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Detection of human, porcine and canine picornaviruses in municipal sewage sludge using pan-enterovirus amplicon-based long-read Illumina sequencing

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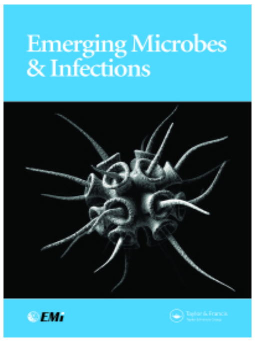

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Detection of Human, Porcine and Canine Picornaviruses in Municipal Sewage Sludge Using Pan-Enterovirus Amplicon-based Long-read Illumina Sequencing

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To the Editor: In situations where most pathogenic, human-infecting virus infections do not result in clinical manifestations, such as with Enteroviruses (EVs) [1], case-based surveillance (CBS) systems lack early detection capacity which is central for mitigating outbreaks before they result in significant morbidity and mortality. Considering most infected people shed viruses (or virus components such as nucleic acid) in large quantities in feces and consequently into wastewater, wastewater-based epidemiology (WBE) has consistently demonstrated capacity to function as an early warning system [2-3] and result in significant time and resource savings by facilitating surveillance of hundreds to thousands of people per sampling event.

We investigated the feasibility of using sludge from different stages of conventional wastewater treatment (primary sludge [PS], waste activated sludge [WAS] and dewatered sludge [centrifuged cake or CC]) for virus surveillance using EVs as a prototype virus. EVs are members of the genus *Enterovirus* (which has over 300 distinct types classified into 15 species) in the family *Picornaviridae*. EVs infect both humans and animals and in the USA are responsible for around 15 million human infections and tens of thousands of hospitalizations annually [4]. Though, over 90% of EV infected individuals are asymptomatic, all infected individuals excrete about 10^8 virus particles/gram of feces (and consequently into wastewater) and shedding continues intermittently for weeks [1,5]. EVs are naked viruses with icosahedral symmetry that are very stable for elongated periods in the environment [1].

In June 2020, nine total sewage sludge samples [PS, WAS and CC] were collected (three per week), over three weeks (figure 1a) from the Morris Forman Water Quality Treatment Center in Louisville, Kentucky, which serves a catchment with a population of ~350,000 people. All samples were subjected to RNA extraction and complete EV capsid RT-PCR (Assay 1, Figures 1b and c) [6]. Subsequently, EV presence per sample was ascertained using assay 2 alongside Sanger sequencing (Figures 1b and c) [6]. This identified five samples as reliably containing EVs (Table

S1). Three contained *Enterovirus Species G* (EV-G) members while each of the remaining two contained CVA11 (EV-C) and multiple peaks (suggestive of more than one EV type, Figure S1), respectively.

Assay 1 amplicons from these five confirmed EV positive samples were subjected to assay 3 and Long-read Illumina sequencing. Seventy-three long-read contigs were recovered from the five EV positive samples (Table S2). Though more variants were recovered using LRIS, both SS and LRIS were congruent with respect to the EV types detected in four (samples 5, 6, 7, and 8) of the five samples (Tables S1 and S2). SS showed multiple peaks in the fifth sample (Sample 3, Tables S1) while LRIS delineated the different virus types (Figure 1a and Table S2) and variants present in the sample. LRIS also showed the presence of two canine picornavirus variants in the sample (Table S2 and S3).

Since unlike for EV-A and EV-C, the enterovirus genotyping tool (EGT) [7] does not resolve EV-G species members into types (Table S3), we used a combination of phylogenetic and pairwise identity analysis to type the EV-Gs, and found them belonging to genotypes 1, 2, 9 and 15 (Figure S2). Pairwise identity analysis showed that the EV-Gs detected in this study were ~20% divergent (Table S4 and Figure S3) from the most similar sequence in GenBank (even those detected in California, USA in 2018 [8] [Figure S3]) suggesting these might have circulated undescribed for around two decades (at an evolutionary rate of 1×10^{-2} substitutions per site per year [*i.e* ~1% divergence per year] [9]). A similar observation was made for the EV-Cs (CVA11 and CVA24) which were 16% to 20% divergent (Table S4) from the most similar sequence in GenBank. The

EV-A (CVA2) was different in that the most similar sequence in GenBank was ~3% divergent (MT641397; found in a respiratory specimen in the UK in 2018) (Table S4).

Phylogenetic analysis of the two CanPV contigs (Figure S4) showed that they belong to a group of unclassified canine picornaviruses that (based on publicly available sequence data in GenBank) have not been previously described in the USA. They have however been described in dogs in the United Arab Emirates (UAE), China and Hong Kong for over a decade (2008 to 2019) [10-12] and more recently in Foxes in Australia [13] but <10 sequences are publicly available in GenBank as of the 23rd of March 2022. Since CanPV detection as described above was serendipitous, to confirm it was truly present in our sample, we designed assay 4 (Figure 1d) and subjected both assays 1 and 3 amplicon from sample 3 to the assay (assay 4, Figure 1b). We succeeded in amplifying the ~950 bp amplicon from both (Figure S5) and Sanger sequencing confirmed that CanPV was, in fact, present. This suggests that CanPV amplification occurred first in assay 1. In fact, we have subsequently recovered multiple variants of CanPV (with the same contig size) in an independent study using samples from another state in the USA (unpublished data) in which we sequenced products from assay 1 using Illumina technology. This confirmed that near complete CanPV capsid region could be amplified using assay 1 and showed divergence bordering ~20% between CanPV capsid variants circulating in the USA between 2019 and 2021 (unpublished data).

