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ABSTRACT: Canine distemper virus (CDV) is a highly contagious pathogen that principally infects wildlife and domestic carnivores. Peridomestic species such as raccoons (*Procyon lotor*) experience outbreaks with high mortality. Clinical signs of infection include anorexia, fever, respiratory infection, and neurologic complications. Although not zoonotic, CDV poses a high risk to unvaccinated domestic animals and the conservation of endangered species. During 2013–16, we opportunistically collected wild and domestic carnivore specimens through a rabies surveillance program in northern Colorado, US. Brainstem and cerebellar tissue samples were independently tested for rabies and CDV by fluorescent antibody test. We tested a total of 478 animals for CDV, comprised of 10 wild and domestic carnivore species. A total of 15% (72/478) of all animals sampled tested positive for CDV, consisting of 24% (71/300) of raccoons and 4% (1/26) of coyotes (*Canis latrans*), but coinfection with rabies virus was not observed among CDV-positive animals. We extracted RNA from positive tissues, and a reverse-transcription PCR was used to create complementary DNA. We amplified and sequenced the hemagglutinin gene from 60 CDV-positive tissues, and a median joining network and maximum likelihood phylogenetic tree revealed two major lineages among samples. Phylogenetic analysis indicated that our sequences were most similar to the America-2 (*n*=55) and the America-3 (*n*=5) CDV lineages circulating in North America. Our results indicated two distinct and distantly related clades of CDV overlapping geographically and temporally among raccoon populations in northern Colorado.

Key words: Canine distemper virus, coyote, hemagglutinin, mesocarnivore, prevalence, raccoon, strain.

INTRODUCTION

Canine distemper virus (CDV) belongs to the genus *Morbillivirus*, family *Paramyxoviridae*. Like all viruses from this genus, CDV has enveloped virions and a single-stranded, negative-sense RNA genome (Barrett 1999; Elia et al. 2006) with approximately 15,690 nucleotides that encode six structural proteins (Yi et al. 2012). Canine distemper virus is highly contagious in carnivores and other wildlife species, and the major route of transmission is through respiratory excretions of aerosolized virus. Other bodily secretions, such as urine or feces, have also been shown to be infectious (Tipold et al. 1992; Deem et al. 2000; Williams 2001). The virus typically enters the host through the respiratory tract, spreads to the lymphatic system, then to the central nervous system. Clinical signs vary by species but typically include oculonasal discharge, vomiting, diarrhea, anorexia, fever, depression, respiratory infection, digital hyperkeratosis, and later in infection, neurologic complications and death (Deem et al. 2000; Williams 2001). Clinical signs of CDV, such as incoordination and other neurologic complications, can resemble clinical signs of rabies infection (Hoff et al. 1974; Williams 2001; Hamir 2011).

Canine distemper virus is not stable in the environment and relies on a supply of susceptible hosts to persist among animal populations. Canine distemper virus infections have been reported in terrestrial and aquatic carnivores as well as in artiodactylids and primates (van Moll et al. 1995; Williams 2001). Some host species of CDV maintain large territories and exist at low densities, such

as wolves (*Canis lupus*), mountain lions (*Puma concolor*), and coyotes (*Canis latrans*), whereas other host species are more ubiquitous and peridomestic such as raccoons (*Procyon lotor*) and striped skunks (*Mephitis mephitis*; Almberg et al. 2010). The combination of different spatial, behavioral, and demographic characteristics of multiple host species allows CDV to persist and avoid exhausting low-density host populations (e.g., Craft et al. 2008).

