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Molecular surveillance of canine distemper virus in diarrhoetic puppies in northeast China from May 2014 to April 2015

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ABSTRACT. To trace the prevalence of canine distemper virus (CDV) in diarrhoetic dogs, a total of 201 stool samples were collected in the Heilongjiang province of northeastern China from May 2014 to April 2015. The 201 fecal samples were subjected to the detection of CDV by using RT-PCR targeting the partial *N* gene, phylogenetic analysis based on the complete *H* gene, and co-infection analysis. Results indicated that 24.88% (50/201) of the samples were positive for CDV. The fifty CDV samples exhibited an overall co-infection rate of 94% (47/50) with four enteric viruses (82%, 41/50) and five bacteria (72%, 36/50). The positivity rate of CDV exhibited differences among regions, seasons, ages and immunization status. Phylogenetic analysis of the complete *H* genes (n=6) revealed that the CDV strains identified in our study belonged to the Asia-1 group, and showed genetic diversities. These data provide evidence that there are a number of genetically diverse CDV Asia-1 strains circulating in diarrhoetic dogs in northeastern China; the CDV-affected animals exhibit the high co-infection with other enteric viruses and bacteria.

KEY WORDS: canine distemper virus, diarrhoetic dog, epidemiology, genetic diversity

Canine distemper virus (CDV) is the etiological agent of canine distemper which is characterized by gastrointestinal, nervous, and/or respiratory symptoms [1]. Recently, CDV infections attracted more attention as a result of its re-emergence, increased activity and genetic diversity worldwide [3, 6, 13]. Canine infectious diarrhea is challenging for veterinarians because of its pathogenic variability and the concurrent presence of viral, bacterial, and protozoan co-infections [7, 9, 11, 16]. Recently, Gizzi *et al*. (2014) reported that CDV showed prevalence rates of 1.0 to 8.7% in diarrhoetic dogs from Brazil, U.S.A., Australia, Canada, the U.K. and Japan [8]. At present, there is still little information on the prevalence of CDV in diarrhoetic dogs in China. In this study, the molecular surveillance of canine distemper virus in diarrhoetic puppies in northeast China from May 2014 to April 2015 was carried out. Resulting data indicated the prevalence, genetic evolution and co-infection of the CDV strains circulating in diarrhoetic dogs in China.

In our study, a total of 201 fecal samples were collected in the form of rectal swabs from diarrhoetic dogs in animal hospitals in the Harbin, Daqing, and Mudanjiang districts of Heilongjiang province in northeastern China from May 2014 to April 2015. All rectal swab samples were stored at −80°C, and their information had been reported in our previous studies [7, 11, 13, 16]. For CDV detection, the viral RNAs were extracted from each sample by using the TIANamp Virus RNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. For bacterium detection, the bacterial genomic DNAs were extracted by using TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's instructions. The extracted viral RNA and genomic DNA were stored at −80°C.

The detection of CDV was performed by using the nested RT-PCR targeting N gene according to the report described by Budaszewski Rda *et al*. [3]. Briefly, first-strand cDNA was synthesized by using Moloney murine leukemia virus (RNase H-) reverse transcriptase (Novoprotein Scientific Inc., Shanghai, China) according to the manufacturer's instructions. PCR amplification conditions were the same as those described by Budaszewski Rda *et al*. [3]. In our study, EmeraldAmp PCR Master Mix (2 × Premix) (TaKaRa Biotechnology Co., Ltd., Dalian, China), and Applied Biosystems GeneAmp PCR System 9700 thermal cycler (Thermo Fisher Scientific, Waltham, MA, U.S.A.) were used for PCR amplification of the target genes. Of the 201 samples, 24.88% (50/201) were positive for CDV (Table 1). The CDV-positive rate differed among the three districts of Heilongjiang province (32.62% for Harbin, 10% for Daqing, 5% for Mudanjiang) (Table 1). Of the CDV-positive animals, 44% had a vaccination history, 42% were aged 3–4 months, 28% were aged 2–3 months, and 66% of the samples were collected from

