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Worldwide diversity of hepatitis A and E viruses in urban wastewater

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Article

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Worldwide diversity of hepatitis A and E viruses in urban wastewater

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Keywords (max 5): Hepatitis A virus, Hepatitis E virus, Rocahepevirus ratti, Wastewater based epidemiology, One Health.

Abstract

Hepatitis A and E viruses (HAV, HEV) are the main causes of enterically transmitted hepatitis. Many infections remain undiagnosed due to their mild clinical course or asymptomatic presentation, and limited testing. Yet, knowledge of circulating genotype diversity is needed to understand their epidemiology and guide interventions. Wastewater surveillance may complement clinical monitoring by capturing infections missed through routine diagnostics.

We applied capture-based metagenomic sequencing to characterize HAV and HEV genetic diversity in wastewater from 62 cities across 38 countries (2017-2019*),* complemented with longitudinal sampling from five European cities (2020-2021). *Rocahepevirus ratti* (rat HEV) genotype C1 was detected in 70% of cities, extending its known geographic range by 12 countries. Rat HEV sequences clustered by city and country, though some lineages spanned multiple continents. *Paslahepevirus balayani* (human HEV), predominantly genotype 3, was most prevalent in European cities. HAV genotype distribution largely reflected regional endemicity, although some subgenotypes were detected in regions where they are rarely reported clinically.

Faecal source analysis suggested that rat HEV detected in wastewater originates primarily from rodent contamination rather than human infection. These findings reveal the global distribution of HAV and HEV genotypes and the widespread occurrence of rat HEV, demonstrating the value of wastewater metagenomics for population-level monitoring of both human and zoonotic hepatitis viruses.

Introduction

Viral hepatitis is among the leading causes of infectious disease mortality worldwide and can be caused by five hepatotropic viruses (hepatitis A-E). Hepatitis B, C and D viruses are primarily transmitted through exposure to infected blood or other bodily fluids and account for most deaths, with chronic hepatitis B and C alone affecting more than 300 million people globally. [1] Hepatitis E virus (HEV) and hepatitis A virus (HAV) are the principal causes of enterically transmitted hepatitis worldwide. HAV is transmitted through contaminated water or food and person-to-person contact, whereas HEV transmission includes both waterborne and zoonotic foodborne routes. [2,3] Although most infections are self-limiting, both viruses can cause severe hepatitis characterized by jaundice, anorexia and malaise, and in some cases progress to fulminant hepatic failure.

Outbreaks of enterically transmitted non-A, non-B hepatitis were first recognised in India in the late 1970s[4] and the causative agent was subsequently identified during an outbreak among Soviet troops in Afghanistan[5]. The identified agent, *Paslahepevirus balayani* (formerly *Orthohepevirus A*), [6,7] is a positive-sense, single-stranded, non-enveloped RNA virus classified within the subfamily *Orthohepevirinae* of the family *Hepeviridae*. Eight genotypes are recognised (HEV-1 to HEV-8), differing in host range and transmission route. Among the eight genotypes, HEV-1 and HEV-2 are restricted to humans and cause large waterborne outbreaks particularly in regions with limited sanitation infrastructure, whereas HEV-3 and HEV-4 are zoonotic, with domestic pigs and wild boar as the main reservoirs.[8,9] Human infection with HEV-3 is commonly linked to the consumption of raw or undercooked pork, wild boar meat or venison, as well as products derived from these animals such as liver sausage.[10–13] Reports of alternative transmission routes, including blood transfusion and possibly waterborne exposure, underscore the complexity of its epidemiology.[14,15] The reported host range of HEV-3 and HEV-4 continues to expand, with confirmed infections in other hosts such as deer and rabbits (which harbour a distinct zoonotic subtype, HEV-3ra), and serological or molecular virus detection in goats, cattle and sheep[16,17]. Genotypes HEV-5 and HEV-6 have only been detected in wild boars in Japan[18,19]. Both HEV-7 and HEV-8 have been detected in camels: HEV-7 has been found in dromedary camels and in a human infection linked to consumption of camel meat and milk[20], and HEV-8 in Bactrian camels.[21] Most human HEV infections are subclinical or cause self-limited acute hepatitis. Disease severity is genotype dependent, with most severe outcomes associated with HEV-1 and 2 infections. Severe outcomes include fulminant hepatitis in pregnant women[22] and individuals with pre-existing liver disease, and persistent infections are mainly seen in immunocompromised patients.[23] Extrahepatic manifestations, including neurological disorders, have also been described.[2]

Beyond *Paslahepevirus balayani*, *Rocahepevirus ratti* (rat HEV, formerly *Orthohepevirus C*), initially considered rodent-restricted, is the only other member of the *Hepeviridae* associated with human

infection and has recently been recognised as an emerging zoonotic pathogen.[24] Since its first identification in 2010, rat HEV has been detected globally in urban rat populations, with additional reports in pigs[25–27] and other mammalian hosts, indicating cross-species transmission potential.[28] The first human case was identified in Hong Kong in 2017 in a liver transplantation patient that developed chronic hepatitis.[29] However, a case report in Canada has shown that immunocompetent individuals can also be infected.[30] Currently, at least 50 cases have been reported, in China, Thailand, France, Spain and Canada.[31] Its true contribution to the hepatitis E burden remains unclear due to the lack of routine diagnostic assays, limited sensitivity of standard HEV RT-PCR (reflecting only ~50–60% nucleotide identity with *Paslahepevirus balayani*), and its similar clinical presentation. Throughout this study, 'HEV' refers collectively to *Paslahepevirus balayani* and *Rocahepevirus ratti*; the species- or genotype-level term is used wherever a specific virus is meant.

HEV infections are not notifiable in all countries, and surveillance practices differ, meaning reported case numbers are not directly comparable. However, modelled estimates suggest 19.4 million acute HEV infections occurred globally in 2021, predominantly in South and Southeast Asia and sub-Saharan Africa, with incidence projected to rise by 2030.[32] Clinical detection captures only a fraction of true incidence as most infections are asymptomatic or self-limiting, and routine diagnostic testing is limited. Population-based screening, including of European blood donors, has confirmed frequent silent circulation of HEV.[33,34] Similar diagnostic and reporting gaps affect HAV.

