First Report of Concurrent Infection of Canine Kobuvirus and Canine Distemper Virus in a Diarrheic Dog in India

Agnihotri, D.,[1] Maan, S.,[1]* Batra, K.,[1] Kumar, A.,[1] Singh, Y.,[1] and Mor. S.K.[2]

[1] College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana 125 004, India.
[2] Molecular Diagnostic Development Lab, Veterinary Diagnostic Laboratory, Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, 1333 Gortner ave., Saint Paul, MN-55108, USA.

* **Corresponding author:** Dr. Sushila Maan Professor and Head, Department of Animal Biotechnology, LUVAS, Hisar, Haryana, India-125 004, India. Email: sushilamaan105@gmail.com

ABSTRACT

Canine Kobuvirus (CKoV) has been recently reported in many countries such as United Kingdom, China, Thailand, United States of America and Italy. In the present study, dogs suffering from gastroenteritis were screened for the presence of Canine Distemper Virus (CDV) in their fecal samples using RT-PCR. The dogs found to be positive for the presence of CDV in the diarrheic fecal samples were subjected to next generation sequencing (NGS) for the whole genome analysis. From one sample the whole genome of Canine Kobuvirus (CKoV) along with partial genome sequences of CDV was obtained. Phylogenetic analysis based on the complete nucleotide genome sequence of CKoV, revealed that the virus had 91-95% nucleotide identity with the Chinese, Japanese and UK strains. Similarly, the phylogenetic analysis based on the partial genome sequence of the CDV isolate showed 97% nucleotide identity with other Indian isolates and Chinese strains. To the best knowledge of the authors, this is the first report of detection and molecular characterization of CKoV in a domestic dog in India. Our result highlights the concerns to veterinarians that diarrhea in dogs may also be due to Canine Kobuvirus infection in addition to the other potential pathogens, and should not be ignored.

Keywords: Canine Kobuvirus; Canine Distemper Virus; Next Generation Sequencing (NGS); Whole Genome.

INTRODUCTION

The *Kobuvirus* which has been identified as a new genus in the family *Picornaviridae*, consists of three species, Aichivirus A (formerly Aichivirus) (1), Aichivirus B (formerly bovine kobuvirus) (2) and Aichivirus C (porcine kobuvirus) (3). The species Aichivirus A consists of four types: Aichi virus 1, canine kobuvirus 1 (4), feline kobuvirus 1 (5) and murine kobuvirus 1 (6). The species Aichivirus B consists of three types: bovine kobuvirus 1 (2), ferret kobuvirus 1 (7) and ovine kobuvirus (8). The species Aichivirus C consists of a single type: porcine kobuvirus 1 (3). Recently, a distinct group of kobuviruses, designated caprine kobuviruses (CKOVs) was proposed as a new candidate species, Aichivirus D, within the genus (9).

Canine Kobuvirus (CKoV) is believed to have originated from the Aichi virus, 20-50 years ago. It belongs to Aichivirus A and is considered to be a genotype (CaKV type 1) distinct from murine kobuvirus (MuKV type 1) and human AiV (AiV type 1) (10). CKoV has a single strand (ss) RNA (+) genome and one large open reading frame (ORF) encoding a single polyprotein that is cleaved into 3 structural capsid proteins (VP0, VP1, and VP3) and 7 non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (4).

Several infections of viral origin are known to affect the

health and wellbeing of dogs (11).These include Canine Parvovirus (CPV), Canine Distemper virus (CDV), Canine Corona virus (CCoV), Canine Herpesvirus (CHV), Canine Adenovirus (CAV) and CKoV to name just a few. Out of these CDV causes a well-known, highly infectious viral disease with mortality in dogs, while current knowledge of kobuvirus infections in carnivores is extremely limited. CDV causes fever, vomiting, diarrhoea, respiratory symptoms, seizures and paralysis in the host. It is distributed globally with a broad host range, including many mammalian species of the families *Canidae*, *Mustelidae*, *Procyonidae*, *Ursidae* and *Viverridae* (12). The duration of the disease is based on the immune response to the CDV by the infected animal.

CKoV is very difficult to isolate and the diagnosis is mainly based on molecular methods. RT-PCR has been developed for the detection of CKoV in feces but further investigation is required for clarification of its pathogenesis (13, 14). The epidemiologic surveillance and genome characterization of CKoVs might help to clarify the global distribution of the virus and its possible association with enteric disease in dogs.

In the present study, we report the concurrent detection of two viral pathogens, Canine Distemper Virus and Canine Kobuvirus in a diarrhoeic sample of a Golden Retriever dog, which was brought to the Veterinary Clinical Complex of College of Veterinary Sciences, LUVAS, Hisar, for disease diagnosis and treatment. The dog showed clinical signs of gastroenteritis and was suspected to be suffering from Canine Distemper. Initial confirmation of the CDV infection was carried out using RT-PCR followed by conventional sequencing. Next-generation sequencing was used to characterize the viruses present in the diarrhea specimen. The study represents the first detection of Canine Kobuvirus in India from a diarrhoeic pet dog.

MATERIAL AND METHODS

For the molecular study, 50 faecal samples were collected from dogs brought to the Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS) clinics from October 2015 to September 2016. The cases presented for routine investigation or deworming and vaccination constituted the healthy control group. The faecal samples were collected by introducing sterile swabs into the rectum of dogs and were preserved in sterile PBS (1ml), vortexed and stored at -20˚C.

