMDTDDVVRFEVGLHECGSRVQVTDNALVYSTFLIHS
Fox         FGTGKLIRPADLTLGPENCEPLASMDTDDVVRFEVGLHECGSRVQVTDNALVYSTFLIHS
Cat         FGTGRLVRPADLTLWPENCEPLISGDSDDTVRFEVELHKCGNSVQVTEDALVYSTFLLHN
Pig         FGTGKLIRPADLSLGPAKCEPLVSQDTDAVVRFEVGLHECG-SLQVTDVALVYSTFLRHD
Human       FGTGKLIRAADLTLGPEACEPLVSMDTEDVVRFEVGLHECGNSMQVTDDALVYSTFLLHD
Mouse       FGTGKLVQPGDLTLGSEGCQPRVSVDT-DVVRFNAQLHECSSRVQMTKDALVYSTFLLHD
            ****:*::..** * .  *:*  * *   .***:. **:*.  :*:*. ****:*** *.

Stoat       PRPAGNLSILRTNRAEIPIECHYPRHRNVSSQAILPTWVPFRTTMLSEEKLVFSLRLMEE
Ferret      PRPVGKLSILRTNRAEIPIECHYPRHRNVSSQAILPTWVPFRTTMLSEEKLVFSLRLMEE
Dog         PRPAGNLSILRTNRAEVPIECHYPRHSNVSSQAILPTWVPFRTTMLFEEKLVFSLRLMEE
Fox         PRPAGNLSILRTNRAEVPIECHYPRHSNVSSQAILPTWVPFRTTMLFEEKLVFSLRLMEE
Cat         PRPMGNLSILRTNRAEVPIECRYPRHSNVSSEAILPTWVPFRTTMLSEEKLAFSLRLMEE
Pig         PRPAGNLSILRTNRAEVPIECHYPRQGNVSSWAILPTWVPFRTTGVSEEKLVFSLRLMEE
Human       PRPVGNLSIVRTNRAEIPIECRYPRQGNVSSQAILPTWLPFRTTVFSEEKLTFSLRLMEE
Mouse       PRPVSGLSILRTNRVEVPIECRYPRQGNVSSHPIQPTWVPFRATVSSEEKLAFSLRLMEE
            *** . ***:****.*:****:***: **** .* ***:***:*   ****.********

Stoat       DWGSEKRSPTFQLGDVAYLQAEVHTGSHVPLRLFVDHCVATLT--PDRSVSPRHTIVDFH
Ferret      DWGSEKRSPTFQLGDVAYLQAEVHTGSHVPLRLFVDHCVATLT--PDRSISPRHTIVDFH
Dog         DWGSEKQSPTFQLGDIAHLQAEVHTGSHMPLRLFVDHCVATLT--PDRNAFPHHKIVDFH
Fox         DWGSEKQSPTFQLGDIAHLQAEVHTGSHMPLRLFVDHCVATLI--PDRNAFPHHKIVDFH
Cat         DWGSEKQSPTFQLGDLAHLQAEVHTGRHIPLRLFVDYCVATLT--PDQNASPHHTIVDFH
Pig         NWSAEKMTPTFQLGDRAHLQAQVHTGSHVPLRLFVDHCVATLT--PDWNTSPSHTIVDFH
Human       NWNAEKRSPTFHLGDAAHLQAEIHTGSHVPLRLFVDHCVATPT--PDQNASPYHTIVDFH
Mouse       NWNTEKSAPTFHLGEVAHLQAEVQTGSHLPLQLFVDHCVATPSPLPDPNSSPYHFIVDFH
            :*.:** :***:**: *:***:::** *:**:****:****    ** .  * * *****

Stoat       GCLVDGLSDASSSFKEPRPRPETLQFTVDMFHFANDSRNMIYITCHLKVTLADRVPDQLN
Ferret      GCLVDGLSDASSSFKEPRPRPETLQFTVDMFHFANDSRNMIYITCHLKVTLADRVPDQLN
Dog         GCLVDGLYNSSSAFKAPRPRPETLQFTVDVFHFAKDSRNTIYITCHLKVTPADRVPDQLN
Fox         GCLVDGLYNASSAFKAPRPRPETLQFTVDVFHFAKDSRNTIYITCHLKVTPADRVPDQLN
Cat         GCLVDGLSDASSAFKAPRPRPETLQFTVYTFHFANDPRNMIYITCHLKVTPASRVPDQLN
Pig         GCLVDGLTEASSAFKAPRPGPETLQFTVDVFHFANDSRNTIYITCHLKVTPADRVPDQLN