HAV is a positive-sense, single-stranded, non-enveloped RNA virus and a member of the *Hepatovirus* genus of the *Picornaviridae* family. Historically, HAV genotyping has been based on sequence variation within a 168-nucleotide fragment of the VP1/2A junction region.[35] Subsequent analysis of the complete VP1 gene refined this classification into six genotypes: three infecting humans (I, II, III), each comprising two subgenotypes (IA, IB; IIA, IIB; IIIA, IIIB), and three restricted to non-human primates (IV, V, VI).[36,37] HAV causes an estimated 1.4 million infections annually, with patterns of prevalence shaped by socioeconomic development and access to improved sanitation and water. In regions with limited sanitation infrastructure, infection typically occurs in early childhood, when disease is largely asymptomatic, resulting in high seropositivity rates by adolescence and lifelong immunity.[38] As many low- and middle-income regions undergo a shift in HAV endemicity due to improvements in sanitation and clean water access, fewer children acquire HAV infection in early childhood, which paradoxically increases the risk of symptomatic, potentially severe disease and outbreaks among susceptible adolescent and adult populations.[39,40]

Wastewater-based epidemiology offers a non-invasive complementary approach by detecting both symptomatic and asymptomatic infections at the population level, independent of healthcare access.

HAV and HEV have been repeatedly detected in sewage, surface waters, and shellfish [41] using targeted PCR-based assays.[42–44] However, recent advances in high-throughput metagenomic and hybrid-capture sequencing enable analysis of the genomic composition of complex viral and microbial communities, allowing simultaneous detection and genomic characterization of a broad range of viral pathogens in wastewater. [45,46]

In this study, we assess the prevalence and diversity of HAV and HEV (including rat HEV) in wastewater samples collected from 62 cities across 38 countries on six continents between 2017 and 2021 using metagenomic sequencing with targeted hybrid-capture enrichment. Because hepatitis virus sequences detected in wastewater may originate from human as well as animal sources, we explore their potential contribution by analysing co-detected astrovirus sequences, classified by host, as a proxy for the faecal sources present in each sample.

Results

Global detection of HEV and HAV in urban wastewater

To assess the presence of HEV and HAV in wastewater, sequencing reads were mapped to inhouse genus/subfamily specific databases. HEV reads were detected in 79% of wastewater samples (359/457), with a median of 262 mapped reads per sample (IQR = 61–669). To further characterize the detected HEV, reads from *Orthohepevirinae*-positive samples were assembled into contigs. In total, 5,709 contigs were obtained from 275 samples (60% of all samples), originating from 52 cities across 38 countries and six continents. The remaining positive samples yielded insufficient coverage for contig assembly or did not meet the quality criteria. Of the assembled contigs, 19% belonged to the genus *Paslahepevirus,* and 81% to the genus *Rocahepevirus* (two near complete genomes, >=6400 bp could be assembled).

HAV reads were detected in 37% of wastewater samples (170/457), with a median of 27 mapped reads per positive sample (IQR = 2–131). After assembly and quality filtering, 258 confirmed HAV contigs were obtained from 85 samples (18%), representing 29 cities across 27 countries and six continents.

HEV diversity and geographic distribution

Paslahepevirus balayani was detected in 42% of the cities (26/62). Three *Paslahepevirus balayani* genotypes were identified: HEV-1 (subtypes 1f, 1g), HEV-3 (subtypes 3a, 3ra, 3c, 3e, 3f1, 3f2, 3g, 3h3, 3i, 3j, 3_uc5), and HEV-4 (subtype 4b) (Fig 1. and 2). The prevalence of *Paslahepevirus balayani* was highest in Europe (61%), exceeding that in Africa (18%, p = 0.03), North America (13%, p = 0.04), and Oceania (25%, p = 0.29) (Fig. 1). Across samples collected between 2017 and 2019, genotype HEV-3 predominated on all continents except Africa. Among HEV-3 subtypes, HEV-3c was the most common, being detected in 13 of 14 *Paslahepevirus*-positive cities. HEV-3e and HEV-3f1 were also frequently observed, whereas HEV-3a, HEV-3f2, and HEV-3i occurred sporadically. Subtype distribution varied geographically, with several genotypes and subtypes showing region-specific occurrence. For example, HEV3c, HEV-3f2, HEV-3g, and HEV-3i were detected only in European cities, whereas HEV-4b, was exclusively detected in Guangzhou (China). Budapest, Vienna, and Valby exhibited the highest HEV-3 subtype diversity. HEV-1 was found in wastewater from five countries across four continents: Togo (Africa), Malaysia (Asia), France and Germany (Europe), and Australia (Oceania).

Rocahepevirus was detected in 70% of the cities (44/62) across multiple continents. Detection rates were comparable in Europe (83%), Africa (82%), and Asia (73%), but lower in North America (38%, p = 0.03) and Oceania (25%, p = 0.04) (Fig. 1) showing that *Rocahepevirus* is more evenly distributed globally compared to *Paslahepevirus*. Given the high prevalence of both *Paslahepevirus balayani* and

Rocahepevirus in European wastewater, together with the substantial HEV-3 subtype diversity, we next examined longitudinal datasets to assess temporal patterns in this region.

[Figure 1]

HEV diversity in European longitudinal wastewater

We analysed longitudinal wastewater samples collected between March 2020 and February 2021 from Bologna, Budapest, Copenhagen (Amager and Avedøre), Rome, and Rotterdam.

Paslahepevirus balayani was detected in 43.3% (58/134) of longitudinal samples and *Rocahepevirus* in 82.8% (111/134). Among *Paslahepevirus balayani* detections, HEV-3 again predominated, with HEV-3c accounting for 79.3% (46/58) of positive samples (Fig. 2). Detection patterns varied between cities: *Paslahepevirus balayani* was detected only sporadically in Bologna, Copenhagen, and Rome, but persistently throughout the sampling period in Budapest and Rotterdam. These two cities also differed markedly in subtype diversity: as many as 11 HEV-3 subtypes co-circulated in wastewater from Budapest, predominantly HEV-3c, HEV-3a, and HEV-3e, whereas a single subtype, HEV-3c, predominated in Rotterdam. *Rocahepevirus* was detected consistently across all five cities and throughout the sampling period. Bologna was the only city that harboured HEV-1g and the rabbit-associated HEV-3ra. No clear seasonal patterns were observed.