Phylogenetic studies of CDV strains worldwide have revealed twelve distinct clades linked to geographic regions (McCarthy et al. 2007; Nikolin et al. 2011; Riley and Wilkes 2015). Five of these clades have been documented in domestic or wild animals in the US and are commonly termed: America-1, America-2, America-3, America-4, and Arctic-like (McCarthy et al. 2007; Nikolin et al. 2011; Riley and Wilkes 2015). The America-1 strain is represented by vaccine strains and was observed in raccoons living near a zoo in Chicago, Illinois (Lednicky et al. 2004a; McCarthy et al. 2007). Dogs (*Canis lupus familiaris*), raccoons, javelina (*Tayassu tajacu*), and captive large felids including black leopard (*Panthera pardus*) and Chinese leopard (*Panthera pardus japonensis*) have hosted the America-2 strain (Harder et al. 1996; Bolt et al. 1997; Cherpillod et al. 1999; Lednicky et al. 2004a, b; Schumaker et al. 2012). The European Wildlife strain was previously reported in an isolated population of fishers (*Martes pennanti*) in California (Keller et al. 2012) and in dogs at a breeding facility in Missouri (Pardo et al. 2005). Additional work identified this group as a new strain (America-3) circulating in North America (Gámiz et al. 2011; Wilkes et al. 2014; Riley and Wilkes 2015; Pope et al. 2016). The America-4 strain has been observed in raccoons, foxes, and dogs from South Carolina, Tennessee, Virginia, and West Virginia (Wilkes et al. 2014; Riley and Wilkes 2015). The Arctic-like strain has only been observed in the US in dogs at the same breeding facility in Missouri previously mentioned (Pardo et al. 2005).

Distemper is a major cause of mortality in wild raccoons in North America and Europe (Roscoe 1993; Hamir 2011). Outbreaks of CDV in peridomestic wildlife, such as raccoons, increase the exposure to domestic and captive animals. A better understanding of CDV outbreaks in wildlife can assist in managing threats to pets, zoos, and agriculture. The majority of published research on CDV in Colorado has been limited to serologic surveys on coyotes and swift foxes (*Vulpes velox*) at the US Army Piñon Canyon Maneuver Site in Las Animas County (Gese et al. 1991, 2004) and the Denver metropolitan area (Malmlov et al. 2014). The discovery of a canine distemper epizootic during our rabies surveillance project provided us with the opportunity to investigate genetic variation of CDV isolates across multiple species in northern Colorado over time.

MATERIALS AND METHODS

Sample collection

A total of 478 samples from animals of 10 different species were tested for CDV (Table 1). Brain stem and cerebellar tissues were collected opportunistically from animals during a rabies surveillance project in northern Colorado during June 2013 through March 2016 (Fig. 1). The study area was then experiencing a rabies epizootic in striped skunk populations (Pepin et al. 2017). Animals tested in this study had been euthanized by local authorities, or collected postmortem, as part of routine public health surveillance for rabies or as part of enhanced surveillance efforts. Thus, animal specimens collected during this study were comprised of two general categories, those with: 1) direct exposure to humans or domestic animals, with or without clinical display of neurologic illness (i.e., collection due to suspicion of rabies); and 2) no documented exposure to humans or domestic animals, with or without clinical display of neurologic illness (i.e., collected for enhanced surveillance). The type of surveillance was recorded for all animals (i.e., public health versus enhanced), but the details of clinical presentation were not available for the majority of specimens. Carcasses were primarily obtained from Boulder and Larimer counties, Colorado, but additional carcasses and tissue samples were supplied by Colorado Parks and Wildlife and the Wyoming State Veterinary Laboratory. One sample from Washakie County, Wyoming was included due to the geographic similarity between northeastern Colorado and central northern Wyoming (Bailey 1983). Washakie County, Wyoming and northern

TABLE 1. Summary of sampling effort, by species of mesocarnivore and canine distemper virus prevalence (percent positive), in northern Colorado, USA during June 2013 through March 2016. Samples were tested by fluorescent antibody test on brain tissue collected postmortem.