History, Physical and Clinical Observation

Fifty dogs up to the age of one year with clinical signs and history of vomition, diarrhea and nervous symptoms were included in the study. Complete history of the affected cases regarding the duration of illness, appetite, frequency of vomition and diarrhea, colour and consistency of vomitus and faeces, deworming and vaccination status, name and type of the vaccine administered, any previous treatment administered and other relevant data was recorded. The blood and serum biochemical parameters samples were fully analysed for complete hematological examination using automated Hematology cell counter (MS4s, Melet Schlosing Laboratories).

RNA Extraction and cDNA preparation

Commercially available live attenuated multi-component vaccine for CDV (Vencomax8®, Vencofarma, Londrina, Brazil) served as a positive control. The total viral (ss-RNA) was extracted from fifty diarrheic faecal samples suspected to be having canine distemper viral RNA as well as from the vaccine by using the combination of Trizol reagents and RNeasy plus universal mini kit (Qiagen, Germantown, Maryland USA). Samples after Trizol extraction were treated with DNase I (0.5 U/µl) at 23˚C for 15 min. This extracted RNA was quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Waltham, Massachusetts, USA). The extracted RNA was further used for library preparation for Next Generation sequencing. cDNA was also synthesized from extracted faecal RNA samples using "Revert Aid first strand cDNA synthesis kit" (K1622, Thermo-scientific® Waltham, Massachusetts, USA) as per manufacturer's instructions and stored at -20°C till further use.

RT-PCR screening for the presence of CDV:

The faecal samples were screened for the presence of viral RNA by amplifying the cDNA in a thermocycler (Veriti, Applied Biosystems Waltham, Massachusetts, USA) prepared by conventional RT-PCR using published primer pair pCD /N/ (F) – ACA GGA TTG CTG AGG ACC TAT and pCD /N/(R)- CAA GAT AAC CAT GTA CGG TGC having an amplicon size of 287 bp targeting N gene of CDV. The reaction mixture and cycling conditions were used as per the methods described (15).

Sequencing of N gene amplicons of CDV:

The 287 bp amplicons obtained by PCR were purified using gel extraction kit (Qiagen, Germantown, Maryland USA) as per the manufacturer's instructions. The purified PCR amplicons CDV/IND/HSR/2016 isolate was sequenced directly using ABI 3130 XL Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) using CDV N gene specific PCR primers as sequencing primers. The contigs of forward and reverse nucleotide sequence obtained from this data were analysed using NCBI BLAST online software tool available on the internet (http://www.ncbi.nlm.nih.gov/BLAST/).

Whole genome sequencing and Library preparation of positive samples:

The whole genome viral library was prepared using10 ηg/μl concentration of total RNA. The cDNA library was prepared using Nextera XT DNA Library Prep Kit (Illumina Way, San Diego, CA, USA) using the standard protocol https://support.illumina.com/content/dam/illumina (support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-05.pdf). The purity and integrity of nucleic acid of samples were measured using Fragment Analyzer System (AATI, Newark, Delaware, United States). The Whole-genome sequencing was performed from the appropriate library prepared and sequenced with an Illumina MiSeq instrument (Illumina Way, San Diego, CA, USA) using 500 cycles, 250 paired end-sequencing protocol. The sequencing reads (Fastaq files) were assembled using the CLC workbench and *de novo* approach in the University of Minnesota using their in house developed NGS data analysis pipeline. The FASTQ files were analysed using in-house bioinformatics pipeline for trimming to remove Illumina adapters using Trimmomatic with a minimum quality score of 20 (v 0.39, https://github.com/usadellab/Trimmomatic). Then, host contamination was removed using bowtie2 (v 2.4.4, https://github.com/BenLangmead/bowtie2). The SPAdes (v3.15.2, https://github.com/ablab/spades) with k-mer values of 21, 31, 41, 51, 61, and 71 and the options --care was used for assembly of unmapped reads. Extracted contigs were analysed using BLASTx at NCBI to determine taxonomy. ORFs of assembled contig/genome were predicted using Vgas tool with default parameters (16, 17, 18, 19).

Phylogenetic analysis

Multiple sequence alignment of the whole genome of CKoV and partial N gene (287 bp) of CDV were carried out with the respective DNA sequences retrieved from NCBI database using Clustal W software implemented in Bio-Edit program (20). MEGA 6 software was used to construct a phylogenetic tree to show the genetic relatedness of CKoV and CDV from different origins (21). Bootstrap probabilities were calculated with 1,000 replicates. Neighbour joining (NJ) phylogenetic trees were constructed using default parameters of different nucleotide sequences of CKoV and CDV separately.

RESULTS

Canine Kobuvirus and Canine Distemper virus in domestic dogs in India

The domestic dogs suffering from gastroenteritis of up to one year of age which were brought to the Veterinary Clinical Complex of Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana for treatment were investigated for the presence of viral origin gastroenteritis. In a previous study, it was found that dogs less than 12 months of age were at increased risk of developing these diseases due to non-vaccination or underdeveloped immune system. Therefore, dogs of one year of age or less were taken into this study (22, 23). Out of these samples, only one sample revealed whole genome of Canine Kobuvirus (CKoV) along with partial genome sequences of CDV.