Our findings show that sludge from different stages (PS, WAS and CC) of conventional wastewater treatment can be used for virus surveillance. We recovered porcine (EV-G), canine (CanPV) and human (EV-A and EV-C) picornaviruses demonstrating this approach provides an avenue that facilitates surveillance of both human viruses and animal viruses and a *One-Health* framework [14]. In addition, our findings document the existence of both human and animal virus (with potential to cause significant morbidity and mortality) lineages that have been circulating in the USA for around two decades undetected. Finally, we document (based on publicly available sequence data in GenBank) the first detection of CanPV in the USA and the first detection globally using wastewater-based epidemiology. Considering the dearth of information on CanPV (with <10 sequences publicly available in GenBank as of 23rd March 2022) we describe a new CanPV assay (figure 1d) targeting the capsid protein gene region that can be used for CanPV detection and molecular epidemiology globally, especially in resource limited settings and thereby facilitate our understanding of its global dynamics.

References

1. Nathanson N, Kew OM. From emergence to eradication: the epidemiology of poliomyelitis deconstructed. Am J Epidemiol. 2010;172(11):1213-29. Available from: https://ncbi.nlm.nih.gov/pubmed/ 20978089.
2. Manor Y, Handsher R, Halmut T, et al. Detection of poliovirus circulation by environmental surveillance in the absence of clinical cases in Israel and the Palestinian authority. J Clin Microbiol. 1999;37(6):1670-5. Available from: https://ncbi.nlm.nih.gov/pubmed/ 10325305.
3. Peccia J, Zulli A, Brackney DE, et al. Measurement of SARS-CoV-2 RNA in wastewater tracks community infection dynamics. Nat Biotechnol. 2020;38(10):1164-1167. Available from: https://ncbi.nlm.nih.gov/pubmed/ 32948856.
4. CDC Non-Polio Enterovirus. (accessed on 24 March 2022); Available online: https://www.cdc.gov/non-polio-enterovirus/index.html.

5. Asghar H, Diop OM, Weldegebriel G, et al. Environmental surveillance for polioviruses in the Global Polio Eradication Initiative. J Infect Dis. 2014;210 Suppl 1:S294-303. Available from: https://ncbi.nlm.nih.gov/pubmed/ 25316848.
6. Faleye TOC, Bowes DA, Driver EM, et al. Wastewater-Based Epidemiology and Long-Read Sequencing to Identify Enterovirus Circulation in Three Municipalities in Maricopa County, Arizona, Southwest United States between June and October 2020. Viruses. 2021; 13(9):1803. Available from: https://ncbi.nlm.nih.gov/pubmed/ 34578384.
7. Kroneman A, Vennema H, Deforche K, et al. An automated genotyping tool for enteroviruses and noroviruses. J Clin Virol. 2011;51(2):121-5. Available from: https://ncbi.nlm.nih.gov/pubmed/ 21514213.
8. Ramesh A, Bailey ES, Ahyong V, et al. Metagenomic characterization of swine slurry in a North American swine farm operation. Sci Rep. 2021;11(1):16994. Available from: https://ncbi.nlm.nih.gov/pubmed/ 34417469.
9. Jorba J, Campagnoli R, De L, et al. Calibration of multiple poliovirus molecular clocks covering an extended evolutionary range. J Virol. 2008;82(9):4429-40. Available from: https://ncbi.nlm.nih.gov/pubmed/ 18287242.
10. Woo PC, Lau SK, Choi GK, et al. Complete genome sequence of a novel picornavirus, canine picornavirus, discovered in dogs. J Virol. 2012;86(6):3402-3. Available from: https://ncbi.nlm.nih.gov/pubmed/ 22354943.
11. Woo PCY, Lau SKP, Choi GKY, et al. Molecular epidemiology of canine picornavirus in Hong Kong and Dubai and proposal of a novel genus in Picornaviridae. Infect Genet Evol. 2016;41:191-200. Available from: https://ncbi.nlm.nih.gov/pubmed/ 27051044.

12. Campbell SJ, Ashley W, Gil-Fernandez M, et al. Red fox viromes in urban and rural landscapes. Virus Evol. 2020;25;6(2):veaa065. Available from: https://ncbi.nlm.nih.gov/pubmed/33365150.
13. Li C, Li Y, Li H, et al. Genomic characterization and phylogenetic analysis of a new canine picornavirus variant in the mainland of China. Virus Res. 2021;296:198351. Available from: https://ncbi.nlm.nih.gov/pubmed/ 33639222.
14. CDC One Health. (accessed on 24 March 2022]; Available online: https://www.cdc.gov/onehealth/

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Data Availability Statement: Sequences generated from this study are available in NCBI GenBank under accession numbers OK554433 – OK554505 and OM782676.

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Loop Genomics for help with Long-Read Illumina sequencing. We especially thank Tuval Ben Yehezkel for post-sequencing support.

Conflicts of Interest: E.M.D and R.U.H. are cofounders of AquaVitas, LLC, 9260 E. Raintree, Ste 130, Scottsdale, AZ 85260, USA, an ASU start-up company providing commercial services in wastewater-based epidemiology. R.U.H. is the founder of OneWaterOneHealth, a non-profit project of the Arizona State University Foundation.

