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ORIGINAL ARTICLE

Assessment of enteric viruses during a hepatitis outbreak in Detroit MI using wastewater surveillance and metagenomic analysis

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caliciviruses, enteric viruses, hepatitis, metagenomics, qPCR, wastewater surveillance.

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Abstract

Aims: This study investigates enteric viruses in wastewater during an outbreak of acute hepatitis caused by hepatitis A virus (HAV) in a large metropolitan area. Emphasis is given to caliciviruses and HAV.

Methods and Results: Metagenomic analysis was performed to characterize enteric viruses excreted by the population of Detroit MI, during a hepatitis A outbreak that occurred in 2017 and 2018. Additionally, HAV, norovirus GII, and sapovirus were quantified, using qPCR, in 54 untreated wastewater samples collected over the course of 4 months. Correlation analysis was performed to identify associations between the number of disease cases and HAV concentrations in wastewater. HAV obtained the highest relative abundance among other enteric viruses detected in wastewater metagenomes. Metagenomic analysis also detected several other enteric viruses including astrovirus, enterovirus and hepatitis E virus. Average sapovirus concentrations of $1{\cdot}36 \times 10^6$ gc l^{-1} were significantly greater than norovirus GII concentrations ($2{\cdot}94 \times 10^4$ gc l^{-1}). Additionally, norovirus GI and GII along with sapovirus GI.1 were detected using metagenomics. HAV loads in wastewater were significantly correlated with the number of disease cases reported 1 week after wastewater sampling.

Conclusions: Surveying untreated wastewater is a promising method for detecting early signs of hepatitis A outbreaks and for routine environmental monitoring of enteric viruses circulating in the environment.

Significance and Impact of the Study: Authors demonstrate the usefulness of metagenomics for genogrouping and enteric viral surveillance.

Introduction

Gastrointestinal disease could be caused by a wide variety of pathogens, including viruses, and symptoms alone may not be enough to distinguish the causative agent. Untreated wastewater harbours a wealth of information about the community in the sewage catchment area. Centralized wastewater treatment facilities have the capacity to collect wastewater from thousands or millions of inhabitants per day revealing valuable information about the serviced population. Broad monitoring of viruses in wastewater may provide a means of identification of the causes of endemic disease and of occasional outbreaks (McCall *et al.* 2020).

Among common causes of gastrointestinal disease are enteric viruses. Such viruses include rotavirus, astrovirus, adenovirus, enterovirus, norovirus, sapovirus and others. Noroviruses are among the leading causes of outbreaks of viral acute gastroenteritis (AGE) worldwide (Glass *et al.* 2009; Hall *et al.* 2013). Although not as notorious as norovirus, sapoviruses are also important pathogens in AGE cases. Norovirus and sapovirus are nonenveloped positive-sense single-stranded RNA viruses in the Caliciviridae family. Norovirus contain at least 10 different genogroups (GI–GX) where GI, GII and GIV are known to infect humans (Chhabra *et al.* 2019). Norovirus GII predominates in sporadic cases and outbreaks globally (Glass *et al.* 2009). Genetic sequences of norovirus samples from

outbreak investigation and sporadic cases occurring between 2005 and 2016 identified 92% of sequences as belonging to norovirus GII, less than 10% of sequences were classified as GI and less than 1% as GIV (van Beek *et al.* 2018). Similarity, Cannon *et al.* (2017) found norovirus GII to be responsible for approximately 82% of norovirus outbreaks in the United Stated between 2013 and 2016. Currently, there are 19 known sapovirus genogroups (GI–GIX) where GI, GII, GIV and GV are known to infect humans (Oka *et al.* 2015; Diez-Valcarce *et al.* 2018). Sapovirus GI has been described in several diarrhoeal cases and sporadic outbreaks of AGE worldwide (Fioretti *et al.* 2016; Kumthip *et al.* 2018; Sánchez *et al.* 2018).

Although not a common cause of AGE, hepatitis A virus (HAV) is an important enteric virus causing significant outbreaks of acute hepatitis worldwide. HAV is a nonenveloped single-stranded RNA virusbelonging to the Picornaviridae family. The burden of hepatitis A outbreaks has had a detrimental impact on communities and healthcare infrastructures (Snyder *et al.* 2019). There are approximately 1·5 million cases of hepatitis A reported annually. According to the World Health Organization, HAV infections resulted in 11 000 deaths in 2015 (WHO 2017). Previous studies have evaluated wastewater surveillance of hepatitis A in communities (Gharbi-khelifi *et al.* 2007; La Rosa *et al.* 2014; Yanez *et al.* 2014; Manor *et al.* 2017; Bisseux *et al.* 2018; Chen *et al.* 2019) using PCR and comparing the detection rate of positive HAV sewage samples to the incidence of clinical cases reported in the catchment area. However, detection rates were, at times, not correlated with clinical records. Several factors influence the detection of viruses in wastewater including the sensitivity of the method, environmental conditions and the inherently low levels of viral pathogens in water systems. This is especially a challenge with qualitative (presence/absence) tests. Hellmer *et al.* (2014) used qPCR to detect HAV in sewage samples in Scandinavia and estimated prevalence according to virus concentration. Findings suggests that wastewater surveillance is a promising tool for monitoring HAV infections in communities. However, the study highlights the difficulties of establishing correlations between concentration and clinical data for viral infections.

This study investigates the distribution of enteric viruses in wastewater during a hepatitis A outbreak in a large metropolitan area in the United States using metagenomics and qPCR. In addition, the authors quantified HAV, sapovirus and norovirus GII in wastewater samples and identified norovirus and sapovirus genogroups and genotypes in wastewater metagenomes using untargeted next generation sequencing (NGS). Furthermore, measured concentrations of HAV in wastewater were correlated with the number of cases reported within the service community.

Methods

Study area and wastewater sample collection

Wastewater samples were collected from the water resource recovery facility (WRRF) located in Detroit, Michigan. The Detroit WRRF is the largest single-site wastewater treatment plant in the United States and treats wastewater from an estimated 3 million inhabitants (GLWA 2018). It has a primary and secondary treatment capacity of 1700 and 930 MGD, respectively, with an average daily flow of 650 MGD. Detroit's WRRF has a combined sewer system, which collects and treats stormwater along with residential, industrial and commercial waste. It services the three largest counties, by population, in Michigan. These are Wayne, Oakland and Macomb counties with a residential land use of 43, 55 and 49%, respectively (Jones *et al.* 2015). The percentage of municipalities serviced by the WRRF in each county is 50, 52 and 49% for Wayne, Oakland and Macomb counties respectively (Fig. 1). The remaining municipalities are served by local or decentralized treatment facilities. The WRRF receives wastewater from its service municipalities via three main interceptors (sewers): North Interceptor-East Arm (NI-EA), Detroit River Interceptor (DRI) and Oakwood-Northwest-Wayne County Interceptor (O-NWI).

