

High prevalence of antibodies against canine adenovirus (CAV) type 2 in domestic dog populations in South Africa precludes the use of CAV-based recombinant rabies vaccines[☆]

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A B S T R A C T

Rabies in dogs can be controlled through mass vaccination. Oral vaccination of domestic dogs would be useful in the developing world, where greater vaccination coverage is needed especially in inaccessible areas or places with large numbers of free-roaming dogs. From this perspective, recent research has focused on development of new recombinant vaccines that can be administered orally in a bait to be used as adjunct for parenteral vaccination. One such candidate, a recombinant canine adenovirus type 2 vaccine expressing the rabies virus glycoprotein (CAV2-RG), is considered a promising option for dogs, given host specificity and safety. To assess the potential use of this vaccine in domestic dog populations, we investigated the prevalence of antibodies against canine adenovirus type 2 in South African dogs. Blood was collected from 241 dogs from the Gauteng and KwaZulu-Natal provinces. Sampled dogs had not previously been vaccinated against canine adenovirus type 1 (CAV1) or canine adenovirus type 2 (CAV2). Animals from both provinces had a high percentage of seropositivity (45% and 62%), suggesting that CAV2 circulates extensively among domestic dog populations in South Africa. Given this finding, we evaluated the effect of pre-existing CAV-specific antibodies on the efficacy of the CAV2-RG vaccine delivered via the oral route in dogs. Purpose-bred Beagle dogs, which received prior vaccination against canine parvovirus, canine distemper virus and CAV, were immunized by oral administration of CAV2-RG. After rabies virus (RABV) infection all animals, except one vaccinated dog, developed rabies. This study demonstrated that pre-existing antibodies against CAV, such as naturally occurs in South African dogs, inhibits the development of neutralizing antibodies against RABV when immunized with a CAV-based rabies recombinant vaccine.

Keywords:
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1. Introduction

Rabies is a classical viral zoonosis and one of the oldest infectious diseases known to affect man. The major etiological agent, rabies virus (RABV), is the type species of the *Lyssavirus* genus, family *Rhabdoviridae*, order *Mononegavirales*. In the developing world, particularly on the continents of Africa and Asia, domestic dogs are the principal hosts and vectors of RABV. Although effective

vaccination programs are crucial to the control of rabies in dogs and the prevention of rabies in humans, such programs are largely neglected in developing regions of the world. In Africa, insufficient priority, funding, infrastructure, access to vaccines and other constraints are encountered commonly [1,2]. Rabies vaccination of dogs is through parenteral vaccination with inactivated or live-attenuated vaccines [3–5]. However, the demographic and social structures in many developing countries allow for large numbers of free-roaming dogs (owned or community). Such dogs are often harder to reach during vaccination campaigns. Safe and efficacious oral vaccines could find application as an adjunct to parenteral vaccination and may contribute towards achieving the required vaccination coverage that would ensure herd immunity in such settings [6–8]. In the developed world, oral vaccination remains a critical tool in the control of wildlife rabies [9].

Given the above considerations, efforts in rabies vaccine research were directed to the development of effective oral vaccines. Oral vaccination of wildlife was the main focus in such

Abbreviations: CAV2-RG, recombinant canine adenovirus type 2 vaccine expressing rabies virus glycoprotein; CAV1, canine adenovirus type 1; CAV2, canine adenovirus type 2; RABV, rabies virus.

[☆] The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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programs. Several oral vaccines have been used successfully to vaccinate wildlife in Europe and in North America [10,11]. Concerning the potential application of oral rabies vaccines in dogs, SAG2 and SAD B19 vaccines have been shown to be effective orally in multiple species including dogs, raccoons and skunks [6,12–15]. However vaccine stability in harsh environments has been a concern [16,17]. Such vaccines, in lyophilized form may be prohibitively expensive limiting the potential use in developing countries.