Figure [2]

Phylogenetic analysis of *Rocahepevirus*

Rocahepevirus accounted for the majority of HEV contigs detected in this study but is not classified beyond genus level by the HEV typing tool of the National Institute for Public Health and the Environment (RIVM). To characterize their genomic diversity, maximum-likelihood trees were constructed for two genomic regions: ORF1 (RNA-dependent RNA polymerase, nt 4351–4851; 187 wastewater contigs, 533 reference sequences) and ORF2 (capsid protein, nt 5701–6201; 156 contigs, 226 reference sequences). In both trees (Supplementary Fig. 1 and 2), all wastewater contigs clustered within genotype C1 of *Rocahepevirus ratti* (rat HEV), the genotype associated with all confirmed human infections reported to date. This study increases the known geographic range of *Rocahepevirus ratti C1* by 12 countries with no previously published C1 sequences in public databases, namely Australia, Croatia, Ecuador, Israel, Ivory Coast, India, Morocco, Poland, Slovakia, Slovenia, Tanzania and Uganda (Fig.3). In two additional countries with no previously reported rat HEV (Senegal and Togo), *Rocahepevirus* was detected as a single contig (~400 bp each), whose closest relatives were genotype C1 references (>= 88% nucleotide identity), although the type could not be assigned by a proposed rat HEV typing scheme (Fig. 3).[47]

To resolve within-C1 diversity, genotype C1 maximum likelihood trees were constructed for ORF1 and ORF2. In both trees, wastewater sequences were interspersed with sequences from rats and clinical isolates from humans, indicating the absence of clear host-specific clustering. Nearest-neighbour analysis showed strong local clustering in both the ORF1 and ORF2 trees: 90% and 89% of wastewater contigs, respectively, had another wastewater contig from the same city as their phylogenetically closest neighbour. When reference sequences from rodents and humans were included, 92% of contigs clustered most closely with a sequence from the same country. Some sub-lineages were primarily found within the same geographic region (e.g., Rotterdam wastewater contigs clustering with a rat HEV sequence from a black rat in the Netherlands Fig. 4), whereas others were detected across multiple regions (e.g., a cluster containing European and African sequences; Supplementary Fig. 3). Sequences from Budapest belonged to multiple distinct sub-lineages, indicating the co-circulation of genetically divergent C1 lineages within the city (Fig. 4).

HAV diversity and geographic distribution

HAV was detected in 29 of 62 cities (47%), with detection rates of 73% in Africa (8/11 cities), 57% in Europe (13/23), 33% in Asia (5/15), 25% in Oceania (1/4), 12% in North America (1/8), and 100% in South America (1/1) (Fig. 5). The 258 HAV contigs comprised four subgenotypes: IA (n=130, 50%), IB (n=83, 32%), IIA (n=24, 9%), and IIIA (n=9, 4%). Within subgenotypes IA and IB, six subgenotype clusters were identified (IA_ab, IA_ac, IA_ad, IB_ad, IB_ae, IB_ai), although the majority of contigs (83%) could not be assigned beyond the subgenotype level because they fell outside of the typing region. Subgenotype IA was detected on five continents, while IB showed a distinct continental pattern with detection in 8 of 11 African cities but only 4 of 23 European cities. Subgenotype IIA was exclusively detected in Yaounde (Cameroon), and IIIA in Kochi (India), Regina (Canada), and Melbourne (Australia). Phylogenetic placement of genotype IA contigs against the RIVM HAV typing tool reference sequences showed clustering with three distinct strains associated with the 2016–2017 multi-country men who have sex with men (MSM) hepatitis A outbreak in Europe (Supplementary Fig. 4).[48] In the longitudinal dataset (2020–2021 samples), HAV was rarely detected (3/134 samples, 2%), and only genotype IB was identified in samples from Budapest (Hungary) and Copenhagen (Denmark).

Exploring animal and human sources of hepatitis viruses in wastewater using astrovirus host composition profiles

Depending on the genotype, HEV detected in wastewater may reflect human infections, or animal contributions, or a combination thereof. To further explore potential animal contributions, we analysed astrovirus sequences from the same samples (Fig.1 and Fig.2). We compared astrovirus host composition between HEV-positive and HEV-negative samples and assessed co-occurrence at the city

level. If animal sources contributed substantially to a particular HEV type, we expected a corresponding proportional shift of astroviruses associated with that host.

Rat HEV detection was significantly associated with increased rodent astrovirus abundance (ALDEx2 effect size = +0.45, FDR < 0.001, Fig. 6) and reduced relative human astrovirus abundance (effect = -0.24, FDR < 0.01). No significant associations were observed with other host categories. At the city level, rodent astroviruses were detected in 84% (37/44) of *rat HEV*-positive cities compared to 21% (4/19) of negative cities. For HEV-3, no association with porcine astroviruses was observed (effect = -0.08, n.s.). Instead, HEV-3-positive samples were enriched for carnivore (effect = +0.30, FDR < 0.001) and rodent (effect = +0.28, FDR < 0.001) astroviruses, with a negative association with avian astroviruses (effect = -0.26, FDR < 0.05). No significant associations were observed for HEV-1 or HAV-I.

Discussion

This study provides the first global-scale assessment of HEV and HAV diversity in wastewater. Our findings reveal widespread circulation of rat HEV and distinct geographic patterns in the distribution of HEV and HAV genotypes.

HEV-3 was the predominant human HEV genotype detected in wastewater, with highest prevalence in Europe, where subtype 3c accounted for most detections. This pattern is consistent with the marked increase in autochthonous HEV-3 infections across the continent between 2005-2015.[49–51] Its enzootic circulation[48] in pig populations is sustained by current farming practices (high animal densities, faecal environmental contamination) and animal trade. [52,53]Europe is the world's second-largest pork producer and among the highest per-capita consumers[54] (Supplementary Fig. 5) which likely contributes to the high prevalence of HEV-3 detected in European wastewater.

Hungary (Budapest) and Austria (Vienna), both among the top five pork-consuming countries (Supplementary Fig. 5), showed particularly high HEV3 prevalence and diversity. In Hungary, studies conducted before 2010 only reported subtypes HEV-3a and HEV-3e in humans, swine, boar and deer [55,56], wheras more recent clinical surveillance studies (2014-2017 and 2018-2025) showed higher genetic diversity.[57,58] Dencs *et al*., suggested that this expansion may in part be explained by two concurrent developments: an increase in pork imports between 2004 and 2024 following EU accession, primarily from Germany, Spain, Poland, and the Netherlands which was further substantiated by a national Porcine Reproductive and Respiratory Syndrome Virus eradication program (2014–2022) that required complete herd replacement with animals imported from Slovakia, the Netherlands, Germany, and the Czech Republic (HEV3c predominance[59]), thereby likely introducing HEV subtypes previously not present in the country.[58]

The HEV-1 detection in Africa (Togo) was consistent with ongoing waterborne transmission in this region[60]. In Europe (Berlin, Nantes, Bologna), Oceania (Melbourne) and Malaysia (Kuala Lumpur), autochthonous HEV-1 transmission has not been established, and detection may reflect population mobility from endemic regions. HEV-4, which replaced HEV-1 as the dominant genotype in China since 2000, was detected exclusively in Guangzhou.

