A Comprehensive Molecular Survey of Viral Pathogens Associated with Canine Gastroenteritis During 2018-21

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Research Article

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Abstract

Viral pathogens account for major aetiology of canine gastroenteritis in India. However, till date, there was no structured comprehensive study reported on the viral aetiology of canine gastroenteritis in India. To elucidate this, 475 rectal swabs collected over a period of three years (2018-2021) from clinical canine gastroenteritis cases were screened by real-time PCR for the presence of six majorly known enteric viruses (Canine parvovirus -2 (CPV-2), canine distemper virus (CDV), canine adenovirus-2 (CAAdV-2), canine corona virus (CCoV), canine astrovirus (CaAstV), canine rotavirus (CRV)). 71.6% of the samples were found to harbour at least one of the five viruses tested (CPV-2/2a/2b/2c, CDV, CAAdV-2, CCoV and CaAstV), whereas, there was no evidence of CRV. The overall incidence rate for each virus was found highest at 64.8% for CPV-2/2a/2b/2c (2a: 21.1%, 2b: 77.4%, 2c: 1.5%), followed by 8% for CDV, 7.2% for CaAstV, 5.9% for CCoV and 4.6% for CAAdV-2 with 16.8% incidence for coinfections ranging from two to four viruses in different combinations. Further, for the first time we report the whole genome sequences of CDV, CCoV and CaAstV Indian isolates from canine gastroenteritis cases. Phylogenetic analysis of whole genome revealed that CDV clustered with Asia-1 lineage, CaAstV clustered with group III that consists of China and Brazil isolates, and CCoV clustered with group II that consists of China and Taipei isolates. This study adds to our knowledge on the prevalence of these pathogens in the Indian subcontinent and highlights the need for relevant and routine vaccine prophylactic measures. It is alarming to note that 70.3% of animals vaccinated with DHPPI were found positive for at least one virus. Hence, regular molecular analysis of the prevalent viruses is crucial to address vaccination failures.

Highlights

1. Viral gastroenteritis is a common problem in canines despite routine vaccinations.
2. Exhaustive studies on viral prevalence were sparse, with none reported from India till date.
3. We report molecular survey of enteric viruses from gastroenteritis cases during 2018-21.
4. CPV-2b is the predominant virus, followed by CPV-2a, CPV-2c, CDV, CAAdV-2, CCoV, CaAstV.
5. We further report whole genome sequence of CCoV, CaAstV and CDV isolates from dogs in India for the first time.
6. Our study significantly contributes to the knowledge on viral canine gastroenteritis and highlights the need for a dedicated and relevant routine vaccine prophylaxis.

Introduction

Dog has been the best common companion animal of all the domesticated animals and like humans, they are also prone to several diseases. Gastroenteritis, the irritation of the stomach and intestines is one among them resulting in vomiting and diarrhoea. This can progress quickly and can become very dangerous to the pet's health and, if left untreated, lead to death, especially in young animals. Gastroenteritis can occur for a variety of reasons *viz.*, dietary indiscretion, tumours, metabolic disorders, toxins, and most important of all, infectious agents such as bacteria (Enany et al. 2021), parasites (Headley et al. 2013; Gerardi et al. 2018), and viruses (DiGangi et al. 2019). Viruses were reported to be detected in up to 60% of diarrhoeic faecal samples (Gizzi et al. 2014).