Common name	Scientific name	Positive	Total	Prevalence (95% confidence interval)
Black bear	*Ursus americanus*	0	13	0
Bobcat	*Lynx rufus*	0	3	0
Coyote	*Canis latrans*	1	26	4% (1–19%)
Dog	*Canis lupus familiaris*	0	24	0
Gray fox	*Urocyon cinereoargenteus*	0	1	0
Canada lynx	*Lynx canadensis*	0	1	0
Mountain lion	*Puma concolor*	0	3	0
Raccoon	*Procyon lotor*	71	300	24% (19–29%)
Red fox	*Vulpes vulpes*	0	34	0
Striped skunk	*Mephitis mephitis*	0	73	0
Totals		72	478	

Colorado both belong to the Dry Domain and Steppe Division levels of ecosystem region delineation as described by Bailey (1983). Tissues were tested separately for rabies virus and CDV by fluorescent antibody test at Colorado State University Veterinary Teaching Hospital Veterinary Diagnostic Laboratory, Fort Collins, except for the single Wyoming sample which was tested by the Wyoming State Veterinary Laboratory.

Age determination

Whenever possible, samples were collected from raccoon carcasses for age determination. Year of age was determined from 92% (276/300) of raccoons tested for CDV. The majority of raccoons sampled were less than 1 yr old (60%, 165/276). The mandible was removed from the skull of each animal, cleaned of tissue, then split into two halves. Mandibles were processed by submerging in water within labeled Whirl-Pak bags, then placed in a hot water bath at 60 C–90 C for 2–6 h, depending on size. We removed the canine tooth and the first premolar tooth, ensuring that the root remained intact. Each tooth was placed in its own uniquely labeled paper envelope. Batches of teeth were randomized and each tooth given an identification number for a completely random and blind age comparison analysis. All samples were shipped to Matson's Laboratory (Manhattan, Montana, USA) for year-of-age determination.

Viral RNA isolation

Unfixed portions of brain tissue were homogenized with 100 μL of lysis buffer in a Retsch® MM301 mixer mill (Haan, Germany) at 20 Hz for 4 min. Canine distemper virus RNA was isolated from the tissue homogenate using TRIzol® Reagent (Life Technologies Corporation, Carlsbad, California, USA) digestion, chloroform separation, isopropanol precipitation, and ethanol wash according to manufacturer's guidelines (Chomczynski and Sacchi 1987).