In Europe, SAD-B19 has been used in oral vaccination of foxes but several vaccine-induced rabies cases led to questioning about the safety of traditional attenuated vaccine strains [18,19]. Other SAD derived vaccines such as SAD Berne, ERA-BHK21 and SAD P5/88 have also been implicated in vaccine-associated rabies cases in wildlife as well as domestic animals [20–22]. A recombinant Vaccinia-virus (V-RG) construct, that expresses the RABV glycoprotein, has been used widely and successfully in North America, and parts of Europe to control rabies in wildlife. The potential risk of infection with V-RG in non-target species, including humans remain a concern, particularly when the prevalence of immunodeficiencies, or the medical use of potent immunosuppressives, is high; these are conditions which increase the risk of severe outcomes from vaccinia infections [23,24]. This concern is especially salient in countries where high numbers of the human population are HIV positive. Despite the extensive use of oral vaccination to control the spread of rabies in the developed world, there is a need for more ideal oral rabies vaccines particularly with respect to cost, efficacy, safety and related factors applicable to canine rabies endemic regions of the world [25].

One approach in the search for new and improved oral rabies vaccines was the development of a canine adenovirus type 2 recombinant vaccine that expresses a RABV glycoprotein gene (CAV2-RG). Modified, live CAV2 is already used worldwide with great success and safety in the routine vaccination of dogs against both CAV1 and CAV2 [26,27]. This virus was used as a vector in the construction of a recombinant CAV2 that expresses the RABV glycoprotein [28]. Various studies showed that such constructs were immunogenic in animals through the intramuscular, intranasal and oral routes [28–30]. Oral administration of CAV2-RG to raccoons and skunks also protected these animals against lethal challenge with RABV [25].

Use of canine adenoviruses candidates may pose less of a challenge regarding safety, given their widespread use in veterinary medicine. Canine adenoviruses are double-stranded DNA viruses of the family *Adenoviridae*. CAV1 is the cause of infectious canine hepatitis, through the infection of vascular endothelial cells as well as hepatic and renal parenchymal cells of dogs [31–33]. CAV2 preferentially infects respiratory tract epithelium and to a lesser degree the epithelial cells of the intestinal tract and is associated with mild respiratory disease including tonsillitis, pharyngitis, tracheitis, bronchitis and bronchopneumonia, collectively referred to as infectious tracheobronchitis (ITB) or “kennel cough” [31,32,34]. Due to mucosal and airborne transmission, CAV2 spreads rapidly in dog populations lacking immunity. The antigenic relatedness and cross-reactivity between CAV1 and CAV2 has led to the widespread routine use of modified – live CAV2 vaccine as protection against infection with both CAV1 and CAV2, although little is known about their natural distributions, globally [35].

Recent serological surveys conducted in Thailand found that 9.2% of healthy dogs presented for sterilization at a public veterinary clinic had antibodies against CAV [36]. Serosurveys among wild canids in Scandinavia reported up to 60% seropositivity against CAV in free-roaming wild canids [37]. Aside from the above studies, very little data are available regarding the actual abundance of these viruses in dog populations (whether free-roaming or owned), and no studies from Africa are described. If CAVs are prevalent, pre-existing immunity could compromise the efficacy of a CAV2-based

recombinant rabies vaccine. However, various reports on the effect of pre-existing immunity against the vector virus are contradictory [26,29,30,38]. To assess the potential application of CAV2-RG as a component of rabies control programs in South Africa, our objective was to gauge the extent of seropositivity against CAV in various canine populations in South Africa. Secondly, we evaluated the effect of pre-existing immunity against CAV on the efficacy of CAV2-RG in dogs.

2. Materials and methods

2.1. Serosurvey of domestic dog populations

Owned dogs from two distant regions in South Africa, within the provinces of Gauteng (metropolitan area) and KwaZulu-Natal (rural, peri-urban areas) were targeted (Fig. 1).

In the KwaZulu-Natal province of South Africa, blood was collected from animals presented at mobile primary healthcare as well as sterilization clinics, which are organized by the department of veterinary services as part of an ongoing program for dog rabies control. Pet owners bring their dogs and cats to these clinics to receive treatment against endo- and ectoparasites, combined vaccines, as well as rabies vaccines, and in the case of sterilization clinics, animals are sterilized. Dogs in these rural communities serve primarily as hunting animals or as protection of property and are often free-roaming. Animals in these communities are rarely presented at these clinics. Animals younger than approximately 2–3 years of age were selected to minimize the chance of previous vaccination with a multivalent vaccine that would among other diseases also protect dogs against infection with either CAV 1 or 2.