Human       GCLVDGLTDASSAFKVPRPGPDTLQFTVDVFHFANDSRNMIYITCHLKVTLAEQDPDELN
Mouse       GCLVDGLSESFSAFQVPRPRPETLQFTVDVFHFANSSRNTLYITCHLKVAPANQIPDKLN
            ******* :: *:*: *** *:******  ****:.** :********: *.: **:**

Stoat       KACSFIKSSRRWSPVEGTADICRCCNKGSCGLPGRSRRLSRLERRGRKSASQTRNRRHVT
Ferret      KACSFIKSSRRWSPVEGTADICRCCNKGSCGLPGRSRRLSRLERRGRKSASQTRNRRHVT
Dog         KACSFIKSTKRSYPVEGSADICRCCNKGSCGLPGRSRRLSHLERGWRRSVSHTRNRRHVT
Fox         KACSFIKSTKRWYPVEGSADICRCCNKGSCGLPGRSRRLSHLERGWRKSVSHTRNRRHVT
Cat         KACSFIKSSNRWFPVEGPADICNCCNKGSCGLQGRSWRLSHLDRPWHKMAS--RNRRHVT
Pig         KACSFSKSSNRWSPVEGPAVICRCCHKGQCGTPSLSRKLSMPKRQ-----SAPRSRRHVT
Human       KACSFSKPSNSWFPVEGPADICQCCNKGDCGTPSHSRRQPHVMSQWSRSAS--RNRRHVT
Mouse       KACSFNKTSQSWLPVEGDADICDCCSHGNCSNSSSSQFQIHGPRQWSKLVS--RNRRHVT
            ***** *.:.   **** * ** ** :*.*.  . *             *  *.*****

Stoat       EEAEITVGPLIFLGKAGDPGAEGSTSPHAS--VMLGLGLATVLSLTLATLVLVLSRRRRA
Ferret      EEAEITVGPLIFLGKAGDPGAEGSTSPHAS--VMLGLGLATVLSLTLATLVLVLSRRRRA
Dog         EEAEITVGPLIFLGKASDHGIEGSTSPHTS--VMLGLGLATVVSLTLATIVLVLAKRHRT
Fox         EEAEITVGPLIFLGKASDHGIKGSTSPHTS--VMLGLGLATVVSLTLATIVLVLAKRHST
Cat         EEADITVGPLIFLGKAADRGVEGSTSPHTS--VMVGIGLATVLSLTLATIVLGLARRHHT
Pig         DEADVTVGPLIFLGKTSDHGVEGSTSSPTS--VMVGLGLATVVTLTLATIVLGVARRRRA
Human       EEADVTVGPLIFLDRRGDHEVEQWALPSDTSVVLLGVGLAVVVSLTLTAVILVLTRRCRT
Mouse       DEADVTVGPLIFLGKANDQTVEGWTASAQTS-VALGLGLATVAFLTLAAIVLAVTRKCHS
            :**::********.::  *   :  : .  :  * :*:***.*  ***::::* ::::  :

Stoat       ASRSVICPVSVPQ
Ferret      ASRSVICPVSVPQ
Dog         ASHPVICPASVSQ
Fox         ASHPVICPASVSQ
Cat         ASRPMICPVSASQ
Pig         AAH-LVCPVSASQ
Human       ASH----PVSASE
Mouse       SSY----LVSLPQ
            ::      .* .:
```

Figure 2. ClustalX (Jeanmougin et al. 1998) alignment of the deduced partial stoat ZPA amino acid sequence (bold) to the ZPA proteins of domestic dog, red fox, domestic cat, domestic pig, human and laboratory mouse obtained from the Genbank protein database. Shown within the alignment are gaps (-) introduced to maximise the alignment; and below are the positions of fully conserved amino acid residues (*), 'strongly' conserved amino acid groups (:) and 'weaker' conserved amino acid group (.) substitutions.