Viral RNA sequencing

The hemagglutinin gene (H gene) was targeted for sequencing due to the high variability of this gene and its frequent use in phylogenetic analyses of CDV (Lednicky et al. 2004a; Demeter et al. 2007). Sample H gene sequences were amplified by reverse-transcription PCR (RT-PCR) using a one-step RT-PCR kit (Invitrogen Corporation, Carlsbad, California, USA) to create complementary DNA from sample RNA. Primers for RT-PCR and DNA sequencing were obtained from previous CDV studies (Lan et al. 2005; Pardo et al. 2005; Demeter et al. 2007; Martella et al. 2007; Table 2). Thermal cycler conditions consisted of reverse transcription performed at 54 C for 30 min, an initial denaturation at 95 C for 15 min, 40 cycles of heat denaturation at 94 C for 45 s, primer annealing at 54 C for 45 s, and DNA extension at 68 C for 1 min followed by a final extension at 68 C for 10 min. Amplification of the H gene was verified using agarose gel electrophoresis and visualized by ethidium bromide staining. Exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT™, Affymetrix, Cleveland, Ohio, USA) was used to clean PCR products following manufacturer's protocols with the modification of using 1.0 μL per sample to remove unincorporated primers and deoxyribonucleotide triphosphates. The H gene was sequenced following labeling with BigDye® Terminator Cycle Sequencing chemistry (Applied Biosystems, Foster
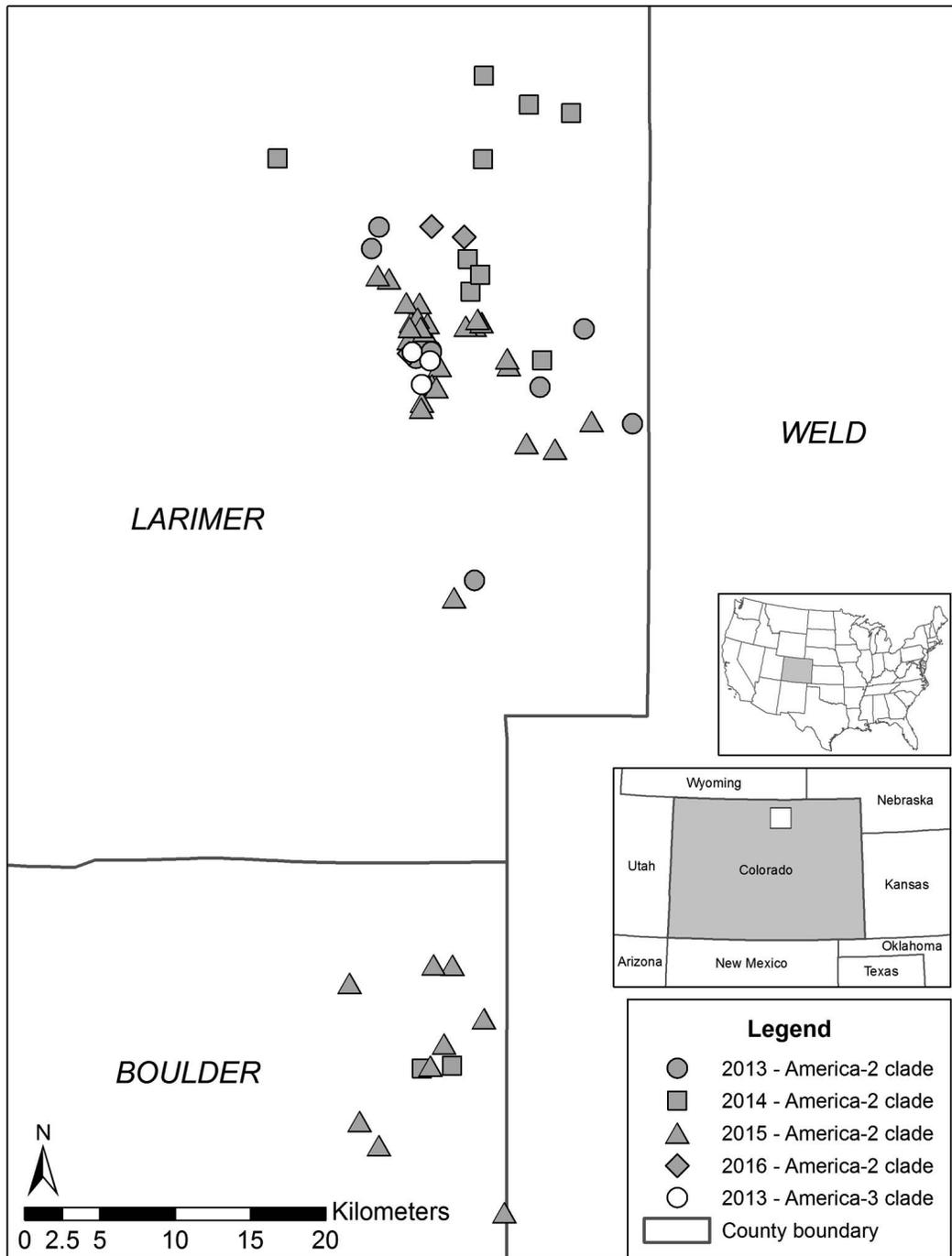
FIGURE 1.   Collection locations of raccoons (*Procyon lotor*) and one coyote (*Canis latrans*) that tested positive for canine distemper virus in northern Colorado, USA during June 2013 through March 2016. Sample shade and symbol describe the year collected and genetic clade of each sample. The names of clades are based on phylogenetic analysis.