HEV detected in wastewater may originate from multiple sources: human faecal shedding, direct animal excretion into the sewer system, or indirect contributions such as slaughterhouse or agricultural discharge. To explore potential animal contributions to HEV in wastewater, we examined associations with astrovirus host composition as a proxy for faecal source inputs. The astrovirus composition in wastewater is predominantly human, reflecting the primary faecal source in urban

wastewater. Samples containing rat HEV, however, showed a compositional shift, with increased rodent- and reduced human associated astroviruses. This pattern is expected when an additional non-human faecal source is present in the wastewater matrix, in this case rat faecal material containing both rat HEV and rodent astroviruses. In contrast, HEV-3 was not associated with porcine astroviruses. However, it was associated with carnivore and rodent astroviruses. Several factors may contribute to these associations like the frequent co-detection of HEV-3 and rat HEV in the same samples and the broad mammalian host range of HEV-3, which extends to carnivores such as cats and dogs based on serological evidence.[61] In these animals, infection could occur through consumption of raw-meat-based diets[62,63]. As expected for a strictly human-restricted virus, HAV-I showed no association with non-human astroviruses, nor with human astroviruses, the latter reflecting the limited compositional variation of this dominant background source.

A limitation of this method is that host assignment based on capsid sequence relies on sequences in public databases, which typically reflect the species from which the virus was sampled, a species which may not represent its exclusive reservoir or contemporary host range. In particular, the reliability of bat host assignments is reduced as bat astroviruses are known to be phylogenetically interspersed among other mammalian astrovirus lineages rather than forming host-specific clusters.[64] In addition, astroviruses may occasionally cross species barriers, further complicating host assignment.[65,66] Taken together, these limitations mean that host-based source attributions should be treated as supporting rather than conclusive evidence.

Rat HEV was detected in 70% of cities, with broad geographic distribution and high relative abundance. All sequences belonged to *Rocahepevirus ratti* genotype C1, consistent with previous wastewater studies.[67–71] Rodents, particularly *Rattus norvegicus* and *Rattus rattus*, are the primary reservoir, and our compositional analysis supports rat excreta as the primary source of rat HEV in wastewater. Urban rats frequently inhabit sewer systems [72,73] and viral RNA prevalence in urban rodent populations ranges between 6 and 26%.[74–77] Transmission to humans can occur through exposure to environments or food contaminated by rats, as most confirmed human cases report no direct rodent contact[31], although human-to-human transmission cannot be excluded.

Serological evidence of exposure to rat HEV has been documented across populations in Asia and Europe, with higher rates associated with older age, rural residence, and rodent contact[78–81] Molecular studies have identified rat HEV as an underrecognized cause of acute hepatitis in cases lacking defined aetiology [79,82], suggesting that infections are more common than reported. Rat HEV RNA has been detected beyond influent wastewater, including in treated effluent wastewater, downstream surface water, and bivalve mollusks[83] intended for human consumption, indicating potential exposure

routes.[84] Our phylogenetic analyses showed geographical clustering at both continental and city levels, consistent with previous observations.[85] In Budapest, the presence of multiple co-circulating lineages indicated sustained local circulation within the urban rat population. However, some clusters contained closely related sequences from geographically distant regions, possibly reflecting rat movement via international maritime trade networks, a similar mechanism that has been proposed for the global dispersal of Seoul orthohantavirus.[86,87] Under a proposed typing scheme most rat HEV sequences fell within genotype C1, Clade I subtype A, a subtype that predominates in Europe, the Americas, and Africa.[85] Notably, our study added rat HEV genotype C1 sequences from at least 12 countries with no previously available sequences, extending the documented geographic range of this genotype.

The high HAV detection rate in African cities is expected given high endemicity of hepatitis A in this region and sustained transmission, primarily among young children. In contrast, the similarly high European detection rate was unexpected given the relatively low reported HAV incidence across most of Europe. HAV in European wastewater likely reflects a combination of factors such as foodborne outbreaks linked to contaminated food products imported from regions with higher HAV endemicity[88,89] and infections among travellers or resident communities with ties to such regions. Notably, the global sampling period (2017-2019) coincided with heightened HAV activity in Europe including a multi-country genotype IA-associated MSM outbreak. [48], suggesting that this may have contributed to the high detection of HAV across Europe in our study. This is further supported by identifying genotype IA wastewater sequences from several European cities and Melbourne that clustered with multi-country outbreak associated strains.[48]

The geographic distribution of HAV subgenotypes was largely consistent with known patterns of endemicity, with globally widespread IA and a predominance of IB in African cities.[90] However, wastewater sequencing also identified genotypes that are infrequently reported through routine surveillance in the regions where they were detected. IIA, which is rarely reported in clinical surveillance, with most documented cases originating from West and Central Africa and occasional cases in Europe, was detected in Yaoundé.[91,92] In addition, IIIA, predominant in South Asia, was identified in Melbourne and Regina. The presence of these genotypes outside their primary regions of circulation suggests that wastewater surveillance captures aspects of population mobility and demographic composition, or low-level local circulation that is underrepresented by clinical surveillance data. Consistent with this, a wastewater study from Calgary showed that HAV abundance correlated with the proportion of residents born in highly endemic countries.[93]

Some limitations should be considered. First, capture-based metagenomic sequencing has lower sensitivity than targeted RT-PCR. In Yaoundé, we dit not detect rat HEV, despite reported detection rates of 26% by nested RT-PCR.[94] Second, a large fraction of the global data is primarily based on biannual sampling (2017–2019), which captures a snapshot of circulating virus types; (sub)types present at other timepoints may be missed, potentially underestimating the true diversity.

Wastewater-based metagenomic sequencing may complement clinical surveillance by capturing viral diversity not detected by routine diagnostics. This is particularly relevant for rat HEV, an emerging zoonotic pathogen for which surveillance remains limited. In addition, wastewater surveillance revealed genotype introductions linked to population demographics and mobility, as well as circulation of outbreak-associated strains, that were not captured in publicly available clinical data from low-endemicity countries.