Untreated wastewater samples were collected at the WRRF from sampling points located at each of the three interceptors. To investigate HAV concentrations during peak hepatitis A outbreak conditions, samples were collected approximately bi-weekly between November 2017 and February 2018 resulting in six sampling events (n = 54). Viruses were isolated from untreated wastewater using electropositive NanoCeram column filters following the EPA's virus adsorption–elution protocol (U.S. EPA 2001). Sewage samples were collected in triplicates for each interceptor. For each replicate, wastewater was passed through a column filter until fouling occurred resulting in filtered volumes of 24–44 L per interceptor. Additionally, 1 L grab samples were collected in triplicates from each sampling site to assess wastewater physio-chemical characteristics (pH, temperature, conductivity). Direct measurements of pH, temperature and conductivity were taken on-site using the YSI Professional Plus handheld device. Average pH, temperature and conductivity were 7·2, 13·5°C and 1031 μS cm^{-1} respectively. NanoCeram column filters, which contained viral particles, were immediately stored on ice and transported to the Environmental Virology Laboratory at Michigan State University (MSU) and stored in −20°C until further processing. Grab samples were stored on ice and immediately

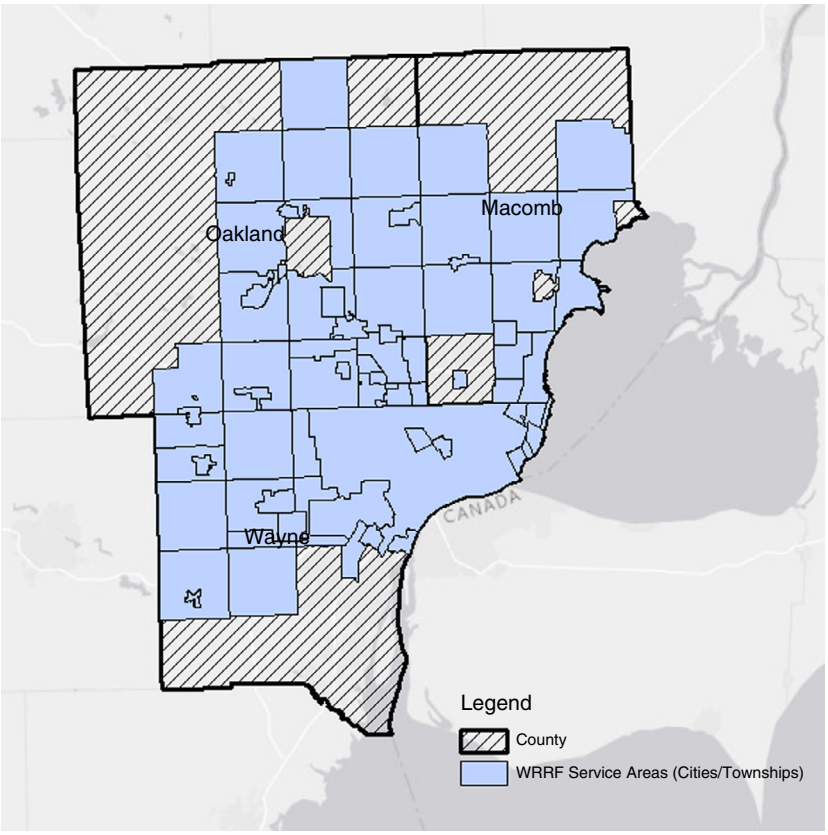

Figure 1 Detroit water resource recovery facility (WRRF) service municipalities in Wayne, Oakland and Macomb counties in Michigan. Service municipalities are based on the 2018 Great Lakes Water Authority sewer map for the DWSD (GLWA 2018). County borders and areas are represented by solid black lines, shaded regions represent service areas.

transferred to the laboratory, preserved at pH 2, and stored in −20°C.

Sample processing and virus isolation

Following wastewater sampling, NanoCeram cartridge filters were eluted within 24 h with 1·5% w/v beef extract (0·05 mol l^{-1} glycine, pH 9·5) according to the EPA's protocol (U.S. EPA 2001). In short, filters were eluted with 1 L of beef extract for a total of 2 min. The pH of the solution was adjusted to 3·5 ± 0·1 and flocculated for 30 min before centrifugation at 2500 ***g*** for 15 min at 4°C. Supernatant was discarded and pellets were resuspended in 30 ml of 0·15 mol l^{-1} sodium phosphate (pH 9·0–9·5) followed by a second round of centrifugation carried out at 7000 ***g*** for 10 min at 4°C. The supernatant was neutralized (pH ~ 7·25) using hydrochloric acid and subjected to filtration using to 0·45 and 0·22 μm syringe filters to eliminate bacterial contamination. Extraction of nucleic acid was performed on 140 μl of purified virus concentrate using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and eluted in 80 μl of elution buffer. Nucleic acid was stored at −80°C until further processing.

Metagenomic Analysis

Random amplification and next generation sequence processing

Purified nucleic acid from each biological replicate was pooled together for a total of 18 samples representing genetic material from all three interceptors during each of the six sampling dates. Nucleic acid from each sample was reverse transcribed and subjected to random amplification as previously described (Wang *et al.* 2003) to evaluate both RNA and DNA viruses. Eighteen samples of viral cDNA were sent to the Research Technology Support Facility Genomics Core at Michigan State University for whole-genome shotgun sequencing (WGS). The Illumina TruSeq Nano DNA Library Preparation Kit was used for all cDNA samples. Library preparation was performed on a Perkin Elmer Sciclone G3 robot according to the manufacturer's recommendations. This was followed by sequencing on an Illumina HiSeq4000 platform generating 150 bp paired-end reads.

Sequence analysis and taxonomic annotation

Sequencing reads generated from WGS were processed on a Unix system through the MSU High Performance

Computing Center. Raw sequences were analysed for quality using FastQC, a quality control tool for sequencing data (Andrews 2010). Sequencing adapters and reads with an average quality score below 20 were removed using Trimmomatic (Bolger *et al.* 2014). Trimmed reads were assembled with IDBA-UD, a short-read de novo sequence aligner for metagenomic data. Reads were assembled into contigs using an iterative k-mer approach with k-mer sizes ranging between 40 and 120 in increments of 10. The remaining parameters were run at default conditions. Assembled contigs used in this study are available via MG-RAST under project name assessment of enteric viruses.