History, clinical profile and laboratory findings of dog diagnosed with CD and CKoV gastroenteritis:

The only dog that was found positive for CDV and CKoV in this study was a two month old male golden retriever. This pup was born in tehsil Adampur, District Hisar, State Haryana. The dog was unvaccinated and born to a non-vaccinated mother. There was no history of movement of pup outside the country and any contact possible with the other dogs as it was separately housed with mother. However, the mother of dog was imported from U.S.A six years previously where CKoV was highly prevalent. The pup may have been infected from CKoV due to close contact with mother who might have acted as asymptomatic active carrier for this virus with low pathogenicity (10). The pup was showing signs of anorexia, lethargy, bilateral purulent

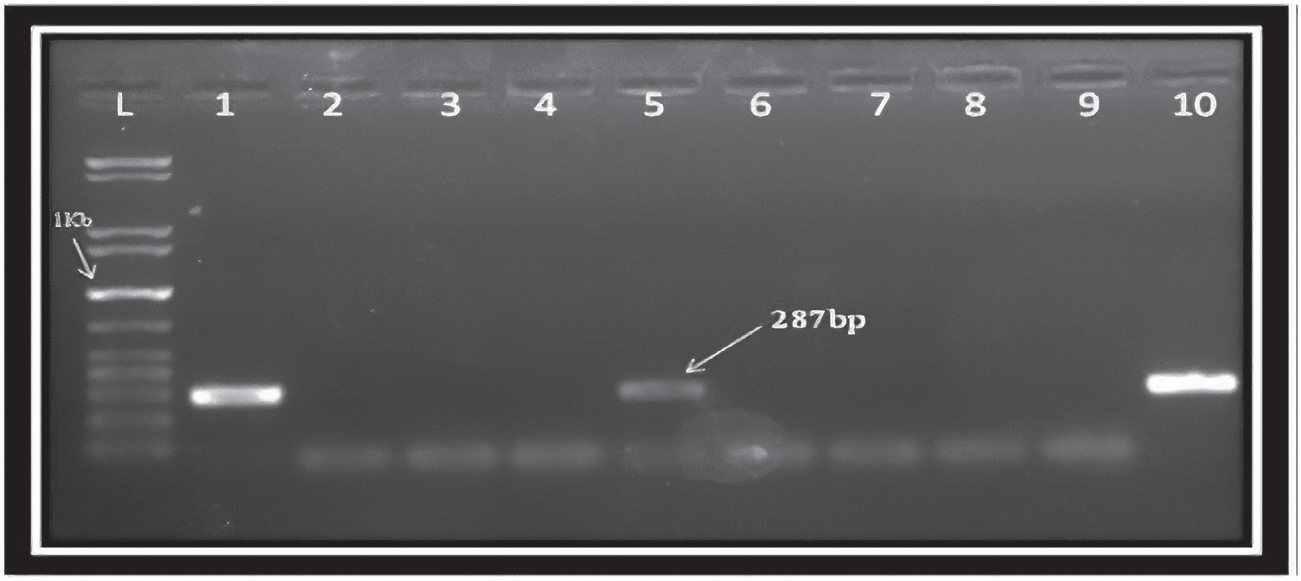

Figure 1. Agarose gel electrophoresis of 287 bp size PCR product of Canine Distemper virus positive sample. L: 1Kb ladder) Lanes 1 and 10: positive control) Lane 5: field sample (positive) Lanes 2 to 4 and 6 to 9: field samples (negative).

Table 1: Biochemical profile of a dog diagnosed with CD and CKoV gastroenteritis

Parameters	Dog affected with CD	Normal Reference range (Source: The Merck's Veterinary Manual, 11th edition)
AST (IU/L)	41.50	13-15
ALT (IU/L)	59.30	10-109
TP (g/dl)	4.80	5.4-7.5
Albumin (g/dl)	3.12	2.3-3.1
Globulin (g/dl)	1.68	2.7-4.4
BUN (mg/dl)	50.40	8-28
Creatinine (mg/dl)	1.68	0.5-1.7
Sodium (mEq/L)	127.50	142-152
Potassium (mEq/L)	4.35	3.9-5.1
Chloride (mEq/L)	102.10	110-124

ocular discharge, photophobia, fever, diarrhoea, dehydration, depression, pustules on abdomen and twitching of facial muscles. Haematological findings were suggestive of leucocytosis (29.50 10^3/µL) with relative neutrophilia (89%) and lymphopenia (9%) as compared to the mean control values for the respective parameters. The amount of haemoglobin (9.00g/dl), packed cell volume (29%) and total platelet count were also observed to be lower than the mean control reference values, which were 11.9-18.9 g/dL, 35-57% and 211-621x10^3/µL respectively. There were increased values of AST, ALT, BUN and serum creatinine, while a decrease in the values of total protein, albumin, globulin and serum electrolytes (sodium, potassium and chloride) in this dog (Table 1).

Phylogenetic analysis of canine distemper virus based on partial N gene sequences:

Rectal swabs were tested for the presence of CDV using RT-PCR as described in material and methods using published primers targeting N gene. The agarose gel electrophoresis revealed an expected band size of 287 bp (Figure 1). The partial sequence of N gene was submitted to GenBank under accession number MN128876.1. Phylogenetic analysis based on a partial N gene sequence of CDV was conducted by comparing this sequence with the commercial vaccine strain and other CDV strain N gene sequences available on GenBank. CDV strain from Hisar showed maximum nucleotide identity of 97% with other Indian strain (Accession Number MH536200.1) and 97.15 % with the CDV isolate from Hebei, China (Accession Number KC427278) (Figure 2). CDV strain from Hisar had 12 nucleotide differences with Rockborn strain over a sequence length of 287 bp. The field CDV sample had 95.8% nucleotide identity with the Rockborn strain of vaccine virus.

