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Suitability of canine herpesvirus as a vector for oral bait vaccination of foxes

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Abstract

Studies were conducted to evaluate the feasibility of using canine herpesvirus (CHV) as a vaccine vector for bait-delivered oral vaccination of wild foxes. To test the viability of CHV in baits, CHV was freeze-dried, incorporated into different baits, stored, and the remaining viral infectivity tested in cell culture after varying periods of time at different storage temperatures. Experimental baits (mouse carcasses) and commercial baits (FOXOFF and PROBAIT) were prepared with either liquid or freeze-dried CHV and tested in two fox trials for their capacity to induce CHV-specific antibodies following oral baiting. Freeze-drying and storage temperatures below 0 °C had a stabilizing effect to virus infectivity. When stored at –20 °C, freeze-dried CHV retained its full infectivity for up to 3 months in PROBAIT baits, the remaining infectivity in FOXOFF baits was 100-fold less. Oral baiting with CHV induced antiviral serum antibodies in all vaccinated foxes (20/20). None of the vaccinated foxes became ill or shed infectious virus into the environment although viral DNA was detected in body secretions as evaluated by PCR. The results indicate that CHV can be freeze-dried and stored over extended periods of time without losing much of its infectivity. This is the first report of CHV being used for oral bait vaccination of foxes. It appears that CHV is well suited for use as a recombinant vector for wild canids.

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1. Introduction

The majority of veterinary antiviral vaccines currently in use for domestic dogs has been developed by the classical laboratory methods of virus attenuation or inactivation. These vaccines have proven to be

safe and highly effective in combating many important viral diseases of domestic dogs such as distemper, parvovirus or rabies (Carmichael, 1999). Rabies vaccines were successfully used also in wild canids, in particular foxes, coyotes and racoons, for which immunization via oral baits is the only practical, large-scale method (Woldehiwet, 2002). However, in addition to the well-known infectious epidemics, wildlife management is likely to face novel challenges in the future, for example, from newly emerging

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diseases such as severe acute respiratory syndrome among various wildlife species in China, Nipah virus epidemics in pigs in Malaysia or recent zoonotic avian influenza cases in South East Asia (Bengis et al., 2004; Daszak et al., 2001; Peiris et al., 2004; Audsley and Tannock, 2004). Development of vaccines against such emerging infections will be time-consuming and costly, especially when conventional methods such as virus attenuation or inactivation are applied.

Overabundance of certain wildlife species is another challenge for wildlife management. Antifertility vaccination (also known as immunocontraception) is currently being developed in an attempt to address problems associated with overabundant wildlife such as foxes in Australia (Reubel et al., 2005). Here, the overabundance of introduced European red foxes poses not only a major threat to the survival of endangered native fauna, but also considerably impacts on lamb production. Antifertility vaccination is particularly appealing for use in foxes because of its humane, non-lethal approach and the potential to reduce the size of pest animal populations by reducing recruitment rather than increasing mortality (for review see (Ferro, 2002)). Research on antifertility vaccines is fundamentally different from the development of antiviral vaccines. The aim of antiviral vaccination is to confer protective immunity against a viral pathogen whereas the intention of antifertility vaccination is to cause an immune response in the vaccinated individual in a way that critical reproductive processes are interrupted. To elicit such immune responses, the antifertility vaccine needs to contain an antigen with contraceptive properties. This can be achieved by either manufacturing the vaccine using appropriate components derived from native proteins or from transgenic proteins that were harvested and purified from bacterial or fungal sources. A more widely used approach is the genetic manipulation of a live vaccine vector in which the antifertility component of the vaccine is supplied, for example, by a genetically engineered virus (Shellam, 1994). Genetic engineering of suitable viral vectors has proven to be a successful method to overcome some of the shortcomings of conventional vaccine development (Jackson et al., 1998). However, of all vaccines currently used in wildlife on a wide geographical scale only one has been developed

using biotechnological genetic engineering. This vaccine is based on a genetically altered vaccinia virus that contains the immunogenic components of rabies virus (Brochier et al., 1991). Unfortunately, the potential of vaccinia virus to indiscriminately infect a wide range of species including humans impedes its general use as a vaccine vector, for example, for applications such as antifertility vaccination of wildlife. It is for this reason that alternative, more species-specific vectors such as canine herpesvirus (CHV) or canine adenovirus (CAAdV) are currently being researched.

The use of live viral vectors to deliver vaccine antigens has already been used for various antifertility vaccine prototypes (Jackson et al., 1998; Redwood et al., 2005; Kerr et al., 1999). A number of biological criteria need to be considered that influence the choice of virus as a vaccine vector (Shellam, 1994; Boyle, 1994). Among them are, for example, the species-specificity of the vector, the ability of the virus to disseminate within a population, the potential of pre-existing population immunity to the vector, the intended route of immunization, and, most critically, the suitability of the vector genome to accommodate additional heterologous DNA without disruption of essential viral gene functions. In the case of wildlife oral vaccination, practical aspects such as the virus stability in baits need also be considered.

Based on serological evidence, European red foxes appear to be susceptible to many viral infections commonly described in domestic dogs (Truyen et al., 1998; Garcelon et al., 1992). With regards to the potential of these viruses as vaccine vectors for wild canids, however, very few appear to be suitable candidates. We have focused our attention on CHV as potential vaccine vector for wild foxes for several reasons: In a previous study we have shown that foxes can be experimentally infected with CHV which caused a long-lasting seropositivity in all foxes (Reubel et al., 2001). Orally infected foxes did not shed CHV into the environment and transmission to in contact foxes was not observed. Although viral DNA was detected at the site of latency, the spinal ganglia of infected foxes, no reactivation of virus could be observed even after high dose corticosteroid treatment (Reubel et al., 2004). In conjunction with this, antibody prevalence in domestic dog populations has been reported to reach up to 88% (Gaskell and

