Carnivore parvovirus ecology in the Serengeti ecosystem: vaccine strains circulating and new host species identified

Running Title: Ecology of carnivore parvoviruses in the Serengeti ecosystem

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Abstract

Carnivore parvoviruses infect wild and domestic carnivores and cross-species transmission is believed to occur. However, viral dynamics are not well understood nor the consequences to wild carnivore populations of the introduction of new strains into wild ecosystems. To clarify the ecology of these viruses in a multi-host system such as the Serengeti ecosystem and identify potential threats for wildlife conservation we analyzed, through real-time PCR, 152 samples belonging to 14 wild carnivore species and 62 samples from healthy domestic dogs. We detected parvovirus DNA in several wildlife tissues. Of the wild carnivore and domestic dog samples tested, 13% and 43%, respectively, were positive for carnivore parvovirus infection, but little evidence of transmission between the wild and domestic carnivores was detected. Instead, we describe two different epidemiological scenarios with separated routes of transmission: first, an endemic feline parvovirus (FPV) route of transmission maintained by wild carnivores inside the Serengeti National Park (SNP); and second, a canine parvovirus (CPV) route of transmission among domestic dogs living around the periphery of the SNP. Twelve FPV sequences were characterized, new host-virus associations involving wild dogs, jackals and hyaenas were discovered and our results suggest mutations in the fragment of the *vp2* gene were not required to infect different carnivore species. In domestic dogs, six sequences belonged to the CPV-2a strain, whilst 11 belonged to the CPV-2 vaccine-derived strain. This is the first description of a vaccine-derived parvovirus strain being transmitted naturally.

Importance of this study

Carnivore parvoviruses are widespread among wild and domestic carnivores, which are vulnerable to severe disease under certain circumstances. The findings from this study, which further the understanding of carnivore parvovirus epidemiology, suggest that feline parvoviruses are endemic in wild carnivores in the Serengeti National Park (SNP); further, that canine parvoviruses are present in the dog population living around the SNP, with little evidence of transmission into wild carnivore species; and finally, that the detection of vaccine-derived virus (described here for the first time circulating naturally in domestic dogs) highlights the importance of performing epidemiological research in the region.

1. Introduction

The species *Carnivore protoparvovirus I*, known colloquially as the *Carnivore parvoviruses*(1, 2), is a member of the Parvoviridae family and includes the antigenic variants feline and canine parvovirus (FPV and CPV). Carnivore parvoviruses infect a wide variety of host species with complex pathological and epidemiological outcomes. They have a broad tropism for mitotically active cells and, depending on the strain, presence of coinfection with other pathogens, and specific characteristics of the host, such as age, species and host immunity, can cause sub-clinical, acute or, especially in young animals, lethal disease(3, 4).

72 Carnivore parvoviruses have a global distribution and are present in
73 apparently healthy individuals from almost all wild and domestic carnivore
74 populations tested (5 – 7). In contrast, there are reports that implicate the
75 introduction of these viruses into wild ecosystems with the decrease in naive
76 populations (e.g. wolves *(Canis lupus)* (8, 9). Despite this apparent ubiquity and
77 variable pathogenicity, understanding of Carnivore parvovirus evolution, strain
78 succession and spread is based upon a limited number of studies, mostly
79 involving diseased captive wild and domestic animals(3). These complexities
80 illustrate the difficulties of predicting the consequences of infection at an
81 individual and a population level, especially in wild ecosystems where multiple
82 potential hosts may reside. In order to better understand which wild species are
83 at risk of infection and optimize conservation measures, it is necessary to
84 further investigate the dynamics of Carnivore parvoviruses.
85 Although FPV and CPV share a recent common ancestor from the early
86 1900s(10) and are differentiated by only small genetic changes, they show
87 several important differences. Known since the 1920s(11), FPV is primarily
88 associated with infection in felines rather than canines (with the exception of
89 foxes), and has also been shown to infect Herpestidae, Mustelidae and
90 Procyonidae(5, 12–14). In contrast, canine parvovirus (CPV), which was first
91 reported in the 1970s(15), shows signs of a recent population expansion and,
92 whilst infection is intimately linked with domestic dog *(Canis lupus familiaris)*
93 populations(16), the virus has been described in a wide range of species,
94 including felines(13, 14, 16–18).

CPV infection in dogs have resulted in the emergence of different antigenic variants or strains: the first strain, designated CPV-2(15), appeared in 1978 and was unable to infect felines. It spread globally and within a few months killed many naive domestic dogs (19, 20). A further strain named CPV-2a appeared in 1980 and rapidly substituted CPV-2 worldwide. Whilst only differentiated from CPV-2 by a few amino acid substitutions, the CPV-2a strain regain the ability to infect felines(21). The most recent strains CPV-2b and 2c emerged in 1984 and 2000, respectively, and have only one amino acid substitution each relative to CPV-2a(4). Today, FPV coexists in different parts of the world with CPV-2a, 2b and 2c with unknown consequences for wild carnivore populations.

In Africa, molecular studies of FPV and CPV in domestic animals were carried out in a limited range of countries (South Africa, Morocco, Tunisia, Ghana and Nigeria)(22–25) and results are consistent with findings from other regions of the world: CPV-2a, 2b, and 2c strains were circulating within domestic dogs, whilst the original strain CPV-2 has not been detected. Further, virus sequences generated from these studies showed a high similarity with strains circulating in the rest of the world, suggesting a similar epidemiological scenario exists in Africa as elsewhere.

Even fewer studies have focused on the role that African wild animals play in Carnivore parvovirus ecology and these have been limited to serological analyses(26–29). These studies have played an important role demonstrating that African wild carnivore species can be infected with Carnivore parvoviruses. However, unlike genetic analyses, serological studies do not enable strain characterization and, because strong antigenic cross-reactions occur among

Carnivore parvoviruses, the presence of antibodies does not enable conclusions to be drawn about the strain of the infecting virus(30, 31). Therefore, despite some studies concluding wild carnivores have been infected by CPV, it is perhaps more appropriate to conclude that seropositive wild carnivores have at some point been infected by an unspecified Carnivore parvovirus strain.

Genetic techniques provide an opportunity to investigate Carnivore parvovirus ecology with more precision(12). As with human parvovirus infection, Carnivore parvovirus DNA is likely to persist after the clinical period(32, 33), increasing the potential for detecting viral infections in archived animal samples. Indeed, the polymorphic *vp2* gene, which encodes the protein responsible for binding the transferrin receptor (TfR) used in Carnivore parvovirus host cell attachment, provides a good candidate for molecular analyses and has been used for strain discrimination and to trace viral origins(19, 34).