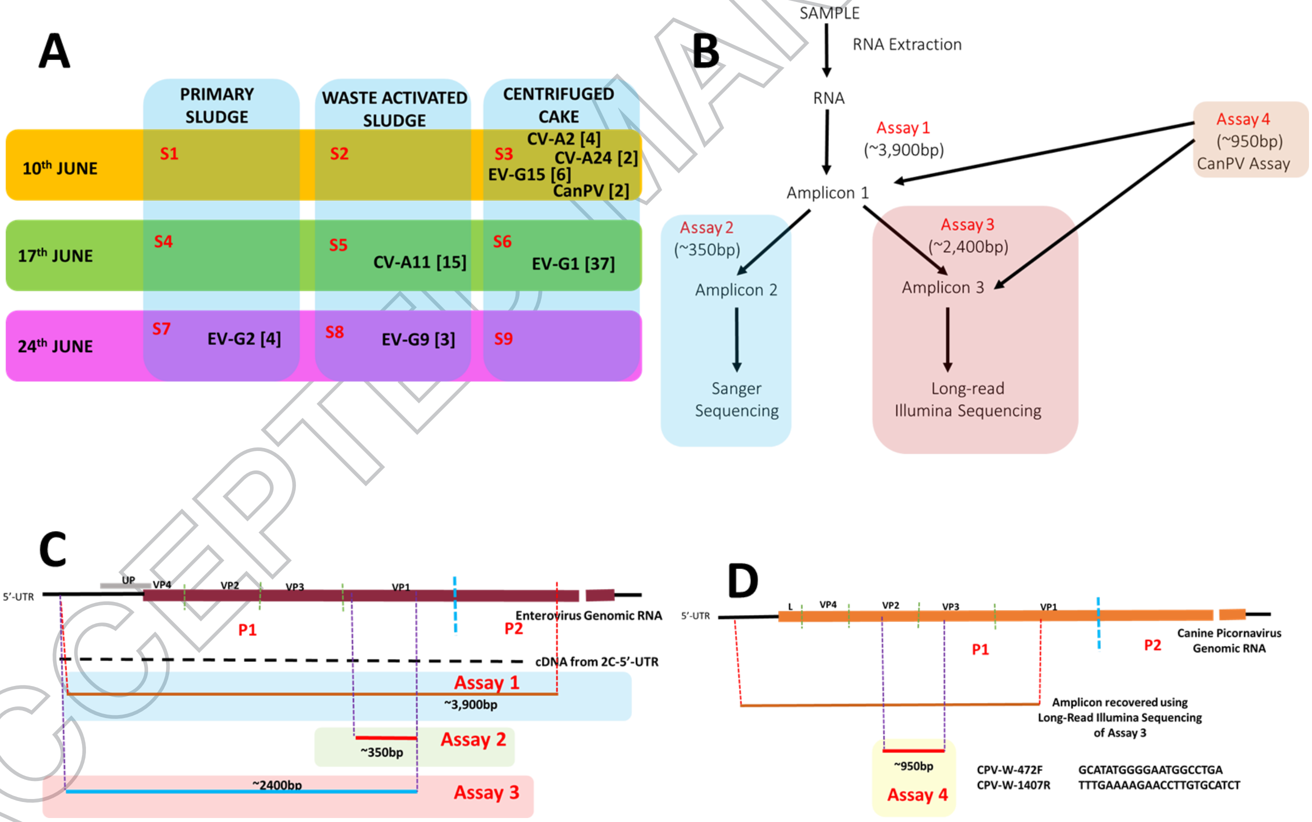

Figure 1: A) Virus types detected in this study. S1-S9 refer to Samples 1-9. Numbers in bracket refer to the number of variants per virus type. B) Schematic representation of the workflow used

in this study, C) EV genomic region amplified by Assays 1, 2 and 3, respectively (D) and CanPV genomic region recovered by long-read Illumina sequencing of amplicon from assay 3 and amplified by assay 4.

APPENDIX A: SUPPLEMENTARY METHODS

1.1. Sample Collection

We collected and analyzed sewage sludge from the Morris Forman Water Quality Treatment Center (wastewater treatment plant [WWTP]) in Louisville, Kentucky, USA in June 2020. The WWTP sampled serves a catchment with a population of ~350,000 people. Three samples [PS, WAS and CC] were collected per day on the 10th, 17th and 24th of June. After collection, the samples were shipped overnight on ice to the Biodesign Institute, Arizona State University, Tempe, Arizona for processing.

1.2. Sample processing, RNA extraction and RT-PCR

We aliquoted 2.5 mL of each sample into a 50 mL centrifuge tube containing 1 g of plastic beads and 7.5 mL of nuclease free water. The mixture was vortexed for 20 minutes and afterwards centrifuged at 4,000 rpm for 20 minutes. The supernatant was collected and stored in 1 mL aliquots at -80^{o}C.

Precisely, 280 µL (140 µL x 2) of each sample was subjected to RNA extraction using the QiaAmp RNA extraction kit (Qiagen, Hilden, Germany) following manufacturer's recommendation. Subsequently, the RNA extract was subjected to the EV detection workflow described in Faleye *et al.* [1] with a slight modification (Figure S1). Specifically, 5 µL of RNA extract was subjected to the one-step RT-PCR assay (assay 1, Figure S1) described in [2]. The amplicon product of the assay was then used as template in two independent second round PCR assays yielding ~350 bp (assay 2, Figure S1) and ~2,400 bp (assay 3, Figure S1) amplicons, respectively as previously described [1]. Assay 2 and 3 amplify VP1 and 5'-UTR to VP1 regions of the EV genome respectively (Figure S2). Assay 2 was used to cost effectively streamline samples subjected to assay 3 and consequently, long-read Illumina sequencing. Please note that all sequencing reactions described here were outsourced to commercial facilities. Hence the Sanger and long-read Illumina sequencing results were also used as controls for each other.