Willoughby, 1999), indicating that the chance of virus transmitting between foxes or from foxes to other canids is low. In contrast to the high seroprevalence in dogs, only a very low percentage of wild foxes that were investigated in Australia (T. Robinson, CSIRO SE, Canberra, Australia, personal communication) and parts of Europe (Truyen et al., 1998) had antibodies to CHV. This lack of population immunity against the vaccine vector may be of critical advantage over other potential canid viruses, such as CA₂V which are common in foxes (Truyen et al., 1998; Garcelon et al., 1992; Riley et al., 2004). Furthermore, it has previously been shown that the CHV genome is capable of accommodating heterologous transgenes from other pathogens such as rabies virus or *Neospora caninum*, and able to confer protective immunity in domestic dogs (Nishikawa et al., 2000; Xuan et al., 1998). Finally, the development of a bacterial artificial chromosome system for CHV has recently been reported (Strive et al., 2005). This system can be expected to greatly facilitate genetic manipulations of the viral genome, enabling quick modification of traits such as insertion of different antigens or removal of essential genes to generate replication defective, non-disseminating viruses.

The aim of the present study was to explore some practical aspects of using CHV as vaccine vector for antifertility vaccination of wild foxes. The laboratory part of the experiments was concerned with the suitability of CHV to be freeze-dried and used in various experimental and commercial baits, and with its storage at different conditions. The CHV-baits were then tested in two fox trials for their potential to induce CHV-specific immunity following oral baiting.

2. Material and methods

2.1. Virus and cell culture

Canine herpesvirus, strain CHV-AUS2, was kindly provided by Prof. Michael Studdert, Department of Veterinary Science, The University of Melbourne, Parkville, Vic., Australia. The use of CHV-AUS2 in fox experiments and some of its molecular characteristics have previously been described (Reubel et al., 2001, 2002). CHV was grown in Madin Darby canine kidney cells (MDCK, American Type Culture

Collection number CCL-34). Cell culture passages 21 and 23 were used for laboratory experiments and bait trials.

2.2. Freeze-drying and storage of CHV

In two preliminary experiments, the stability of freeze-dried CHV (fd-CHV) was compared with that of liquid CHV (l-CHV) at different storage conditions. Potential stabilizing effects of sucrose (Merck, Australia), bovine serum albumin (BSA, Astral Scientific, Australia) and fetal calf serum (FCS, Invitrogen, Australia) on the freeze-drying process was also tested. In experiment A, aliquots of CHV ($10^{7.20}$ tissue cell culture infectious dose 50% per ml, TCID₅₀/ml) were stored either in liquid or freeze-dried form for various periods of time (up to 24 months) at different storage temperatures (37, 20, 4, –20 and –70 °C). CHV was freeze-dried for 24 h in a Flexi-Dry MP bench top lyophilizer (FTS Systems, Stoneridge, NY, USA) according to the manufacturer's instructions. The remaining infectivity of the virus was determined at different time points by titration in MDCK cells. In experiment B, sucrose, BSA or FCS was added to liquid and freeze-dried CHV ($10^{10.20}$ TCID₅₀/ml before freeze-drying) at a final concentration of 10% (w/v) and the change in virus infectivity measured following different storage conditions. The infectivity of samples stored at 4, –20 and –70 °C was determined after 1 month in storage, that of samples stored at 20 and 37 °C after 24 h. Cell cultures were checked daily for cytopathic effects. Virus titres were calculated using the Spearman–Kaerber method (Kaerber, 1931) and expressed as TCID₅₀/ml.

2.2.1. Baits

Two commercially available fox baits were used in the fox bait trials. PROBAIT (kindly provided by Nicky Marlow, Department of Conservation and Land Management, WA, Australia) is a sausage-formed bait made out of dry kangaroo meat. FOXOFF bait (kindly provided by Linton Staples, Applied Biotechnologies, Melbourne, Vic., Australia) is manufactured as a cubicle pressed from dry dog food material. These two baits were used to evaluate the viability of CHV after incorporation into bait material. For the preliminary bait trial, mouse carcasses were also used as vaccine baits. The mice (*mus domesticus*) were bred in the

Animal House of CSIRO Sustainable Ecosystems, Canberra, Australia.

2.2.2. Stability of freeze-dried CHV in baits

To determine the stability of fd-CHV incorporated into baits, 1 ml aliquots of CHV ($10^{5.5}$ TCID₅₀/ml) were frozen at -70°C and freeze-dried for 24 h in a Flexi-Dry MP bench top lyophilizer (FTS Systems) according to the manufacturers instructions. Bait matrix of PROBAIT and FOXOFF baits was cut into 3 mm thick slices, autoclaved, placed into sterile Petri dishes and evenly covered with 100 mg of fd-CHV. The Petri dishes (three replicates for each sample) were stored for 1, 2 or 3 months at different storage temperatures (4, 20 and -20°C). The remaining virus infectivity was then evaluated by titration on MDCK cells. The CHV-covered bait matrix was homogenised, mixed with 5 ml of sterile cell culture media and incubated for 12 h at 4°C . Samples were centrifuged at 3000 rpm for 15 ms, and the supernatant used for titration. One hundred microlitres of supernatant was added in five replicates to MDCK cells in 96-well cell culture plates and incubated for 5 days. Cell cultures were checked daily for cytopathic effects. Virus titres were calculated using the Spearman–Kaerber method (Kaerber, 1931) and expressed as TCID₅₀/ml.

In a preliminary experiment, possible cytotoxicity of PROBAIT and FOXOFF bait matrix for MDCK cells was examined. Baits were cut into sugar cube sized pieces (approximately 2 cm^3), autoclaved and immersed in cell culture media. Samples were incubated for 48 h at 4°C , homogenised, centrifuged and the supernatant used to inoculate cell cultures which were monitored for cytotoxic effects for 5 days.