A previously described method (McCall *et al.* 2020) was used to detect human viruses in metagenomic samples. Briefly, contigs were aligned against the Viral RefSeq database using tBLASTx with an *e*-value of 10^{-3}. Taxonomy annotation of aligned contigs was performed using the lowest common ancestor algorithm in MEGAN (6.15.0). The top 10% of BLAST alignments with a minimum bit score of 50 and contig coverage of at least 80% were considered in taxonomic analysis with the remaining parameters at default conditions. Contigs assigned to human virus groups were extracted for further analysis of enteric viruses. Extracted contigs were aligned with BLASTX with an *e*-value of 10^{-5} against a custom human virus database ning 5979 human viral proteins in Swiss-Prot database (Boeckmann *et al.* 2003 resented all human viruses in the Swiss-Prot database at the time of retrieval (September 2019). Contigs annotated as norovirus and sapovirus were genotyped using the Norovirus Typing Tool 2.0 (https://www.rivm.nl/mpf/typingtool/norovirus/). This tool is a reliable web-based genotyping platform, which performs a series of steps including BLAST alignment along with phylogenic analysis and bootstrap validation to characterize caliciviruses and enterovirus genotypes (Kroneman *et al.* 2011).

Quantitative PCR

Preparation of Standards and limit of detection

HAV was obtained from ATCC for preparation of standard controls. Nucleic acid was extracted as detailed in the previous section and transformed into One Shot TOP10 chemically competent *Escherichia coli* cells using the TOPO Cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Plasmid DNA containing cloned HAV was extracted and quantified as previously described (Munir *et al.* 2011). The protocol detailed in step 2 of the subsequent section was utilized to prepare a standard curve with 10-fold serial dilutions of positive HAV controls ranging from 10^3 to 10^{10} genome copies/reaction. The standard curve used to estimate HAV concentrations in collected samples obtained a slope and R^2 value of −3·6 and 0·99 respectively.

To establish the assay's limit of detection (LOD), purified nuclease-free water was spiked with twofold serial dilutions of HAV positive control ranging between 12·5 copies/reaction and 100 copies per reaction. Ten replicates of each dilution and negative control were analysed with identical RT-qPCR conditions as described above. The LOD was defined as the lowest copy number belonging to the serial dilution that yielded a positive PCR response in 95% of occurrences (Burns and Valdivia 2008). A PCR response was considered positive if it obtained a quantification cycle (Cq) value paired with a sigmoidal amplification curve. A LOD of 100 viral copies/reaction was obtained as observed in an earlier study (Simmons and Xagoraraki 2011).

Quantitative synthetic norovirus GII RNA and sapovirus RNA was obtained from ATCC. RNA was diluted 10-fold and analysed as described in the following section. Standard curves for norovirus GII and sapovirus obtained R^2 values of >99% and slopes of −3·82 and −3·35 respectively. The LOD for each virus was determined by the lowest point on the standard curve. Norovirus GII and sapovirus obtained detection rates of 10^1 gc μl^{-1}. All virus concentrations were normalized according to sampling volumes and reported as copies per liter.

Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to determine HAV, sapovirus and norovirus GII concentrations in RNA samples. All qPCRs were performed in triplicates on a Mastercycler ep realplex2 (Eppendorf) in 96-well optical plates. Amplification of cDNA was mediated using Lightcycler 480 Probes Master (Roche, Basel, Switzerland) at a concentration of 1× per reaction.

HAV was quantified using a two-step RT-qPCR with previously described primers and probe (Jothikumar *et al.* 2005). Briefly, viral RNA was reverse transcribed using iScript RT-qPCR Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Five microlitres of cDNA, negative control or positive control was transferred to a 15 μl reaction mix containing a final concentration of 250 nmol l^{-1} for each primer, 150 nmol l^{-1} of probe, 1× Lightcycler 480 probes master and sterile nuclease free water. All reactions were performed in triplicates with the following amplification conditions: denaturation at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, 55°C for 20 s and 72°C for 15 s.

Sapovirus was quantified using a two-step RT-qPCR based on a previously described method (Oka *et al.* 2006).

Briefly, viral RNA was reverse transcribed using iScript RT-qPCR Supermix (Bio-Rad) according to the manufacturer's protocol. Sapovirus quantification was carried out in a 25 μl reaction containing each primer and probe. Reactions were performed with the following conditions: 95°C for 15 min, followed by 45 cycles of 94°C for 15 s, 62°C for 1 min and 72°C for 15 s. Norovirus GII was quantified using a one-step RT-qPCR as previously described (Le Guyader *et al*. 2009). In short, the RT-qPCR was carried out in a 25 μl reaction mixture containing primers and probe, 2 μl of iScript RT-qPCR Supermix and 5 μl of viral RNA, negative control or positive control. Reactions were performed with the following conditions: reverse transcription at 25°C for 5 min, 46°C for 20 min and 95°C for 1 min, followed by 45 cycles of 95°C for 15 s, 60°C for 1 min and 65°C for 1 min.

Clinical data collection for HAV

Disease data for hepatitis A for each service county were obtained from the Michigan Department of Health and Human Services. Weekly counts of confirmed hepatitis A cases were extracted from the Michigan Disease and Surveillance System (MDSS) from 1 January 2017 to 1 December 2019. The MDSS is a communicable disease reporting system used to facilitate coordination and sharing of disease surveillance data among multiple shareholders including healthcare providers and medical laboratories (MDHHS 2020a). Michigan requires all physicians, healthcare providers and laboratories to report hepatitis A cases within 24 h directly to the MDSS or local health department (MDHHS 2017). Reported cases per county were obtained as weekly aggregate counts and classified as de-identified health information according to the Health Insurance Portability and Accountability Act (HIPPA) Privacy Rule. The number of weekly disease cases are structured according to the CDC Morbidity and Mortality Weekly Report, which aggregates the number of cases reported from Sunday to Saturday of each week.

Selection of Hepatitis A cases

Virus incubation period, time of peak viral shedding in faeces, wastewater detention time and sampling frequency was used to inform temporal selection of hepatitis A cases for correlation with average HAV concentrations per sampling date. A median incubation period of 28 days (Fiore 2004), and peak viral shedding range of 10–12 days from exposure (CDC 2015) were considered in the selection process. Detention times of wastewater in the three main interceptors were estimated under normal dry weather conditions using Manning's equations to identify significant lags in wastewater transport (Davis 2010).

Majority of the Regional Wastewater Collection System (RWCS) transports wastewater to the WRRF by gravity. A coefficient of roughness of 0·013 was estimated based on pipe material, centrifugally spun concrete (Davis 2010). Average flowrate and interceptor dimensions are taken from the GLWA 2019–2023 Capital Improvement Plan (CIP) (GLWA 2018). Flowrates per interceptor are determined based on the percentage of flow coming from each interceptor as specified in the CIP. The detention time in each interceptor was determined by dividing the length of each interceptor by the estimated average flow velocity. Assuming uniform partial flow conditions, average detention times under dry weather conditions were less than 1 day (4·3–13·7 h) for each interceptor. Given the long incubation period of HAV and aggregated health data, detention time was considered negligible.