TABLE 2.   The primer names, sequences, and citations for the PCR amplification and Sanger sequencing of the canine distemper virus hemagglutinin gene from positive raccoons (*Procyon lotor*) and one coyote (*Canis latrans*) in northern Colorado, USA during June 2013 through March 2016.

Primer name	Sequence (5′→3′)	Primer citation
CHF	AAC TTA GGG CTC AGG TAG TC	Demeter et al. 2007
CDV-HS2	ATG CTG GAG ATG GTT TAA TTC AAT CG	Lan et al. 2005
204	GAA TTC GAT TTC CGC GAT CTC C	Martella et al. 2007
232b	TAG GCA ACA CCA ATA ATT TRG ACT C	Martella et al. 2007
14	GTC CTT CTC ATC CTA CTG G	Pardo et al. 2005
16	AGT GGA GAT CGC GGA AGT	Pardo et al. 2005
21	CAT CTT ATG GGC GGT TGA	Pardo et al. 2005
22	GTG AAC TGG TCT CCT CTA	Pardo et al. 2005

City, California, USA), purified through Sephadex™ G-50 columns (GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania, USA), and loaded on an ABI 3500xl Genetic Analyzer (Applied Biosystems). The DNA sequences were manually trimmed, edited, and assembled using Sequencher v. 5.3 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and exported as FASTA files for sequence analysis.

Data analysis

Sequences representative of the 12 major CDV lineages were selected from GenBank (see Supplementary Table S1) and included in the phylogenetic analysis to provide a broader comparison of samples from this study. Phocine distemper virus was included for outgroup rooting. The CDV H gene sequence alignment was performed using MEGA v. 7 (Kumar et al. 2016). The best-suited nucleotide substitution model for the generated data was selected using jModelTest v. 2.1.7 (Guindon and Gascuel 2003; Darriba et al. 2012). A maximum likelihood phylogenetic tree was created using MEGA with 10,000 replicates and the transversional substitution model with gamma distributed rate variation among sites (Posada 2003). To describe relationships that violated the assumptions of phylogenetic analyses (Posada and Crandall 2001), a median joining network (Bandelt et al. 1999) was created using POPART (Leigh and Bryant 2015). Networks allow relationships among samples to be displayed as a descriptive reticulation rather than a simple bifurcating tree and offer resolution at the scale of a single nucleotide. Standard genetic diversity indices, including gene diversity (Nei 1987) and F_{ST} (Wright 1950), were estimated using Arlequin v. 3.5 (Excoffier et al. 2005; Excoffier and Lischer 2010).

RESULTS

CDV prevalence

Samples positive for CDV included one coyote and 71 raccoons, resulting in virus prevalence of 4% (n=26, 95% confidence interval 1–19%) and 24 (n=300, 95% confidence interval 19–29%), respectively. Within the young of the year cohort of raccoons, 19% (31/165) of samples were CDV positive. The proportion of CDV-positive samples within each age cohort increased to 35% (23/65) and 44% (11/25) among 1-yr-old and 2-yr-old raccoons respectively, which comprised 24% (65/276) and 9% (25/276) of the total sample, before declining to 10% (2/21) among raccoons that were 3 years and older.