The Serengeti ecosystem is an important area for the study of Carnivore parvovirus ecology. First, it hosts large and diverse wild carnivore populations, which can provide critical information about natural infection routes. Second, some species living in the system, such as African wild dogs, are endangered(35) and require protection. Third, as mass dog vaccination programs against rabies, canine distemper and CPV have been performed around the periphery of the Serengeti National Park (SNP) since 1996, this environment provides an opportunity to investigate the implications of mass dog vaccination in wild and domestic carnivore populations.

The principal objective of this study was to investigate the molecular

epidemiology of Carnivore parvoviruses in domestic and wild carnivores of the Serengeti ecosystem. While serological studies have confirmed the presence of Carnivore parvoviruses infection in lions(36), hyaenas(31), jackals(27), wild dogs and domestic dogs(37), no molecular studies have yet been carried out to characterize circulating strains in a wide range of potential host species. Within this objective, we aimed to investigate the natural routes of infection in wild and domestic carnivores, the likelihood of cross-species transmission, and potential transmission of Carnivore parvovirus strains found in vaccinated domestic dog populations.

2. Results

2.1. Results in Wildlife

2.1.1. Presence of infection in wildlife

The presence of Carnivore parvovirus DNA was confirmed in 13.8% (C.I. 8.7-20.3) (n = 21) of samples, and in 9 out of 14 wild carnivore species tested (Table 1). In four of the five species in which Carnivore parvovirus DNA was not detected, the sample size was low (<7) precluding conclusive inference regarding absence. The species with the highest proportion of infected individuals was the African civet *(Civettictis civetta),* in which 80% (C.I. 28.4-99.5) (n = 5) of samples were positive. No infection was detected in the bat-eared fox (*Otocyon megalotis*), despite the relatively large sample size (n = 15). Of the seven different tissues analyzed, positive results were obtained in six (Fig. 1a). The fecal sample from the positive lion, which was added *posteriori*, was positive to infection.

The output from the binomial regression analysis investigating the

167 determinants of Carnivore parvovirus infection in the samples tested is given in
168 Table 2. Liver samples (OR = 17.8 (95% CI 1.8, 218), p = 0.01) and samples
169 collected from Viverridae (OR = 17.6 (95% CI 3.3, 118), p = 0.001) were significant
170 predictors of infection. The year of sample collection was not a predictor of infection
171 (Fig. 1b).

172 There was no association between likelihood of sample infection and
173 distance to the nearest building (OR = 1.0 (95% CI 0.98, 1.04), $p > 0.3$) or the SNP
174 boundary (OR = 1.0 (95% CI 0.99, 1.02), $p > 0.1$) (Fig. 2).

175

176 ***Table 1.*** *Percent of wild carnivore families and species infected with*
177 *Carnivore parvovirus DNA, detected by real-time PCR.*

178 ***Table 2****: The final regression output, investigating the determinants of the*
179 *likelihood of detecting Carnivore parvovirus DNA in the samples, is given.*

180

181 ***2.1.2. Sequence analysis in wildlife***

182 From a total of 21 positive wild carnivore samples, 13 *vp2* gene fragments
183 were sequenced. Ten sequences consisted of 1377 nucleotides, one each of 1311,
184 1088 and 699 nucleotides. Nucleotides previously used for the classification of
185 Carnivore parvoviruses (20, 38, 39) were present in all these isolates (detailed in
186 Table 3): twelve isolates belonged to the FPV strain (three lions, two spotted
187 hyaenas (*Crocuta crocuta*), two African wild dogs *(Lycaon pictus)*, two civets, one
188 genet (*Genetta genetta)*, one white-tailed mongoose (*Ichneumia albicauda*), and
189 one black-backed jackal (*Canis masomelas*)), whilst one isolate belonged to the
190 CPV-2a strain (black-backed jackal). The FPV and CPV-2a isolates detected in

191 black-backed jackals were found in two different individuals. This is the first time
192 FPV DNA has been detected in jackals, hyenas, African wild dogs and white-tailed
193 mongoose. Interestingly, the CPV-2a isolate was detected in the most recently
194 obtained wild carnivore sample (2011). This isolate was the only sequence with
195 intermediate features between FPV-like and CPV-like viruses (detailed in *Section*
196 *3.1.4.*).

197 **Table 3. A s**ummary of the amino acid variation that characterizes the strains
198 of Carnivore parvoviruses and the important mutations that distinguish the
199 sequences from this study is shown. The 459 amino acid fragment of the VP2
200 protein sequenced is represented. Blue color indicates mutations among the
201 domestic dog sequences, possibly introduced by the live virus vaccine strain; violet
202 color indicates important and repeated mutations found among the wildlife
203 sequences;. *Incomplete strains. Clf, Canis lupus familiaris (domestic dog); Cm,
204 Canis mesomelas (black-backed jackal); Pl, Panthera leo (lion); Cc, Crocuta
205 crocuta (spotted hyaena); Civ. civ., Civettictis civetta (African civet); IA, Ichneumia
206 albicauda (white-tailed mongoose), Lp, Lycaon pictus (wild dog); Gg, Genetta
207 gennetta (genet)
208
209 ***2.1.3. FPV in wildlife***
210 Comparison of FPV sequences from this study showed nucleotide identities
211 of 99.5-99.9% (mean 99.75, SD 0.09) and amino acid identities of 99.1-100%
212 (mean 99.61, SD 0.23). This compares with a global blast search in GenBank® in
213 which no identical FPV sequences were found. Following comparison with the
214 template strains from different years and locations, nucleotide identity was 98.0-

99.2% and amino acid identity was 96.9 - 99.8%. The maximum amino acid variability of the study sequences was 0.9%, whilst the maximum amino acid variability between the study and the template sequences was 3.1%, suggesting the study sequences to be more closely related with each other than with sequences found elsewhere.

The FPV sequences detected in wild carnivore species in the Serengeti ecosystem shared two mutations at two different residue positions that distinguish them from FPV sequences described elsewhere. These mutations were located at amino acid position 303, where a Tyr replaced a Phe residue (F303Y), and at position 101, where a Thr substituted an Ile residue (I101T) (detailed in Table 3). Following comparison with the most similar FPV strains found in GenBank®, the first mutation F303Y was only found in a cougar *(Puma concolor)* (USA, 1989, GenBank® accession Nº EU659113) and could have arisen independently in this individual. Residue 303 is located in the capsid surface area that contacts with the host cell receptor, and as such this position is subjected to evolutionary selective pressures(34). The second mutation, I101T, which emerged during the differentiation of CPV-2a from CPV-2(41), has occasionally been reported in FPV sequences extracted from wild and domestic species from different years and locations (e.g. GenBank® accession Nº MF069447, FJ440714, KP682520). Polymorphic residue 101 lies just below the capsid surface and, together with residue 87, alters the antigenic structure and influences the binding to feline and canine cells(40). Together, amino acid residues 303 and 101 determine host-range and the mutations detected in this study form a geographic cluster, as demonstrated by the phylogenetic network (see below).