Table 2: Pairwise comparison of percent nucleotide identity of different genes of Indian CKoVs and worldwide distributed other isolates

Viruses (Country, Year)	Accession number	L gene	VP0 gene	VP3 gene	VP1 gene	2A gene	2B gene	2C gene	3A gene	3B gene	3C gene	3D gene
CKoV CH-1 (China, 2012)	JQ911763.1	95.53	94.93	93.58	88.78	98.20	95.96	95.62	96.13	95.06	97.61	97.02
CKoV CU_101 (Thailand, 2018)	MK201777.1	94.16	94.58	94.05	85.80	97.31	95.15	96.12	96.13	95.06	97.26	96.90
CKoV AH-1/CHN/2019, (China, 2019)	MN449341.1	94.16	95.01	93.58	85.70	97.01	95.15	96.12	95.42	95.06	96.58	97.64
CKoV 12D049 (South Korea, 2013)	KF924623.1	92.22	93.43	92.71	92.52	97.26	95.15	95.52	95.42	95.06	96.24	95.30
CKoV strain CU_249 (Thailand, 2018)	MK201778.1	95.14	93.96	93.58	86.07	97.90	94.95	96.02	96.13	93.83	96.24	97.64
CKoV_CE9_AUS_2012, (Australia, 2012)	MH052678.1	92.22	91.69	92.11	90.38	97.01	95.15	95.22	94.68	95.06	96.24	95.91
CKoV SMCD-59 (China, 2017)	MF062158.1	94.36	93.96	93.43	84.73	97.01	95.76	95.72	95.07	95.06	96.15	97.02
CKoV US-PC0082 (USA, 2011)	JN088541.1	92.55	91.34	92.56	92.27	96.11	93.54	94.52	94.37	95.06	95.12	95.41
CKOV CU_716 (Thailand, 2018)	MK201779.1	94.36	93.16	93.45	85.07	95.81	93.74	95.12	95.42	95.06	96.24	97.39
CKoVdog/AN211D/USA/2009 (USA, 2009)	JN387133.1	92.65	91.16	92.56	91.07	96.11	92.73	94.82	92.25	95.06	94.95	95.53
CKoV CaKoV-26 (Brazil, 2018)	MH747478.1	93.19	92.48	92.54	87.51	96.71	94.14	94.42	95.77	95.06	94.70	95.91
CKoV 1 isolate DD2 (Tanzania, 2015)	KM068048.1	93.58	91.16	92.69	89.76	94.91	92.73	92.94	94.37	90.12	94.02	95.29
CKoV S272/16 (Germany, 2019)	MN337880.1	92.02	90.99	93.75	89.90	94.61	92.32	93.63	96.13	95.06	94.92	95.66
CKoV 1 isolate B103 (Africa, 2015)	KM068051.1	91.63	90.90	91.94	89.05	96.41	92.73	92.25	92.61	95.06	94.53	95.04
CKoV UK003 (UK, 2013)	KC161964.1	94.94	92.65	93.90	91.21	95.81	91.52	94.72	93.29	95.06	95.30	96.40

Phylogenetic analysis of Indian Canine Kobuvirus based on whole genome sequence:

The next generation sequencing yielded major reads containing whole genome of Canine Kobuvirus (CKoV). The final sequence of CKoV (CKoV/IND/HSR/2016) was 7975 nucleotides long. The whole genome sequence obtained has been deposited in the GenBank database with accession no. MT610361. Phylogenetic analysis was conducted by comparing whole genome sequences of CKoV with other available sequences present in Genbank. The CKoV under study (CKoV/IND/HSR/2016) revealed 95% nucleotide identity with Canine Kobuvirus CH-1, Chinese strain (Accession Number JQ911763.1) and 94% nucleotide identity with Canine Kobuvirus strain CU_101 from Thailand (Accession Number MK201777.1) (Figure 3). This Indian strain has 371 base pair difference from the Canine Kobuvirus CH-1, Chinese strain. The CKoV under investigation was closely related to Aichivirus A, containing Kobuviruses isolated from dogs, bats, cats and humans. The Indian strain revealed 94.59%, 94.01% and 93.68%, nucleotide identity with Korean, Australian and USA strains, respectively. Based on whole genome analysis, the Indian strain (CKoV/IND/HSR/2016) clustered closely to the virus isolated from China, Thailand and differed from sub-cluster from the viruses from the USA, Brazil, Australia, Japan and Germany. On the basis of VP1 gene, this strain revealed, 92.54 %, 92.29% and 91.23%, nucleotide identity with Korean, USA and UK strains respectively. The pairwise comparison of other genes of Indian strain with other worldwide distributed viruses is given in Table 2.

Genetic analysis of Indian Kobuvirus based on whole genome sequence

The whole genome obtained for CKoV (CKoV/IND/HSR/2016) was 7975 nucleotide long and contains an open reading frame (ORF) of 7335 nucleotides (602-7936) encod-