The amplicon product of assays 1 and 3 were independently used as template for assay 4 (Figure S1) which amplifies an ~ 950bp region of the CanPV genome (Figure S3) spanning VP2-VP3 (nucleotide 1089 to 2067 relative to JN831356). Specifically, 2uL of each was added independently to a PCR master mix containing 12.5uL of GoTaq green, 0.25uL (100uM) each of forward and reverse primers and 10uL of PCR-grade water. Amplification was done in a Bio-Rad C1000 thermal cycler as follows; 94^{o}C for 2 minutes, 35 cycles of 94^{o}C for 15 seconds, 55^{o}C for 30 seconds and 60^{o}C for 60 seconds. This was followed by 68^{o}C for 5 minutes and held at 4^{o}C until stopped. The amplicons (~350 bp, ~950 bp and ~2400 bp) were resolved on 1% or 2% gels stained with GelRed (Biotium Inc., Fremont, California, USA).

1.3. Sequencing

The ~350 bp and ~ 950bp amplicons were Sanger sequenced at the Genomics Core at Arizona State University while the ~2,400 bp amplicon was indexed, normalized and pooled using Loop Genomics long-read sequencing kit (San Jose, California, USA) as recommended by the manufacturer. The pooled product was then shipped to Loop Genomics where library preparation and sequencing was done using their long-read sequencing technology on a HiSeq 4000 Illumina sequencer (2 × 150 paired-end). Assembly of short reads to long reads was also done using Loop Genomics pipeline [3] and Spades [4].

1.4. Genotyping

The EV contigs were identified using the enterovirus genotyping tool (EGT) version 1.0 [5] and duplicate contigs were removed using the '*find duplicates*' tool in Geneious Prime v2020.1.2 (Biomatters Ltd., Auckland, New Zealand). For the EVs, a local database was created using sequences recovered in this study and similar sequences from GenBank [6]. Specifically, the EV contigs recovered in this study were subjected to a BLASTn [7] search of the GenBank database, the top 100 hits of each were recovered, pooled and duplicate sequences removed. Multiple sequence alignments were done using ClustalW in MEGAX [8] and Maximum-Likelihood (ML) trees were generated in MEGAX using 1,000 bootstrap replicates. Pairwise Identity analysis was performed using SDT v1.2. [9].

The CanPV contigs were identified using both the EGT and BLASTn search of the GenBank database. Subsequently, a local database was created as described above for EVs and subsequently *in silico* translated using the *orfFinder* web server; a National Center for Biotechnology Information (NCBI) resource [10]. The *in silico* translated database was then used to make ML phylogenetic trees as described above. Sequences generated in this study have been deposited in GenBank under the accession numbers OK554433 – OK554505 and OM782676.

References

1. Faleye TOC, Bowes DA, Driver EM, et al. Wastewater-Based Epidemiology and Long-Read Sequencing to Identify Enterovirus Circulation in Three Municipalities in Maricopa County, Arizona, Southwest United States between June and October 2020. Viruses. 2021; 13(9):1803. Available from: https://ncbi.nlm.nih.gov/pubmed/ 34578384.
2. Majumdar M, Martin J. Detection by Direct Next Generation Sequencing Analysis of Emerging Enterovirus D68 and C109 Strains in an Environmental Sample From Scotland. Front Microbiol. 2018;9:1956. Available from: https://ncbi.nlm.nih.gov/pubmed/30186268.
3. Callahan BJ, Grinevich D, Thakur S, et al. Ultra-accurate microbial amplicon sequencing with synthetic long reads. Microbiome. 2021;5;9(1):130. Available from: https://ncbi.nlm.nih.gov/pubmed/34090540.
4. Bankevich A, Nurk S, Antipov D, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19(5):455-77. Available from: https://ncbi.nlm.nih.gov/pubmed/22506599.
5. Kroneman A, Vennema H, Deforche K, et al. An automated genotyping tool for enteroviruses and noroviruses. J Clin Virol. 2011;51(2):121-5. Available from: https://ncbi.nlm.nih.gov/pubmed/ 21514213.
6. Sayers EW, Cavanaugh M, Clark K, et al. GenBank. Nucleic Acids Res. 2021;49(D1):D92-D96. Available from: https://ncbi.nlm.nih.gov/pubmed/ 33196830.
7. Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10. Available from: https://ncbi.nlm.nih.gov/pubmed/2231712.
8. Kumar S, Stecher G, Li M, et al. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol. 2018;35(6):1547-1549. Available from: https://ncbi.nlm.nih.gov/pubmed/ 29722887.

9. Muhire BM, Varsani A, Martin DP. SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. PLoS One. 2014;9(9):e108277. Available from: https://ncbi.nlm.nih.gov/pubmed/ 25259891.
10. Sayers EW, Barrett T, Benson DA, et al. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 2011;39(Database issue):D38-51. Available from: https://ncbi.nlm.nih.gov/pubmed/ 21097890.

APPENDIX B: SUPPLEMENTARY FIGURES

Figure S1: Samples of Sanger sequencing chromatogram data showing usable and unusable data and multiple peaks.