239 A further mutation at position 20, where a Thr substituted an Ala residue
240 (A20T), was present in three of eight FPV sequences containing this amino acid
241 (belonging to two lions and a mongoose, detailed in Table 3). This mutation was
242 also found in four of the CPV sequences described in dogs sampled in this study
243 (see below). Mutation A20T was not found in any of the most similar FPV strains
244 found in GenBank® and information regarding this residue was lacking in the
245 literature reviewed. We hypothesize that, because residue 20 was located only a
246 few residues from the primer sequence, this mutation could be a sequencing error.
247 Five other single FPV sequence mutations were found (V83I, Q159H, H222P,
248 V250M, Q296H), each occurring in one sample only. Residues involved were not
249 strain type determinant and no previous studies determining the effects of these
250 substitutions were found.
251 Among the FPV sequences from this study, two pairs of amino acid
252 sequences were pairwise identical, (i) H414 (lion, liver, 2004) and H284 (white-
253 tailed mongoose, spleen, 2008) and (ii) H450 (hyaena, liver, 2007) and H253
254 (civet, spleen, 2009)). Furthermore, five amino acid sequences (H414, H284,
255 H450, H253 and H440) were only differentiated by a nucleotide at a single position
256 (number 58), which encodes the amino acid residue at position 20, discussed
257 above.
258
259 ***2.1.4 CPV-2a from the black-backed jackal H398***
260 Sequence H398 clustered phylogenetically with the CPV-2a sequences from
261 dogs (see below). However, a single mutation at amino acid position 323 (Asp
262 residue substituted the CPV-2a-typical Asn or Glu (Table 3)) was present. As the

263 amino acid at this position is exposed on the surface of the virus and controls the
264 interaction with the canine transferrin receptor (TfR)(6), it is possible that this viral
265 mutation would favor the binding to a feline transferrin receptor.
266 Furthermore, we described four additional amino acid mutations in this
267 sequence: A20T, R80T, D99H, D125Y (detailed in Table 3). Of these mutations,
268 substitution A20T is shared by eight of our wildlife and domestic dog isolates.
269
270 ***2.1.5. Phylogenetic analysis in wildlife***
271 The phylogenetic tree (Fig. 3) indicated that the FPV strains detected in the
272 wildlife species in this study have a common ancestor, formed a geographic cluster
273 separated widely from other published isolates, and are closely related suggesting
274 cross-species transmission.
275
276 **2.2. Results in Domestic Dogs**
277 ***2.2.1. Presence of infection in dogs***
278 The presence of Carnivore parvovirus DNA was detected in 42.9% (C.I. 30.5-
279 56.0) ($n = 26$) of the domestic dog samples assayed and in six of the eight villages
280 (75%) in which sampling took place. The villages with the highest proportion of
281 infected individuals were Merenga (2008) and Kitawasi (2005), in which 87.5%
282 (C.I. 47.3-99.7) and 83.3% (C.I. 35.9-99.6) of dogs sampled were infected,
283 respectively (Figs. 2 and 4). None of the factors studied (village, year of sampling,
284 age or gender of dog) were significant predictors of infection ($p > 0.4$).
285
286 ***2.2.2. Sequence analysis in dogs***

From a total of 26 positive domestic dog samples, 13 isolates of 1377 nucleotides and four of approximately 700 nucleotides were obtained. Of these, 11 were classified as CPV-2 and six as CPV-2a. Of the CPV-2 strains, three were found in samples from the village of Kitawasi (2005), six from Merenga (2008), one from Nyamburi (2009), and one from Piyaya (2009). Of the CPV-2a strains, four were detected in Nyamburi (2005, 2006 and 2009), one in Piyaya (2004), and one in Kitawasi (2005) (Figs. 2 and 4).

CPV-2 and CPV-2a strains were differentiated using the amino acid positions 87, 101, 219, 300, 305, and 375, which are considered determinant residue positions for the classification of the CPV strains(41, 42). Sixteen of the seventeen sequences obtained from domestic dogs fulfilled this classification with no intermediate virus-like features. An exception was the CPV-2 sequence obtained from isolate H493 (Table 3), which, apart from position 101 in which a Thr was substituted by Ile, presented all the residues that characterize the CPV-2 strain. Position 101 determines the antigenic structure and binding capabilities of the capsid(42), and a Thr at this position is typical of the CPV-2a, 2b and 2c strains but has also been described in FPV sequences from GenBank® and in all the FPV sequences described in this study (detailed in Section 3.1.3.). We conclude therefore that the CPV-2 sequence found in H493 presented an intermediate virus-like feature at position 101.

In addition to the six amino acid residues used to differentiate CPV2 from 2a, three further common mutations that differentiate CPV-2 from CPV-2a strains were found. These mutations were located in amino acid positions 219, 297 and 386. Substitution S297A was first detected in 1987 in CPV-2a strains and is reported to

311 be distributed globally(43). All the CPV-2a isolates from this study had this
312 mutation. Mutations I219V and Q386K were found in all the CPV-2 isolates.
313 Although these two mutations were not found in any of the template strains, they
314 were found in the live virus vaccine strain contained in the Nobivac® Puppy DP
315 vaccine, which has been used in mass dog vaccination programs conducted in the
316 study area (GenBank® accession Nº MG264079). These substitutions (of Ile by Val
317 at position 219 and Gln by lysine at position 386) were patented by the
318 manufacturer (US 9,186,398 B2)(44) and introduced in order to attenuate the virus.
319 Sequence comparisons of the CPV-2 strains obtained in this study showed
320 nucleotide identities of 98.8-100% and amino acid identities of 98-100%. Two
321 nucleotide CPV-2 sequences from different villages and years (H503 from
322 Merenga in 2008 and H506 from Kitawasi in 2005) were identical and a third
323 sequence (H469 from Merenga in 2008) was translated into the same amino acid
324 sequence. Specific mutations differentiating the strains are detailed in Table 4.
325
326 ***Table 4.*** The 459 amino acid fragment of the VP2 protein that characterizes
327 Carnivore parvoviruses was sequenced in this study. Amino acid variation is
328 shown. Amino acids used to differentiate CPV-2 from CPV-2a are not included.
329 Clf, Canis lupus familiaris; Cm, Canis mesomelas.
330
331 A blast search identified similar and identical CPV-2 sequences in different
332 continents. A nucleotide sequence described in a dog in Italy in 2005 (accession
333 Nº FJ222824)(45) was found to be identical to sequences H503/H506 and to the
334 Novibac® Puppy DP vaccine strain described in Ecuador (MG264079)(46). This is