2.2.3. Foxes

Twenty-four adult wild European red foxes (6 males, 18 females, aged from 1 to 3 years) were selected for the study. The foxes were purchased as cubs from farmers in New South Wales and Victoria and reared in the animal facilities of CSIRO Sustainable Ecosystems in Canberra, Australia. All foxes were vaccinated against canine distemper virus, canine parvovirus and canine parainfluenza virus with a commercially available vaccine (ProtechTMC3, Webster, Australia). Prior to, and after the bait trials, foxes were fed daily with canned and dry dog food and had unrestricted access to drinking water. Foxes were

housed individually in outdoor cages equipped with wooden shelter boxes. The project was approved by CSIRO Sustainable Ecosystems' Animal Ethics Committee (01/02-17).

2.2.4. Fox bait trial 1

In the preliminary trial, FOXOFF baits and mouse carcasses were prepared with 10^6 TCID₅₀ of either l-CHV or fd-CHV and fed to eight female foxes (two foxes per group). FOXOFF baits were prepared by making six indentations into the surface of the bait which were filled with l-CHV, and the bait frozen at -20°C until use in the trial. To apply fd-CHV, FOXOFF baits were rolled in milk powder containing 10^6 TCID₅₀ of the virus and stored at -20°C . Mouse carcass baits were prepared by making six subcutaneous incisions into the rump of the mouse which were filled with milk powder containing fd-CHV. Liquid CHV was applied to the carcasses by subcutaneous injections of virus at six sites. Two control foxes each were given the same dose of fd-CHV, either intranasally or perorally. Foxes were kept in individual cages to allow for verification of bait consumption. To increase the likelihood of foxes eating the baits, foxes were starved 24 h prior to the beginning of the baiting experiment. Foxes received one bait per day on 3 consecutive days. This procedure was repeated after 14 days. For the first 10 days the foxes were monitored daily for signs of clinical disease, and body temperatures and body weights were recorded on a regular base. Blood was collected every 2 weeks and the serum antibody response to CHV determined by Focus-ELISA (FELISA) as described (Reubel et al., 2001). Mouth, nose and eye secretions (one combined swab) and vaginal secretions were collected over a period of 23 days to determine the presence of infectious CHV and viral DNA by virus isolation and PCR. Sterile cotton swabs (Sarstedt, Germany) were used to collect secretions from eye, nose and throat (swab one) and from vaginal or prepuce secretions (swab two). Prior to sampling, swabs were kept in sterile tubes containing 1.0 ml of sterile phosphate buffered saline (PBS). After sampling, the fluid was squeezed out of the cotton and stored at -70°C .

2.2.5. Fox bait trial 2

For the second bait trial, FOXOFF and PROBAIT baits were prepared with 10^6 TCID₅₀ of fd-CHV and

fed to 12 individually kept foxes (6 males and 6 females). Baits were rolled in milk powder containing 10^6 TCID₅₀ of the virus and stored at -20°C . Prior to the start of the actual vaccination trial, foxes were fed “empty” baits (not containing virus) for 3 days to adjust them to eating the baits. During the actual trial, foxes received one bait per day on 3 consecutive days. This procedure was repeated once after 3 weeks. Clinical, virological and serological evaluation was performed as described for the first trial.

2.2.6. Serology

Antibodies to CHV were determined in plasma samples by FELISA as described (Reubel et al., 2001).

2.2.7. Virus isolation

MDCK cells were grown in 48-well microtiter plates and inoculated in duplicate with 30 μl of each swab sample. Inoculated cell cultures were incubated at 37°C and 5% CO₂ for 5 days and checked daily for cytopathic effects. Supernatants of wells with visible

cytopathic effects were harvested and assayed by PCR for the presence of CHV DNA.

2.2.8. PCR

Two different PCR assays were used in the trials. Samples in the first bait trial were analysed by conventional nested PCR for the detection of the CHV thymidine kinase (TK) gene as described (Reubel et al., 2001). For the second bait trial, a real-time PCR was developed and applied. Real-time PCR was performed using a Corbett Rotor Gene 2000 (Corbett Research, Sydney, Australia), and data analysis was performed with the software provided by the manufacturer. Primers for real-time PCR were designed using the web-based version of the LuxTM Designer software (Invitrogen, Melbourne, Australia). The primer pTK-143FU (5'-GGCATTCTGGCGTAT-CATCCT-3' and the fluorogenic reverse primer pTK-175RL (5'-gtacaagCGCAGCTTCCGGTCTTGtAC-3') amplify a 74 nucleotide region within the thymidine kinase region of the CHV genome. PCR reactions were set up according to the supplier's

Table 1

Effect of storage conditions (temperature and time) on the infectivity of liquid and freeze-dried CHV (empty fields = not done)

Storage time (weeks)	Infectivity (TCID ₅₀ /ml) at storage temperature									
	CHV liquid					CHV freeze-dried				
	-70°C	-20°C	4°C	20°C	37°C	-70°C	-20°C	4°C	20°C	37°C
0	7.20	7.20	7.20	7.20	7.20	7.20	7.20	7.20	7.20	7.20
1					0.00					4.45
2					0.00					3.95
3					0.00					2.95
4	6.70	5.45	4.95	0.00	0.00	6.20	5.20	4.70	0.00	2.70
5					0.00					2.45
6										3.45
7										2.95
8	6.45					6.45				
11										2.45
15										2.20
16			3.45					2.45		
19										2.20
20			2.95					2.20		
21										2.20
23										0.00
28	6.45	4.20	2.20			5.70	5.20	2.20		
32			2.20					0.00		
56	6.20	3.45				5.20	5.95			
64	6.20	2.45				5.70	5.70			
80	6.20	3.95				6.20	5.70			
96	6.20	2.45				6.20	5.45			

instructions. Cycle conditions included denaturation for 2 min at 95 °C, 35 repeats of denaturing for 5 s at 95 °C, annealing for 5 s at 95 °C and extension for 10 s at 72 °C, followed by a final extension at 72 °C for 1 min. A plasmid containing one copy of the TK gene, pCMH500 (Strive et al., 2005), was used as quantitation standard. The real-time PCR was able to detect as little as 10 copies of target DNA.