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Table 1. Further analysis of the CDV-positive samples

Classification		Harbin (n=141)	Daqing (n=20)	Mudanjiang (n=40)	Total (n=201)
Positive rate for CDV (%)		32.62 (46/141)	10 (2/20)	5 (2/40)	24.88 (50/201)
Vaccination (%)					
Yes		43.48 (20/46)	50 (1/2)	50 (1/2)	44 (22/50)
No		36.96 (17/46)	—	50 (1/2)	36 (18/50)
Collection data (%)	Jan. to Mar.	4.35 (2/46)	50 (1/2)	50 (1/2)	8 (4/50)
	Apr. to Jun.	—	—	—	—
	Jul. to Sep.	26.09 (12/46)	50 (1/2)	—	26 (13/50)
	Oct. to Dec.	69.57 (32/46)	—	50 (1/2)	66 (33/50)
Age (%)	1M <Age ≤2M	6.52 (3/46)	—	—	6 (3/50)
	2M <Age ≤3M	30.43 (14/46)	—	—	28 (14/50)
	3M <Age ≤4M	41.30 (19/46)	—	100 (2/2)	42 (21/50)
Co-infection of 50 CDV samples with other enteric viruses (%)					
Viruses		82.61 (38/46)	100 (2/2)	50 (1/2)	82 (41/50)
	CPV-2	32.61 (15/46)	—	—	30 (15/50)
	CCoV	10.87 (5/46)	100 (2/2)	—	14 (7/50)
	CaKV	4.35 (2/46)	—	50 (1/2)	6 (3/50)
	CBoV	2.17 (1/46)	—	—	2 (1/50)
	CPV-2+CCoV	8.69 (4/46)	—	—	8 (4/50)
	CaKV+CCoV	8.69 (4/46)	—	—	8 (4/50)
	CBoV+CPV-2	2.17 (1/46)	—	—	2 (1/50)
	CaKV+CPV-2	2.17 (1/46)	—	—	2 (1/50)
	CPV-2+CaKV+CCoV	10.87 (5/46)	—	—	10 (5/50)
Bacteria		73.91 (34/46)	—	100 (2/2)	72 (36/50)
	Diarrheagenic *E. coli*	36.96 (17/46)	—	100 (2/2)	38 (19/50)
	Vibrio cholerae	34.78 (16/46)	—	—	32 (16/50)
	Shigella spp.	19.57 (9/46)	—	—	18 (9/50)
	Campylobacter spp.	13.04 (6/46)	—	—	12 (6/50)
	Salmonella enterica	2.17 (1/46)	—	—	2 (1/50)
	Diarrheagenic *E. coli*+*Shigella* spp.	8.69 (4/46)	—	—	8 (4/50)
	Diarrheagenic *E. coli*+*Vibrio cholerae*	6.52 (3/46)	—	—	6 (3/50)
	Diarrheagenic *E. coli*+*Vibrio cholerae*+*Shigella* spp.	4.35 (2/46)	—	—	4 (2/50)
	Diarrheagenic *E. coli*+*Vibrio cholerae*+*Campylobacter* spp.	4.35 (2/46)	—	—	4 (2/50)
	Diarrheagenic *E. coli*+*Shigella* spp. +*Campylobacter* spp.	2.17 (1/46)	—	—	2 (1/50)
Viruses+Bacteria		95.65 (43/46)	—	100 (2/2)	94 (47/50)
Co-infection analysis among vaccined and unvaccined dogs (%)					
Viruses	Vaccination	95.00 (19/20)	100 (1/1)	100 (1/1)	95.45 (21/22)
	Unvaccination	70.58 (12/17)	—	0 (0/1)	66.67 (12/18)
Bacteria	Vaccination	85.00 (17/20)	0 (0/1)	100 (1/1)	81.82 (18/22)
	Unvaccination	64.71 (11/17)	—	0 (0/1)	61.11 (11/18)
Viruses+Bacteria	Vaccination	100 (20/20)	100 (1/1)	100 (1/1)	100 (22/22)
	Unvaccination	94.12 (16/17)	—	100 (1/1)	94.44 (17/18)

October to December (Table 1). The detailed information about the CDV positive dogs was shown in Supplemental Table 1.