```
Dog         MACKQKGDSGSPSSRFSADWSTYRSLSLFFILVTSVNSVGVMQLVNPIFPGTVICHENKM
Fox         MAYKQKGDGGSPSNWFSADWSTYRSLSLFFILVTSVNSVGVMQLVNPIFPGTVICHENRM
Stoat       MACRQKGACGSPSSWFRADWSTYRSLSLFFILVTSVSSTGAFQLGDPVFPGTVICNENRM
Cat         MASRQKGDSGSPSSWFNADWSTYRSLFLLFILVTSVNSIGVLQLVNPVFPGTVTCYETRM
Pig         MACRHRGDSGRPLSWLSASW---RSLLLFFPLVTSVNSIGVNQLVNTAFPGIVTCHENRM
Human       MACRQRGGSWSPSGWFNAGWSTYRSISLFFALVTSGNSIDVSQLVNPAFPGTVTCDEREI
Mouse       MARWQRKASVSSP----CGRSIYRFLSLLFTLVTSVNSVSLPQSENPAFPGTLICDKDEV
            **  ::     .     ..    * : *:* **** .* .  *  :. *** : * : .:

Dog         TVEFPRDLGTKKWHASVVDPFSFELLNCTSILDPEKLTLKAPYETCSRRVLGQHQMAIRL
Fox         TVEFPSDLGTKKWHASVVDPFSFELLNCTSILDPEKLTLKAPYETCSRRVLGQHQMAIRL
Stoat       VVEFPSNLGAEKWHASVVDPTSFKSWNCISILDSEKLTLNVPYETCTKRVHGQHLIAIGL
Cat         AVEFPSDFGTKKWHTSVVDPFSFELLNCTYILDPENLTLKAPYETCTRRTLGQHRMIIRL
Pig         VVEFPRILGTKIQYTSVVDPLGLEMMNCTYVLDPENLTLKAPYEACTKRVRGHHQMTIRL
Human       TVEFPSSPGTKKWHASVVDPLGLDMPNCTYILDPEKLTLRATYDNCTRRVHGGHQMTIRV
Mouse       RIEFSSRFDMEKWNPSVVDTLGSEILNCTYALDLERFVLKFPYETCTIKVVGGYQVNIRV
             :**.   . :   .****. . .  **   ** *.:.*. .*: *: :. * : : * :

Dog         TDNNAASRHKAFMYQISCPVMQTEETHEHAGSTICTKDSMSFTFN-IIPGMADEN---TN
Fox         TDNNAASRHKAFMYQISCPVMQTEETHEHAGSTICTKDSMSFTFN-IIPGMADEN---TN
Stoat       LDNTTALPSTTFIYHIRCPVAQAEETQEHAGSTICTKDSMSFTFN-VIPDMADEN---SD
Cat         KDHNAASRHNSLMYQINCPVMQAEETHEHAGSTICTKDSMSFTFN-VIPGLADEN---TD
Pig         IDDNAALRQEALMYHISCPVMGAEGPDQHSGSTICMKDFMSFTFN-FFPGMADENVKRED
Human       MNNSAALRHGAVMYQFFCPAMQVEETQGLSASTICQKDFMSFSLPRVFSGLADDS---KG
Mouse       GDTTTDVRYKDDMYHFFCPAIQA-ETHEISEIVVCRRDLISFSFPQLFSRLADEN----Q
             : .:       :*:: **.  .  ..  :  .:* :* :**::  .:. :**:.