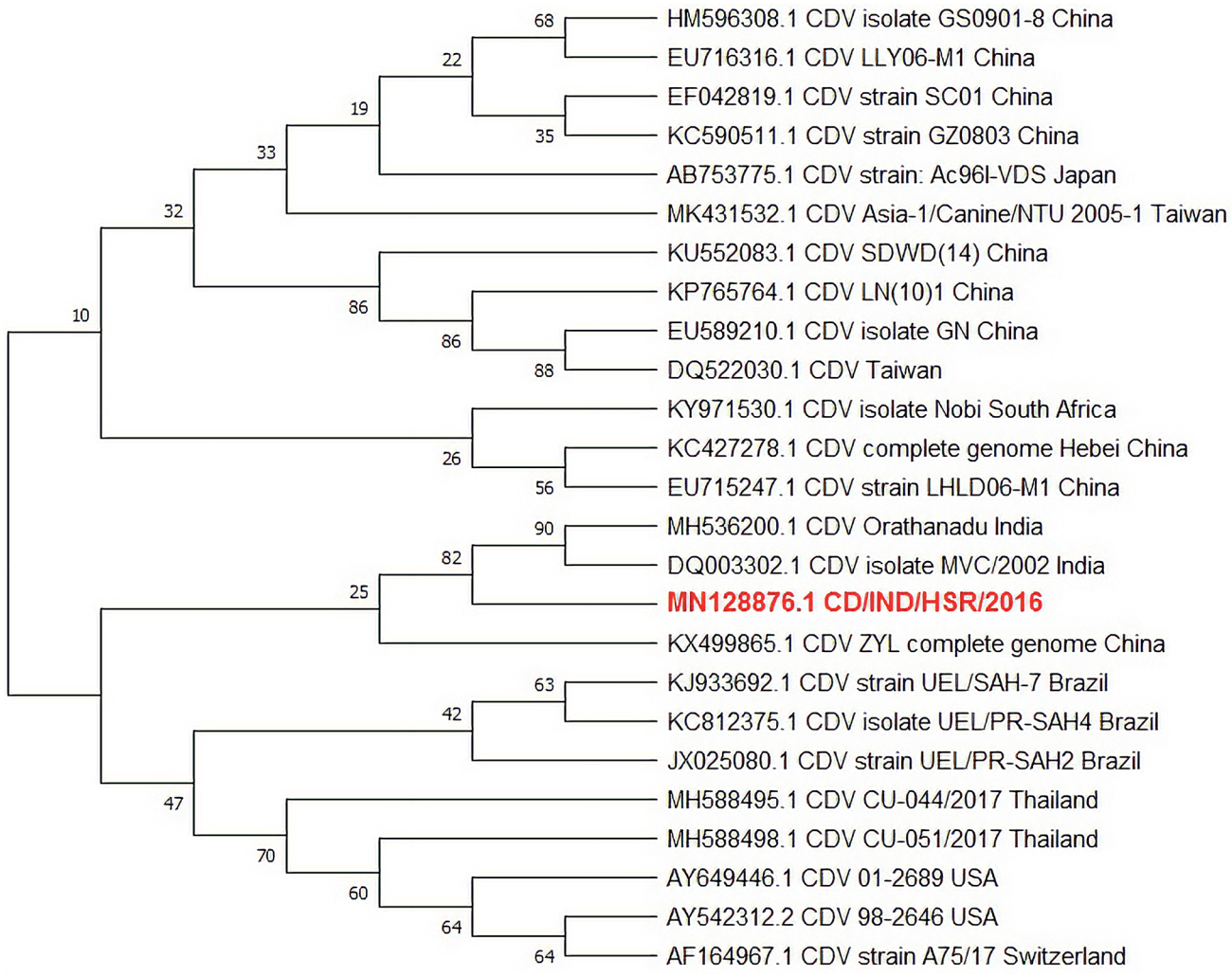

Figure 2. Phylogenetic analysis of CDV strain using the nucleotide sequences of the partial N gene of CDV. The Indian Kubvirus strain is indicated in red.

ing a putative polyprotein precursor of 2444 amino acids (aa). The full genome of CKoV (CKoV/IND/HSR/2016) had a high G+C content (58%) compared to some other kobuviruses (52–59%).

DISCUSSION

Viruses such as Parvovirus, Distemper virus, Coronavirus, Rotavirus, Adenovirus, Herpesvirus, Influenza virus and Parainfluenza virus have been reported as potential canine pathogens (24,25,26) as these are highly infectious viral pathogens and cause high morbidity and mortality in affected dogs. Diseases caused by CKoV infection in domestic dogs have remained unclear until now. Some workers have found that detection of CKoV RNA was not a major cause of diarrhoea in dogs (14) while CKoV was detected in outbreaks of acute gastroenteritis in canine shelters in the United States (4). The authors have also detected CKoV in dogs suffering from diarrhoea with the concurrent infection of canine coronavirus, canine parvovirus-2 and canine bocavirus (14, 27).

In the current study, the CKoV was detected in a domestic pup suffering from gastroenteritis of viral origin. During this investigation, the dogs having clinical signs of inappetence, diarrhea, vomition, nervous symptoms and dehydration were studied. Out of 50 screened samples for viral gastroentertitis infections, we found 25 dogs positive for CPV, 4 for CCoV and one for CDV. Routine diagnosis of CDV is done by IFA, ELISA and SN assays, virus isolation on canine cells but these are time consuming and do not