3. Results

3.1. Influence of freeze-drying and additives to the stability of CHV

Cell culture infectivity of both l-CHV and fd-CHV decreased more rapidly at higher storage temperatures (20 and 37 °C) than at 4, –20 and –70 °C (Table 1). When stored at 37 °C, l-CHV lost its infectivity after 1 week, whereas it remained infectious for up to 3 months when stored at 37 °C in a freeze-dried form. Freeze-drying appeared to preserve CHV infectivity also at a storage temperature of –20 °C. At –20 °C, the virus infectivity of l-CHV decreased 100-fold after 28 weeks of storage and another 100-fold by week 96. In contrast, the infectivity of fd-CHV decreased 10-fold by week 28 but then remained stable until the end of the experiment. There was no substantial difference in virus infectivity between l-CHV and fd-CHV that were stored at –70 °C. Both preparations retained most of their infectivity for up to 24 months (10-fold reduction of virus titres).

Freeze-drying generally reduced the infectivity of CHV approximately 10-fold, independently of which

stabilizer was added to the virus solution. Addition of sucrose to CHV before freeze-drying increased the stability of the virus at each of the five storage temperatures. Sucrose was also beneficial to virus infectivity when the virus was stored as a liquid (Table 2).

3.2. Stability of freeze-dried CHV in baits

After 48 h of exposure, visible cytotoxic effects of either bait matrix alone (no virus) on the cell cultures were not observed. There were clear differences, however, between the two baits with regards to their virus inactivating properties over time (Fig. 1). Adsorption of CHV to FOXOFF matrix at 20 °C resulted in complete loss of infectivity as early as 1 month after exposure (100,000-fold reduction of virus infectivity), whereas the same process in PROBAIT caused a 100-fold reduction which did not substantially change after 2 and 3 months. Similarly, when CHV was exposed to FOXOFF for 1 month at 4 °C, virus infectivity was reduced 1000-fold whereas little change in infectivity was noticed after exposure to PROBAIT. Storage at the same temperature for 2 months resulted in a 10,000-fold reduction of infectivity for FOXOFF and a 100-fold reduction for PROBAIT. Three months of exposure to FOXOFF at 4 °C completely destroyed the infectivity of CHV whereas in CHV in PROBAIT baits retained an infectivity of $10^{2.45}$ TCID₅₀/ml (100-fold reduction). Storage at –20 °C was more beneficial to the virus stability in both baits. After 1 and 2 months of contact with FOXOFF, the infectivity of CHV decreased about 10-fold and an additional 10-fold when exposed for a total of 3 months. In contrast, after

Table 2

Effect of sucrose, bovine serum albumin (BSA) and fetal calf serum (FCS) on the storage stability of liquid and freeze-dried CHV

Virus preparation	Additive	Infectivity (TCID ₅₀ /ml) at storage temperature				
		–70 °C	–20 °C	4 °C	20 °C	37 °C
CHV liquid	No additive	7.70	9.20	6.95	8.45	4.95
	Sucrose	10.20	9.95	7.20	8.70	6.70
	BSA	8.70	8.20	7.45	8.45	5.45
	FCS	8.95	7.95	8.20	7.45	6.95
CHV freeze-dried	No additive	6.70	5.70	5.95	6.70	5.20
	Sucrose	9.70	7.45	6.95	8.70	7.20
	BSA	6.45	5.95	5.95	6.70	6.70
	FCS	6.95	6.20	5.45	5.70	5.70

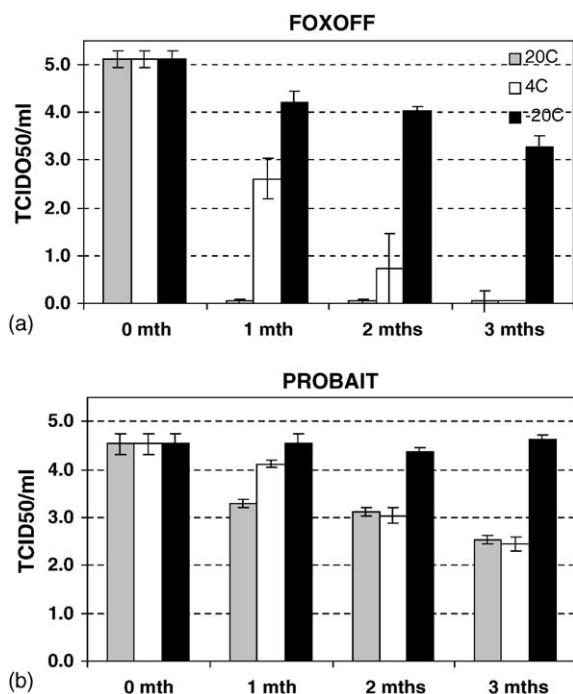


Fig. 1. Tissue culture infectivity of freeze-dried CHV after exposure to (a) FOXOFF and (b) PROBAIT bait matrix over time at different storage temperatures (20, 4 and -20°C). TCID₅₀/ml, tissue culture infectious dose 50% per ml; average of three replicates, standard error mean is indicated by error bars.

exposure to PROBAIT, CHV infectivity remained unchanged over the entire trial period (3 months).

3.3. Fox bait trial 1

3.3.1. Bait acceptance

At the first baiting, foxes appeared to have individual preferences regarding the palatability of baits. Some baits were eaten within the first 24 h after distribution, whilst others were not touched until the second day of baiting. However, each fox eventually ate all the six baits that were offered (Table 3). At the second baiting, every fox ate the bait within 24 h after it was distributed into the cage.