The CDV-positive rate in feces of diarrhoetic dogs has been reported to be 8.7% (9/104), 1.4% (110/7,829), 2.3% (12/526), 1.6% (46/2,855), 2.5% (7/674), 1.0% (5/486) in Brazil, the U.S.A., Australia, Canada, the U.K. and Japan by using real-time PCR [9]. The CDV-positive rate identified in our study was significantly higher than that of other countries. CPV-2, CCoV, CaKV and CBoV had been reported to be associated with severe enteritis in puppies [4, 5]. In our previous studies, the positivity rates of CPV-2, CCoV, CaKV and CBoV had been revealed to be 47.26% (95/201), 28.36% (57/201), 17.91% (36/201) and 7.46% (15/201), respectively, within the same 201 samples investigated in the current study [7, 11, 13, 16]. The CDV-positive rate (24.88%) within these samples is nearly equal to CCoV's positivity rate. These data demonstrate that there is a very high prevalence of CDV in diarrhoetic dogs in northeastern China, making it one of main enteric pathogens in this region.

Continuation of Table 1.

Classification		Harbin (n=141)	Daqing (n=20)	Mudanjiang (n=40)	Total (n=201)
Identity analysis of 6 complete *H* genes (%)					
	Nuleotides	97.0–99.7	—	97.6	96.7–99.0
	Amino acids	96.7–99.0	—	97.9	96.1–99.0
Comparison of 6 complete *H* genes with reference srtains (%)					
Onderstepoort vaccine strain	Nuleotides	90.7–91.5	—	90.7–91.2	90.7–91.5
	Amino acids	89.8–90.7	—	89.9–90.6	89.8–90.7
Lederle vaccine strain	Nuleotides	90.6–91.4	—	90.5–91.0	90.5–91.4
	Amino acids	89.0–90.3	—	89.1–89.8	89.0–90.3
Rockborn vaccine strain	Nuleotides	95.1–95.7	—	95.3–95.8	95.1–95.8
	Amino acids	94.6–95.6	—	94.2–95.6	94.2–95.6
Convac vaccine strain	Nuleotides	90.7–91.4	—	90.5–91.0	90.5–91.4
	Amino acids	89.1–90.1	—	89.0–89.6	89.0–90.1
Snyder Hill vaccine	Nuleotides	90.5–91.2	—	90.4–90.9	90.4–91.2
	Amino acids	90.0–90.8	—	89.8–90.5	89.8–90.8
CDV3 vaccine strain	Nuleotides	90.6–91.3	—	90.5–91.0	90.5–91.3
	Amino acids	90.1–91.1	—	90.0–90.6	90.0–91.1
Asia 1 strain	Nuleotides	97.5–97.9	—	97.5–97.6	97.5–97.9
	Amino acids	97.2–98.0	—	96.9–97.7	96.9–98.0
Asia 2 strain	Nuleotides	91.7–92.4	—	92.3–92.6	91.7–92.6
	Amino acids	91.9–92.8	—	91.6–92.8	91.6–92.8
Asia 3 strain	Nuleotides	88.8–89.2	—	89.1–89.4	88.7–89.4
	Amino acids	85.0–85.7	—	84.7–85.7	84.7–85.7
Asia 4 strain	Nuleotides	92.7–93.3	—	92.8–93.1	92.7–93.3
	Amino acids	93.4–94.7	—	93.1–94.4	93.1–94.7
European Wildlife strain	Nuleotides	93.4–94.1	—	93.7–94.2	93.4–94.2
	Amino acids	93.8–94.6	—	93.4–94.6	93.4–94.6
Arctic like strain	Nuleotides	93.5–93.9	—	93.8–94.0	93.5–94.0
	Amino acids	92.9–93.8	—	92.8–93.8	92.8–93.8
Africa strain	Nuleotides	93.5–93.9	—	93.4–93.9	93.4–93.9
	Amino acids	93.6–94.6	—	93.3–94.6	93.3–94.6
America 2 strain	Nuleotides	92.7–93.4	—	92.9–93.6	92.7–93.6
	Amino acids	91.9–92.8	—	91.8–92.9	91.8–92.9