Methods

Wastewater sample collection and sequencing

Wastewater samples were collected between 2017 and 2021 as part of the Global Sewage Surveillance project within the Versatile Emerging Infectious Disease Observatory (VEO) network, following a sample collection protocols described in detail elsewhere.[46,95] Briefly, untreated urban sewage was obtained from main pipelines or treatment plant inlets, either as 24-h composite samples or pooled grab samples. Sampling followed either a biannual global design or a longitudinal sampling design for selected sites. The 2017–2019 sampling campaign (hereafter referred to as Global Sewage dataset) included samples from cities worldwide and was sequenced using Illumina MiSeq and is available from the European Nucleotide Archive (ENA) under study accession PRJEB87273. The 2020–2021 campaign (hereafter referred to European longitudinal dataset) focused on five European cities and was sequenced using using both MiSeq and NextSeq instruments. All samples were processed using the GastroCap hybrid-capture approach, which targets viruses associated with gastroenteric disease, including *Adenoviridae, Astroviridae, Caliciviridae, Hepeviridae, Parvoviridae, Picornaviridae, Spinareoviridae and Sedoreoviridae.*[96]

Bioinformatics analysis

A wastewater sequence analysis workflow was generated using Snakemake (version 7.19.1).[97] In short, raw reads were deduplicated using CD-HIT-dup (-u 50)[98], which groups and removes duplicated reads based on the first 50 nucleotides. Deduplicated reads were quality trimmed using FastP (version 0.23.4) where read ends were trimmed to a mean quality Phred score of 25 with a sliding window of 5. Reads shorter than 50 nucleotides were discarded[98]. To enable virus-specific analyses, two reference databases were generated: one containing *Orthohepevirinae* sequences and one containing *Hepatovirus* sequences derived from GenBank. For each, sequences >= 200 nucleotides were included and deduplicated at 99% identity using CD-HIT-dup. [98] Reads were then mapped separately against the two databases using bbmap (BBTools version 39.19, minid = 0.7, ambiguous = all) and mapped reads were assembled using metaSPAdes (Spades version v3.13.0, --meta). Taxonomic annotation of the resulting HEV and HAV contigs was performed with DIAMOND blastx (version 2.1.10) against the NCBI non-redundant protein database (release 2024-07-01), assigning taxonomy based on the highest bitscore normalized by contig length. Reads were subsequently mapped back to these contigs with BWA-MEM[99] (version 0.7.18) per contig reads counts were obtained with SAMtools[100](version, -bF 2052) and seqkit (version 2.3.0, seqkit bam). For downstream analyses, only contigs ≥300 bp with an average coverage ≥3× were retained. The resulting HEV contigs had a median length of 472 bp (IQR 389–649) and HAV contigs 471 bp, (IQR = 393–682) Relative abundance was expressed as RPM,

435 calculated by dividing the number of reads mapped to a contig by the total number of deduplicated
436 reads per sample, multiplied by 10^6.

437 Taxonomic assignment at the genotype level

438 For taxonomic assignment at the genotype level, the HEV and HAV typing tools of the RIVM were used.
439 In brief, these tools use blastn and phylogenetic methods against a set of well curated HEV and HAV
440 reference sequences to assign genotypes.[101,102] The HAV typing tool additionally assigns subclusters
441 within subgenotypes (e.g., IA_ab, IA_ac). To visualize the phylogenetic placement of subgenotype IA
442 HAV wastewater contigs with a subcluster assignment (≥300 bp, ≥3× coverage), these were added as
443 fragments to the tool's curated IA reference alignments using MAFFT v7 and maximum-likelihood
444 trees were inferred with IQ-TREE 2 (1,000 ultrafast bootstrap replicates) and visualized in iTOL v6.[103]

445 To validate the genotype assignments for short contigs, we performed an *in silico* fragmentation
446 analysis. Thirty-six complete HEV reference genomes covering all *Paslahepevirus balayani* subtypes
447 (HEV-1a–1g, 2a–2b, 3a–3m, 3ra, 4a–4i, 5a, 6a, 7a, 8a) and one *Rocahepevirus ratti* genome were
448 fragmented into overlapping segments of five sizes (300, 500, 1,000, 3,000, and 6,000 bp) at 100-bp
449 intervals, yielding 3,274 fragments. Each fragment was submitted to the RIVM HEV Genotyping Tool
450 and the assigned genotype was compared to the known classification of the source genome
451 (Supplementary Fig. 6). *Rocahepevirus* contigs could not be assigned beyond genus level; genotype
452 assignment for these contigs was therefore based on phylogenetic analysis.

453 Statistical analysis

454 Differences in virus prevalence between continents were assessed using Fisher's exact test. P-values
455 below 0.05 were considered statistically significant. All statistical analyses were performed in R
456 (version 4.3.3).

457 *Rocahepevirus* phylogenetic analysis

458 Maximum-likelihood trees were constructed for two 500-bp regions: ORF1 (RNA-dependent RNA
459 polymerase, nt 4351–4851) and ORF2 (capsid protein, nt 5701–6201), selected to maximize the
460 number of wastewater contigs spanning each window (positions relative to NC_038504). Reference
461 sequences for the genus *Rocahepevirus* were obtained from NCBI Virus (taxonomy ID 2948883).
462 Sequences were aligned using MAFFT v7, and wastewater contigs spanning the target windows were
463 added to the reference backbone. Trees were inferred using IQ-TREE 2 automatic model selection
464 *(ModelFinder;* best-fit models: TIM2+F+I+R5 for ORF1; GTR+F+I+R4 for ORF2) with 1000 bootstrap
465 replicates. To improve resolution within genotype C1, a separate ORF2 tree was constructed using
466 only C1 sequences, with a genotype C2 sequence (LC057247) as outgroup. Trees were mid-point

rooted and visualized in iTOL v6.[103] Countries with a *Rocahepevirus* detection that was neither placed in the phylogenetic trees nor previously reported in publicly available databases were treated as potential new records; their longest contigs were analysed with a proposed rat HEV typing tool[47] and by BLASTn against the NCBI nucleotide (nt) database.

Astrovirus animal typing database

To assess potential animal faecal contributions to HEV in wastewater, we analysed astrovirus sequences from the same samples. Astroviruses infect a broad range of vertebrate hosts, are largely host-specific[104], are shed in faeces, and are captured by the GastroCap enrichment panel, allowing host-classified astrovirus reads to serve as a proxy for the faecal source composition of each sample. We constructed a host-annotated reference database based on the astrovirus ORF2 (capsid) region. All publicly available Astroviridae capsid sequences were retrieved from the NCBI Virus portal (downloaded September 2025). For each record, the host's scientific name was mapped to a broad host category (Human, Pigs/Suids, Rodents, Bats, Carnivores, Birds, Ruminants, and others) using a curated lookup table. Sequences were clustered at 100% amino-acid identity using MMseqs2, retaining the host composition for each cluster. Wastewater-derived Astroviridae contigs were screened against this database using DIAMOND v2.1 blastx (--evalue 1×10^{-5}, alignment length ≥80 amino acids). A weighted-vote procedure was used to infer the most probable host for each contig: the DIAMOND bitscore of each hit was proportionally distributed across the host categories represented in the matched reference cluster, and cumulative weights were summed across all hits. The host category with the highest cumulative weight was reported as the predicted host. Assignments with a relative weight share ≥70% were classified as high-confidence, 60–70% as medium, and <60% as low. Only high-confidence assignments were used for the primary analysis.