To determine which weeks to select for comparison, sampling days were used as a reference point for creating a timescale. The authors back-calculated from the reference point to infer the day of exposure for infected persons excreting HAV in faeces during the day of sampling. The median incubation period was positioned on the timescale based on the day of exposure with a range of 15–50 days (Fiore 2004). A 2-week selection window spanning 1 week before and 1 week after the median incubation period was selected based on weekly aggregated health data and to account for the wide variation in incubation times. Based on this mechanistic approach, HAV in wastewater could be correlated with cases reported approximately 1 week after sampling (Fig. 2).

Statistical analyses

All statistical analyses were performed in R (R Core Team 2019). Bonferroni's corrected Dunn's nonparametric pairwise test was used to assess significant differences in HAV concentrations between sampling dates and interceptors. Furthermore, the nonparametric Spearman's rank correlation analysis was performed to evaluate the agreement between HAV concentrations in collected samples for each sampling date and clinical cases selected. $P < 0{\cdot}05$ were consider statistically significant. A one-way analysis of variance (ANOVA) and Tukey's HSD *post hoc* tests were used to investigate significance between mean concentrations of norovirus GII and sapovirus in wastewater samples.

Results

Metagenomic detection of enteric viruses in wastewater

Illumina sequencing generated a total of 624·4 million reads that were subject to quality trimming resulting in 595·2 million reads. An average of approximately 0·18%

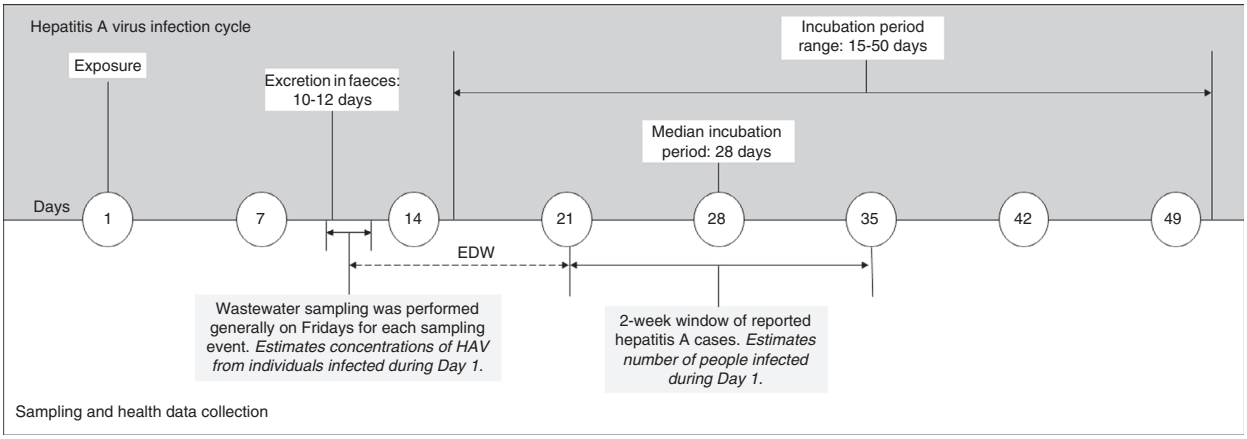

Figure 2 Selection approach for correlating HAV concentrations in wastewater and hepatitis A cases in the service community. Time scale is in days with 1-week increments. EDW = early detection window.

(0·05–0·78%) of viral affiliated contigs were assigned to human viral taxa. Average sequence lengths per sample of human-associated contigs were between 685 and 1222 bp. Metagenomic analysis detected several enteric viruses in wastewater samples belonging to the Adenoviridae (*mastadenovirus*), Astroviridae (*mamastrovirus*), Caliciviridae (*sapovirus, norovirus*), Hepeviridae (*orthohepevirus*), Parvoviridae (*bocaparvovirus*) and Picornaviridae (*parechovirus*, *enterovirus*, *hepatovirus*) families (Fig. 3).

Calicivirus detection in wastewater

Concentrations of norovirus GII and sapovirus were measured and compared to understand the occurrence of these caliciviruses in wastewater samples. Sapovirus and norovirus detection rates in wastewater were 94·4 and 100% respectively. Concentrations of sapovirus range between $1{\cdot}10 \times 10^5$ and $4{\cdot}66 \times 10^6$ gc l^{-1} ($1{\cdot}36 \times 10^6$ gc l^{-1}). Sapovirus concentrations were significantly greater than norovirus GII concentrations, which were $1{\cdot}16 \times 10^3$ to $1{\cdot}15 \times 10^5$ gc l^{-1} ($2{\cdot}94 \times 10^4$ gc l^{-1}) ($P < 0{\cdot}0001$) (Fig. 4). There was no significant difference in calicivirus concentrations between interceptors ($P > 0{\cdot}05$). Although sapovirus concentration remained largely the same between dates, norovirus concentrations varied significantly ($P < 0{\cdot}0001$).

Norovirus and sapovirus related contigs were detected in 50% of metagenomic samples with average contig lengths of 570 bp and 28% of samples with average lengths of 542 bp respectively (Fig. 3). Protein sequences related to norovirus GII (NCBI accession no. P54634) and sapovirus GI (NCBI accession no. Q69014) were identified with > 90% identity in 8/9 and 4/5 samples respectively. Norovirus GI (accession no. Q04544) and GII were both detected in the NI-EA interceptor during the 14 December 2017 sampling date. Sapovirus GI contigs were typed as GI.1.

HAV detection in wastewater

HAV was detected in all locations sampled during each sampling date (Fig. 5). Average HAV concentrations per interceptor range between $9{\cdot}49 \times 10^7$ and $3{\cdot}09 \times 10^4$ gc l^{-1}. Average HAV concentrations per sampling date range from $2{\cdot}42 \times 10^6$ and $6{\cdot}09 \times 10^7$ (median $5{\cdot}77 \times 10^6$) gc l^{-1}. Dunn's nonparametric pairwise test found a significant difference in HAV concentrations between O-NWI and DRI interceptors ($P < 0{\cdot}05$) and the 17 November 2017 and 16 February 2018 sampling dates ($P < 0{\cdot}001$).

HAV correlation to clinical cases

In late 2016 the CDC declared a multi-state hepatitis A outbreak with the primary mode of transmission being person to person (CDC 2020; Hofmeister *et al.* 2020). Among the states affected are Michigan resulting in 920 cases, 80% hospitalizations and 30 deaths (CDC 2020; MDHHS 2020b) as of December 2019. The peak of the hepatitis A outbreak in Michigan occurred between August 2017 and December 2017 with a steady decline thereafter.