Dog         P-SGGKWMMEVDD-AKAQNLTLREALMQGYNFLFDS-HRLSVQVSFNATGVTHYMQGNSH
Fox         P-SGGKWMMEVDD-AIAQNLTLREALIQGYNFLFDS-HRLSVQVSFNATGVTHYMQGNSH
Stoat       SKTLLRWILEIGDGAKVQTLTLQEAVTRGYSIFFDEKQKLSIQMPFNAIGVTDYVQGNRH
Cat         IKNPMGWSIEVGDGTKAKTLTLQDVLRQGYNILFDN-HKITFQVSFNATGVTHYMQGNSH
Pig         SKQRMGWSLVVGDGERARTLTFQEAMTQGYNFLIEN-QKMNIQVSFHATGVTRYSQGNSH
Human       TKVQMGWSIEVGDGARAKTLTLPEAMKEGFSLLIDN-HRMTFHVPFNATGVTHYVQGNSH
Mouse       NVSEMGWIVKIGNGTRAHILPLKDAIVQGFNLLIDS-QKVTLHVPANATGIVHYVQESSY
                  * : :.:   .: *.: :.: .*:.::::. :::..::. :* *:. * * . :

Dog         LYTVPLKLIHTSPGQKIILTTRVLCMSDP-VTCNATHMTLTIPEFPGKLQSVRFENTNFR
Fox         LYTVASEAYTHISWEKIILTTRVLCMSDP-LTCNATHMTLTIPEFPGKLQSVRFENRNFA
Stoat       LYTAPLKLIQESTGQKLILTTRVLCISDP-VNCNATHVTVTIPEFPWKLTSVSFENRSFA
Cat         LYMVPLKLIHESLGQKIILTTRVLCMSDA-VTCNATHVTLTIPEFPGKLKSVSSENRNFA
Pig         LYMVPLKLKHVSHGQSLILASQLICVADP-VTCNATHVTLAIPEFPGKLKSVNLGSGNIA
Human       LYMVSLKLTFISPGQKVIFSSQAICAPDP-VTCNATHMTLTIPEFPGKLKSVSFENQNID
Mouse       LYTVQLELLFSTTGQKIVFSSHAICAPDLSVACNATHMTLTIPEFPGKLESVDFGQWSIP