3.3.2. Clinics, virus isolation and PCR

None of the foxes that were fed CHV-spiked baits and none of the control foxes that were manually vaccinated either perorally or intranasally showed clinical signs of illness. The body temperatures and bodyweights of the foxes did not change substantially following exposure to CHV (data not shown). None of the foxes including the controls shed infectious virus in their mouth/nose/eye or vaginal secretions as determined by virus isolation in cell culture. In contrast, CHV DNA was readily detected by PCR for the CHV TK gene in secretions of all baited foxes on several days during the trial period (Table 4). Mouth/

Table 3
Experimental design and results of bait uptake in fox bait trial 1

Fox ID	Sex	Treatment	Baits taken							
			Baiting 1				Baiting 2			
			Day 0	Day 1	Day 2	Total day 3	Day 14	Day 15	Day 16	Total day 16
F0140	F	FOXOFF CHV freeze-dried	No	No	2/3	3/3	Yes	Yes	Yes	12/12
25D14	F		No	Half	2/3	3/3	Yes	Yes	Yes	
3325B	F	FOXOFF CHV liquid	Half	Half	Yes	3/3	Yes	Yes	Yes	12/12
72713	F		No	No	Half	3/3	Yes	Yes	Yes	
5766	F	Mouse carcass CHV freeze-dried	Yes	Yes	Yes	3/3	Yes	Yes	Yes	12/12
14F3B	F		Yes	Yes	Yes	3/3	Yes	Yes	Yes	
45E02	F	Mouse carcass CHV liquid	No	No	3/3	3/3	Yes	Yes	Yes	12/12
37126	F		Yes	Yes	Yes	3/3	Yes	Yes	Yes	
COA58	F	CHV freeze-dried peroral	Control							
80670	F									
61B6C	F	CHV freeze-dried intranasal	Control							
F445A	F									

Table 4

PCR detection of CHV DNA in mouth/nose/eye secretions (A) and genital secretions (B) of foxes after baiting with FOXOFF or mouse baits spiked with freeze-dried or liquid CHV

Fox ID	Sex	Treatment	Days After Baiting																								
			B	B	B												B	B	B								
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
A Mouth/nose/eye secretions																											
F0140	F	FOXOFF	-	-	-	-	-	+	-	-	+	+	-	-	-	-	+	+	ND	ND	-	+	-	+	+		
25D14	F	CHV freeze-dried	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	ND	ND	-	+	+	-	+		
3325B	F	FOXOFF	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	+	ND	ND	-	+	-	-	-		
72713	F	CHV liquid	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	+	-	ND	ND	+	-	-	-		
5766	F	Mouse carcass	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	ND	ND	-	-	+	-	-		
14F3B	F	CHV freeze-dried	-	-	-	+	-	+	-	+	+	-	-	-	-	-	-	-	ND	ND	-	-	+	+	-		
45E02	F	Mouse carcass	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	ND	ND	-	-	+	+	-		
37126	F	CHV liquid	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	ND	ND	-	-	-	-	-		
COA58	F	CHV freeze-dried	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	ND	ND	+	+	+	+	+		
80670	F	Peroral	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	ND	ND	+	+	+	+	+		
61B6C	F	CHV freeze-dried	-	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	+	ND	ND	+	+	+	+		
F445A	F	Intranasal	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	ND	+	+	+	+	-		
B Genital secretions																											
F0140	F	FOXOFF	-	-	-	-	ND	-	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
25D14	F	CHV freeze-dried	-	-	-	-	ND	-	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
3325B	F	FOXOFF	-	-	-	-	ND	-	-	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
72713	F	CHV liquid	-	+	-	+	ND	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
5766	F	Mouse carcass	-	-	+	-	ND	-	-	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
14F3B	F	CHV freeze-dried	-	-	-	-	ND	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
45E02	F	Mouse carcass	-	+	-	-	ND	+	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
37126	F	CHV liquid	-	-	+	-	ND	-	-	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
COA58	F	CHV freeze-dried	-	+	+	-	ND	-	-	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
80670	F	Peroral	-	-	-	-	ND	+	-	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
61B6C	F	CHV freeze-dried	-	-	-	-	ND	+	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
F445A	F	Intranasal	-	+	+	-	ND	-	+	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		

Results for perorally and intranasally inoculated control foxes are also displayed. B, baiting days; +, positive; -, negative; ND, not done.

nose/eye secretions of the perorally and intranasally inoculated control foxes were PCR-positive as early as day 1 post inoculation. Viral DNA was detectable for an extended period of time in all four foxes (up to 14 consecutive days after the first inoculation), and also consistently after the second inoculation. PCR positivity was not as frequent and also not as evenly distributed in the bait-fed foxes compared to the manually inoculated foxes, although some individuals had CHV DNA in their mouth/nose/eye secretions for up to 9 consecutive days after the first baiting. After the second baiting, CHV DNA was only occasionally detected in those animals. In the four manually vaccinated foxes, PCR was positive on average on 19.5 days out of 21 days tested, compared to an average of 7.6 days/21 days tested in the baited foxes. However, the individual PCR positivity of these eight foxes varied considerably (between 3/21 and 13/21). Except for fox 14F3B, CHV DNA was also detected in vaginal secretions taken from baited or manually vaccinated foxes although at a much lower frequency (for logistic reasons samples were taken only during for the first 7 days of the trial).

3.3.3. Serology

Both perorally and both intranasally inoculated control foxes seroconverted 3 weeks after the CHV administration (Fig. 2a). Feeding of CHV-spiked mouse carcass and FOXOFF baits (prepared with liquid and freeze-dried virus) also induced specific anti-CHV serum antibody responses in 7/8 foxes although it was not as strong as that caused by manual peroral and intranasal vaccination of the control foxes (Fig. 2b and c).

3.4. Fox bait trial 2

3.4.1. Bait acceptance

It generally appeared that foxes preferred PRO-BAIT sausage baits over FOXOFF baits (Table 5). This was first observed in the adaptation pre-baiting period with “empty” baits where foxes in the PROBAIT group ate 5/6 baits on the first day, 6/6 on the second and 4/6 on the third. This compares with 1/6 baits on the first day, 1/6 on the second and 1.5/6 on the third day for foxes in the FOXOFF group. However, at the fourth day of pre-baiting all foxes in both groups had eaten all baits. A similar preference