Exploring potential sources of HEV in wastewater

To explore potential animal sources of hepatitis viruses detected in wastewater, we compared the astrovirus composition of samples in which a given target virus was detected to samples in which it was absent. Astroviruses infect a broad range of vertebrate hosts and are largely host-specific with each type typically infecting one or a few closely related species. As they are shed in faeces, and are captured by GastroCap, host-classified astrovirus reads could function as a proxy for the faecal source composition of each sample. If a target virus originates from a specific animal source, samples containing that virus may show a corresponding astrovirus presence from that host. For each sample, the mapped read counts of astrovirus contigs were summarized into seven host categories based on the high-confidence host assignments described above. Samples were then classified as positive or negative for each virus type (HEV-1, HEV-3, *Rocahepevirus*, and HAV-I). HEV-4, HEV-3ra, HAV-II, and

HAV-III were excluded due to insufficient detections across cities (each detected in fewer than three cities)). To test for compositional differences between groups, we applied ALDEx2[105] a differential abundance method that accounts for the compositional nature of sequencing data.[106] ALDEx2 models count uncertainty through Dirichlet-multinomial Monte Carlo sampling (128 instances), applies a centred log-ratio transformation, and performs Wilcoxon rank-sum tests on the transformed values. Positive ALDEx2 effect sizes indicate that a given astrovirus host category makes up a larger proportion of the astrovirus community in samples where the hepatitis virus was detected, while negative values indicate a smaller proportion. P-values were corrected for multiple testing across all 28 tests (4 virus types × 7 host categories) using the Benjamini–Hochberg method and reported as False Discovery Rate (FDR) *q*-values. Only samples with at least one astrovirus detection were included in this analysis (n = 403). Additionally, city-level co-detection rates were calculated as the proportion of cities positive for a given hepatitis virus in which astroviruses from each host category were also detected.

Data Availability

The raw sequencing datasets, metagenome assemblies and analysis results are available on the European Nucleotide Archive under project: PRJEB87273.

Code Availability

The metagenomic workflow used in this study is made available at https://github.com/EMC-Viroscience/Worp_etal_Worldwide_diversity_HAV_HEV_wastewater.

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Author Contributions Statement

M.D.G. M.P.G.K. and F.M.A. conceived the study, secured funding and provided overall supervision. N.W. drafted the initial manuscript with input from M.P.G.K., M.D.G & B.B.O.M. M.D.G & B.B.O.M. supervised manuscript preparation and data analysis. S.G. coordinated sample collection at

Waterboard Hollandse Delta. P.A.F. performed sample collection, and contributed to data interpretation. C.M.E.S and N.W performed NGS. N.W. performed bioinformatic data analyses. E.E.B.J. and C.B. provided methodological guidance on CoDa. N.W. produced the figures. D.F.N, P.A.F., R.W.I.L., D.K.P., C.M.E.S., C.B., E.E.B.J., E.N.H., P.M., R.S.H., I.C., D.R., F.M.A., M.P.G.K, M.D.G. & B.B.O.M., critically read and contributed to the manuscript. The Global Sewage Consortium authors carried out sewage sampling, filled in metadata and shipped the samples.

Competing Interests Statement

The authors declare no competing interests.

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Figures

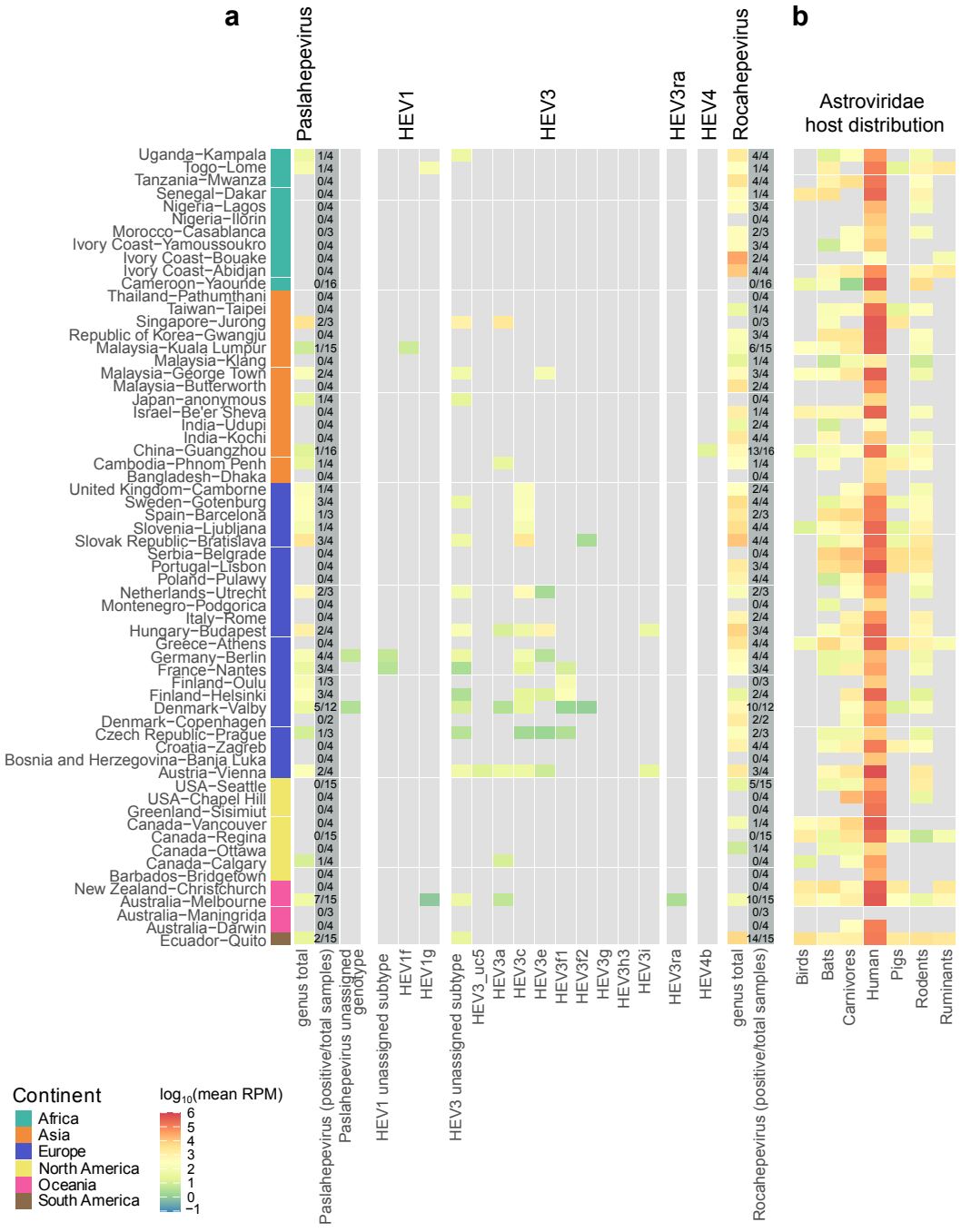
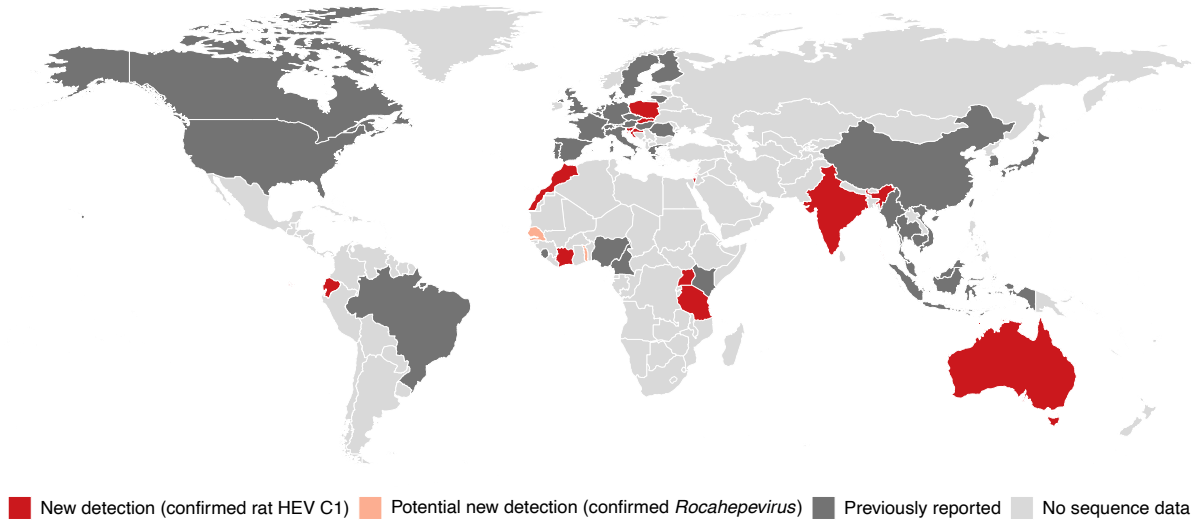