            ** .  :       :.::::::: :* .*  : *****:*::***** ** **   . .:

Dog         VSQLHNHGIDKEELNGLRLHFSKSLLKMNSSEKCLLYQFYL
Fox         VSQLHNHGIDKEELNGLRLHFSKSLLKMNSSEKCLPYQFYL
Stoat       LSQLHDHGIHKEESKGLRLHFSKTLLKIKSSEKCLPYQFYL
Cat         VSQLHNNGIDKEESSGLTLHFSKTLLKMEFSEKCLPYQFYL
Pig         VSQLHKHGIEMETTNGLRLHFNQTLLKTNVSEKCLPHQLYL
Human       VSQLHDNGIDLEATNGMKLHFSKTLLKTKLSEKCLLHQFYL
Mouse       EDQWHANGIDKEATNGLRLNFRKSLLKTKPSEKCPFYQFYL
             .* * :**. *  .*: *:* ::*** : ****  :*:**
```

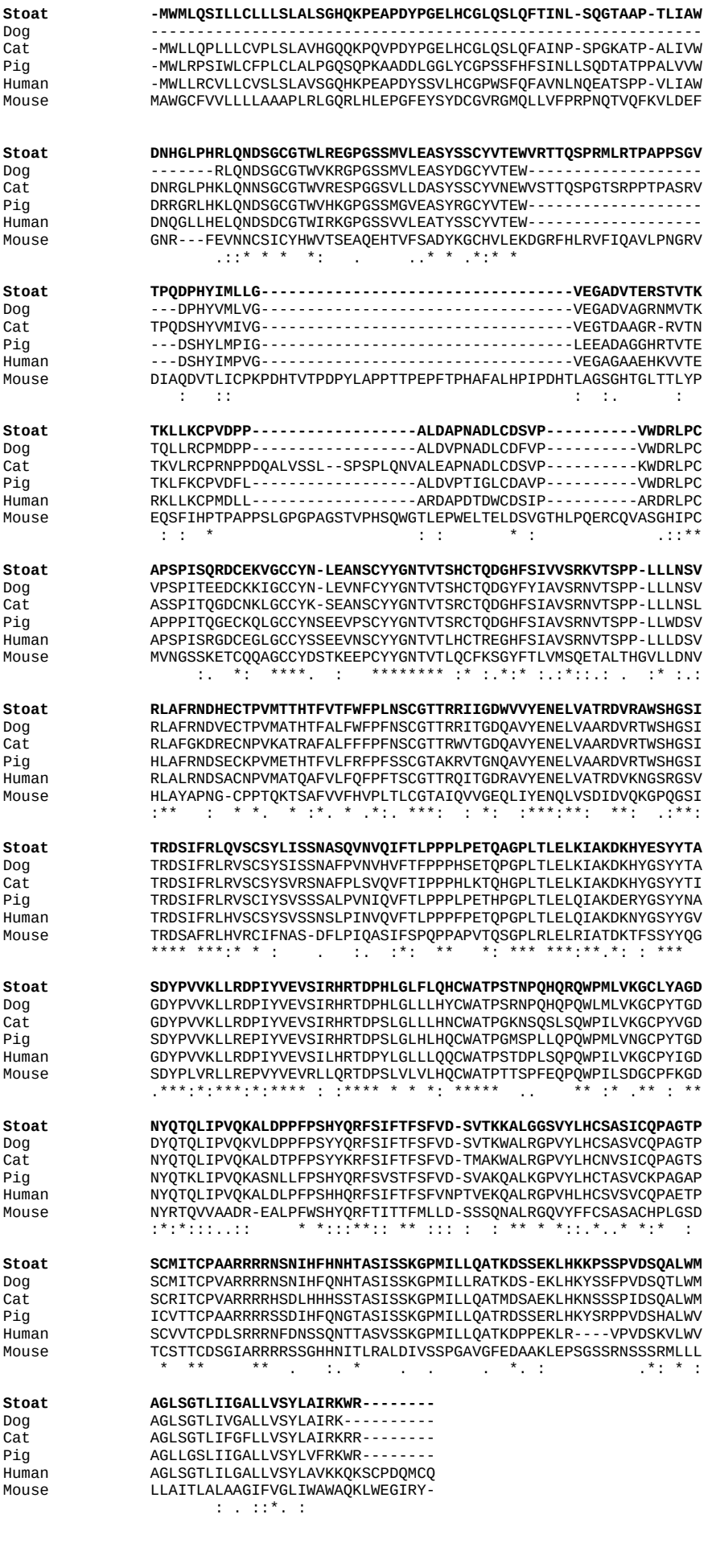

Figure 3. ClustalX (Jeanmougin et al. 1998) alignment of the deduced stoat ZPB amino acid sequence (bold) to the ZPB proteins of domestic dog (partial), domestic cat, domestic pig, human and laboratory mouse obtained from the Genbank protein database. Shown within the alignment are gaps (-) introduced to maximise the alignment; and below are the positions of fully conserved amino acid residues (*), 'strongly' conserved amino acid groups (:) and 'weaker' conserved amino acid group (.) substitutions.