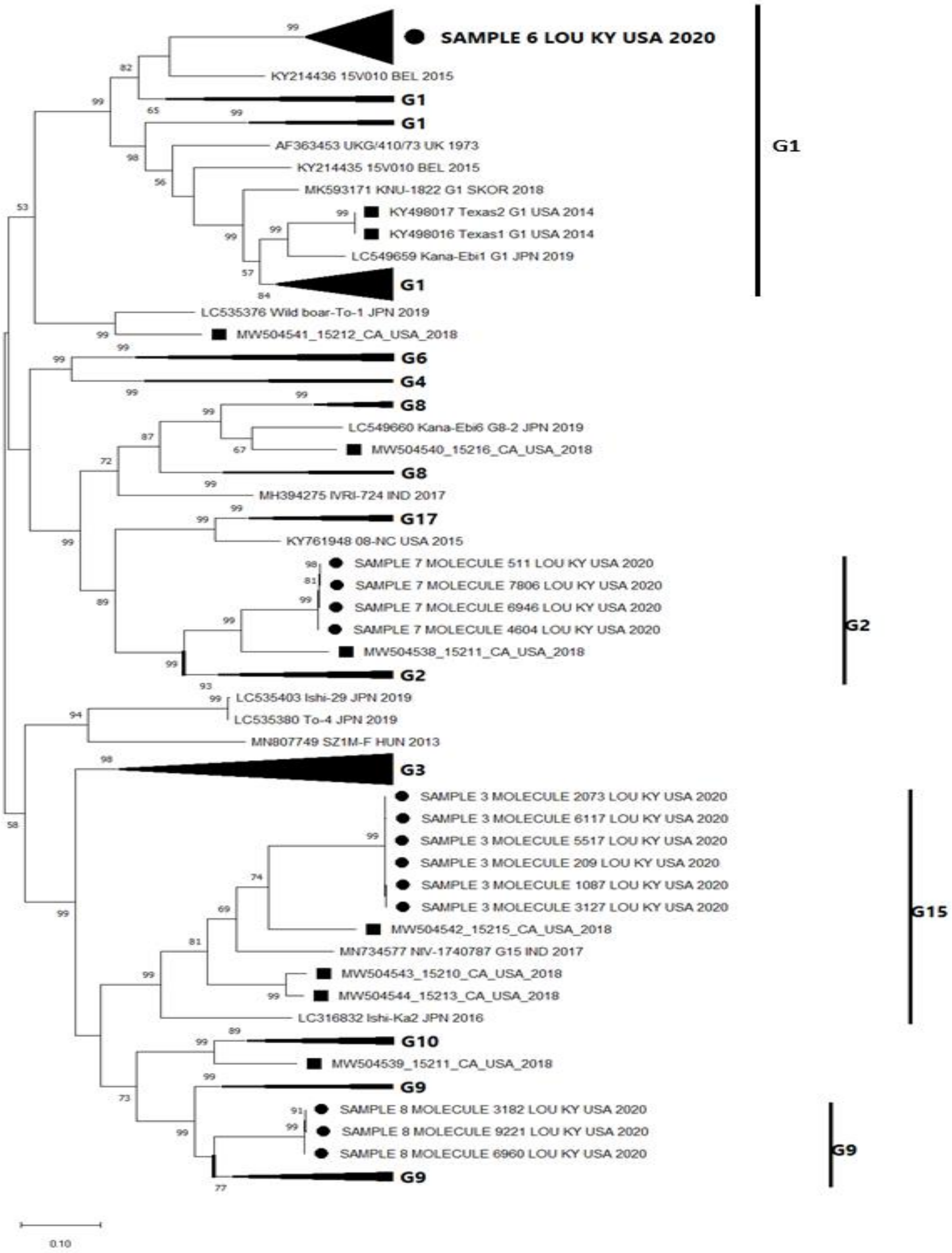

Figure S2. Identification of EVG contigs recovered in this study using the 2,400 bp contigs. Sequences described in this study and those previously described in the USA are labelled with black circle and square, respectively. Types bordered with black vertical lines (G1, G2, G9 and

G15) connote types detected in this study. Bootstrap values are shown if >50%. Collapsed taxa denote all sequences therein belong to the same EVG type.