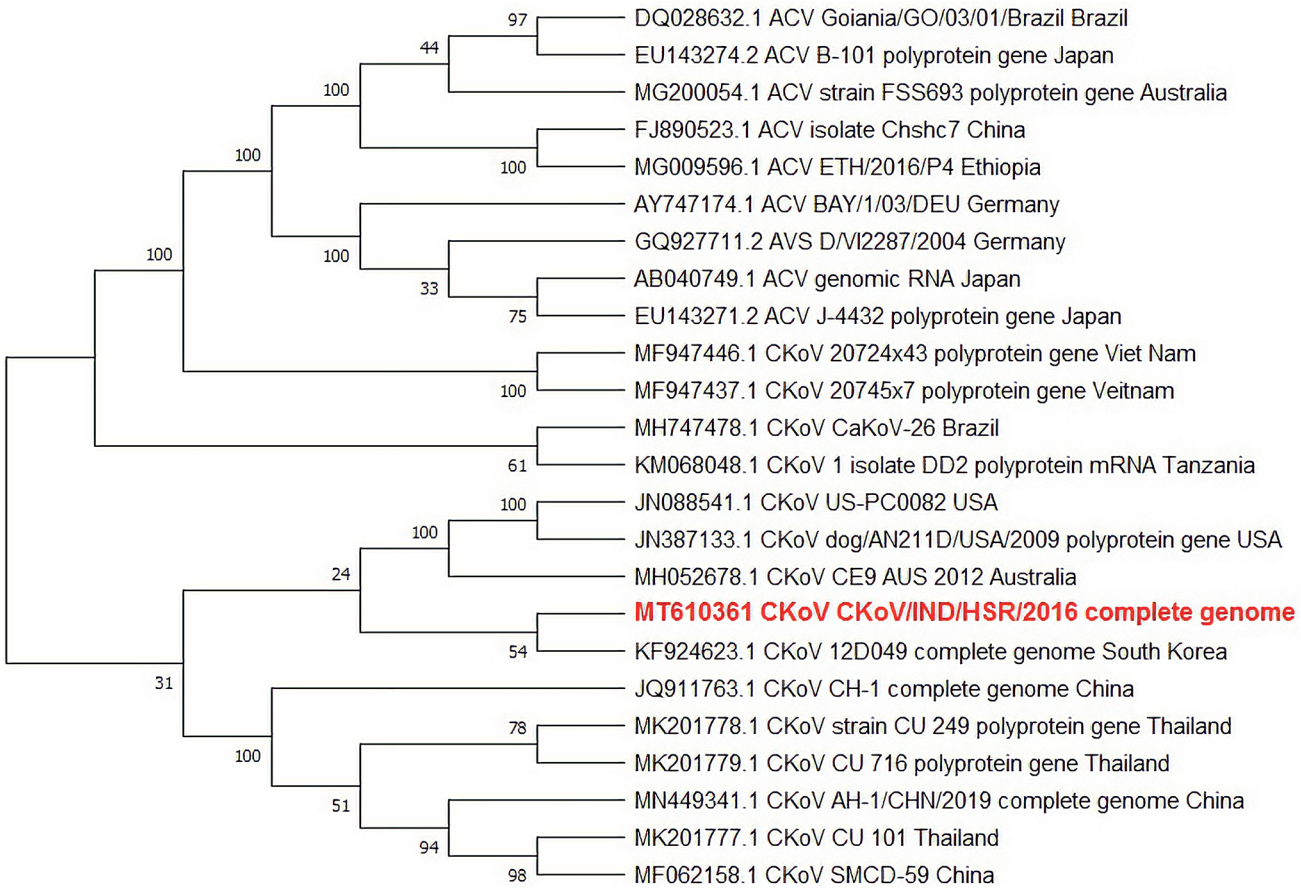

Figure 3. NJ tree of the completed genome of CKoVs. The phylogenetic tree was constructed by using MEGA v6.0 using P distance method. Values on branches represent bootstrap values. The Indian Kubvirus strain is indicated in red.

provide definitive diagnosis (28). Therefore, next generation sequencing analysis was done to obtain a definitive diagnosis and characterization of CDV genome that is circulating in dogs (29). In addition, NGS analysis from a single sample has the potential to identify multiple pathogens present in a single sample. The present study involving NGS of a sample showed the simultaneous presence of CKoV genome in that diarrheic faecal sample of a golden retriever dog which was also found positive for CDV in RT-PCR.

CKoV has been recognized in several countries in domestic dogs and wild animals. The prevalence of CKoV in domestic dogs has been reported to be 2.34% in Italy, 17.9% in China, 1.25% in UK and 19% and 13.2% of diarrheic and healthy dogs in Korea, respectively (10, 14, 30, 31). In Japan, 37.2% of diarrheic dogs and 48% of clinically healthy kennelled dogs were found to be positive for CKoV (32). There has been no prior report of infection of CKoV in dogs in India. The detection of CKoV in a young pup of two months of age corroborates with the previous observation that the CKoV may be frequently detected in younger dogs (32,33). In contrast to the earlier observations supporting the detection of CKoV in both diarrheic and non-diarrheic dogs (31, 32, 33), the dog found positive in this study for CKoV was suffering from gastroenteritis and also CDV infection. The findings of the present study are in accordance with the previous studies (10,30, 31, 32), which support that CKoV may also be the cause of enteric diseases. There are many viruses responsible for diarrhea either alone or in connection with other viruses (co-infection), which seems to be the case here.

NGS is the best indicator of the relationship between samples collected in different geographical regions and may help in understanding the antigenic differences between different biological samples. It could have implications in

vaccine differentiation studies to control a particular pathogenic agent (34). Phylogenetic analysis of CKoV showed that Indian strain (CKoV/IND/HSR/2016) is closely related to Chinese and Thai strains with 95% and 94% nucleotide identity in complete genome sequences respectively. The CKoV strain from India (CKoV/IND/HSR/2016) had a nucleotide identity of 94.2% with Thailand isolate, 94% with South Korean isolate and 93% with Brazilian isolates. It is genetically different from isolates of Ethiopia, Germany and Vietnam sharing only 78% nucleotide identity.