In contrast to the rural setting and peri-urban setting in KwaZulu-Natal, the target area in Soweto (Johannesburg, Gauteng province) is an urban area. The domestic dog population in Soweto that was sampled in this study composed of community and owned animals that were selected based on the lack of health care, as part of a larger study that focuses, among others, on the impact of poor health care on the efficacy of rabies vaccines in dogs. These dogs are often subject to high parasite loads as well as other diseases such as canine distemper and parvovirus infection and were most unlikely to have ever received any vaccinations or primary health care. In Gauteng, the blood collection was done as part of a larger study of dog ecology and welfare.

Sera were recovered through centrifugation and heat-inactivated at 56°C for 30 min. Neutralization tests for the assessment of CAV antibody titers were conducted in 96 well microtitre plates. Working dilutions of the stock virus was back titrated with each set of plates, with the inclusion of negative (MEM and serum) and positive controls. In brief, two-fold dilutions of serum from 1:2 to 1:2048 were made in minimal essential media with Earl salts. Each serum was tested in duplicate. Approximately 100 TCID₅₀ (50 µl) of the CAV2 stock virus was added to each well. The serum/virus mixture was incubated for 90 min at 37°C with 5% CO₂. Madin-Darby kidney cells were added at a concentration of approximately 5 × 10⁴ cells per well (50 µl per well). The end-points were determined after 3 days of incubation at 37°C and 5% CO₂. Any neutralization below 1:8 dilution was considered as negative (personal communication, Dr. E. Dubovi, Cornell University).

2.2. Vaccination trial in dogs with pre-existing immunity

2.2.1. Dogs

Sixteen 3-month old purpose-bred Beagles were obtained from commercial sources. None of the dogs had been vaccinated previously against rabies. All dogs received multivalent

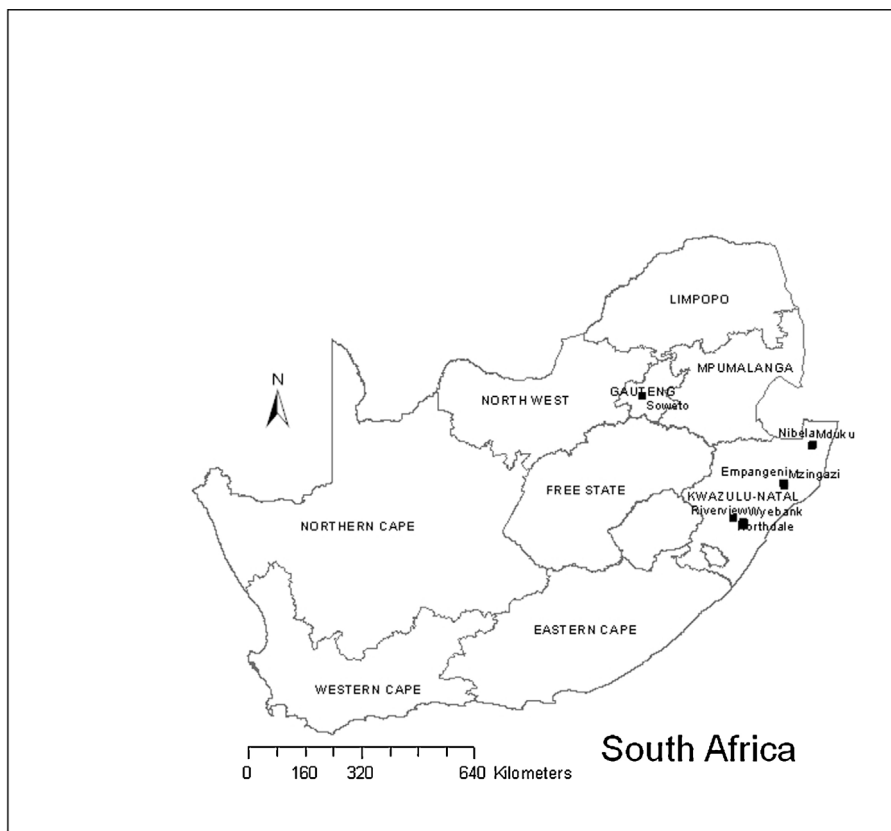


Fig. 1. Map of South Africa with provinces indicated in capital letters. Sample collection sites in Gauteng and KwaZulu-Natal are shown.

vaccination against canine parvovirus, canine distemper virus and CAV approximately 5 months before start of trial, with a booster dose administered 6 weeks before start of trial. Dogs were identified by a unique tattoo and were group-housed. Dogs were quarantined a minimum of 30 days for general health observations, prior to commencement of this study. All animal care and experimental procedures were performed under an approved protocol in compliance with CDC Institutional Animal Care and Use Guidelines.