Canine parvovirus (CPV) (Caddy 2018), Canine distemper virus (CDV) (Quintero-Gil et al. 2019), Canine adenovirus (CAAdV) (Ramidi et al. 2020), Canine coronavirus (CCoV) (Buonavoglia et al. 2006), Canine astrovirus (CaAstV) (Fauquet and Fargette 2005), Canine rotavirus (CRV) (Ortega et al. 2017) were frequently reported as a cause for viral gastroenteritis in dogs. CPV belonging to *Parvoviridae* family is highly contagious and was first identified in dogs suffering from severe hemorrhagic gastroenteritis and myocarditis as CPV-2 variant (Appel et al. 1979; Hoang et al. 2019); later on replaced by three antigenic variants namely CPV-2a, CPV-2b, and CPV-2c (Zhong et al. 2014; Sharma et al. 2016; Chiang et al. 2016; Zhou et al. 2017b). CDV belonging to *Paramyxoviridae* family is responsible for high mortality rates in dogs worldwide (Quintero-Gil et al. 2019). Haemagglutinin gene of CDV was known to be evolved under genetic drift and was responsible for introduction of genetically different CDV strains (Gardon and Stöckli 1985). It was reported that at least fifteen different lineages of CDV are circulating worldwide (Bhatt et al. 2019; Kodi et al. 2020). CAAdV belongs to *Adenoviridae* family and circulates as two distinct serotypes, CAAdV-1 and CAAdV-2 (Mohammadi et al. 2011; Ramidi et al. 2020). Despite routine vaccinations, re-emergence of CAAdV-2 was documented throughout the world (Balboni et al. 2014). CCoV belongs Alphacoronavirus 1 of *Coronaviridae* family, causes mild to moderate enteritis in dogs characterized by high morbidity and low mortality (Buonavoglia et al. 2006). The Alphacoronavirus 1 also include transmissible gastroenteritis virus

of swine, porcine epidemic diarrhea virus, feline coronaviruses, and human coronavirus 229E (Decaro and Buonavoglia 2008). Canine astrovirus (CaAstV) belonging to Mamastrovirus of *Astroviridae* family (Fauquet and Fargette 2005) has been detected in multiple countries from diarrheic dogs (Toffan et al. 2009; Martella et al. 2012; Choi et al. 2014; Zhou et al. 2017a). It is mostly known to be associated with mixed viral enteric infections, especially in young dogs (Martella et al. 2011; Zhou et al. 2017a). CRV belongs to *Reoviridae* family and causes neonatal diarrhea (Ortega et al. 2017).

The aim of the present study is to investigate and delineate the prevalence of major enteric viruses in stool samples from symptomatic dogs. Furthermore, first genome sequences of CDV, CCoV and CaAstV from Indian isolates were also reported along with their phylogenetic analysis.

Materials And Methods

Clinical sample collection and processing

Rectal swabs were collected from clinically ill dogs presented with gastroenteritis at Teaching veterinary clinical complex, College of Veterinary Science, Hyderabad and various private clinics in this region. The case histories of 475 samples screened in this study were presented in Table S1. Rectal swabs were collected over a period of three years (2018-2021). The swabs were homogenized in 3 ml of 0.1 M PBS (pH 7.4) containing antibiotics (100 IU/ml Benzyl Penicillin, 100 µg/ml Streptomycin sulphate), centrifuged at 6000 rpm for 10 min at 4°C and the supernatant filtered through 0.22 µ syringe filter was used for further analysis.

Viral Nucleic Acid Extraction And Reverse Transcription

Viral nucleic acid extraction and reverse transcription

The viral RNA was extracted using TRIZOL® reagent (Ref:15596018; Ambion®) and the cDNA was synthesized using PrimeScript first strand cDNA Synthesis kit (Cat No.6110A; TaKaRa) following the manufacturer's protocol. The viral DNA was extracted using phenol chloroform and isoamyl alcohol method as described by Sambrook and Russel (Sambrook and Russell 2001).

Screening of clinical samples for viral etiology by real time PCR

The cDNA or viral DNA served as a template for PCR. The real time PCR was carried out with SYBR® Premix Ex Taq™ PCR Master Mix (Cat.No.RR420A; TaKaRa) in Step One Plus real-time PCR system (Applied Biosystems). The primers used for initial screening of six viruses in individual reactions were listed in the Table 1; along with the real-time PCR reaction conditions. The antigenic typing of CAdV was done into CAdV-1 and CAdV-2 based on the difference in melting temperature (Balboni et al. 2015). The antigenic typing for CPV-2/2a/2b/2c was done by a modified version of TARMS-PCR reported before (Dema et al. 2021).