Figure 1. **Global detection of hepatitis E viruses and host distribution of astrovirus sequences in urban wastewater collected between 2017-2019.** A. Detection of hepatitis E viruses in wastewater samples collected from 62 cities worldwide. Locations are ordered by continent, country, and city. Colour intensity represents $\log_{10}$-transformed mean reads per million (RPM) per location, with grey indicating absence of detectable reads. Columns correspond to viral taxa within the *Paslahepevirus* and *Rocahepevirus* genera, grouped by genotype and subtype. Fractions indicate the number of positive samples relative to the total number analysed for each city. B. Distribution of astrovirus sequences by host category across locations. Columns represent host categories, and colour intensity reflects $\log_{10}$(mean RPM), with grey indicating locations with no detectable astrovirus reads



970

971 **Figure 2**. **Diversity of hepatitis E viruses in longitudinally collected wastewater samples from five**
972 **cities in Europe (Bologna, Budapest, Copenhagen, Rome, and Rotterdam) between March 2020 and**
973 **February 2021.** Colour intensity represents $\log_{10}$-transformed mean reads per million (RPM) per
974 month, with grey indicating absence of detectable reads. Rows correspond to viral taxa within the
975 *Paslahepevirus* and *Rocahepevirus* genera, grouped by genotype and subtype. The lower panels show
976 the distribution of astrovirus sequences by host category. Rows represent host categories, and colour
977 intensity reflects $\log_{10}$(mean RPM), with grey indicating locations with no detectable astrovirus reads.



978

979 **Figure 3. Geographic distribution of Rocahepevirus ratti genotype C1: previously reported**
980 **sequences and new detections from this study.** Red, countries with a new genotype C1 detection
981 confirmed by phylogenetic analysis (n = 12). Light red: countries where *Rocahepevirus* was detected
982 but the genotype could not be assigned by the proposed rat HEV typing tool (single contig); BLAST
983 nearest neighbours were genotype C1 (Senegal, Togo). Dark grey, previously reported C1 sequences
984 in public sequence databases. Light grey, no sequence data.