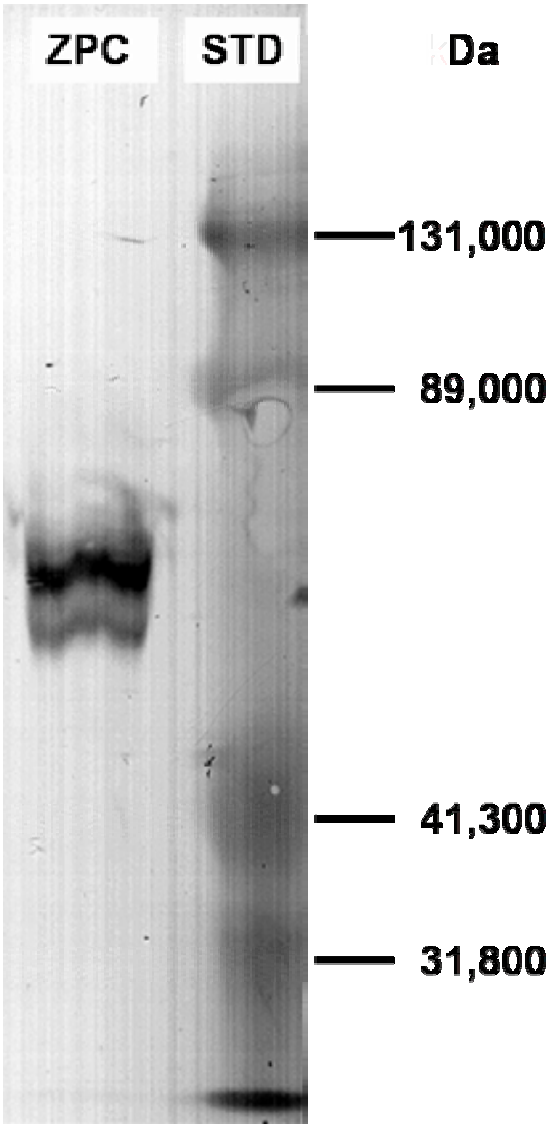

Figure 4. Immuno-blot of transiently expressed stoat ZPC. Lane 1. CV1 cells infected with vaccinia virus vTF7-3 followed by transfection with pTM1-stoatZPC and incubated for 24 hours. The positions of pre-stained protein size standards (kDa) in an adjacent lane are shown.

(Wasserman et al. 1999). The presence of two major immuno-reactive stoat ZPC glycoforms expressed by the transfected CV1 cells is consistent with post-translational modification including signal-peptide cleavage and addition of multiple N- and O-linked oligosaccharides to the 46.9 kDa ZPC primary translation product.

3.3 REVIEW OF VIRAL VECTORS

Table 2 provides a listing of the most important viruses recorded as able to infect mustelids under natural or experimental circumstances, and includes those previously identified (Hinds et al. 2000) as prospectively suitable for use in a VVIC context. Additional comments on factors of likely importance with respect to any such use are provided and, in some cases, elaborated on in section 4.

TABLE 2. MAJOR VIRUSES WHICH CAN INFECT MUSTELIDS.

VIRUS	MUSTELID HOST	COMMENTS
Aleutian mink disease virus (Parvoviridae)	Various, including mink, stoat and ferret	Causes persistent chronic disease in mink, possibly with maternal transmission to kits; also infects ferrets and stoats. Feasible to produce genetically manipulated virus. Global emergence of the canine parvovirus in 1978 from an unknown source, speculated to reflect a host-range shift from a feline-infecting parvovirus, suggests that the risk of cross-species transmission / host range extension of a genetically manipulated form might limit potential use for immunocontraception in mustelids.
Mink enteritis virus (Parvoviridae)	Mink	Other mustelid hosts have apparently not been tested, but are likely to be susceptible. Comments regarding use of genetically modified parvoviruses in an immunocontraceptive context are as above—reinforced in this case by the emergence of the MEV disease in Canada in 1947 (Parrish 1990)—very likely also from a feline virus source.
Aujeszky's disease virus (Suid Herpesvirus 1; pseudorabies) (Herpesviridae)	Mink; probably infects various others	Causes persistent respiratory infection in swine (thought to be its natural host) and produces a usually fatal encephalomyelitis in cattle, sheep, dogs, cats, foxes and mink. Can also infect birds. Feasible to produce genetically manipulated virus, but viral host range would appear to preclude use in mustelid control.