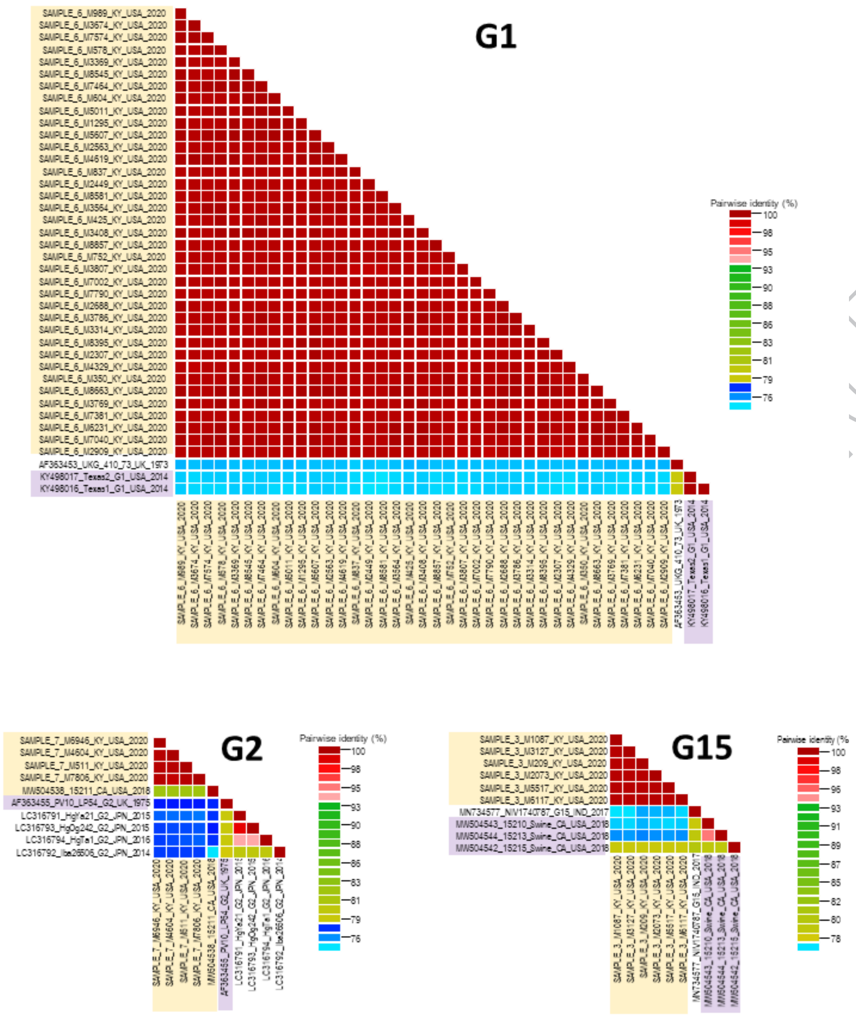

Figure S3. Pairwise identity analysis of EVG types found in this study for which sequences have been previously described in the USA. Sequences described in this study and those previously described in the USA are highlighted with yellow and purple, respectively.

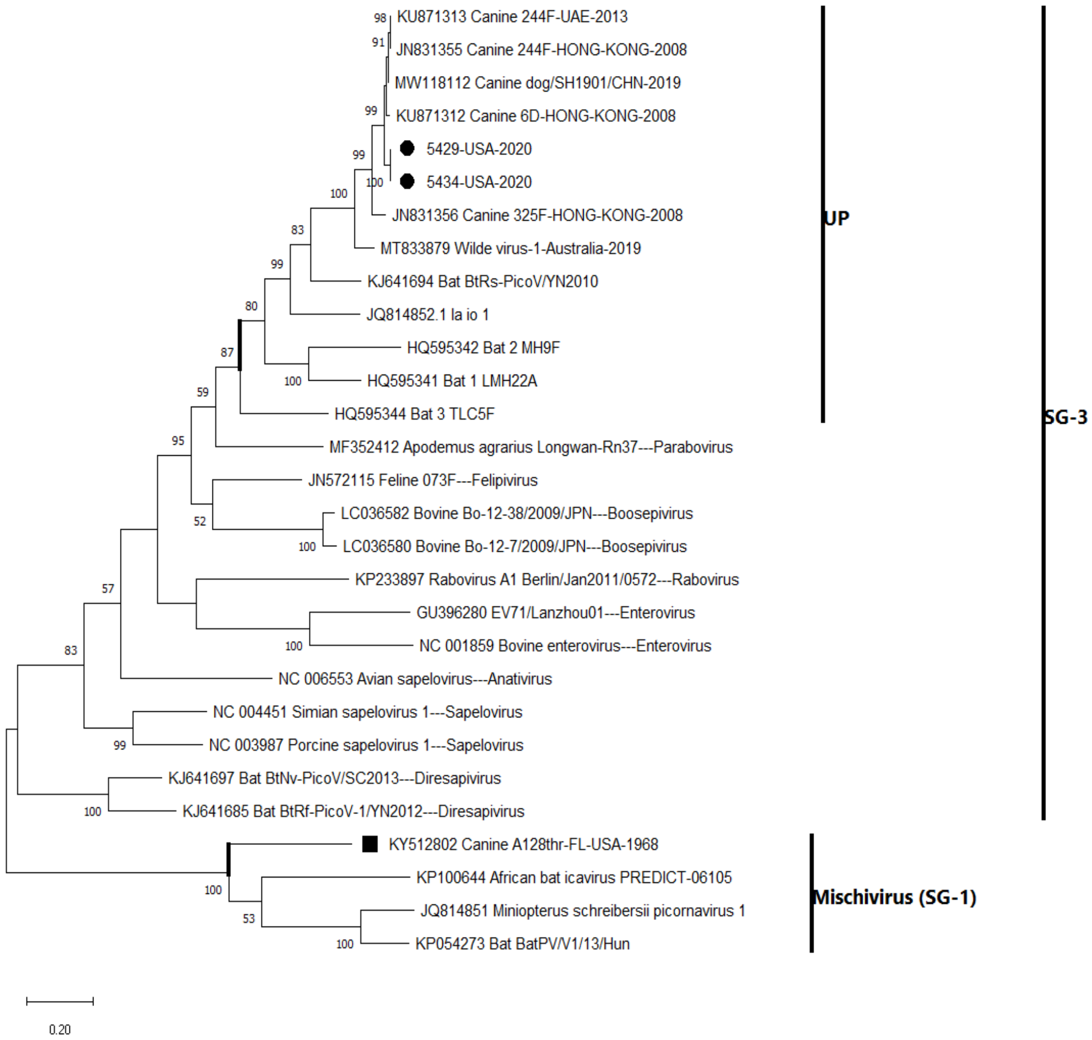

Figure S4. Phylogenetic tree for identification of canine picornavirus contigs recovered in this study. Sequences described in this study and those previously described in the USA are labelled with black circle and square, respectively. Bootstrap values are shown if >50%. Abbreviations: SG - Supergroup; UP - Unclassified Picornaviruses.