2.3. Vaccination

Dogs were assigned randomly to either the test group (12 dogs) or control group (4 dogs). The recombinant vaccine used in this study, CAV2-RG has been described previously [28]. On day 0 (approximately 6 weeks after booster with CPV, CDV, CAV vaccine) test group dogs received 1 ml of CAV2-RG (1×10^8 TCID₅₀/ml) per os, while control dogs received 1 ml of sterile distilled water, administered via needleless syringe. Approximately six weeks after initial vaccination with CAV2-RG, two dogs received booster doses of CAV2-RG (1×10^8 TCID₅₀/ml per os).

2.4. Neutralizing antibody determination

Before vaccination with CAV2-RG, blood was collected from all dogs. After vaccination, dogs were bled weekly. Blood was allowed to clot and serum was recovered by centrifugation. The CAV2 neutralizing antibody levels were determined as described previously. RABV neutralizing antibody (RVNA) levels were determined using the Rapid Fluorescent Focus Inhibition Test (RFFIT) as described [39].

2.5. Rabies virus challenge

The challenge RABV (coyote RABV variant) was prepared from the salivary glands obtained from a naturally infected dog from Texas. Salivary glands were homogenized (10%, w/v) in phosphate buffered saline with 2% fetal bovine serum. The suspension yielded a concentration of approximately 10^7 median mouse intracerebral lethal doses₅₀ per ml.

Approximately 6 weeks after vaccination with CAV2-RG, all dogs were sedated through intramuscular administration of Telazol (9.9–13.3 mg/kg of Tiletamine HCl–Zolazepam HCl). Dogs were inoculated in the masseter muscles with a 0.5 ml suspension of challenge RABV diluted 1:100 in 2% fetal bovine serum as diluent. All dogs were observed daily for any clinical signs suggestive of rabies for a maximum of 60 days post RABV challenge. Any clinical suspect animals were restrained, sedated and euthanized. Euthanasia was performed with an intravenous dose of a concentrated barbiturate solution (approximately 2 ml/kg) while dogs were under sedation. Representative brain tissue samples were collected from each euthanized dog and were kept frozen at -80°C . Brain tissue samples were examined for the presence of RABV antigen by direct fluorescent antibody test (DFA).

3. Results

For this study blood was collected from owned dogs in two provinces in South Africa. A high percentage of dogs from both sampling regions had antibodies against CAV. In KwaZulu-Natal 62% (45/73) of dogs and in Gauteng 45% (76/168) had detectable levels of neutralizing antibodies against CAV2 (Fig. 2). Of the dogs with detectable levels of CAV antibodies, 95% (115 of 121 positive dogs) had high antibody titers equal to or higher than 64.