Canine distemper virus (Paramyxoviridae)	Various, including ferrets, weasels, badgers, otters	Feasible to produce genetically-manipulated virus, but the wide host range of the virus (which includes seals) would potentially preclude use for control of mustelids.
Canine hepatitis (Adenoviridae)	Various, including minks, skunks, otters	Feasible to produce genetically-manipulated virus. Host range in Carnivora is limited to canids, ursids and mustelids, of which only the domestic dog and three mustelids are present in NZ. Vaccination is available and routine for domestic dogs. Unmodified virus is reported to cause rapid death in foxes and skunks, but mild symptomology with strong antibody response in mink (see Cabasso 1981). May warrant further investigation for use as an immunocontraceptive vector (see accompanying text).
Mink coronavirus (Coronaviridae)	Mink	Genetically-manipulated coronaviruses have been constructed, but mink coronavirus has apparently never been directly isolated or grown *in vitro*. Many factors relating to its potential for use remain uncertain/unknown.
Bovine rhinotracheitis virus (Herpesviridae)	Mink, ferret	Host range of the virus (which includes domestic cattle) is likely to preclude use for immunocontraception in mustelids.
Mink calicivirus (Caliciviridae)	Mink	Feasible to produce genetically-manipulated virus, prospectively with antigenic epitopes of interest expressed on the capsid coat protein. Host range of the cultivable mink calicivirus strain (probably a member of the genus *Vesivirus*) beyond mink is apparently unknown. A vesivirus isolated from skunks has been shown to be closely related to San Miguel sea-lion virus. Mink calicivirus was previously recorded as a picornavirus of mink (Long et al. 1980).
Influenza virus (Orthomyxoviridae)	Various	Prospective host range of the virus is likely to preclude use for any immunocontraceptive purpose.
Rabies virus (Rhabdoviridae)	Various	Host range of the virus and associated symptomatology preclude use for any purpose.
Vaccinia virus (Poxviridae)	Various	Host range of the virus appears to preclude use for immunocontraception.
Mink astrovirus (Astroviridae)	Mink	No host range assessments have been made, and the virus has not been grown *in vitro*; many factors relating to potential for use in an immunocontraceptive context thus remain uncertain/unknown.
Badger gamma-herpesvirus (Herpesviridae)	Badger	Host range *in vivo* is unknown; *in vitro* tests show the virus grows in a mink lung cell line, but not in 26 other cell lines of vertebrate origin. Natural infection with gamma-herpesviruses is commonly associated with host malignancies, suggesting limited desirability for use in an immunocontraceptive context.

4. Discussion

4.1 IDENTIFICATION OF AN ANTIGEN

This work reports the first sequences of zona pellucida proteins from members of the carnivore family Mustelidae. The ZPC protein of the stoat shows closest identity to the corresponding ZPC protein of the domestic ferret (98%), followed by relationships of 84%, 83% and 78% identity, respectively, with those of dog, fox and cat. The stoat ZPB protein shows close identity to a partial dog ZPB sequence (80% identity; 84% similarity) and the complete cat ZPB (72%; 80%). Partial amino acid sequence of the stoat ZPA protein showed high levels of relationship to the corresponding regions of dog ZPA (69%; 80%), fox ZPA (66%; 78%), and cat ZPA (67%; 77%).

These results are broadly consistent with long-established taxonomic principles that place mustelids, canids and felids as sister families in the Suborder Fissipedia in the Order Carnivora, and which thus separate them on primarily morphological grounds from other taxa for which zona pellucida sequences are now available.

The relatively high conservation of ZPC amino acid identity between the stoat and porcine molecules (75% identity), and the presence of a number of regions of completely conserved residues, suggests that direct immunisation of stoats with porcine-derived material might present a realistic test of the potential of VVIC using stoat ZPC in a yet-to-be-determined vector. Such trials are currently being undertaken by Landcare Research New Zealand.