335 the same vaccine that has been used in mass dog vaccination programs in the
336 Serengeti ecosystem. It was not reported whether the isolate from Italy was
337 collected from a vaccinated or unvaccinated dog. Other sequences containing one
338 of the two patented vaccine strain mutations (I219V and Q386K) were obtained
339 from foxes and raccoons in China in 2009 (Zhang et Yang, unpublished work,
340 2010, GenBank® accession Nº GU392236 - GU392241) and from a dog in the USA
341 in 1995 (U22186). All showed a nucleotide identity of 99.8% and amino acid identity
342 of 99.5% with the sequence H503/H506.

343 Sequence comparisons of the CPV-2a strains obtained in this study showed
344 nucleotide identities of 98.6-99.5% and amino acid identities of 97.8-99.5%.
345 When the CPV-2a isolate H501 from this study was compared with two similar
346 strains found in GenBank® (from a dog in Italy in 2000 (Accession NºAF30644)
347 and a dog in Thailand in 2004 (Accession NºFJ869128)), maximum nucleotide
348 identities of 99.8% and 99.6% were obtained and a maximum amino acid identity
349 of 100%. Consequently, the H501 isolate from this study was more similar to the
350 isolates found in Thailand and Italy than with the other two CPV-2a isolates found
351 in Tanzania.

352

353 ***2.2.3. Phylogenetic analysis in dogs***

354 Phylogenetic analysis suggests that CPV-2a sequences from this study are
355 closely related with global strains, suggesting that CPV-2a sequences from the
356 Serengeti ecosystem do not form a clear geographic cluster and are closely
357 related to sequences isolated in other continents. Therefore, in contrast to the
358 FPV sequences in wildlife which displayed a localized geographic clustering, the

359 CPV sequences isolated in this study seem to share a common evolutionary
360 process with global sequences.

361 The CPV-2 sequences isolated in this study clustered with two sequences
362 from Italy and China and the Novibac® Puppy DP vaccine strain (GenBank®
363 accessin number MG264079).

364

365 **3. Discussion**

366 We have demonstrated Carnivore parvoviruses to be widely distributed
367 among wild and domestic carnivores in the Serengeti ecosystem. While wildlife
368 was infected with FPV, domestic dogs living around the periphery of the SNP were
369 infected with CPV. With the exception of a jackal infected with CPV-2a, there was
370 no evidence of cross-species transmission, suggesting the existence of two
371 separate epidemiological systems. Given that CPV has been shown to be present
372 in 'wilderness' areas in other continents and that cross-species parvovirus
373 transmission has been documented between domestic and captive and free-living
374 wild carnivores (5, 6, 47), this finding was unexpected.

375

376 ***Viral populations in wildlife***

377 We found parvovirus DNA in 13.2% of the wild carnivores sampled in the
378 Serengeti ecosystem. The samples were collected through a convenient non-
379 random method and most were collected from animals found dead on the primary
380 road traversing the center of the SNP. As such, this is not likely to be a
381 representative sample and does not provide an unbiased prevalence estimate.
382 However, because the percentage of wildlife samples found to be positive did not

change significantly across the ten years studied or across the species tested, this lends weight to the hypothesis that Carnivore parvoviruses are endemic in wildlife species in the Serengeti ecosystem, as may be the case for wildlife in other continents(30). The likelihood of positives was not related to proximity to human habitation, which would be consistent with independent routes of Carnivore parvovirus transmission in wild and domestic carnivores.

Thirteen wild animal samples were found to be positive for Carnivore parvovirus DNA, of which 12 were identified as FPV and one as CPV-2a (detected in a black backed jackal). Important amongst these results was the detection for the first time of FPV infection in wild dogs, jackals and hyaenas. Whilst many species of Carnivora appear to be susceptible to Carnivore parvoviruses, the host range of FPV has been reported to be restricted to foxes, felids and some closely related families such as mustelids(5, 48 (O. Calatayud et al., manuscript under submission)). As such, these findings are notable.

In addition, these findings are of interest as they raise questions concerning the interpretation of previous serological studies, which assumed infection with CPV was responsible for seropositive results in jackals, hyenas and African wild dogs (27, 49). Our results suggest that seropositivity in these earlier studies might have resulted from FPV infection instead, highlighting the importance of strain characterization in understanding Carnivore parvovirus dynamics.

Host susceptibility to Carnivore parvovirus infection is largely driven by the ability of viruses to bind to the carnivore transferrin receptor (TfR) used in host cell attachment(50, 51). Indeed, both FPV and CPV can infect felines because they can bind feline TfR. However, a mutation introduced less than six million years ago

into the TfR gene encoding the N-linked glycosylation site in the apical domain confers resistance to FPV infection in most canine species. This glycan mutation is present in coyotes *(Canis latrans)*, wolves *(Canis lupus)* and domestic dogs(50–52), but has been shown to be lacking in red foxes, bat eared foxes and black-backed jackals (52). As predicted by the lack of the glycan-introducing mutation, we report for the first time natural FPV infection in a jackal (H418). Furthermore, we report for the first time that hyena and African wild dog can also be infected by FPV, suggesting these species might also lack the glycan-introducing mutation. This is consistent with the evolutionary history of hyenas, wild dogs and jackals, which all share a relatively distant common ancestor with wolves, coyotes and dogs(53, 54). It is possible, therefore, that these species diverged before the emergence of the canid glycan-introducing mutation.

Cross-species transmission events of FPV among wildlife species have been previously documented in wild(6, 10) and in captive conditions(55), suggesting that parvoviruses are transmitted between hosts during contact, for example predation and/or scavenging of carcasses. The finding in this study of genetically indistinguishable viruses in sympatric species in the Serengeti ecosystem provides further evidence that FPV can be transmitted between species and that these transmission events occur in this ecosystem. Even where sequence mutations were identified, phylogenetic analysis demonstrated a close relationship among all the sequences described. This clustering is due primarily to two specific mutations (F303Y and I101T) that characterize all identified Serengeti ecosystem strains. The clustering of Serengeti FPV sequences independently of other sequences reported in GenBank® and the stable number of infections across the ten year period studied

431 also suggests that FPV has been present in this ecosystem for a long time and
432 may be endemic.