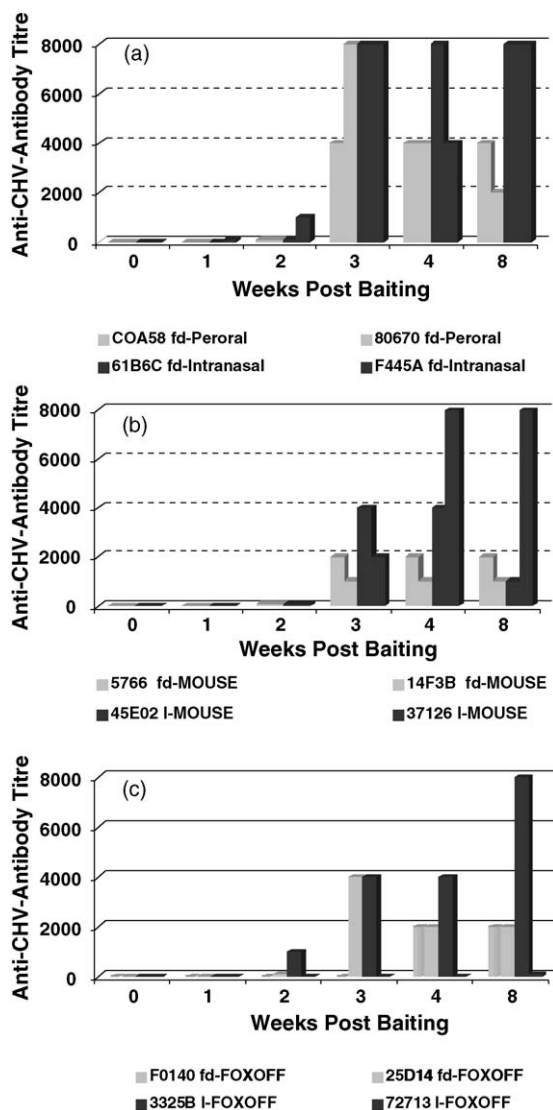


Fig. 2. Anti-CHV serum antibody response in foxes (a) manually inoculated either perorally or intranasally with freeze-dried (fd) or liquid (l) CHV or (b) fed with mouse carcass baits coated with l-CHV or fd-CHV or (c) fed with FOXOFF baits coated with l-CHV or fd-CHV.

for PROBAIT baits was seen when CHV-coated baits were fed. At the end of the first vaccination period, foxes in the PROBAIT group had eaten 18/18 baits compared to 14/18 in the FOXOFF group. At the end of the second baiting period, the numbers were 18/18 for the PROBAIT group and 17/18 for the FOXOFF group.

Table 5
Experimental design and results of bait uptake in fox bait trial 2

Fox ID	Sex	Treatment	Baits taken									
			Pre-baiting			Baiting 1			Baiting 2			
			1	2	3	Day 0	Day 1	Day 2	Day 21	Day 22	Day 23	
24A19	F	PROBAIT	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
83886	F		Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
92313	F		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
53033	M		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
96582	M		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
80036	M		No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Total/day			5/6	6/6	4/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
Total/period					18/18			18/18				18/18
11A6E	F	FOXOFF	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
D4A7C	F		No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
85221	F		Yes	No	Half	No	No	No	Half	No	Yes	Yes
87748	M		No	No	No	Yes	Yes	Yes	Half	Half	Yes	Yes
91093	M		No	No	No	Yes	Yes	Yes	No	Half	Yes	Yes
05682	M		No	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes
Total/day			1/6	1/6	1.5/6	5/6	5/6	4/6	3/6	4/6	6/6	6/6
Total/period					18/18			14/18				17/18

3.4.2. Clinics, virus isolation and PCR

There was no evidence from the average body temperatures or body weights that foxes in either experimental group were clinically affected by eating the CHV-baits (Figs. 3 and 4). However, the body weights and body temperatures of individual foxes fluctuated considerably during the trial period. Infectious CHV was not isolated from mouth/nose/eye secretions (one combined swab) or from genital secretions (second swab) from any of the foxes in either group. However, CHV DNA was detected in secretions of all foxes in the trial when evaluated by real-time PCR (Table 6). Mucosal secretions taken from mouth/nose/eye swabs were positive at several occasions after CHV bait vaccination. A small number of samples (one in each experimental group) was positive for CHV DNA as early as 1 day after the first baiting period. The peak of positive PCR swabs occurred between days 3 and 7 post vaccination, with no substantial difference observed between the two experimental groups. None of the foxes in the FOXOFF group had positive PCR samples during the second baiting period whereas two foxes in the PROBAIT group were again positive on days 21 and 24, respectively. In contrast to the mouth/nose/eye secretions, which were positive for CHV DNA in all

foxes over several days, genital swabs were positive in only 5/12 foxes (3 in the PROBAIT group and 2 in the FOXOFF group), and only infrequently. Five of the six positive swabs originated from male foxes.

3.4.3. Serology

All foxes in both groups, except fox 85221, seroconverted as early as 2 weeks after the first bait vaccination (Fig. 5). Antibody titres increased with time and levelled off around 3–4 weeks post vaccination, reaching average titres of at least 1:6400. A boosting effect by the second vaccination was not observed. Anti-CHV antibody titres were generally higher in foxes that had been fed PROBAIT baits compared to those fed with FOXOFF baits. However, even fox 85221 which had eaten only 1.5 of 6 offered FOXOFF baits and which was the last one to seroconvert (by week 3 post vaccination) maintained an antibody titre of 1:6400 by week 5 post vaccination.