Wastewater collection, performed on Fridays, was used as a reference point for creating the time scale. The authors back-calculated from the reference point to infer the day of exposure for infected persons excreting HAV in faeces during the day of sampling. The median incubation period was positioned based on the day of exposure with a range of 15–50 days (Fiore 2004). A 2-week

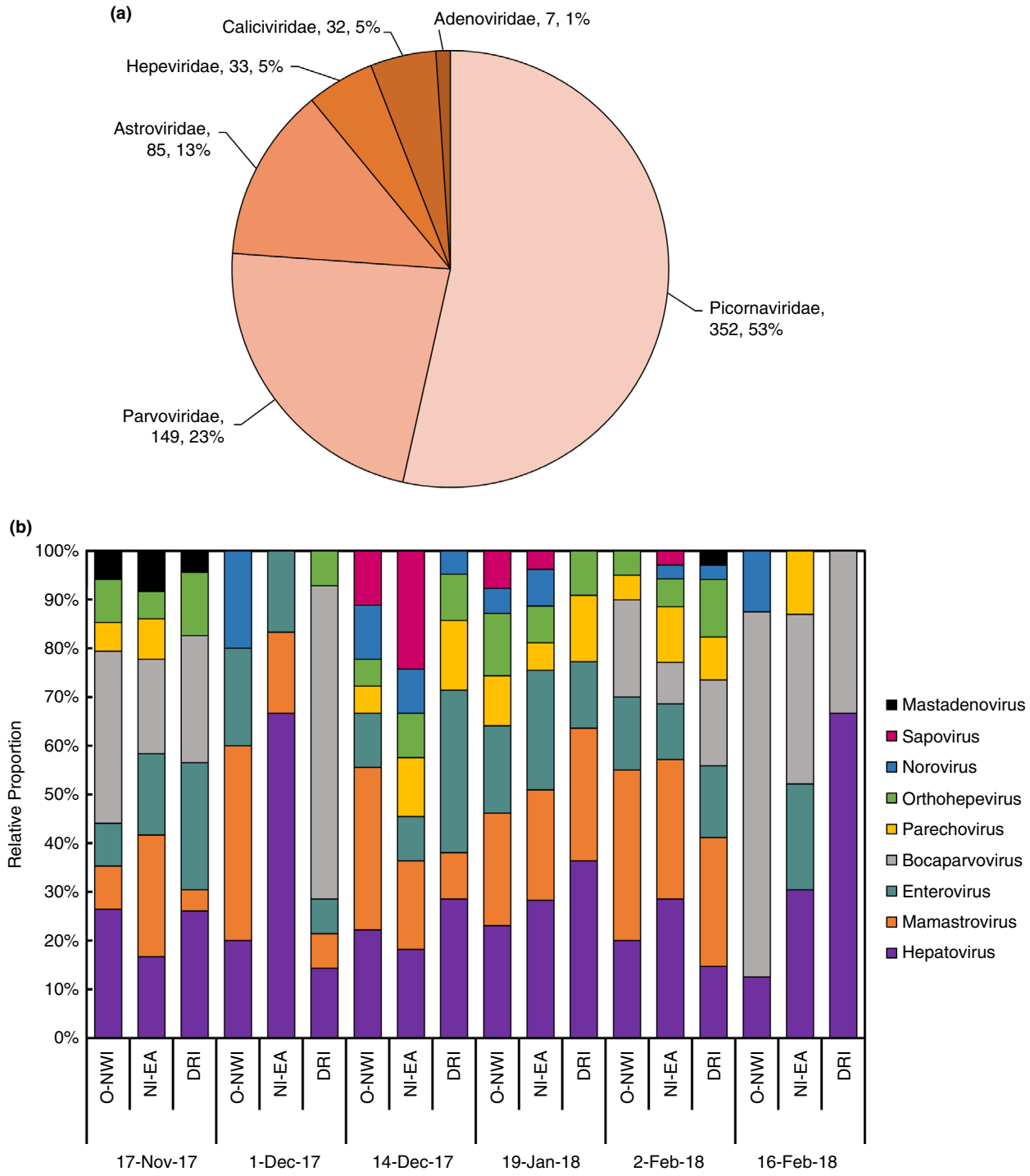

Figure 3 (a) Proportion of contigs associated with enteric virus families. (b) Proportion of enteric viruses detected in wastewater samples. Human viruses are annotated at the genus taxonomic level.

selection window spanning 1 week before and 1 week after the median incubation period was selected based on weekly aggregated health data and to account for the wide variation in incubation times. Spearman's correlation coefficient showed a significant positive correlation between the number of cases reported and HAV concentrations collected approximately 7 days prior ($\rho = 0{\cdot}55$, $P < 0{\cdot}0001$) (Fig. 6b).

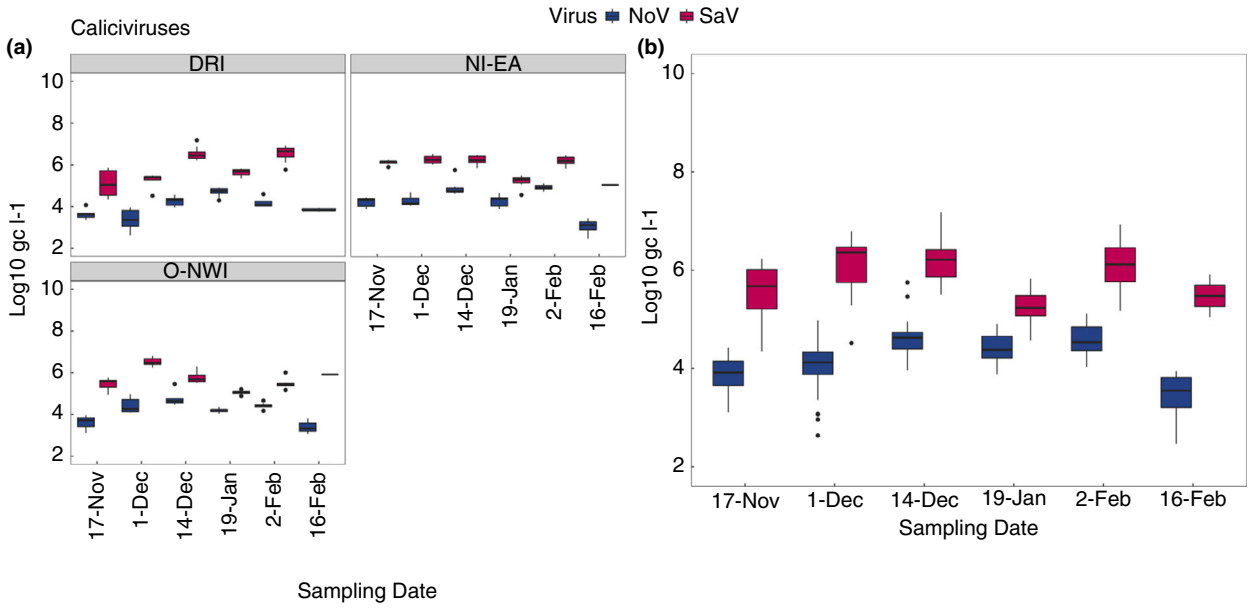

Figure 4 Boxplots for concentrations of norovirus genogroup II (NoV GII) and sapovirus (SaV) in wastewater samples per interceptor (a) along with average concentrations per sampling date (b). Median concentrations are denoted with a horizontal line.