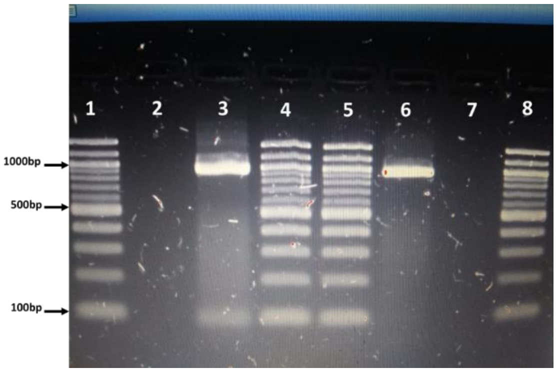

Figure S5: Assay 4 gel electrophoresis result. Lanes 1, 4, 5 and 8 contain 100bp ladder. Lanes 2 and 7 are empty. Lanes 3 and 6 show bands suggestive of successful CanPV detection using assay 4. Band in lane 3 was recovered by subjecting amplicon from assay 1 to assay 4. Band in lane 6 was recovered by subjecting amplicon from assay 3 to assay 4.

Appendix C. SUPPLEMENTARY TABLES

Table S1. Sanger sequencing results for EV. S-ID means sample identification number.

S-ID	Collection Date	Sludge type	EV-Screen	EV-type
1	10th June 2020	Primary	Positive	Poor data
2	10th June 2020	Waste Activated	Negative	NA
3	10th June 2020	Centrifuged Cake	Positive	MP
4	17th June 2020	Primary	Negative	NA
5	17th June 2020	Waste Activated	Positive	CVA11
6	17th June 2020	Centrifuged Cake	Positive	EV-G
7	24th June 2020	Primary	Positive	EV-G
8	24th June 2020	Waste Activated	Positive	EV-G
9	24th June 2020	Centrifuged Cake	Positive	Poor data

Table S2. Illumina sequencing results for EV. S-ID means sample identification number.

S-ID	Short Reads	Assembled long Reads	Virus Long-reads	Enterovirus Long-reads	Virus-type (N)	Total Contigs	Short Reads Range	Coverage
3	2,898,558	6,610	3,483	2,393	CVA24 (2)/CVA2 (4)/EVG (6)/CP (2)	14	1666 – 402	208x – 31x
5	1,175,369	1,667	1,233	1,233	CVA11 (15)	15	1088 – 250	136x – 31x
6	3,035,478	9,002	771	771	EVG (37)	37	1880 – 276	235x – 34x
7	3,336,677	8,512	866	307	EVG (4)	4	1698 – 340	212x – 42x
8	3,137,213	9,427	164	130	EVG (3)	3	1882 – 570	235x – 71x
	13,583,295	**35,218**	**6,517**	**4,834**		**73**		

Table S3. Enterovirus Genotyping tool (EGT) virus identification results. Abbreviations: NA = Not Applicable; EV = Enterovirus, CanPV = Canine Picornavirus, CV = Coxsackievirus, VP = Virus protein.

Amplicon-Unique-ID	Begin	End	RefSeq	BLAST result	BLAST score	Type	Type support	VP1 type	VP1 type support

S3_M1087	594	2935	AF363455	EVG	69.41	NA	NA	NA	NA
S3_M1819	537	2931	NC_001612	EVA	80.91	CV-A2	100	CV-A2	100
S3_M1933	528	2992	NC_002058	EVC	79.46	CV-A24	100	CV-A24	94
S3_M2073	594	2935	AF363455	EVG	69.54	NA	NA	NA	NA
S3_M 209	594	2935	AF363455	EVG	69.54	NA	NA	NA	NA
S3_M2103	528	2992	NC_002058	EVC	79.38	CV-A24	100	CV-A24	95
S3_M3127	594	2935	AF363455	EVG	69.58	NA	NA	NA	NA
S3_M3761	537	2931	NC_001612	EVA	80.87	CV-A2	100	CV-A2	100
S3_M4055	537	2931	NC_001612	EVA	80.78	CV-A2	100	CV-A2	100
S3_M5429	557	2879	JN831356	CanPV	79.93	NA	NA	NA	NA
S3_M5434	557	3030	JN831356	CanPV	79.89	NA	NA	NA	NA
S3_M5517	594	2935	AF363455	EVG	69.50	NA	NA	NA	NA
S3_M5564	537	2931	NC_001612	EVA	80.87	CV-A2	100	CV-A2	100

S3_M6117	594	2935	AF363455	EVG	69.50	NA	NA	NA	NA
S5_M1027	528	2989	NC_002058	EVC	79.16	CV-A11	100	CV-A11	100
S5_M1172	528	2989	NC_002058	EVC	79.24	CV-A11	100	CV-A11	100
S5_M1250	528	2989	NC_002058	EVC	79.04	CV-A11	100	CV-A11	100
S5_M1441	528	2989	NC_002058	EVC	79.08	CV-A11	100	CV-A11	100
S5_M1500	528	2989	NC_002058	EVC	81.30	CV-A11	100	CV-A11	100
S5_M1560	528	2989	NC_002058	EVC	81.52	CV-A11	100	CV-A11	100
S5_M1651	528	2989	NC_002058	EVC	79.08	CV-A11	100	CV-A11	100
S5_M167	528	2989	NC_002058	EVC	81.65	CV-A11	100	CV-A11	100
S5_M207	528	2989	NC_002058	EVC	79.00	CV-A11	100	CV-A11	100
S5_M237	528	2989	NC_002058	EVC	79.08	CV-A11	100	CV-A11	100
S5_M238	528	2988	NC_002058	EVC	81.47	CV-A11	100	CV-A11	100
S5_M248	528	2989	NC_002058	EVC	79.12	CV-A11	100	CV-A11	100