4. Discussion

The aim of the present studies was to investigate some practical aspects of using CHV as a vaccine vector for oral baiting of wild foxes. A variety of

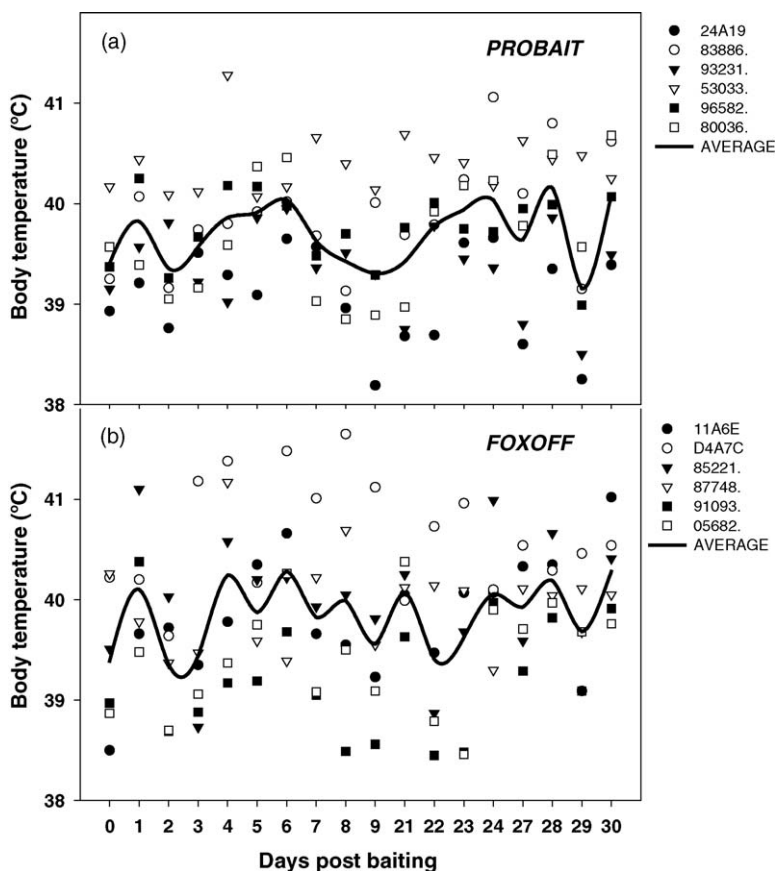


Fig. 3. Body temperatures of foxes following uptake of (a) PROBAIT or (b) FOXOFF baits coated with freeze-dried CHV. Individual foxes are depicted as symbols. Averages are shown as black lines.

biological criteria need to be considered that influence the choice of virus as a vaccine vector (Shellam, 1994; Boyle, 1994). Among them are for example the species-specificity of the vector, the potential of pre-existing population immunity to the vector, and the intended route of immunization. For wildlife vaccines which are usually delivered perorally in baits, the stability of the virus at different environmental conditions and the suitability of the virus for freeze-drying and incorporation into baits are of particular importance. Predictions of the *in vivo* efficacy of live antiviral vaccines can be made by determining the infectivity of the vaccine virus in cell culture *in vitro* (for review see (Terpstra and Kroese, 1996)). We therefore explored in laboratory experiments suitable conditions for freeze-drying of CHV, and how various storage conditions or vaccine additives would affect

the infectivity of the virus. Because CHV had not yet been used in a freeze-dried form for vaccination or as a component of oral baits, we also tested in fox trials whether peroral application of CHV-coated baits could induce antiviral antibody responses in the baited foxes.

Freeze-drying and storage temperatures below 0 °C had a stabilizing effect on the viability of CHV, both of cell culture virus and of CHV incorporated into baits. There was no difference in virus infectivity between fd-CHV and l-CHV after 96 weeks of storage when the samples were kept at –70 °C. However, freeze-drying considerably preserved virus infectivity at the storage temperature of –20 °C, where virus titres of fd-CHV decreased only 100-fold compared to a 10,000-fold decrease for the liquid virus. Similarly, fd-CHV survived at 37 °C for up to 21 weeks whereas the

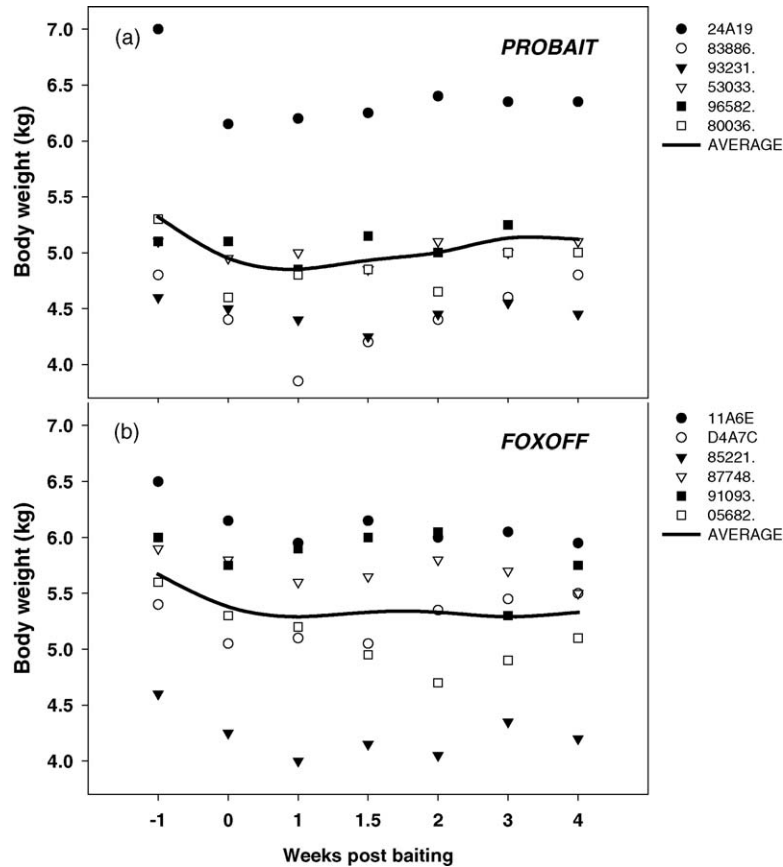


Fig. 4. Body weights of foxes following uptake of uptake of (a) PROBAIT or (b) FOXOFF baits coated with freeze-dried CHV. Individual foxes are depicted as symbols. Averages are shown as black lines.

infectivity of l-CHV was lost after 1 week. The addition of sucrose was also beneficial for the stability of the virus, freeze-dried or liquid. Similar results have been published for other herpesviruses such as varicella zoster or rinderpest virus where both freeze-drying and addition of sugars substantially increased the viability of these viruses (Grose et al., 1981; Harper et al., 1998; Worrall et al., 2000).