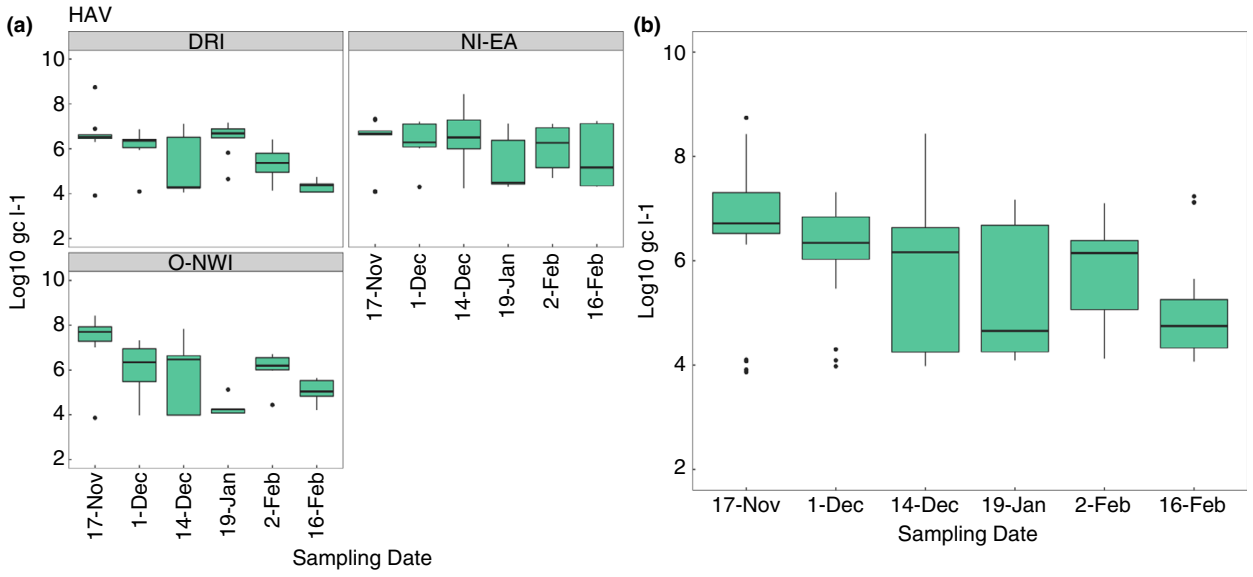

Figure 5 Boxplots for concentrations of HAV in wastewater samples per interceptor (a) along with average concentrations per sampling date (b). Median concentrations are denoted with a horizontal line.

Discussion

Enteric viruses in wastewater

Metagenomic analysis was used to investigate the distribution of enteric viruses in community wastewater during a hepatitis A outbreak. *Hepatovirus* was identified in all 18 samples with the greatest relative abundance compare to all enteric viruses detected. Additionally, hepatitis E virus (HEV) was identified in untreated wastewater samples. Like HAV, HEV is an enteric virus causing acute hepatitis and is transmitted through faecal-oral route and via contaminated water and food. Albeit, HEV has a low fatality rate in immunocompetent individuals, pregnant