S5_M307	528	2989	NC_002058	EVC	81.52	CV-A11	100	CV-A11	100
S5_M352	528	2989	NC_002058	EVC	79.16	CV-A11	100	CV-A11	100
S5_M447	528	2989	NC_002058	EVC	79.04	CV-A11	100	CV-A11	100
S5_M476	528	2989	NC_002058	EVC	79.12	CV-A11	100	CV-A11	100
S5_M554	528	2989	NC_002058	EVC	79.12	CV-A11	100	CV-A11	100
S5_M954	528	2989	NC_002058	EVC	81.52	CV-A11	100	CV-A11	100
S6_M1295	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M2307	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M2449	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M2563	594	2935	AF363455	EVG	76.43	NA	NA	NA	NA
S6_M2688	594	2935	AF363455	EVG	76.52	NA	NA	NA	NA
S6_M2909	594	2935	AF363455	EVG	76.56	NA	NA	NA	NA
S6_M3314	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA

S6_M3369	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M3408	594	2935	AF363455	EVG	76.56	NA	NA	NA	NA
S6_M350	594	2935	AF363455	EVG	76.52	NA	NA	NA	NA
S6_M3564	594	2935	AF363455	EVG	76.52	NA	NA	NA	NA
S6_M3674	594	2935	AF363455	EVG	76.52	NA	NA	NA	NA
S6_M3769	594	2935	AF363455	EVG	76.56	NA	NA	NA	NA
S6_M3786	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M3807	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M425	594	2935	AF363455	EVG	76.57	NA	NA	NA	NA
S6_M4329	594	2935	AF363455	EVG	76.43	NA	NA	NA	NA
S6_M4619	594	2935	AF363455	EVG	76.43	NA	NA	NA	NA
S6_M5011	594	2935	AF363455	EVG	76.56	NA	NA	NA	NA
S6_M5607	594	2935	AF363455	EVG	76.52	NA	NA	NA	NA

S6_M578	594	2935	AF363455	EVG	76.60	NA	NA	NA	NA
S6_M604	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M6231	594	2935	AF363455	EVG	76.60	NA	NA	NA	NA
S6_M7002	594	2935	AF363455	EVG	76.52	NA	NA	NA	NA
S6_M7040	594	2935	AF363455	EVG	76.60	NA	NA	NA	NA
S6_M7381	594	2935	AF363455	EVG	76.52	NA	NA	NA	NA
S6_M7464	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M752	594	2935	AF363455	EVG	76.43	NA	NA	NA	NA
S6_M7574	594	2935	AF363455	EVG	76.56	NA	NA	NA	NA
S6_M7790	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M837	594	2935	AF363455	EVG	76.30	NA	NA	NA	NA
S6_M8395	594	2935	AF363455	EVG	76.52	NA	NA	NA	NA
S6_M8545	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA

S6_M8581	594	2935	AF363455	EVG	76.39	NA	NA	NA	NA
S6_M8663	594	2935	AF363455	EVG	76.48	NA	NA	NA	NA
S6_M8857	594	2935	AF363455	EVG	76.43	NA	NA	NA	NA
S6_M989	594	2935	AF363455	EVG	76.60	NA	NA	NA	NA
S7_M4604	594	2935	AF363455	EVG	78.38	NA	NA	NA	NA
S7_M511	594	2935	AF363455	EVG	78.42	NA	NA	NA	NA
S7_M6946	594	2935	AF363455	EVG	78.46	NA	NA	NA	NA
S7_M7806	594	2935	AF363455	EVG	78.38	NA	NA	NA	NA
S8_M3182	594	2932	AF363455	EVG	71.87	NA	NA	NA	NA
S8_M6960	594	2932	AF363455	EVG	71.74	NA	NA	NA	NA
S8_M9221	594	2932	AF363455	EVG	71.74	NA	NA	NA	NA

Table S4. Top two BLASTn hits of representatives of each EV type recovered from sludge in Kentucky, USA

			Top 2 BLASTn Hits			
Amplicon-Unique-ID	**BLAST result**	**Type**	**Accession number**	**% Identity**	**Accession number**	**% Identity**
S3_M1087	EVG	G15	MN734577.1	78.80	KT265894.2	76.76
S3_M6117	EVG	G15	MN734577.1	78.71	KT265894.2	76.88
S3_M1933	EVC	CV-A24	MW373950.1	80.74	EF015036.1	79.77
S3_M2103	EVC	CV-A24	MW373950.1	80.66	EF015036.1	79.69
S3_M3761	EVA	CV-A2	MT641397.1	96.86	KP289361.1	85.47
S3_M4055	EVA	CV-A2	MT641397.1	96.73	KP289361.1	85.35
S3_M5429	CanPV	NA	KU871312.1	84.84	MW118112.1	84.53
S3_M5434	CanPV	NA	KU871312.1	84.80	MW118112.1	84.48
S5_M248	EVC	CV-A11	JF260918.1	80.95	JF260919.1	80.92
S5_M307	EVC	CV-A11	AF465512.1	81.38	AF499638.1	81.26
S6_M989	EVG	G1	LC316790.1	78.93	MZ679264.1	78.43
S6_M2909	EVG	G1	LC316790.1	78.97	MZ679264.1	78.41
S7_M6946	EVG	G2	MW504538.1	82.38	LC316793.1	79.05
S7_M7806	EVG	G2	MW504538.1	82.27	LC316793.1	78.92
S8_M3182	EVG	G9	KT265894.2	83.70	KT265961.2	83.40
S8_M6960	EVG	G9	KT265894.2	83.65	KT265961.2	83.36