433 It is noteworthy that, of all the wild carnivore species tested, we only detected
434 CPV in one species, a jackal. This is in contrast with reports describing CPV
435 infection in wild carnivores in other parts of the world(6, 56). Although the Serengeti
436 ecosystem is considered a relatively intact wilderness (57), there are populations
437 of humans and domestic animals, including many dogs, living around its periphery
438 and incursions frequently occur (58). As a result, it seems likely that wild carnivores
439 would be exposed to CPV in the Serengeti. The lack of detection of CPV in wild
440 carnivores in the Serengeti could arise because wild carnivores are resistant to
441 infection (possibly as a result of FPV within the Serengeti ecosystem creating an
442 immunological barrier), or have been clearing infection, or have been dying in small
443 and imperceptible epidemic waves. This latter explanation seems possible given
444 that most parvoviruses causing disease in large cats have been described not as
445 FPV but as CPV(59). Indeed, a recent analysis of long-term serological data to
446 investigate the transmission ecology of CPV in the Serengeti ecosystem indicates
447 that infection cycles in lions are coupled with those in dogs, providing some
448 evidence of cross-species transmission(60). However, as CPV and FPV are
449 antigenically similar and difficult to distinguish serologically, it is likely seropositive
450 lions were infected with FPV, complicating the interpretation of the serological data.
451 While the genetic analyses provide no evidence for cross-species transmission,
452 the different conclusions drawn by the serological and genetic studies are not
453 mutually exclusive. Our study suggests that it is likely that FPV is circulating as an
454 endemic infection in lions, however it is also possible that transient outbreaks of

CPV may also occur as a result of spill-over from domestic dogs. The integration of data from multiple sources and from more comprehensive sampling will clearly be needed to allow a more complete understanding of a complex epidemiological picture.

The detection of Carnivore parvovirus DNA in a range of tissues supports the hypothesis that, similar to human parvoviruses(33), Carnivore parvovirus DNA remains in the body following initial infection, as has been shown in previous studies(5, 6, 47). These results further highlight the potential value for carnivore parvovirus epidemiological research of tissue samples collected from carcasses and should encourage analysis of such samples collected from other ecosystems across Africa and elsewhere.

Viral populations in dogs and vaccine shedding

With just under half of the domestic dog blood samples being positive, the results indicated that CPV has been circulating widely in the villages adjacent to the SNP during ten years, suggestive of endemicity. This finding was unexpected given that all of the sampled dogs appeared healthy. Our results suggest that CPV DNA persists in blood for longer periods than previously thought(61) with no clinical signs.

Surprisingly, 65% of the sequenced viruses from dogs were CPV-2, even though this strain has been replaced in most areas of the world by the newer antigenic types 2a, 2b and, more recently, 2c(41). The detection of this strain in several different villages over a four-year period generates confidence in this finding. Several lines of evidence suggest that a modified-live vaccine virus was

479 the source of this CPV-2 strain and, because all the samples used in this study
480 belonged to unvaccinated individuals, transmission from vaccinated to
481 unvaccinated dogs may have occurred. First, all the CPV-2 sequences described
482 contained two genetic markers patented by the vaccine manufacturer and
483 artificially introduced to attenuate the vaccine virus(44). Second, three amino acid
484 sequences were identical to the vaccine strain. Third, this vaccine has been used
485 in annual mass dog vaccination programs in the region. Consequently, we
486 conclude that these findings represent cases of natural transmission of vaccine-
487 derived CPV-2 (vdCPV) in domestic dogs. This is the first time that this has been
488 demonstrated empirically.

489 Although this phenomenon has not been demonstrated before, the potential
490 for this event and the route of transmission has been reported by previous studies.
491 Two studies demonstrated that 23% of dogs immunized against CPV using a
492 modified-live virus vaccine shed virus DNA in their feces during at least 20 days(61,
493 62). A third experimental study demonstrated that, following contact with
494 vaccinated dogs, unvaccinated dogs became seropositive without showing signs
495 of disease(63). Consequently, it is possible that, following transmission of virus
496 from vaccinated individuals, naive dogs are becoming infected. It is also possible
497 that these infections might result in a protective immunity against CPV.

498 Although we did not find evidence of cross-species transmission of vdCPV in
499 the Serengeti ecosystem, the host range might not be restricted to domestic dogs.
500 Indeed, the same artificial mutations have been reported in samples obtained from
501 foxes and raccoons from China in 2009 (Zhang et Yang, unpublished work,
502 GenBank® accession Nº GU392236 - GU392241), suggesting that vdCPV could

503 be transmitted to wild carnivores. However, because we do not know which brand
504 of vaccine was used in China, we are not able to draw conclusions regarding
505 whether vaccine transmission resulted following vaccination with Nobivac Puppy
506 DP vaccine or if it can be triggered by use of other modified-live parvovirus
507 vaccines.

508 A concern of live vaccine viruses is the potential for reversion to virulence(64,
509 65), although there are no reports in the literature of this happening with vdCPV.
510 Because recombination(66) or novel mutations might lead to a loss of the benign
511 phenotype, surveillance to monitor for such an eventuality would have merit.

512 The circulation of vaccine-derived strains CPV-2a, CPV-2b and CPV-2c
513 would be of more concern than the CPV-2 strain because it has been shown that
514 field strains 2a, 2b and 2c are able to cause disease in felids and in other wildlife
515 species(10, 20). For this reason, vaccine shedding from CPV-2a, 2b and 2c live
516 vaccines could have an impact on wild carnivores and we recommend further
517 investigation to assess the risks of the use of these vaccines in proximity to wildlife
518 protected areas.

519

520 ***Intermediate features***

521 Two sequences from this study showed coding mutations at strain-
522 determinant positions. Intermediate features between FPV and CPV have been
523 described only once in a wild carnivore (a red fox from Germany(67)), and
524 intermediate mutations between different CPV strains have been previously
525 described in raccoons(56), probably as a result of host-adaptation.