Table 1
Primers used for initial screening of viruses

S. No.	Primer name	Primer sequence	Target gene	Virus	Amplicon size	PCR conditions	Reference
1.	CPV-F CPV-R	5'AAACAGGAATTAATACTATACTAATATATTTA3' 5'AAATTTGACCATTGAT AAAT3'	VP-2	CPV	90	Initial denaturation at 95°C for 5 min followed by 4 cycles of 95°C for 30 sec and 60°C for 1 min. Melt curve was set starting at 50°C to 95°C with a ramp speed of 1%.	(Decaro et al. 2005)
2.	CAdV-F CAdV-R	5'AGTAATGGAAACCTAGGGG3' 5'TCTGTGTTTCTGTCTTGC3'	E3	CAdV	166		(Balboni et al. 2015)
3.	CDV-F CDV-R	5'AGCTAGTTTCATCTTAATACTATCAAATT3' 5'TTAATCTCTCCAGAAAATCATGC3'	N	CDV	87		(Elia et al. 2006)
4.	CaAstV-F CaAstV-R	5'GTACTATACCRTCTGATTTAATT3' 5'AGACCAARGTGTCATAGTTCAG3'	ORF1b	CaAstV	293		(Martella et al. 2011)
5.	CCoV-F CCoV-R	5'TTGATCGTTTTTATAACGGTTCTACAA3' 5'AATGGGCCATAATAGCCACATAAT3'	M	CCoV	99		(Decaro et al. 2004)
6.	CRV-F CRV-R	5'TTAGATACTACAAGTAATGGAATCGGATGT3' 5'TGGGTGTCATTTGATACAACTTCA3'	VP7	CRV	76		(Logan et al. 2006)

Isolation Of Virus In Cell Lines

DMEM containing 1% FBS was used for maintenance of Madin-Darby Canine Kidney (MDCK), A-72 (Canine fibroblast) or Epstein-Barr virus-transformed marmoset B lymphoblastoid (B95a) cell lines at 37°C under 5% CO₂. The cells were kept in maintenance media during virus propagation. For virus isolation, the faecal samples were emulsified in PBS and the clear supernatant was used as a seed for virus culture. The virus isolation for CAdV-2, CPV-2/2a/2b/2c and CaAstV were carried out in MDCK cells, whereas for CDV and CCoV, B95a, and A-72 cells, respectively were used. The cells were incubated at 37°C under 5% CO₂ and were observed for CPE such as granulation, rounding or detachment of cells in clusters disturbing the confluent monolayer. The cells were freeze-thawed three times and the viral supernatant was used as a seed for subsequent passages.

Whole Genome Sequence Analysis

The viral RNA was sent to the sequencing facility MedGenome Labs Ltd., Karnataka, India for whole genome sequencing. Briefly, the whole genome was sequenced using HiSeqX (Illumina). Around 12.3 Gb data was generated with 81 million reads for CaAstV and 14 Gb data was generated with 95 million reads for CDV and CCoV. The average Q30% of above 80% is considered. The reads were first aligned to canine genome (GCF_000002285.3_CanFam3.1) and the unaligned reads were then aligned to respective reference viral genome. *De novo* assembly was performed using metaspades to obtain scaffolds. The scaffolds were subjected to gene prediction using Prodigal and the predicted ORFs are subjected to Blastx. The sequences were deposited in the NCBI database using online BankIt submission form.

Phylogenetic Analysis

Predicted viral genome was subjected to BLASTn. At least 15 sequences from the Blastn results were randomly collected based on percentage identity. Multiple sequence alignment was performed using MUSCLE algorithm in MEGAX software and was exported to MEGA file format. The phylogenetic tree was reconstructed using Neighbour Joining algorithm in MEGAX software by setting the test of phylogeny as bootstrap method and number of replicates as 1000 (Kumar et al. 2018). The clades were divided according to

geographical distribution or as per relativeness in to different lineages/groups. Further, the phylogenetic tree was reconstructed based on the individual gene sequences of CDV, CaAstV and CCoV as similar to above. Sequences of ten isolates per gene distributed geographically were collected from Genbank and all the genes of the virus were run in single analysis for better pictorial representation.

Results And Discussion

Most of the clinical presentations in dogs involve gastroenteritis, more often with viral aetiology. In the present study, we screened the rectal swabs from 475 clinically suspected dogs for the presence of either of six viruses namely, CPV-2/2a/2b/2c, CDV, CAdV-2, CCoV, CRV and CaAstV that are the most common aetiology of viral gastroenteritis.