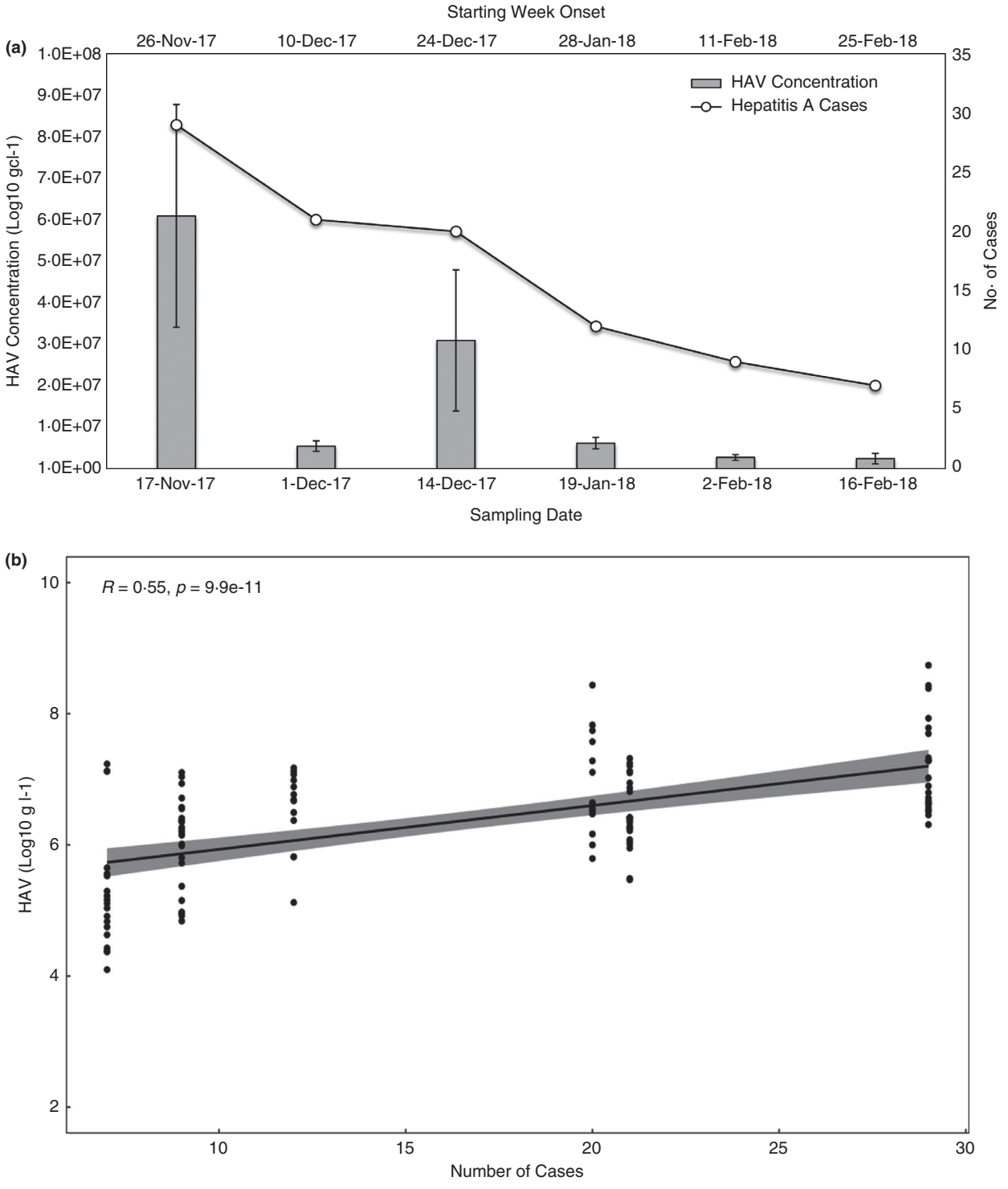

Figure 6 (a) Temporal correlation between selected reported hepatitis A cases in service counties and average measured HAV concentrations in wastewater samples. Error bars represent the standard error of measured concentrations for each date. (b) Resulting plot from Spearman's correlation analysis.

women and individuals with suppressed immune systems could face chronic infections and higher death rates (Kamar *et al.* 2014). Despite, HEV infections being rare in the United State, metagenomic findings indicate the presence of the virus in 72% of untreated wastewater with seven cases reported in the service community in

2017 and 2018 combined (data not shown). It is possible that asymptomatic infections have occurred in the study community or the virus persist in the environment and deserves further investigation.

Among other enteric viruses detected, adenoviruses and astroviruses are common causes of diarrhoeal illness alongside caliciviruses. Adenoviruses belong to the mastadenovirus genus and are abundant in wastewater with subgroups F and G being a causative agent of gastroenteritis, mainly in children (Ghebremedhin 2014). Indeed, the relatively low detection of adenoviruses (4/18) was surprising since these viruses are abundant in wastewater and persistent in the environment (Bofill-Mas *et al.* 2006; Fong *et al.* 2010; Elmahdy *et al.* 2019). This is likely a result of inherent bias when reverse transcribing the samples for NGS since our previous study detected adenoviruses in the same set of samples with a 100% positive detection rate using qPCR (McCall *et al.* 2020). Nonetheless, subgroups F and G were detected in positive samples with >90% similarly to the reference gene (data not shown). Astroviruses, belonging to the *mamastrovirus* genus, were detected in 83% of samples and was the second most abundance enteric virus next to *hepatovirus*. Astroviruses have been detected in raw wastewater in previous studies using metagenomics or PCR techniques (Meleg *et al.* 2006; Ng *et al.* 2012). Astroviruses are a leading cause of gastroenteritis in children accounting for up to 9% of acute nonbacterial gastroenteritis in children globally (Bosch *et al.* 2014). Additionally, in a previous study, astroviruses were the second most frequently detected viruses next to norovirus in stool samples collected from adults with acute diarrhoea (Arena *et al.* 2014). Results here suggest a significant circulation of astrovirus infections within the service community during sampling.

Lastly, human bocavirus (HBoV), of the *bocaparvovirus* genus, and *parechovirus* (HPeV) were detected in 55 and 67% of samples, respectively, and are also associated with diarrhoeal illnesses (Guido *et al.* 2016; Olijve *et al.* 2018). However, further work is needed to confirm what role HBoVs play in gastroenteritis-associated cases as they are commonly present alongside known aetiological agents like adenovirus, rotavirus and norovirus (Guido *et al.* 2016). Parechovirus and bocavirus have been detected in raw sewage and stool and in various geographical locations (Victoria *et al.* 2009; Räsänen *et al.* 2010; Cantalupo *et al.* 2011; Iaconelli *et al.* 2016; Hamza *et al.* 2017; Rikhotso *et al.* 2020).

Calicivirus quantification in wastewater

Quantification of norovirus GII and sapovirus was carried out on wastewater samples to determine and compare the occurrence of these caliciviruses in community wastewater. Detection rates between sapovirus and norovirus were comparable. Norovirus obtained positive detection rates similar to previous studies (Campos *et al.* 2016; Teixeira *et al.* 2020). Sapovirus detection rates in raw wastewater ranging between 12·4 and 100% have been noted (Kiulia *et al.* 2010; Di Bartolo *et al.* 2013; Murray *et al.* 2013; Fioretti *et al.* 2016; Kaas *et al.* 2016; Kitajima *et al.* 2018; Mancini *et al.* 2019) with a positive detection rate of 92% observed in a recent study conducted in Arizona, United States (Kitajima *et al.* 2018). This agrees with the detection rates seen here demonstrating prevalence of sapovirus in the environment. Concentrations of sapovirus were significantly greater than norovirus GII during both sampling periods. Consistent with this study, sapovirus concentrations of 10^6 gc l^{-1} have also been observed in untreated wastewater samples from Arizona (Kitajima *et al.* 2014) and Brazil (Fioretti *et al.* 2016) using qPCR. Kaas *et al.* (2016) observed a similar trend between norovirus and sapovirus with mean concentrations of sapovirus at $7{\cdot}9 \times 10^6$ gc l^{-1} as compared to norovirus GII $1{\cdot}09 \times 10^6$ gc l^{-1}, although both viruses were detected in 100% of untreated wastewater samples. In contrast, a recent study investigating seasonal patterns of enteric viruses found sapovirus concentrations to be generally lower than noroviruses although both viruses displayed similar seasonal trends (Farkas *et al.* 2018). Clinical records were investigated for incidences of norovirus infections reported during the sampling period. Due to infrequent reporting of norovirus cases, correlations could not be observed between norovirus cases and norovirus GII concentrations in wastewater (data not shown).

Genogroup and genotype classification of norovirus and sapovirus

Norovirus and sapovirus genogroups were identified in wastewater metagenomes using a reliable genotyping tool. Although less sensitive to genotype level variations as compare to amplicon sequencing, untargeted whole-genome sequencing has demonstrated practicality in this area with enhanced concentration or metagenomic approaches (Strubbia *et al.* 2019). The benefit of untargeted sequencing is the capability of detecting a multitude of human pathogens without prior knowledge of the sample's microbiome. While we were not able to identify genotypes for norovirus in this study, metagenomics discovered genogroups I and II. Norovirus Typing Tool classified several sapovirus contigs as genotype GI.1. Further confirmation is needed to confirm the presence of norovirus and sapovirus genotypes in wastewater samples. Still results here indicate that untargeted NGS and metagenomics with optimized virus enrichment

techniques is a promising approach to broad classification of calicivirus genogroups.