526 The first, a vdCPV from domestic dog H493 sampled in 2009, had the amino

527 acid substitution I101T, common to the CPV-2a strain and to the FPV strains
528 detected in the Serengeti ecosystem. Position 101 is variable and this mutation
529 has been previously described in raccoons and domestic cats (56, 68), however
530 this is the first time an intermediate CPV/CPV-2a strain has been reported in
531 dogs(6). Although it is not clear whether this substitution arose after a
532 recombination or a mutation event, an evolving modified-live virus is of concern.
533 The second intermediate mutation was described in the black backed jackal
534 sequence H398 in 2011. Although we classified this strain as CPV-2a, it presented
535 an Asp replacing an Asn residue at amino acid position 323, which is typical of the
536 FPV strain(41). Amino acid position 323 is located on a raised region of the capsid
537 surrounding the three-fold spike which contacts the TfR(51). Although this
538 mutation is predicted to reduce replication in canine cells, it is possible that this
539 viral mutation would favor the binding to a feline transferrin receptor present in
540 jackals (52).
541
542 In summary, this study has demonstrated that, whilst Carnivore parvovirus
543 infection occurs in numerous species living in the Serengeti ecosystem, there
544 appears to be separated transmission routes involving wild and domestic
545 carnivores. Furthermore, whilst FPV appears endemic in wild carnivore
546 populations living in the Serengeti ecosystem (including canids and hyenas),
547 CPV-2 and CPV-2a appear to be circulating almost exclusively in domestic dog
548 populations, with CPV-2 infection likely arising as a result of vaccine shedding.
549

4. Materials and methods

4.1. Sampling

Archived (-20ºC) biological samples collected between 2002 and 2011 were used in this study. These were:

i) tissue samples (n = 112) collected during wild carnivore post-mortem examinations carried out in the Serengeti National Park (SNP). Linked GPS coordinate data was available for most of the samples. The cause of death for each of the sampled wild carnivores was unknown;

ii) blood samples (n = 40) collected opportunistically from wild carnivores during general anesthesia immobilization;

iii) uncoagulated (EDTA) blood samples (n = 62) collected during mass dog vaccination programs from manually restrained healthy unvaccinated (< 1.5 years old) domestic dogs living in villages around the periphery of the SNP. These villages were Nyamburi, Merenga, Pinyinyi, Malambo, Kitawasi, Engarasero, Arash and Piyaya (Fig. 4).

The taxonomic family, tissue type, age and geographic provenance of each sample in Sections i and ii are detailed in Table 5.

Table 5. *Taxonomic families and species from which the samples analyzed in this study originated. Sp=spleen; Bl=blood; LN=Lymph Node; In=Intestine; Li=Liver; SG=salivary gland; Suba=subadult; ND=no data;*

573 *SNP=Serengeti National Park; NPA= neighbouring protected areas;*
574 *BBJ=Black-backed jackal.*
575
576 One lion (*Panthera leo)* feacal sample was available for testing. It
577 belonged to a spleen-positive adult animal (H440) and was excluded from the
578 statistical analysis.
579
580 ***4.2. Molecular analysis***
581 DNA was purified from 50mg of tissue or 200*µ*L of blood in a series of
582 rapid "wash-and-spin" steps, using the High Pure PCR Template™ Preparation
583 Kit (Roche® Diagnostics Gmbh, Mannheim, Germany following the
584 manufacturer´s recommendations. Purified DNA was stored at -80ºC until
585 further use.
586 To determine the presence of parvovirus DNA (case ascertainment), a
587 previously described real-time PCR(69) (qPCR) was performed. It targeted a
588 conservative parvovirus region of 163 nucleotides and it did not discriminate
589 between different parvovirus strains. The primers used in this study are detailed
590 in Table 6 and the thermal cycling conditions in Table 7. In this qPCR, the final
591 mixture of 25*µ*L contained 0.4 *µ*M of primers, 10*µ*L of Quantiprobe™ (Qiagen®
592 GmbH, New York, USA), 0.4 *µ*M of probe, 3.5 *µ*L of template DNA and free-
593 nuclease water. The assay was performed on a StepOne™ Real-Time PCR
594 System (Foster City, USA).
595

596 ***Table 6.*** *Primers used in the present study. *Nucleotides numbered*
597 *according to Reed et al., 1988*(69)*. **c, complementary.*
598 ***Table 7****. PCR thermal cycling conditions*
599
600 For the characterization of the Carnivore parvovirus strains, a 1377 portion
601 of the *vp2* gene (total length: 1755bp) was amplified with four nested PCRs.
602 The final mixture of 25μL contained 0,5 μL of primers, 0,25 μL of FastStart
603 Universal Master Mix (Roche™), 1.5 μL of Cl_2Mg, 2.5 μL of buffer 10x, 0.5 μL
604 dNTP 10 μM, 4 μL of template DNA and sterile free-nuclease water. Three
605 internal nested PCR reactions amplified three segments of different length
606 (Table 6), which were subsequently purified. Five primers *(*P1, P3, P4, Forward,
607 Reverse, in Table 6) were used to sequence five overlapping fragments
608 covering a total of 1377 nucleotide residues with the automated Sanger
609 sequencing method.
610 A 10^{-2} commercial CPV vaccine dilution (Nobivac® Puppy DP, MSD
611 Animal Health, Carbajosa de la Sagrada, Spain), containing attenuated live
612 CPV-2 virus, was added as a positive control, and sterile free-nuclease water as
613 a negative (blank) control to each step of the molecular analysis.
614
615 ***4.3 Sequence data***
616 Blast searches in GenBank® were performed for each sequence
617 generated. Nucleotide sequences were translated into the putative amino acid
618 sequences and position sites were numbered(70). Specific amino acids were
619 used for classification of the FPV, CPV-2, CPV-2a, CPV-2b and CPV-2c strains

620 (20, 38, 39). Nucleotide and amino acid sequence pairwise identity was
621 calculated using the online software SIAS® (Sequence Identity and
622 Similarity)(71). Only sequences > 1300bp were used in this analysis. In
623 addition, template sequences of Carnivore parvoviruses were randomly
624 selected from 1990, 2007 and 2015 and were included for comparison
625 (GenBank® accession no.: M38246, EU145593, KX434462). The 1990 sequence
626 was the oldest one found in the GenBank® database and was included in the
627 study to assess viral diversity over time.

628 Sequences were aligned with the Clustal W method using the MEGA7®
629 software(72). A maximum-likelihood phylogenetic tree was inferred and the
630 reliability was evaluated with the bootstrap method based on 1000 replicates
631 using the same software.