The detailed information of the reference strains as follows: Onderstepoort vaccine strain from U.S.A. (AF378705); Lederle vaccine strain from Hungary (DQ903854); Rockborn vaccine strain from Denmark (GU266280); Convac vaccine strain from Denmark (Z35493); Snyder Hill vaccine from Russia (AF259552); CDV3 vaccine strain from China (EU726268); Asia 1 strain from China (FJ423608); Asia 2 strain from Japan (AB040768); Asia 3 strain from China (EU743935); Asia 4 strain from China (KJ437594); European Wildlife from Denmark (Z47759); Arctic Like strain from Denmark (Z47760); Africa strain from South Africa (FJ461703); America 2 strain from U.S.A. (AY649446).

To investigate the co-infection of CDV with other pathogens, the 50 CDV-positive samples were subjected to detection of canine enteric viruses and bacteria. For viruses, canine parvovirus-2 (CPV-2), canine kobuvirus (CaKV), canine coronavirus (CCoV), and canine bocavirus (CBoV) were detected by either PCR or RT-PCR according to previously described protocols or reports [7, 10, 11, 16]. For bacteria, the main bacterial pathogens were detected by using the multiplex PCR panels described by Sjöling *et al.* (2015) on basis of their specific virulence genes, including *Salmonella, Shigella* spp. (*S. dysenteria, S. sonnei,* and *S. flexneri*), *Campylobacter* spp. (*C. coli,* and *C. jejuni*), *Vibrio cholerae* (*V. vulnificus, V. parahaemolyticusm,* and *V. cholerae*), *Yersinia enterocolitica, Aeromonas hydrophila, enteroaggregative Escherichia coli* (EAEC), *enteropathogenic E. coli* (EPEC), *enterotoxigenic E. coli* (ETEC), *enterohemorrhagic E. coli* EHEC and *enteroinvasive E. coli* (EIEC) [14]. The detailed information about co-infections of the CDV-positive dogs in northeast China was shown in Supplemental Table 1.

In our study, 94% (47/50) of CDV-positive samples exhibited co-infection with at least one enteric pathogen (Table 1). The co-infections of CDV with CPV-2, CCoV, CaKV, and CBoV accounted for 82% (41/50), in which 30% were positive for CPV-2, 14% were positive for CCoV, 6% were positive for CaKV, 2% were positive for CBoV, 8% were positive for CPV-2 and CCoV, 8% were positive for CaKV and CCoV and 10% were positive for CPV-2, CCoV and CaKV (Table 1).

Co-infections between CDV and enteric bacteria were observed in 72% of the cases (36/50) (Table 1). Of the co-infections, 38%