Sequence data

Due to the opportunistic nature of obtaining carcasses and tissue samples, and the complex networks of organizations involved in the study, only 68 of the 72 CDV-positive tissue samples were available for genetic analysis. Among the 68 CDV-positive tissue samples, 60 samples yielded complementary DNA suitable for sequencing whereas eight samples were of unsatisfactory quality. The CDV H gene sequences were trimmed to 1,933 nucleotides, among which 157 (8.1%) were polymorphic. Sample CDV H gene sequences were uploaded to GenBank (accession nos. MF953415–MF953474). Thirty-one unique haplotypes were observed, ranging from one to 12 individuals per haplotype (Fig.
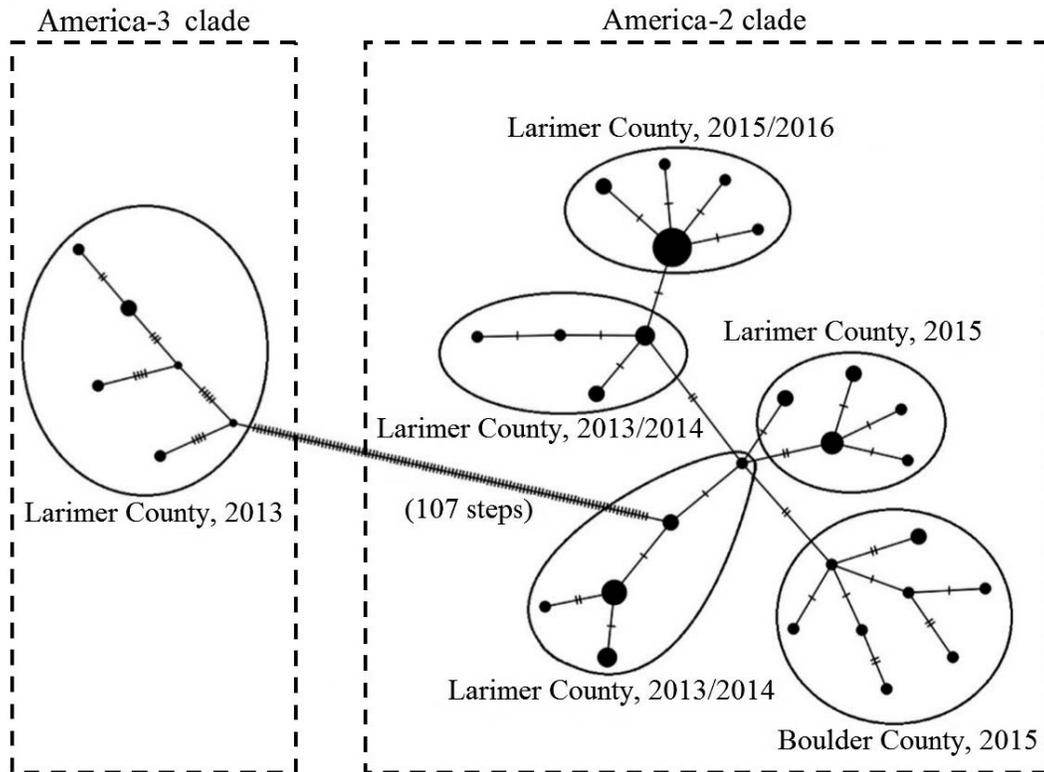
FIGURE 2.   Median joining network showing relationships among canine distemper virus hemagglutinin gene sequences collected from positive raccoons (*Procyon lotor*) and one coyote (*Canis latrans*) in northern Colorado, USA during June 2013 through March 2016. Each tick-mark on lines connecting samples represents a single nucleotide difference. Dashed boxes identify the samples belonging to the America-2 and America-3 clades. The America-3 clade includes two inferred ancestral nodes within the network.

2). Phylogenetic and network analyses of H gene sequences revealed the presence of two distinct groups (Figs. 2, 3). The median joining network included two inferred ancestral nodes within the America-3 clade (Fig. 2).

Phylogeny

A phylogram was created by combining the 60 H gene sequences from this study with 37 additional H gene sequences obtained from GenBank (Fig. 3; see Supplementary Table S1 and Supplementary Figs. S1, S2). Sample H gene sequences were trimmed to 1,815 nucleotides to match sequence length with reference sequences from GenBank. Among the 60 sequences analyzed from this study, 55 samples grouped with the America-2 clade (92%; see Supplementary Fig. S1) and five samples aligned with the America-3 clade

(8%; see Supplementary Fig. S2). Among the 55 America-2 sequences, 26 unique haplotypes were observed. Four novel haplotypes were observed from our study samples within the America-3 clade. The gene diversity values among the 55 America-2 sequences and the five America-3 sequences from this study were 0.9367 ± 0.0215 and 0.9000 ± 0.1610, respectively. The pairwise F_{ST} value between the two clades was 0.96 (P-value=0.0 ± 0.0).