Prevalence Rate Of Gastroenteritis Causing Viruses

Being a highly sensitive, rapid, and specific technique that can detect low titre viruses, real-time PCR was successfully being used for molecular screening of clinical samples for viruses (Decaro et al. 2005). Initial screening for viral aetiology showed that around 71.6% of gastroenteritis cases were infected with either one or a mixture of the five tested viruses, whereas none of them tested positive for the presence of CRV (Table S1). Based on a previous molecular survey in symptomatic dogs, viral aetiology in gastroenteritis cases can go up to 93% (Zobba et al. 2021). Fig. 1a and Table S2 show the detailed prevalence rate for the viruses that tested positive upon initial screening by real-time PCR. The overall incidence rate for each virus was found highest at 64.8% for CPV-2/2a/2b/2c, followed by 8% for CDV, 7.2% for CaAstV, 5.9% for CCoV and 4.6% for CAdV-2. Further, the antigenic typing of highly prevalent CPV-2/2a/2b/2c by TARMS-PCR revealed CPV-2b as the most prevalent antigenic type followed by CPV-2a, and CPV-2c (Fig. 1b and Table S3). It was previously reported that the prevalence rate for CPV-2/2a/2b/2c would be in the range of 50-70% (Kumar and Nandi 2010; Abedi et al. 2018), for CDV to be around 2% (Ashmi et al. 2017; Agnihotri et al. 2018), for CaAstV in the range of 9-40% (Takano et al. 2015; Zhou et al. 2017a; Li et al. 2018), for CCoV in the range of 8-65% (Ntafis et al. 2013; Wang et al. 2016; Agnihotri et al. 2018), and around 60% for CAdV (Balboni et al. 2014; DiGangi et al. 2019). The prevalence rate of viruses may depend on age of the dog (1m- 5years), geographical distribution and environmental factors. We observed that apart from CPV-2/2a/2b/2c all other infections occurred mostly in combination with other viruses, especially with CPV-2. The incidence rate for coinfections ranging from two to four viruses in different combinations accounted for 16.8%. It was previously reported that the mixed infections with different viral combinations was much common in dogs with gastroenteritis (Pratelli et al. 2001; Damián et al. 2005; Chvala et al. 2007; Headley et al. 2013; Zobba et al. 2021). In conjunction with previous reports (Pratelli et al. 2001; Headley et al. 2013; Zobba et al. 2021), we suggest that the gastroenteritis cases may be screened for mixed infections for effective treatment. Most of the pet owners failed to provide proper history as there was no pet health record maintained with them. Of the animals for which the vaccination history was available, it was interesting to note 70.3% of the animals vaccinated with DHPPIL were found positive for at least one of the viruses in question. This observation alerts the need to focus on updating the vaccine with circulating strains of the viruses. Moreover, it highlights the need for regular molecular and serological screening of prevalent viruses to address the vaccination failures.

Recovery Of Virus Isolates In Cell Lines

Because of the presence of mixed infections, especially with CPV-2 a highly virulent virus (Nandi and Kumar 2010), it was difficult to obtain virus isolates from MDCK cells alone. Hence for isolation of CDV and CCoV we used specialized cell lines such as B95a (Pawar et al. 2011) and A-72 (Pratelli et al. 2003). The virus isolates were recovered by infecting the respective cell lines as described in previous section. We observed CPE after 5 dpi for CDV in B95a cells, 3 dpi for CPV-2/2a/2b/2c, CaAstV and 5 dpi for CAdV-2 in MDCK cells, 5 dpi for CCoV in A-72 cells (Fig. 2).

Whole Genome Sequencing Based Evolutionary Dynamics

Worldwide, there were limited reports of whole-genome sequences of CDV, CCoV and CaAstV isolated from dogs (Caddy and Goodfellow 2015; Romanutti et al. 2020; He et al. 2020); and we found no reports from India, till date. Hence, the whole genome of