As expected, norovirus GII was the most frequently detected genogroup in metagenomic samples. It is well known that norovirus GII is the predominant strain in norovirus-associated outbreaks and sporadic cases (Glass *et al.* 2009; Harada *et al.* 2009; Rajko-Nenow *et al.* 2013; Lun *et al.* 2018) and has a global prevalence in wastewater (Ueki *et al.* 2005; Iwai *et al.* 2009; Farkas *et al.* 2018; Fumian *et al.* 2019). In a recent study, amplicon sequencing was used to characterize norovirus strains from isolates obtained from sporadic cases and outbreaks between 2014 and 2016 in Australia and New Zealand and compare that to shifts in norovirus strains identified in wastewater. The authors found norovirus GII to dominate in clinical specimens compared to GI, with Sydney GII.4 detected in over 40% of norovirus positive isolates in both regions throughout the study period. Similar temporal patterns for GII.2 were seen in raw wastewater samples collected from Australian cities as in clinical samples with about a 2 month lead time indicating the predictive nature of wastewater surveillance (*Fumian et al.* 2019). Previous studies have also observed norovirus GII to be most commonly detected in wastewater during the winter months in comparison to GI, which is seen during warmer climate (Nordgren *et al.* 2009; Kamel *et al.* 2010). On the contrary, Fumian *et al.* 2019, detected norovirus GI more frequently during the colder seasons compared to warmer months (Fumian *et al.* 2019). Norovirus and sapovirus are both known to have distinct seasonal distribution (Oka *et al.* 2015; Robilotti *et al.* 2015), but year around sampling is required to draw such conclusions from this study. Sapovirus GI.1 was the only genotype classified in wastewater samples. Similarly, a recent study detected sapovirus GI.1 in 83% of wastewater samples considered using amplicon-based sequencing (Mancini *et al.* 2019). Even further, sapovirus GI was the prevalent genogroup in wastewater collected from several geographical regions including Brazil (Fioretti *et al.* 2016), the United States (Kitajima *et al.* 2018), Italy (Di Bartolo *et al.* 2013), South Africa (Murray *et al.* 2013), Tunisia (Varela *et al.* 2018) and Japan (Kitajima *et al.* 2011).

Environmental surveillance of HAV and connection to health data

Concentrations of HAV in wastewater samples collected during a hepatitis A outbreak were utilized to investigate associations between viral concentrations in wastewater and clinical data. HAV was detected in all sampling locations per sampling date. Previous studies have reported negative or low detection rates in wastewater in low endemic regions in cases where no reports of hepatitis A were circulating in the community. For example, qPCR was performed to survey enteric viruses in a sewage treatment plant in the United Kingdom. HAV went undetected in all samples and there were no reports of hepatitis A cases within the study area during the time of sampling (Farkas *et al.* 2018). Furthermore, low detection rates, <10%, were reported in intermediate endemic regions using RT-PCR and nested PCR techniques (Kokkinos *et al.* 2011). These conclusions suggest that the detection rate reported in this study was indicative of increased viral disease patterns in the surrounding community.

Although the presence of viruses in wastewater may be a good indicator of circulation within the community, detection in wastewater samples is confounded by sample processing regimes and environmental conditions. Previous studies observed low detection rates of HAV in raw wastewater samples despite high clinical incidence in the service community (Gharbi-khelifi *et al.* 2007; Kamel *et al.* 2010). Additionally, qualitative approaches could pose challenges in distinguishing between baseline and outbreak conditions in endemic regions where circulation of the virus in the environment is expected. Methods used in this study were able to capture high expected detection rates of HAV in collected samples suggesting the efficiency of the sampling method and sensitivity of quantitative PCR assays. Furthermore, there were significant positive correlations between concentration and cases indicating that wastewater surveillance of HAV could aid public health officials in tracking early signs of hepatitis A outbreaks. Indeed, in order to make reliable inferences about disease prevalence an increase in the frequency and number of samples collected is necessary.

There was a significant difference in HAV concentrations between O-NWI and DRI interceptors. According to Detroit Water and Sewerage Department (DWSD) personnel, the DRI carries more industrial waste. Additionally, the DRI interceptor transports a greater portion of stormwater during wet weather events as compared to the NI-EA and O-NWI interceptors. Therefore, it holds the potential to have a greater dilution effect. Differences between interceptors are less likely attributed to variations in pH, conductivity or wastewater temperature since wastewater characteristics observed in this study were consistent across interceptors (data not shown). Previous studies have also reported negligible effects of these physio-chemical parameters on viral concentrations (Farkas *et al.* 2018; Sidhu *et al.* 2018). Findings indicate that the presence of industrial waste or dilution due to stormwater may lower viral concentrations in influent wastewater and influence correlations between virus concentrations and disease cases.

Metagenomic analysis and qPCR was used to evaluate norovirus, sapovirus and HAV and the presence of other enteric viruses in untreated wastewater. Sapovirus concentrations were significantly greater than norovirus GII concentration throughout the study period suggesting a higher environmental prevalence of sapoviruses in the community during the time of sampling. Norovirus GII and sapovirus GI were prevalent in metagenomic samples. Metagenomic analysis also detected the presence of other important enteric pathogens and even ones possibly contributing significantly to gastrointestinal illness cases in the service community. This study highlights the need for routine monitoring of sapovirus infections and demonstrates the usefulness of metagenomics for viral surveillance.

Generally, molecular methods implemented in this investigation effectively captured HAV loads in wastewater during hepatitis A outbreak conditions. Hepatitis A cases were strongly correlated with viral concentrations in wastewater when accounting for disease patterns and sampling conditions. Increases in hepatitis A incidence in the surrounding community were revealed in wastewater approximately 7 days before cases were reported to healthcare facilities. Despite strong correlations between clinical cases and HAV viral concentrations in wastewater, more frequent and rigorous environmental sampling is needed to fully understand HAV patterns in wastewater under various conditions. Additionally, statistical models used for establishing associations between virus concentrations and disease presence can vary greatly depending on locality. Establishing a baseline for HAV concentrations in wastewater within a given region can help to distinguish between environmental background and outbreak conditions. Such efforts can provide the basis for establishing actionable HAV concentration thresholds in wastewater for public health officials. Molecular and sequencing approaches can work together to identify various enteric viruses circulating in the community, better forecast disease outbreaks and facilitate monitoring strategies for disease prevention.

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Conflict of Interest

No conflict of interest declared.

Author contributions

All authors contributed to all aspects of the work.

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