632 Sequences described in this study were submitted to GenBank®
633 (accession numbers MK251434-MK251461).

634

635 ***4.4. Statistical analysis***

636 Statistical analyses were performed using the exact binomial confidence interval
637 (95% confidence level) for prevalence calculations. Associations between the
638 presence of Carnivore parvoviruses DNA and potential explanatory variables,
639 such as type of tissue, species, family, age (young, juvenile, adult), sex and
640 year of collection, were evaluated by binomial logistic regression using the
641 software R®(73). Variable selection was carried out using manual forward
642 selection based on lowest Akaike information criterion (AIC). Strengths of
643 associations were determined based on odds ratios with 95% confidence

limits(74). Wildlife sample coordinates were used to calculate the distance (km) from the location of sampling of wildlife to the point of nearest human contact, with the location of a) the nearest building and b) the SNP boundary used as proxy measures. QGIS® Geographic information System Software(75) was used for distance calculations and for the representation of sample locations. A binomial logistic regression model was constructed with the proxy measures described above as predictor variables to investigate whether proximity to human habitation and / or the park boundary predicted likelihood of Carnivore parvovirus infection.

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928 ## FIGURE LEGENDS

929 **Fig. 1.** Percentage (and 95% confidence intervals) of wildlife samples that
930 were parvovirus DNA positive (a) for each tissue type (SG = salivary gland; LN =
931 lymph node) and (b) for each time period. The number of samples within each
932 category is represented (n).

933 **Fig. 2**. The map indicates the location of the Serengeti Maasai Mara
934 ecosystem within Tanzania (inset). The shaded areas identify the Serengeti
935 National Park (Serengeti), the Ngorongoro Conservation Area (Ngorongoro)
936 and a number of unnamed game reserves. The location of the villages where
937 domestic dog samples were collected and the location where positive (red dots)
938 and negative (grey dots) wildlife samples were collected is shown.

939 **Fig. 3.** Phylogenetic tree constructe from the VP2 nucleotide sequences
940 described in this study, which are marked with a blue (FPV) and a red circle (CPV),
941 and in other parts of the world. Clf: canis lupus familiaris; Fsc: Felis silvestris catus.
942 All horizontal branches are drawn to a scale of nucleotide substitutions per site

943 **Fig. 4.** Percentage (and 95% confidence intervals) of domestic dog samples
944 from each village that were positive for parvovirus DNA. The number of samples
945 from each village is given.

946

TABLES

	Total	Positives	Percent infected (95% C.I.)
Viverridae (combined)	8	6	75 (34.9-96.8)
African civet	5	4	80 (28.4-99.5)
Genet	3	2	66.7 (9.4-99.2)
Herpestidae	7	1	14.3 (0.4-57.9)
Mongoose	7	1	14.3 (0.4-57.9)
Felidae (combined)	52	6	11.5 (4.4-23.4)
Lion	44	6	13.64 (5.2-27.4)
Cheetah	6	0	0
Leopard	1	0	0
Serval	1	0	0
Canidae (combined)	51	5	9.8 (3.3-21.4)
Black backed jackal	15	2	13.3 (1.7-40.5)
Wild dog	20	3	15 (3.2-37.9)
Bat eared fox	15	0	0
Aardwolf	1	0	0
Hyaenas (combined)	34	3	8.8 (1.9-23.7)
Spotted hyaena	32	2	6.3 (0.8-20.8)
Striped hyaena	2	1	50 (1.3-98.7)
Total	152	21	**13.8% (8.7-20.3)**

Table 1. Percentage of samples from different wild carnivore families and species that were infected with parvovirus DNA, detected by real-time PCR.

		Estimate	Std. Error	z value	Pr(>|z|)
	Intercept	-3.54	0.97	-3.65	0.0003***
Tissue	Brain	1.99	1.03	1.92	0.05
	Intestine	-16.48	2039.21	-0.01	0.99
	Liver	2.88	1.18	2.44	0.01*
	Lymph node	1.48	1.11	1.33	0.18
	Salivary gland	22.11	6522.64	0.003	0.99
	Spleen	0.59	0.92	0.64	0.52
Family	Felidae	0.85	0.75	1.13	0.26
	Hyenidae	-0.05	0.85	-0.06	0.95
	Viverridae	2.87	0.90	3.18	0.001**

Table 2: *The final regression output, investigating the determinants of the likelihood of detecting Carnivore parvovirus DNA in the samples, is given.* Significant codes: 0‘***’ 0.001‘**’ 0.01‘*’ 0.05‘.’ 0.1‘ ’ 1. Null deviance: 117.802 on 149 degrees of freedom

Residual deviance: 90.2 on 140 degrees of freedom. AIC: 110.2

Clade	Strain	Species	Year	Amino acid residue																
				20	80	87	93	101	103	219	297	300	303	305	321	323	347	375	386	426
FPV	Reference strains			A	K	M	K	I (+T)	V	I	S	A	F	D	N	D	A	D	Q	N
CPV-2				A	R	M	N	I	A	I	S	A	F	D	N	E (+N)	T	N	Q	N
CPV-2a				A	R	L	N	T	A	I	A (+S)	G	F	Y	K (+N)	E (+N)	A	D	Q	N
CPV-2b				A	R	L	N	T	A	I	A (+S)	G	F	Y	K (+N)	E (+N)	A	D	Q	D
CPV-2c				A	R	L	N	T	A	I	A (+S)	G	F	Y	N	N	A	D	Q	E
CPV-2	H494	Clf	2005	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H489		2005	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H504		2005	A	R	M	N	I	A	V	S	A	F	D	N	N	T	N	K	N
	H506		2005	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H469		2008	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H499		2008	T	R	M	N	I	A	V	S	-	F	D	N	N	T	N	K	N
	H488		2008	T	R	M	N	I	A	V	S	A	F	D	N	N	T	N	K	N
	H503		2008	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H476*		2008	-	-	-	-	-	-	-	S	A	F	D	N	N	T	N	K	N
	H327		2009	T	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H493		2009	A	R	M	N	T	A	V	S	A	F	D	N	N	T	N	K	N
CPV-2a	H491		2004	A	R	L	N	T	A	I	A	G	F	Y	N	N	A	D	Q	N
	H321		2005	T	R	L	N	T	A	I	A	G	F	Y	N	N	A	D	Q	N
	H501		2009	A	R	L	N	T	A	I	A	G	F	Y	N	N	A	D	Q	N
	H492*		2005	-	-	-	-	-	-	-	A	G	F	Y	N	N	A	D	Q	N
	H323*		2006	-	-	-	-	-	-	-	A	G	F	Y	N	N	A	D	Q	N
	H318*		2006	-	-	-	-	-	-	-	A	G	F	Y	N	N	A	D	Q	N
	H398*	Cm	2011	T	T	L	N	T	A	I	-	-	F	Y	N	D	A	D	Q	N
FPV	H382	Pl	2002	T	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H414	Pl	2004	T	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H227	Civ civ	2004	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H418	Cm	2005	-	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H450	Cc	2007	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H401	Cc	2007	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H439	Lp	2007	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H440	Pl	2008	-	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H284	Ia	2008	T	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H253	Civ.civ	2009	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H410*	Gg	2005	-	-	-	K	-	V	I	S	A	Y	D	N	D	A	D	Q	N
	H272*	Lp	2007	-	-	-	-	-	-	-	S	A	Y	D	N	D	A	D	Q	N
Vaccine CPV-2 (Intervet)	MG264079		2017	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
Vaccine CPV-2 (Pfizer)	EU914139		2006	A	R	M	N	I	A	K	S	A	F	D	N	N	A	D	R	N