the three viruses (CDV, CaAstV, CCoV) was sequenced, extracted and the ORF regions were predicted. The total genomic length was found to be around 15.6 kb for CDV, 6.5 kb for CaAstV, 29 kb for CCoV which was nearly similar to that of the previous reports (Von Messling et al. 2001; Carstens 2010; Mihalov-Kovács et al. 2017). We found six ORFs (N, M, F, H, L, P) for CDV; three ORFs for CaAstV (ORF-1a, 1b, 2); and two overlapping ORFs coding ORF-1a and RdRp genes along with nine other genes (M, S, E, N, ORF-3a, 3b, 3c and ORF-7a, 7b) for CCoV as similar to earlier reports (Von Messling et al. 2001; Martella et al. 2011; Garcia 2016). The complete sequences along with protein-coding regions were deposited in the GenBank database of NCBI using BankIt submission and the accession numbers are MT905031 for CDV, MT894143 for CaAstV and MT955604 for CCoV. The whole-genome sequence of CDV was clustered with Asia-1 lineage that was distant from vaccine lineage (Fig. 3a), CaAstV was clustered with group III that consists of China and Brazil isolates when divided into three groups based on the relatedness (Fig. 3b). CCoV was clustered with group II that consists of China and Taipei isolates when divided into two groups based on the relatedness (Fig. 3c). A previous study suggested that all wild type strains of CDV clustered in a similar way to the groups obtained as in H gene analysis, which is normally used to identify geographically distinct CDV lineages (Romanutti et al. 2020). We observed a similar grouping in this study. Further, we also noticed that all the wild type strains are genetically distant to vaccine strains (Martella et al. 2006; Zhao et al. 2010). For CaAstV and CCoV, we could only find very limited reports of whole genome based phylogenetic analysis based on geographical distribution. A study reported a similar observation for CaAstV where one of the strains they sequenced got clustered with China isolates (Caddy and Goodfellow 2015). Another study reported that the CCoV isolates from five provinces of China during 2018-19 clustered with the earlier isolates from China similar to our observations (He et al. 2020).

Further, to understand the phylogeny with respect to individual *orfs* of each of the viruses, the phylogenetic tree based on individual genes of the respective viruses using ten different geographically distinct sequences for each was reconstructed. To our knowledge, very few of such reports on these viruses were published before (Li et al. 2018). Most of the reports relied on partial or complete sequence of one or two variable genes in total (Swati et al. 2015; Navarro et al. 2017; Zhou et al. 2017a; Li et al. 2018). Hence, it is noteworthy to understand if there exists any difference in evolutionary relationship with respect to individual genes. The isolates were grouped based on the relatedness; the geographical lineages can't be tested as the number of isolates collected were only ten. Fig. 4a shows that the current CDV isolate was clustered under M-2 of 3 groups for *M* gene, L-3 of 3 groups for *L* gene, H-1 of 2 groups for *H* gene, N-2 of 3 groups for *N* gene, F-2 of 3 groups for *F* gene, P/C-1 of 2 groups for *P/C* gene. Though the isolates interchanged in between groups with respect to genes, the current isolate always clustered with China and Europe isolates except for *L* and *F* genes, which got clustered with North America and African isolates. However, the whole genome analysis shows clustering with Asia-1 lineage. Fig. 4b shows that the current CaAstV isolate was clustered under ORF-1a-1 of 3 groups for *ORF-1a*, ORF-2-3 of 3 groups for *ORF-2*. However, the *ORF-1b* of all showed high level of similarity so that all the isolates were clustered together. The variation in molecular evolutionary relationship is varying greatly for individual gene for CaAstV as the current isolate is clustered with China isolates with respect to *ORF-1a* analysis, South America and Europe isolates with respect to *ORF-2* analysis, and all the isolates show similarity with respect to *ORF-1b* analysis. The *ORF-2* phylogeny was similar to that of the whole genome for CaAstV. Fig. 4c shows that the current CCoV isolate was clustered under ORF-1a-2 of 4 groups for *ORF-1a* gene, ORF-3a-3 of 4 groups for *ORF-3a* gene, ORF-3b-4 of 4 groups for *ORF-3b* gene, rdp-2 of 4 groups for *rdp* gene, N-1 of 3 groups for *N* gene, E-1 of 2 groups for *E* gene, S-3 of 3 groups for *S* gene, M-3 of 4 groups for *M* gene, ORF-7b-2 of 2 groups for *ORF-7b* gene, ORF-7a-1 of 3 groups for *ORF-7a* gene, ORF-3c-2 of 4 groups for *ORF-3c* gene. Similar to CDV, there was shuffling of lineages with respect to individual *orfs*. The current isolate always clustered with China and Europe isolates except for *E* and *ORF-3c* genes, which got clustered with North America, China and Europe isolates; and Europe isolates, respectively. However, the whole genome analysis show clustering with group II, which has Chinese isolates. Our observations were consistent with the previous reports on independent evolution of genes and various selection pressures and/or evolutionary constraints for various genomic regions (Lukashov and Goudsmit 2002; Li et al. 2018). From this study we understood that the molecular evolutionary dynamics were changing with respect to the individual genes in some cases.