The median joining network confirmed substantial genetic differentiation between two groups of samples, which were separated by 107 nucleotide differences (Fig. 2). Furthermore, the median joining network resolved the America-2 clade into subclades corresponding approximately with the location and year sampled.
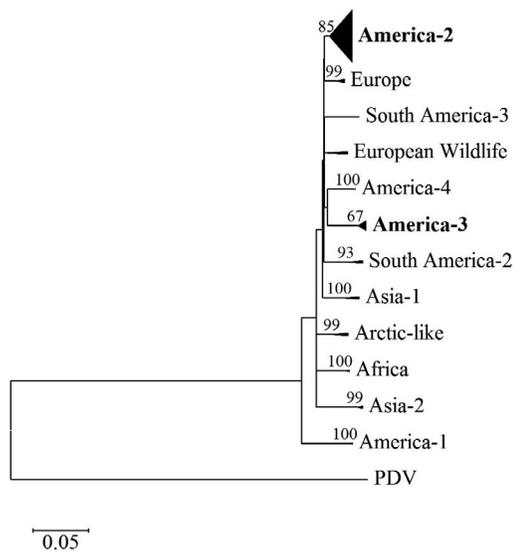
FIGURE 3. Phylogram constructed from canine distemper virus hemagglutinin gene sequences collected from positive raccoons (*Procyon lotor*) and one coyote (*Canis latrans*) in northern Colorado, USA during June 2013 through March 2016, compared to sequences from previous studies. Bootstrap values above 60% are shown. Samples from this study grouped with the bolded clades (America-2, *n*=55; America-3, *n*=5). Phocine distemper virus (PDV) was included as an outgroup. International isolate descriptions and expanded phylograms for America-2 and America-3 clades are provided in Supplementary Table S1 and Supplementary Figures S1 and S2, respectively.

DISCUSSION

Opportunistic sampling of mesocarnivore carcasses did not reveal cases of coinfection with both rabies virus and CDV; however, both diseases were detected throughout the sampling period (Pepin et al. 2017). Concurrent infections of rabies virus and CDV have been documented in populations of raccoons in Florida (Hoff et al. 1974) and New Jersey (Hamir et al. 1998) and red foxes (*Vulpes vulpes*) in Italy (Nouvellet et al. 2013). Previous studies have observed periodicity among CDV epizootics (Roscoe 1993) and CDV and rabies virus epizootics (Hoff et al. 1974), suggesting an interval of approximately 4–5 yr between outbreaks in raccoon populations.

The occurrence of multiple cocirculating strains of CDV is not uncommon in the scientific literature. Pardo et al. (2005) observed two different strains of CDV (America-3 and Arctic-like) in a dog breeding facility in Missouri, Lednicky et al. (2004a) observed two different strains (America-1 and America-2) in raccoons near a Chicago area zoo, and Hashimoto et al. (2001) observed two different strains (Asia-1 and Asia-2) in dogs in Japan. The majority of samples from this study (92%) belonged to the America-2 strain of CDV, although we also documented two CDV strains cocirculating during an epizootic.