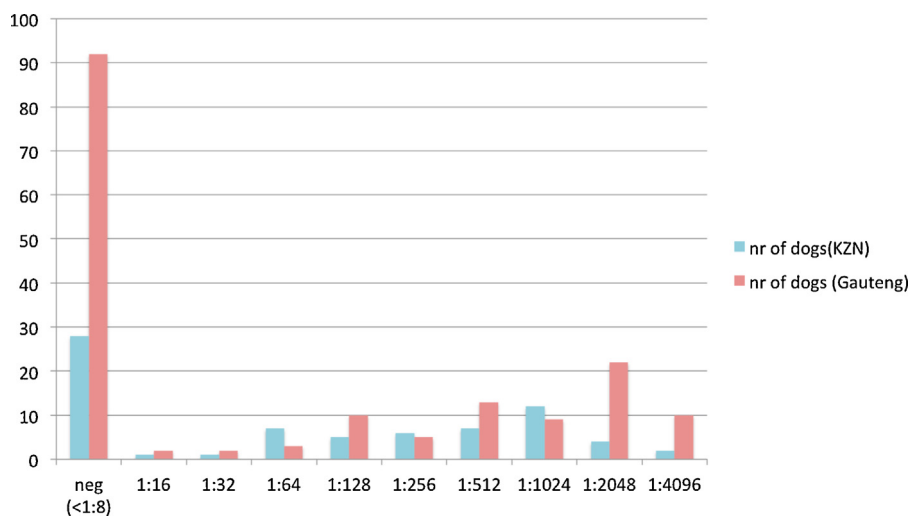


Fig. 2. CAV seropositivity and the distribution of antibody titres in dogs from the KwaZulu-Natal (KZN) and Gauteng (JHB) provinces of South Africa.

Table 1
Response of CAV2-RG in generating RABV NA and in protecting against lethal rabies challenge.

	VNA titers	Vaccinates												Control group			
		Dog1	Dog2	Dog3	Dog 4	Dog 5	Dog6	Dog7	Dog8	Dog9	Dog10	Dog11	Dog 12	Dog1	Dog2	Dog3	Dog4
Baseline after combined vaccine	CAV Ab	128	256	1024	64	256	128	256	128	256	256	256	32	0	0	0	0
	RABV Ab	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 0	CAV Ab	128	256	1024	128	512	128	32	256	256	512	256	32	0	0	0	0
	RABV Ab	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 7	CAV Ab	128	1024	512	2048	256	128	256	1024	256	256	256	2048	0	0	0	0
	RABV Ab	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 14	CAV Ab	32	1024	512	2048	128	64	64	256	128	256	128	1024	0	0	0	0
	RABV Ab	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 21	CAV Ab	32	1024	1024	2048	64	64	32	256	64	256	64	1024	0	0	0	0
	RABV Ab	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 28	CAV Ab	32	1024	512	2048	64	64	32	128	64	512	64	1024	0	0	0	0
	RABV Ab	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 35	CAV Ab	32	1024	2048	2048	512	64	512	512	128	256	128	1024	0	0	0	0
	RABV Ab	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 42 (received booster dose CAV2-RG)		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical signs		+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Survivors		No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No
DFA results		+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+

In subsequent experiments, we evaluated the effect of pre-existing vaccine-induced immunity on CAV2 immunity, using purpose-bred captive dogs. At day 40 after administration of the combination vaccine (canine adenovirus, parvovirus and distemper), all dogs seroconverted and demonstrated detectable levels of virus neutralizing antibodies (VNAb) against CAV, although at levels that were in general considerably lower than those found for naturally infected South African dogs. No VNAb to RABV was detected in any initial baseline sera on day 0 prior to vaccination with CAV2-RG.

Following vaccination with CAV2-RG, a third of the dogs showed an increase in levels of VNAb against CAV2 between days 1 and 14, but by day 35, 7 out of 12 were found to have a titer exceeding their baseline level. Throughout the study, analyses of serum samples from the 10 vaccinated dogs that received a single dose of CAV2-RG, did not detect any VNA against RABV. Of the two individuals that each received a booster dose of CAV2-RG only one dog developed detectable VNAb by RFFIT (0.62 IU/ml). All animals, except one of the boosted vaccinates, displayed signs compatible with clinical rabies in the days following a lethal RV challenge. Rabies diagnosis was confirmed in all suspect animals by the DFA test. The single

survivor was negative for evidence of RABV antigens in the brain after euthanasia at the conclusion of the study (Table 1).

4. Discussion

Canine rabies is a serious and increasing concern in the developing world, responsible for the vast majority of global human rabies exposures and fatalities [8,40]. In contrast, developed nations have proven that mass vaccination of dog populations, achieving herd immunity (approximately 70% coverage), can eliminate dog rabies and prevent human rabies [41,42]. Achieving this goal is a challenge for the remaining dog rabies endemic areas of the world, notably Africa, Asia and South America. There are many reasons for the enormity of this challenge in the developing world. These include the lack of infrastructure and large uncontrolled dog populations, which are major factors preventing effective vaccination coverage. In this regard, the possibility to include oral vaccination in control programs may offer distinct advantages, particularly if such vaccines are specific for dogs. Thus, if appropriate vaccination

coverage in areas with free-roaming dogs is not achievable by parenteral vaccination campaigns, oral vaccination may offer a solution provided that such vaccines are cost effective, safe and efficacious [40]. Arguably, current rabies vaccines suitable for oral vaccination of dogs do not comply fully with all ideal characteristics [16,17,20,21] and from that perspective, the development of new vaccines against dog rabies is a worthwhile objective.