Table 3. A summary of the amino acid variation that characterizes the strains of Carnivore parvoviruses and the important mutations that distinguish the sequences from this study is shown. The 459 amino acid fragment of the VP2 protein sequenced is represented. Blue color indicates mutations among the domestic dog sequences, possibly introduced by the live-virus vaccine strain; violet color indicates important and repeated mutations found among the wildlife sequences;. *Incomplete strains. Clf, Canis lupus familiaris (domestic dog); Cm, Canis mesomelas (black-backed jackal); Pl, Panthera leo (lion); Cc, Crocuta crocuta (spotted hyaena); Civ. civ., Civettictis civetta (African civet); IA, Ichneumia albicauda (white-tailed mongoose), Lp, Lycaon pictus (wild dog); Gg, Genetta gennetta (genet)

Clade	Strain	*Sp.*	20	55	67	81	80	82	99	112	125	134	136	144	156	232	239	241	250	323	401	425
CPV-2	H494	Clf	A	E	R	R	R	V	D	V	D	S	L	E	S	I	D	V	V	N	L	T
	H489		·	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·	·	·	·	·
	H504		·	·	·	·	·	·	·	·	·	·	·	·	·	I	·	S	·	·	·	·
	H506		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	H469		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	H499		T	·	·	·	·	·	·	·	·	·	·	Q	·	·	·	·	·	·	·	·
	H488		T	·	·	·	·	·	·	·	·	·	·	·	F	·	E	·	·	·	·	P
	H327		T	·	·	·	·	·	H	·	·	·	·	·	·	·	·	·	·	·	·	·
	H493		·	·	T	K	·	M	·	·	·	·	F	·	·	·	·	·	·	·	·	·
	H503		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	H476*																·	·	·	·	·	·
CPV-2a	H491		·	Q	·	·	·	·	·	I	·	N	M	Q	·	·	·	·	G	·	·	·
	H321		T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	H501		·	·	·	·	·	·	·	·	·	N	·	·	·	·	·	·	·	·	·	·
	H492*																·	·	·	·	·	·
	H323*															·	·	·	·	·	·	·
	H318*																		·	·	·	·
	H398	Cm	T	·	·	·	T	·	H	·	Y	·	·	·	·	·	·	·	·	D	·	·

Table 4. The 459 amino acid fragment of the VP2 protein that characterizes Carnivore parvoviruses was sequenced in this study. Amino acid variation is shown. Amino acids used to differentiate CPV-2 from CPV-2a are not included. Clf, Canis lupus familiaris; Cm, Canis mesomelas.

Family, Species	Tissue							Age			Location			Total tested
	Sp	Bl	Br	LN	In	Li	SG	Adult	Suba	ND	SNP	NPA	ND	
Canidae														
Wild dog	11	3	3	3						20	1	9	10	20
Bat eared fox	10		2	1	1	1		9	2	4	14	1		15
BBJ	5	4	2	1	1	1	1	11		4	13		2	15
Aardwolf	1							1			1			1
Felidae														
Lion	13	25		3	1	2		23	4	17	41	1	2	44
Cheetah	3		2		1			4	1	1	6			6
Leopard	1							1			1			1
Serval	1							1			1			1
Hyenidae														
Spotted hyena	8	8	7	6	2	1		13	9	10	30	1	1	32
Striped hyaena			1	1				2			1	1		2
Viverridae														
African civet	5							5			4		1	5
Genet	3							2		1	1	1	1	3
Herpestidae														
Mongoose	2		2		2	1		5	2		5	1	1	7
Total	63	40	19	15	8	6	1	77	18	57	119	15	18	152

981 **Table 5.** Taxonomic families and species from which the analyzed samples
982 were obtained. Sp=spleen; Bl=blood; LN=Lymph Node; In=Intestine; Li=Liver;
983 SG=salivary gland; Suba=subadult; ND=no data; SNP=Serengeti National Park;
984 NPA= neighboring protected areas; BBJ=Black-backed jackal.

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Primer	Sequence	Binding site (nt)*
Forward(68)	5'-TGGAACTAGTGGCACACCAA-3´	3456–3473
Reverse(68)	5'-AAATGGTGGTAAGCCCAATG-3'	3636–3655c**
Probe(68)	5'-CAGGTGATGAATTTGCTACAGG-3´	3555–3576
VPF(78)	5'-ATGGCACCTCCGGCAAAGA-3'	2285-2303
VPR(78)	5´-TTTCTAGGTGCTAGTTGAG-3'	4512-4530c
P1(79)	5'-ATGAGTGATGGAGCAGTTC-3'	2788–2807
P3(79)	5'- CCATTTCTAAATTCTTTG-3'	3752–3770
P4(79)	5'-AAGTCAGTATCAAATTCTT-3'	4202–4221c

990 **Table 6.** Primers used in this study are shown. *Nucleotides numbered
991 according to Reed et al., 1988(69). **c, complementary

Reaction	Primers	Cycling conditions	Amplicon length (nt)
qPCR	Forward(68), Reverse(68), Probe(68)	5´ 95ºC, 40x (30" 95ºC, 30" 60ºC)	163
External PCR	VPF(78), VPR(78)	5´ 94ºC, 40x (30" 94ºC, 30" 48ºC, 150" 72ºC) and 7' 72ºC	2209
Internal PCR I	P1(79), Reverse	5´ 94ºC, 40x (15" 94ºC, 15" 58ºC, 110" 72ºC) and 7´ 72ºC	829
Internal PCR II	P3(79), P4(79)	5´ 95 ºC, 30x (30" 95ºC, 30" 44ºC, 45" 72ºC) and 7´ 72ºC	432
Internal PCR IV	Forward, P4	5´ 94 ºC, 40x (30" 95ºC, 30" 50ºC, 96" 72ºC) and 7´ 72ºC	746

992 **Table 7**. PCR thermal cycling conditions used in this study are shown
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