This study documents additional cases of the America-3 strain of CDV, including its first detection in raccoons in the US. The America-2 strain has been documented multiple times in raccoons in the US (Blixenkrone-Møller et al. 1992; Lednicky et al. 2004a, b). The America-3 strain has been documented in dogs from a breeding facility in Missouri (Pardo et al. 2005), wild fishers from California (Keller et al. 2012), dogs from Tennessee, Texas, and Canada (Wilkes et al. 2014; Riley and Wilkes 2015), and an unpublished account of a dog in Wyoming in 2010 (GenBank JF283476). The America-3 strain was not observed to be circulating throughout the duration of the sample collection period. The America-2 and America-3 strains overlapped spatially and temporally early in the study, but the America-3 strain was not detected after 2013 when most (73.3%) of the samples were collected. It is currently unclear whether competitive exclusion between strains may have led to the apparent extinction of the America-3 strain during the study period.

Previous serologic surveys in North America observed a wide range of CDV neutralizing antibody prevalence estimates across mesocarnivore species. Among raccoon surveys, seroprevalence values were 16% in Indiana (Raizman et al. 2009), 22% in New York (Parker et al. 1961), 23% in Illinois (Mitchell et al. 1999), 33% in Nebraska (Bischof and Rogers 2005), 54% in Florida (Hoff et al. 1974), 58% in Wisconsin (Kamps et al. 2015), and 84% in Maryland (Jamison et

al. 1973). Previous surveys of coyotes have observed CDV neutralizing antibody prevalence ranging from 40–57% in Colorado (Gese et al. 1991, 2004; Malmlov et al. 2014), 61% in Nebraska (Bischof and Rogers 2005), and 64% in Wisconsin (Kamps et al. 2015). Serologic surveys use a blood sample from a live animal to screen for pathogen-specific antibodies with a virus neutralization test. In contrast, the fluorescent antibody test that we used was a direct antigen diagnostic test that required brain tissue and, therefore, could only be used postmortem. The observed prevalence among raccoons in our study (24%; Table 1) is within the range of prevalence reported by serologic methods mentioned above. The observed prevalence among coyotes in this study (4%; Table 1) is lower than prevalence reported from prior serosurveys. This difference may be an indication that coyotes were more refractory to infection with the strain circulating in this study and more likely to have experienced subclinical infections and survive encounters with CDV.

The CDV prevalence results from this study may be expected to differ in comparison to antibody prevalence estimates because serosurveys include animals which may survive virus exposure whereas the direct antigen test approach measures prevalence among animals that may have succumbed to the disease. Animals infected with CDV either die or experience a subclinical infection that confers lifelong immunity (Appel 1987; Williams 2001). Our study targeted animals that were likely to display clinical signs compatible with rabies or CDV infections. However, because we collected samples from carcasses or acquired tissue samples postmortem from other sources, detailed records of clinical signs and behaviors were frequently missing. Thus, the calculation of CDV prevalence presented here (Table 1) may be biased upwards, as the sample collection algorithm targeted animals with signs of neurologic illness or that were involved in a possible direct contact exposure with humans or pets (i.e., suspected of rabies).

Studies that examined relationships between seroprevalence and animal age found that both older raccoons (Mitchell et al. 1999) and older coyotes (Gese et al. 1991, 2004) had a greater prevalence of CDV antibodies compared to younger animals, suggesting that older animals were more likely to have been exposed to CDV (although, see Junge et al. 2007). In our study, a greater proportion of CDV cases were detected in younger raccoons, with the highest infection rates observed among the 1-yr-old and 2-yr-old age classes. This was not necessarily inconsistent with the serosurvey studies whereby older animals are more likely to have been exposed to the virus and survived. In contrast, younger animals may not have experienced an initial exposure, and high infection-associated mortality may lead to lower seroprevalence among exposed younger animals. Given the high proportion in our study of raccoons belonging to this age class, but their lower infection rate, our results did not suggest that the young of the year were a critical, susceptible cohort to maintain circulation of the virus.

Our study demonstrates how testing for multiple wildlife viruses during a rabies surveillance study led to a better understanding of CDV concurrently infecting wildlife populations. Pathogen isolation revealed two distantly related lineages of CDV, one previously undescribed in raccoons in northern Colorado.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2017-09-238.

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