4.2 IDENTIFICATION OF A VIRAL VECTOR

The brief review of the viruses listed in Table 2 shows that several can be immediately discounted with respect to practical suitability for use as immunocontraceptive vectors, generally on the basis of their recorded host range. For example, while mustelids are apparently susceptible to infection with rabies and pseudorabies, there is no serious prospect of being able to release such agents into the environment in any form. Similarly, there would appear to be cogent arguments against use of any agent whose host range includes humans, irrespective of the severity or otherwise of any disease likely to result. On these grounds, it can be assumed that vaccinia and influenza viruses would not constitute realistic candidates for use.

Some of the other viruses listed have less obvious but similarly important potential cautionary elements associated with any prospective use in genetically manipulated form. Aleutian mink disease virus has a host range that is apparently restricted to mustelids; however, the known 'fragility' of host range determination in parvoviruses (Hueffer & Parrish 2003), coupled with the documented propensity for parvoviruses to undergo host range shifts, might argue against their use in this context. This assessment is supported by the emergence in 1978 of canine parvovirus from a source

that is unknown, but speculated to be a felid-infecting parvovirus (Parrish 1990). In our assessment, the viral agent of greatest prospective interest for use in a stoat-directed VVIC program is canine hepatitis virus (canine adenovirus-1; CAV-1). This virus was previously identified as a candidate vector by Hinds et al. (2000), and appears to possess many characteristics that would justify its further assessment for use in the role (e.g. Woods 2001). While a systematic program of experimentation will be required to rigorously examine this prospect, the series of observations outlined below support this view:

- The host range of CAV-1 for animals present in New Zealand appears, with the exception of dogs, to extend only to stoats, weasels and ferrets. A survey of 64 river otters (*Lontra canadensis*) in New York State (Kimber et al. 2000) did not reveal the presence of antibodies to CAV-1 in sera from sampled animals, despite its presumptive presence in surrounding canid populations and detection of canine herpesvirus antibodies in the sample population, suggesting that host range even within the Family Mustelidae is limited.
- The virus has a global distribution in canids (including in New Zealand), and protection against infection is routinely provided for domestic dogs through canine vaccines.
- The virus itself appears to be able to induce a strong immune response in experimental tests with some mustelids (e.g. mink (*Mustela vison*); see Cabasso, 1981).
- The virus can be grown *in vitro*, and well-established technologies are available for its genetic manipulation. Infectious recombinant virus could potentially be produced in replication-competent forms (i.e. forms able to replicate and transmit from the host; Morrison et al. 2002) and, prospectively, in replication-incompetent forms (i.e. that are able to replicate to a limited extent only, and unable to transmit between hosts; Kremer et al. 2000). While either form could be delivered in edible baits, if an abortive infection by a replication-incompetent virus was sufficient to induce a strong immune response against the reproductive antigen, it might be possible to limit environmental presence of transmissible virus in situations where this was considered desirable.

The selection of a viral vector for any prospective VVIC approach to stoat control would clearly need to satisfy a range of scientific, regulatory and public interest considerations. At this time there are not sufficient data to propose any virus for this purpose. Nevertheless, the known characteristics of canine adenovirus 1 (CAV-1), as discussed above and in Table 2, appear consistent with those required of any prospective candidate agent for this role, suggesting that further studies of its characteristics of infection in mustelids and its interaction with non-target species in New Zealand would be warranted.

5. Conclusions

Work reported here provides the first record of mustelid zona pellucida ovarian proteins with potential utility in immunocontraception of mustelids, and compares their sequences with homologous proteins from other members of the Carnivora. We also extend and update previous reviews of viral agents potentially suitable for use in the context of immunocontraception, and note that canine adenovirus-1 (CAV-1) displays a number of biological characteristics that identify it as a potential candidate agent. We recommend further studies of the interaction of this virus with mustelid pest species in New Zealand.

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