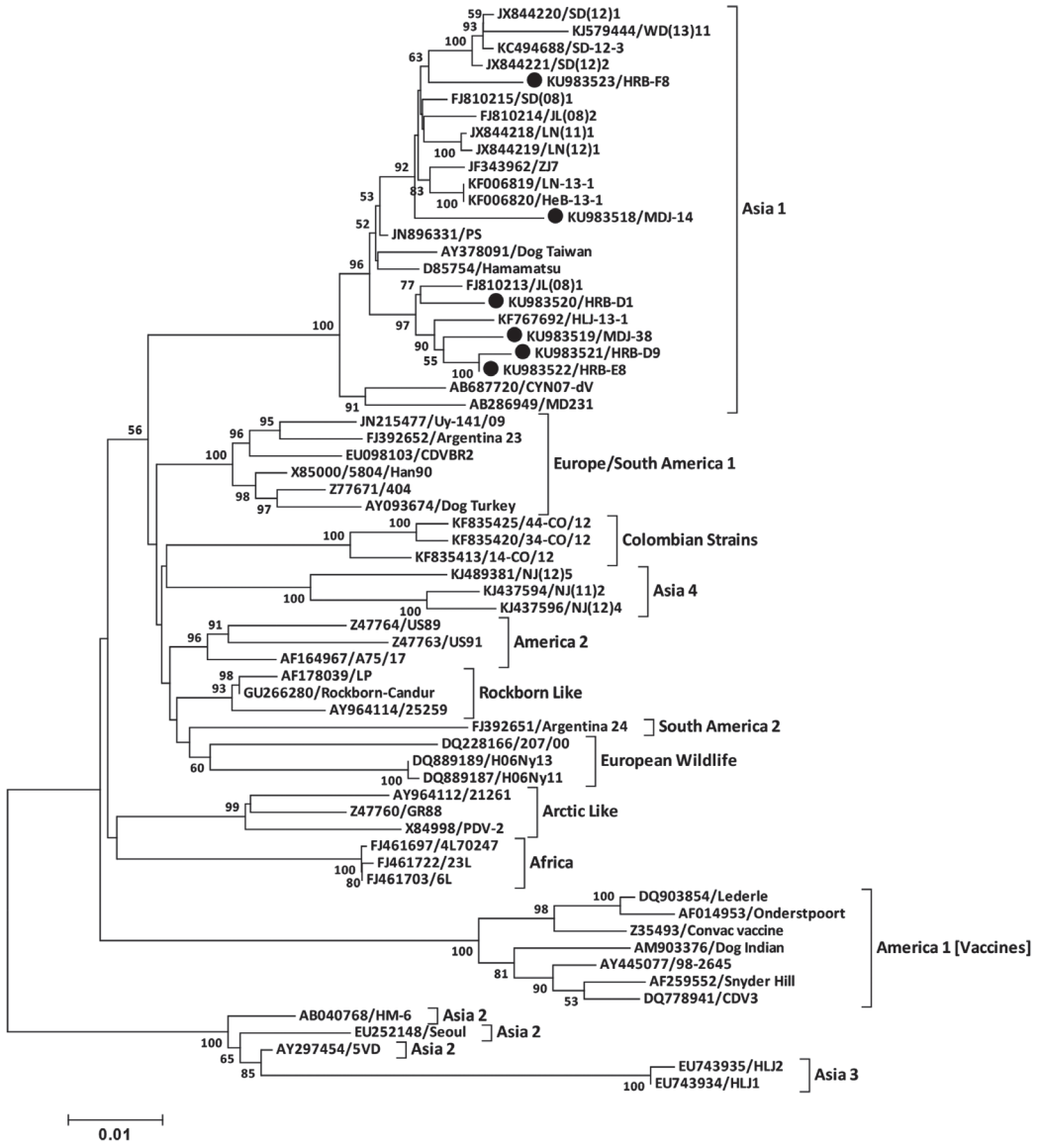

Fig. 1. Phylogenetic analysis of the complete *H* gene of six CDV strains identified in our study. Black circle represents the CDV strains identified in our study.

were positive for diarrheagenic *E. coli* (EIEC/EPEC/EHEC/typical EPEC), 32% were positive for *V. cholerae,* 18% were positive for *Shigella* spp., 12% were positive for *Campylobacter* spp., 2% were positive for *S. enterica*, 8% were positive for diarrheagenic *E. coli* and *Shigella* spp., 6% were positive for diarrheagenic *E. coli* and *V. cholerae*, 4% were positive for diarrheagenic *E. coli*, *V. cholerae* and *Shigella* spp., 4% were positive for diarrheagenic *E. coli*, *V. cholerae* and *Campylobacter* spp., 2% were positive for diarrheagenic *E. coli*, *Shigella* spp. and *Campylobacter* spp. (Table 1).