Adenoviruses have for some years been the target of extensive research related to their application as gene therapy vectors, and more recently as vectors for recombinant vaccines. The CAV2-RG recombinant virus seems an ideal candidate for a new recombinant oral vaccine against dog rabies. The backbone virus exhibits great host specificity, and is globally used safely as an attenuated vaccine against CAV1. CAV2-RG would also offer added safety as a rabies vaccine through the inclusion of a single RABV gene, thereby nullifying the possibility of reversion to a virulent state, which remains a hypothetical concern in the case of rabies virus-based vaccines.

Despite this potential, a wide spread pre-existing immunity against adenoviruses may be a major hurdle in the use of such vectors. Very little data are available as to the prevalence of CAV2 antibodies in dog populations in Africa. As a first step towards an evaluation of the potential of a CAV-based recombinant vaccine for use in the oral vaccination against rabies in domestic dogs, this study aimed to investigate the extent to which South African dogs are naturally exposed to CAV 2. To be representative, we included two geographically and ecologically distinct groups of dogs. In KwaZulu-Natal, up to 62% of the dogs had neutralizing antibodies against CAV2, while the Gauteng group also had a high seropositivity just below 50%. Although no prior data had been available for dogs in South Africa, various studies have reported the presence of antibodies to CAV2 or CAV1 in domestic animals, as well as wildlife, in other parts of the world. For example Olsen et al. [43] reported significant levels of antibodies against CAV1 in 26% of unvaccinated dogs in Sweden. Osterhaus et al. [44] reported up to 41% seropositivity in dogs against CAV1, while Mainka et al. [45] found that 29% of dogs roaming freely through a nature reserve in China had antibodies against CAV2. Recently, Yildirim et al. [33] found that up to 66% of Kars dogs in Turkey showed significant levels of antibodies against either CAV1 or CAV2. A seroprevalence of 23.2% was reported in European red foxes (*Vulpes vulpes*) in Australia [46]. Antibodies against CAV2 have also been detected in free-ranging communities (including raccoons, skunks, mongooses, black bears, wolves and jackals) in Alaska, Canada, North America, and Zimbabwe [47–50]. Our findings are consistent with reports from elsewhere in the world and confirm that there is a high degree of natural exposure to CAV in South African dogs. This scenario is probably true for other countries in Africa, particularly sub-Saharan areas.

Previous studies on the efficacy of CAV2 as oral vaccine in raccoons, skunks and dogs all showed increases in antibody titers against CAV, and in the case of recombinant CAV vaccines against RABV, also seroconversion against RABV [49,51]. However these studies did not determine whether animals included in the study had pre-existing antibodies against CAV. Literature on the effect that pre-existing antibodies against a carrier virus (vector) may have on the levels of vaccine seroconversion (targeted at the foreign gene product) varies greatly. Some reported no effect on immune stimulation [29,30,32]. Others reported inhibition of any response against the foreign gene [26,27]. Oral vaccination of raccoons with V-RG, in the presence of pre-existing antibodies against orthopox viruses, caused a decrease in antibody titers directed against the RABV glycoprotein or complete lack of seroconversion in some animals [52]. Clarifying this issue with respect to CAV2-RG, which appeared to be a promising vaccine candidate for oral vaccination of dogs against rabies, was therefore an important aspect of our study. In avoiding ambiguity, our approach was to evaluate the

efficacy of CAV2-RG administered orally to dogs, in the presence of pre-existing antibodies against CAV2, followed by RABV challenge. Thus, it was clear that CAV2-RG does not elicit RABV neutralizing antibodies in the presence of pre-existing CAV Nab and does not protect dogs against RABV, even after booster immunization. Given the high levels of neutralizing antibodies against CAV in African dog populations, and the lack of RABV-specific seroconversion in the presence of pre-existing immunity to CAV, this vaccine does not appear to be a suitable candidate as a rabies vaccine for dogs.

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