Conclusions

From the present study we conclude that all the three antigenic variants of CPV-2 (CPV-2a/2b/2c), CDV, CaAstV, CAdV-2 and CCoV were circulating in India during the period of 2018-21. CPV-2b was found to be the most prevalent antigenic variant of all the gastroenteritis viruses surveyed in this study. We notice that majority of the gastroenteritis cases presented to the clinics were infected with at least one virus and the number of viruses infecting each dog range from one to four. To our knowledge, this is the

first study to report the whole genome sequences of the CDV, CAstV and CCoV viruses isolated in dogs from India. The phylogenetic analysis shows the isolates were distant to that of the vaccine strains. We strongly emphasize on the need for developing a relevant vaccine with circulating variants of viruses for canine gastroenteritis.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection were performed by Anusha Dema, Mounika Tallapally, Bhagyalakshmi Buddala, Haritha Kodi, Ashwini Ramidi and Sai Spandana Chenji. Analysis was done by Vishweshwar Kumar Ganji, Jaya Laxmi P, Vamsi Krishna S, Narasimha Reddy Yella, Kalyani Putty. The first draft of the manuscript was written by Vishweshwar Kumar Ganji and Kalyani Putty. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

All data generated and analysed during this study are included in this published article and its supplementary information files. *The whole genome sequence datasets generated during the current study are available in the NCBI GenBank database repository as accession no. MT905031 for CDV, MT894143 for CAstV and MT955604 for CCoV.*

Ethics Approval

The current study does not involve human/animal experimentation; hence, ethical approval is not needed. Although dogs were used in this study for collection of rectal swabs, they were not subjected to any form of treatment or experimentation. Rectal swabs were collected by a licensed veterinarian.

Consent to participate

Informed consent was obtained from animal owners for collection of samples

Consent to publish

Not applicable for the current study.

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Figures

Figure 1

Prevalence of canine enteric viruses. a) The prevalence of different canine enteric viruses during the year 2018-21 in the Hyderabad region of Telangana state in India was depicted with bar diagram. The length of the bar indicates number of samples tested positive (Y-axis) for each virus either individually or in mixed infection (X-axis). The percentage above the bar indicates the prevalence rate for that particular virus. CPV* indicates either CPV-2/2a/2b/2c. b) The antigenic prevalence of CPV-2/2a/2b/2c during the year 2018-21 in Hyderabad region of Telangana state in India was depicted with bar diagram. The length of the bar indicates number of samples tested positive (Y-axis) for respective antigenic variant (X-axis). The percentage above the bar indicates the prevalence rate for that particular antigenic variant.

Figure 2

Isolation of canine enteric viruses. Panel A shows healthy cell culture images taken at 20X (total of 200X) magnification whereas panel B shows virus infected cell culture images taken at 20X (total of 200X) magnification. From left to right (i) MDCK/CPV (ii) MDCK/CAdV-2 (iii) A-72/CCoV (iv) B95a/CDV (v) MDCK/CaAstV.

Figure 3

Phylogenetic analysis of the whole genome of current isolates against published sequences from NCBI database. The CDV isolates from current study clustered with Asia 1 lineage (a), CaAstV isolates from current study clustered with group III that consists of China and Brazil (b), CCoV isolates from current study clustered with group II that consists of China and Taipei isolates.

Figure 4

Phylogenetic analysis based on the individual ORF of current isolates against published sequences from NCBI database. The phylogenetic tree was reconstructed for CDV (a), CaAstV (b) and CCoV (c) for individual genes with ten isolates per gene in a single analysis for each virus. The clusters were divided into groups based on relatedness.

Supplementary Files

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