Mixed infections of canine enteric viruses and bacteria frequently occur in diarrheic dogs. Gizzi *et al.* (2014) reported that 68.3% were positive for at least one pathogen in 104 fecal samples of the diarrheic dogs; single infection and co-infection rates were 54.9 and 45.1%, respectively; 65.6% of the animals (21/32) had dual infections, 15.6% of the animals (5/32) had triple infections, and 18.8% of the animals (6/32) had quadruple infections [8]. In our study, the co-infection of CDV with least one other enteric pathogen was revealed to be 94%, of which 82% of co-infections had at least one enteric virus and 72% with at least one enteric bacteria. The most common single co-infection of CDV was with CPV-2 virus (30%), or with diarrheagenic *Escherichia coli* (38%). The co-infection of CDV with CPV-2, CaKV and CCoV reached 10%. The high co-infection rates of CDV positive samples with multiple pathogens are responsible for the high incidence of diarrhea in dogs. However, the effect of multiple pathogens on the disease outcomes remains unclear. Therefore, we suggest that further studies need to better explain the relationship between the high co-infection rates and the severity of clinical symptoms.

To trace evolution of the CDV strains identified in our study, we attempted to perform the sequencing of the complete *H* gene of the 50 identified CDV strains according to the protocol described by Budaszewski Rda *et al*. [3]. The first round of amplification

was conducted by using primer pair RH3-F and RH4-R, and the nested PCR was performed by using inner primer pairs H1F/H1R, H2F/H2RB, CDVF10B/CDVR10 and H3FB/H3R generating overlapping fragments of 789, 700, 870 and 542 bp, respectively. The amplified PCR products for the complete *H* genes were directly subjected to Sanger sequencing. Sequence analysis was carried out by using the EditSeq program in the Lasergene DNASTAR™ version 5.06 software). All nucleotide sequences generated in our study were submitted to GenBank. Furthermore, phylogenetic tree of the complete *H* genes was generated from the ClustalX-generated alignments by MEGA6.06 software using the neighbor-joining method [15]. Result indicated that a total of six complete *H* genes were successfully amplified and sequenced in our study. The sequence comparison revealed that the six complete *H* genes showed nucleotide homologies of 96.7–99% and amino acids homologies of 96.1–99% (Table 1). The six complete *H* genes identified in our study exhibited a high nucleotide similarity (97.5–97.9%) with the CDV Asia 1 reference strain (Table 1). Furthermore, the phylogenetic analysis revealed that the six CDV strains were divided into the CDV Asia 1 group, forming three clades (Fig. 1). At present, CDV strains include ten distinct lineages known as America-1, America-2, Arctic-like, Rockborn-like, Asia-1, Asia-2, Africa-1, European Wildlife, Europe/South America-1 and South America-2 [6]. In our study, only CDV Asia-1 strains were identified, which is in line with most reports from China [2, 12]. Although the type Asia-1 is the most predominant CDV group in China, the CDV Asia-1 strains identified in our study also exhibited genetic diversity (Fig. 1). The genetic diversity of CDV Asia-1 strains may be attributed to the long-term immune pressure which induces the adaptive mutation of CDV strains to varying degrees.

Our results reveal genetic diversity, complex co-infection and high prevalence of CDV Asia-1 strains in diarrhoetic dogs in northeastern China. Our findings suggest that CDV infection plays an important role in the onset diarrhea in dogs in northeastern China. These data increase understanding of the CDV Asia-1 strains as diarrhea-causing agents, and provide valuable etiological information of diarrhea in dog populations. However, further studies are needed to clarify the effect of the highly prevalent co-infections reported in this study on the severity of the clinical symptoms of the diarrhoetic dogs.

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