Clear differences were observed regarding the influence of bait material on virus infectivity after storage. The matrix of both baits was not toxic to cell cultures. However, exposure of fd-CHV to FOXOFF bait over time was more detrimental to virus infectivity than exposure to PROBAIT bait matrix. The reasons for this difference were not established, but FOXOFF baits appear to have a higher moisture content than PROBAIT baits which may have been a contributing

factor for the quicker virus inactivation in this bait. Temperature and moisture are known to be the most important factors affecting virus survival in the environment (Hurst et al., 1980). It was therefore not surprising that temperatures above 0 °C destroyed the infectivity of fd-CHV in some baits within weeks. However, fd-CHV survived considerably longer in PROBAIT baits at those temperatures than in FOXOFF baits where the virus infectivity was completely lost within 1 month when stored at 20 °C, and within 3 months when stored at 4 °C. In contrast, there was still considerable amounts of infectious virus left in PROBAIT baits that were stored at 4 and 20 °C. Most importantly, fd-CHV did not loose any infectivity when stored at –20 °C in PROBAIT baits for 3 months. These findings demonstrate that CHV can be freeze-dried, incorporated into baits and stored for an extended

Table 6

Detection of CHV DNA by real-time PCR in mouth/nose/eye secretions (A) and genital secretions (B) of foxes after baiting with FOXOFF or PROBAIT baits coated with freeze-dried CHV

Fox ID	Sex	Treatment	Days After Baiting																	
			B	B	B										B	B	B			
			0	1	2	3	4	5	6	7	8	9	10	21	22	23	24	27	30	
A Mouth/nose/eye secretions																				
24A19	F	PROBAIT	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
83886	F		-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	-
92313	F		-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
53033	M		-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
96582	M		-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-
80036	M		-	+	-	+	-	+	+	-	-	-	-	-	+	-	-	+	-	-
B Genital secretions																				
24A19	F	PROBAIT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND
83886	F		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND
92313	F		-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	ND	ND
53033	M		-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	ND	ND
96582	M		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND
80036	M		-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	ND	ND
116AE	F	FOXOFF	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	ND	ND
D4A7C	F		-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	ND	ND
85211	F		-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	ND	ND
87748	M		-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	ND	ND
91093	M		-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	ND	ND
5682	M		-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	ND	ND

B, baiting days; +, positive; -, negative; ND, not done.

period of time without losing much of its infectivity and presumably its potency as a vaccine.

The testing of CHV-baits in foxes resulted in several essential observations although due to the low numbers of foxes per experimental group both trials need to be considered preliminary, and hence

statistical significance cannot be deduced. Using mouse carcasses and FOXOFF baits spiked with liquid or freeze-dried CHV it was determined whether foxes would seroconvert upon consuming these baits. We showed for the first time that perorally or intranasally applied fd-CHV induced antiviral anti-

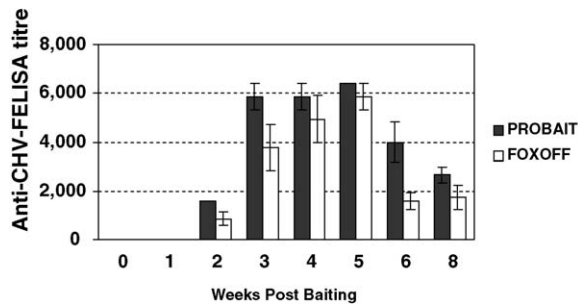


Fig. 5. Anti-CHV antibody titres of foxes following bait vaccination with FOXOFF or PROBAIT baits coated with freeze-dried CHV (mean \pm standard error mean).

body responses in 11/12 foxes. CHV was not pathogenic, and infectious CHV was not shed into the environment. Although manual administration of CHV to control foxes induced higher antibody titres than bait feeding, the results clearly warranted further investigations of CHV administration in baits.

The second fox trial focused on the use of two commercially available baits, FOXOFF and PROBAIT which were coated with fd-CHV. Foxes appeared to have a preference for PROBAIT baits compared to FOXOFF baits which was reflected both in the temporal uptake of the baits and in the overall numbers of baits taken. It was observed that foxes tended to cache FOXOFF baits for possible later consumption whereas PROBAIT baits were usually eaten soon after they were supplied. PROBAIT-baited foxes had higher average anti-CHV antibodies than foxes in the FOXOFF group. This could be explained by a higher intake of infectious CHV in the PROBAIT group which is more likely when the bait is eaten soon after distribution. Exposure of virus to the environment is expected to inactivate the virus over time and thus its potency to induce immune responses will be reduced. As in the first trial, none of the baited foxes became ill or shed infectious virus into the environment although CHV DNA was found in secretions of all foxes. After the first baiting, all mouth/nose/eye secretions were PCR positive for up to 7 days, but genital secretions were only occasionally positive. After the second baiting, viral DNA was detected in secretions of only two foxes. Eleven out of 12 foxes had seroconverted at this time (week 2 post vaccination), and it is likely that newly developed antiviral immunity prevented further viral replication

and viral DNA shedding. Five of the six positive genital swabs were collected from male foxes. Previous studies have shown that systemic virus replication and viremia following oral CHV intake is unlikely (Reubel et al., 2001). It seems rather probable that the PCR positivity of genital swabs was caused by the presence of CHV-containing saliva through grooming and licking. It is noteworthy that fox 85221 ate none of the baits during the first baiting period, but seroconverted by week 3. It could be that by carrying the bait in the mouth for caching and possibly by licking on it, enough of fd-CHV was resorbed through the oral mucosa to cause an antiviral antibody response. Feeding of fd-CHV-coated baits induced anti-CHV antibodies in 12/12 foxes which corroborated the serological findings of the preliminary trial.

This is the first report where CHV was successfully used in a freeze-dried form to orally bait foxes. All baited foxes seroconverted and none became ill. Considering the specificity of CHV to canids, its innocuousness to foxes following oral baiting and the lack of population immunity in this species, CHV appears to have many of the properties that are critically required for a recombinant vaccine vector for wild foxes.

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