The full genome of CKoV encodes for leader protein L, capsid proteins VP0, VP3, VP1 and nonstructural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D (27). In this strain, the composition of different proteins were leader protein L (171 aa), capsid proteins VP0 (381 aa),VP3 (224 aa), VP1 (278 aa) and non-structural proteins 2A (111 aa), 2B (165 aa), 2C (335 aa), 3A (94 aa), 3B (27 aa), 3C (390 aa) and 3D (269 aa). On the basis of deduced amino acid sequence, this isolate also revealed 89 changes at the amino acid level at different positions when compared only with the reference polyprotein sequence (YP_009380518) (27). There were 2 aa change in L protein, 3aa in VP0, 1 aa in VP3, 1 aa in 2B, 2 aa in 2C, 3 aa in 3A, 1 aa in 3B and 6aa in 3C. There was the major change of 70 aa in VP1 protein depicting a mutation in Indian strain encoding for capsid protein. VP1 capsid viral protein has been observed as the most variable gene in various CKoVs identified in different countries (31, 33). Similarly, this isolate also revealed 70 amino acids at the reference protein sequence of VP1. Moreover, the VP1 gene of Indian strain (CKoV/IND/HSR/2016) has more nucleotide identity to USA, Korean and UK strains. Moreover, in this strain, putative proline rich region was present in the portion of VP1 gene, which has also been found in similar studies of different isolates from China and Thailand (33, 35).

On the basis of genetic analysis, there were 89 different amino acids distributed in polyprotein in the Indian strain (CKoV/IND/HSR/2016) which are not present in other worldwide distributed isolates. These unique amino acids will be beneficial for the detection of viral origin and development of strain specific diagnostics. Genetically, these changes in amino acids may have resulted from the mutation of the virus during the course of infection to overcome the immune system of animals.

In conclusion, the present study revealed the presence of CKoV along with CDV in the faecal sample of a young dog suffering from diarrhoea in India. This molecular characterization of the novel CKoV complete genome from India may help in studies related to molecular epidemiology, diagnostics development and vaccine development for these viruses. This is the first report of concurrent infection of CDV and CKoV in diarrheic faecal samples of dogs in India.

CONCLUSION

Concurrent viral infections in canine gastroenteritis cases are possible and may result in the greater severity of the disease. The diagnostic studies conducted herein can prove to be an important tool for studying molecular epidemiology of significant canine viral pathogens.

CONFLICT OF INTEREST

All authors have declared no conflict of interest.

ACKNOWLEDGEMENT

The authors are thankful to the technical and non-teaching staff of the department and also thankful to LUVAS administration for financial support and smooth running of the NGS facility. Authors also appreciate the work of all the researchers whose contribution is referred in this research.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as described on the journal's author guidelines have been adhered to. This manuscript uses samples collected from dogs, which were clinically infected and brought for routine diagnosis and hence no ethical approval was required. A registered and experienced veterinarian collected the samples.

REFERENCES

1. Yamashita, T., Sakae, K., Tsuzuki, H., Suzuki, Y., Ishikawa, N., Takeda, N., Miyamura, T. and Yamazaki, S.: Complete nucleotide sequence and genetic organization of Aichi virus, a distinct member of the Picornaviridae associated with acute gastroenteritis in humans. J. Virol. 72: 8408-8412, 1998.
2. Yamashita, T., Ito, M., Kabashima, Y., Tsuzuki, H., Fujiura, A. and Sakae, K.: Isolation and characterization of a new species of kobuvirus associated with cattle. J. Gen. Virol. 84: 3069-3077, 2003.
3. Reuter, G., Boldizsár, A., Kiss, I. and Pankovics, P.: Candidate new species of Kobuvirus in porcine hosts. Emerg. Infect. Dis. 14: 1968-1970, 2008.
4. Kapoor, A., Simmonds, P., Dubovi, E. J., Qaisar, N., Henriquez, J.

A., Medina, J., Shields, S. and Lipkin, W. I.: Characterization of a canine homolog of human Aichivirus. J. Virol. 85: 11520-11525, 2011.
5. Chung, J. Y., Kim, S. H., Kim, Y. H., Lee, M. H., Lee, K. K. and Oem, J. K.: Detection and genetic characterization of feline kobuviruses. Virus genes. 47: 559-562, 2013.
6. Phan, T. G., Kapusinszky, B., Wang, C., Rose, R. K., Lipton, H. L. and Delwart, E. L.: The faecal viral flora of wild rodents. PLoS pathogens 7: e1002218, 2011.
7. Smits, S. L., Raj, V. S., Oduber, M. D., Schapendonk, C. M., Bodewes, R., Provacia, L., Stittelaar, K. J., Osterhaus, A. D. and Haagmans, B. L.: Metagenomic analysis of the ferret fecal viral flora. PloS one (8): e71595, 2013.
8. Oem, J. K., Lee, M. H., Lee, K. K. and An, D. J.: Novel Kobuvirus species identified from black goat with diarrhea. Vet. Microbiol. 172: 563-567, 2014.
9. Reuter, G., Boros, A., Pankovics, P. and Egyed, L.: Kobuvirus in domestic sheep, Hungary. Emerg. Infect. Dis. 16: 869-870, 2010.
10. Carmona-Vicente, N., Buesa, J., Brown, P. A., Merga, J. Y., Darby, A. C., Stavisky, J., Sadler, L., Gaskell, R. M., Dawson, S. and Radford, A. D.: Phylogeny and prevalence of kobuviruses in dogs and cats in the UK. Vet. Microbiol. 164: 246-252, 2013.
11. Li, L., Pesavento, P. A., Shan, T., Leutenegger, C. M., Wang, C. and Delwart, E.: Viruses in diarrhoeic dogs include novel kobuviruses and sapoviruses. J. Gen. Virol. 92: 2534-2541, 2011.
12. Martella, V., Bianchi, A., Bertoletti, I., Pedrotti, L., Gugiatti, A., Catella, A., Cordioli, P., Lucente, M. S., Elia, G. and Buonavoglia, C.: Canine distemper epizootic among red foxes, Italy, 2009. Emerg. Infect. Dis. 16: 2007-2009, 2010.
13. Choi, S., Lim, S. I., Kim, Y. K., Cho, Y. Y., Song, J. Y. and An, D. J.: Phylogenetic analysis of astrovirus and kobuvirus in Korean dogs. J. Vet. Med. Sci. 76: 1141-1145, 2014.
14. Li, C., Wei, S., Guo, D., Wang, Z., Geng, Y., Wang, E., Zhao, X., Su, M., Wang, X. and Sun, D.: Prevalence and phylogenetic analysis of canine kobuviruses in diarrhoetic dogs in northeast China. J. Vet. Med. Sci. 78: 7-11, 2016.
15. Frisk, A. L., König, M., Moritz, A. and Baumgärtner, W.: Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. J Clin. Microbiol. 37: 3634-3643, 1999.
16. Zhang, K. Y., Gao, Y. Z., Du, M. Z., Liu, S., Dong, C., Guo, F. B.: Vgas: A Viral Genome Annotation System. Front. Microbiol. 10:184, 2019.
17. Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A. and Korobeynikov, A.: Using SPAdes de novo assembler. Curr. Protoc. Bioinformatics. 70: e102, 2020.
18. Bolger, A.M., Lohse, M. and Usadel, B.: Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 30:2114-2120, 2014.
19. Langmead, B. and Salzberg, S.: Fast gapped-read alignment with Bowtie 2. Nat. Methods. 9: 357-359, 2012.
20. Hall, T.A.: BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95-98, 1999.
21. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S.: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30(12): 2725-2729, 2013.
22. Alves, P. A. B., Rosa, I. C. A. S., Modena, C. M. and Caetano, J.: Occurrence of haemorrhagic gastroenteritis in dogs in Belo Horizonte, Minas Gerais, Brazil. Arq. Bras. Med. Vet. Zootec. 50: 661-664, 1998.
23. Castro, T. X., Cássia, N. R., Garcia, C., Luciana, P. S., Erika, G., Costa, E. M., Marcello, G. C. G., Labarthe, N. V. and de-Almeida, M. F.: Clinical, hematological and biochemical findings in puppies with corona virus and parvovirus enteritis. Can. Vet. J. 54: 885-888, 2013.
24. Decaro, N., Martella, V. and Buonavoglia, C.: Canine adenoviruses and herpesvirus. Vet. Clin. North Am. Small Anim. Pract. 38: 799-viii, 2008.
25. Dubovi, E. J.: Canine influenza. Vet. Clin. North Am. Small Anim. Pract. 40: 1063-1071, 2010.
26. Renshaw, R. W., Zylich, N. C., Laverack, M. A., Glaser, A. L. and Dubovi, E. J.:Pneumovirus in dogs with acute respiratory disease. Emerg. Infect. Dis. 16: 993-995, 2010.
27. Kong, N., Zuo, Y., Wang, Z., Yu, H., Zhou, E. M., Shan, T. and Tong, G.: Molecular characterization of new described kobuvirus in dogs with diarrhea in China. SpringerPlus, 5: 2047, 2016.
28. Kim, Y. H., Cho, K. W., Youn, H. Y., Yoo, H. S. and Han, H. R.: Detection of canine distemper virus (CDV) through one-step RT-PCR combined with nested PCR. J. Vet. Sci.2:59-63, 2001.
29. Marston, D. A., Watson, J., Wise, E. L., Ellis, R. J., Bedin, E., Ayalew, G., Abute, M., de Lamballerie, X., Fooks, A. R., Sillero-Zubiri, C. and Banyard, A. C.: Complete Genomic Sequence of Canine Distemper Virus from an Ethiopian Wolf. Genome Announc. 5, e00621-17, 2017.
30. Martino Di, B., Di Felice, E., Ceci, C., Di Profio, F. and Marsilio, F.: Canine kobuviruses in diarrhoeic dogs in Italy. Vet. Microbiol. 166: 246-249, 2013.
31. Oem, J.K., Choi, J.W., Lee, M.H., Lee, K.K. and Choi, K.S.: Canine kobuvirus infections in Korean dogs. Arch. Virol.159: 2751-2755, 2014.
32. Soma, T., Matsubayashi, M. and Sasai, K.: Detection of kobuvirus RNA in Japanese domestic dogs. J. Vet. Med. Sci. 78: 1731-1735, 2016.
33. Charoenkul, K., Janetanakit, T., Chaiyawong, S., Bunpapong, N., Boonyapisitsopa, S., Tangwangvivat, R. and Amonsin, A.: First detection and genetic characterization of canine Kobuvirus in domestic dogs in Thailand. BMC Vet. Res. 15: 254, 2019.
34. Nater, A., Burri, R., Kawakami, T., Smeds, L. and Ellegren, H.: Resolving Evolutionary Relationships in Closely Related Species with Whole-Genome Sequencing Data. Syst. Biol. 64: 1000-1017, 2015.
35. Chen, L., Zhu, L., Zhou, Y. C., Xu, Z. W., Guo, W. Z. and Yang, W. Y.: Molecular and phylogenetic analysis of the porcine kobuvirus VP1 region using infected pigs from Sichuan Province, China. J. Virol. 10, 281, 2013.