985

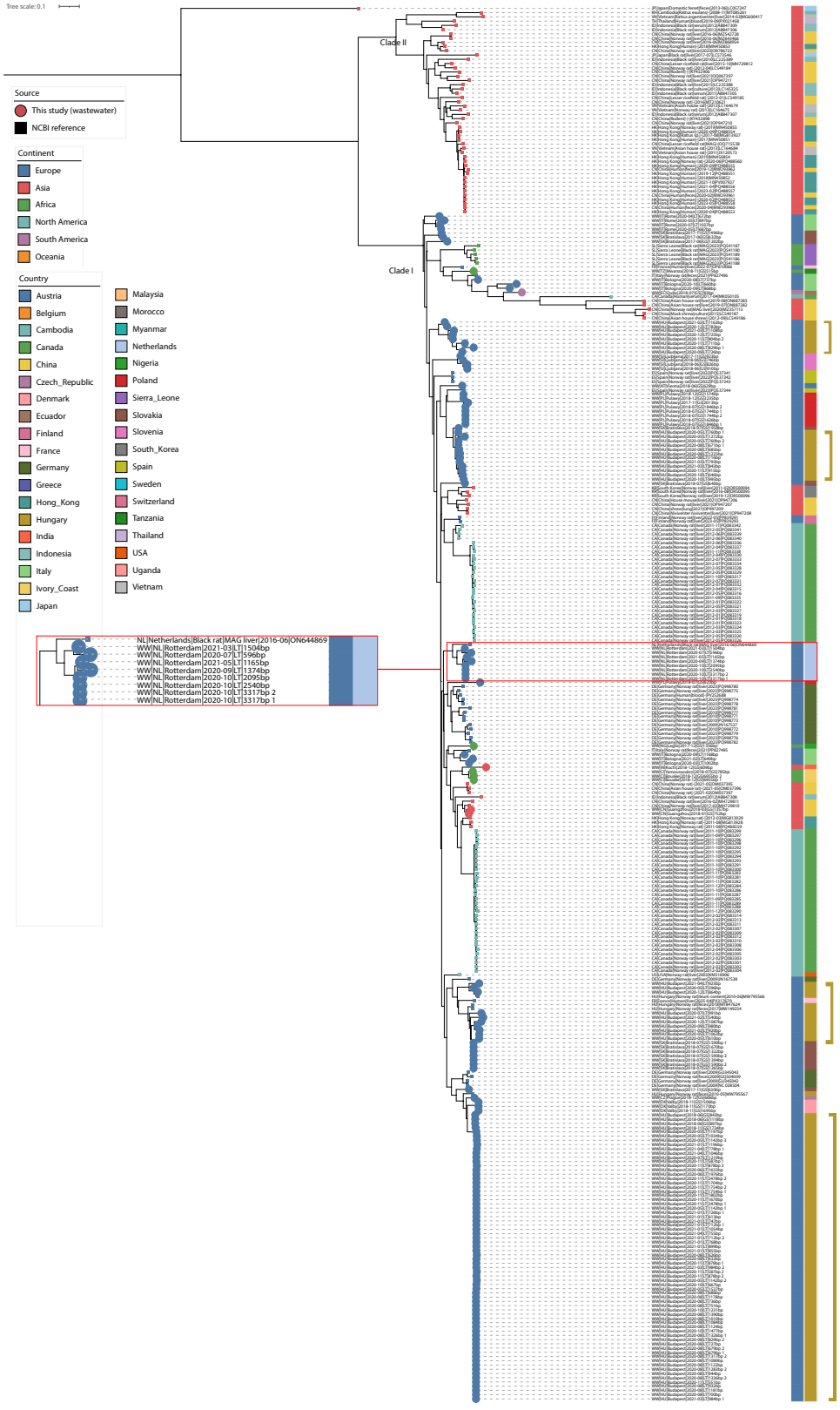


Figure 4. Phylogenetic analysis of *Rocahepevirus ratti genotype C1* sequences detected in wastewater based on partial ORF2 (capsid protein). Maximum-likelihood tree of a 500-bp region (nucleotide positions 5701–6201 relative to *NC_038504.1*), including wastewater contigs (circles, coloured by continent) and GenBank sequences (squares). The bars on the side represent continent and country of origin from left to right.

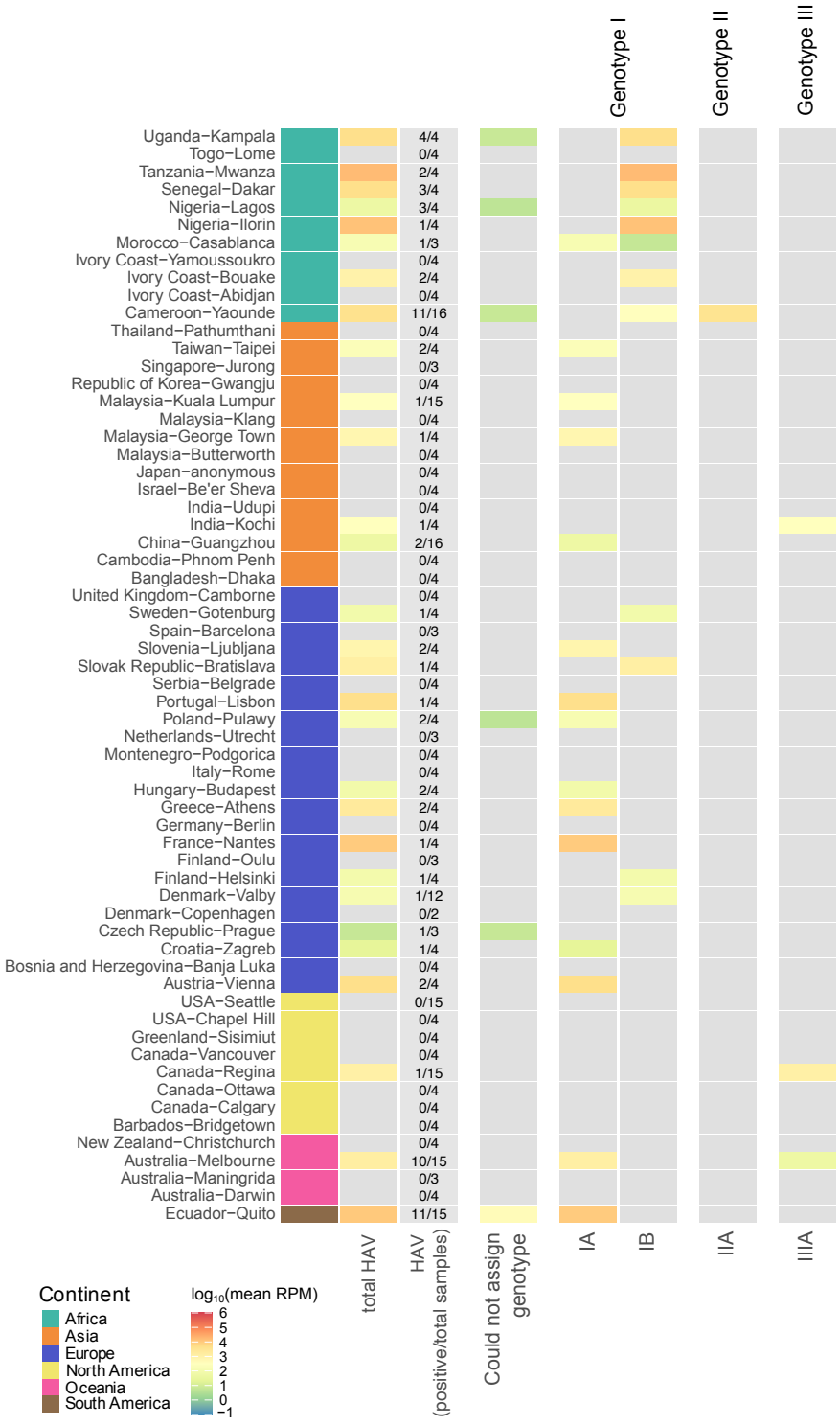


Figure 5. **Global detection of hepatitis A virus in urban wastewater collected between 2017–2019.** Detection of hepatitis A virus (HAV; family *Picornaviridae*, genus *Hepatovirus*) in wastewater samples collected from 62 cities worldwide. Locations are ordered by continent, country, and city. Colour intensity represents $\log_{10}$-transformed mean reads per million (RPM) per location, with grey indicating absence of detectable reads. Columns correspond to HAV genotypes (I, II, III) and subtypes (IA, IB, IIA, IIIA) as assigned by the RIVM HAV Genotyping Tool. Contigs confirmed as HAV by BLAST but not placed phylogenetically are shown under "Could not assign genotype" (n = 12 contigs). Fractions indicate the number of HAV-positive samples relative to the total number analysed per location.

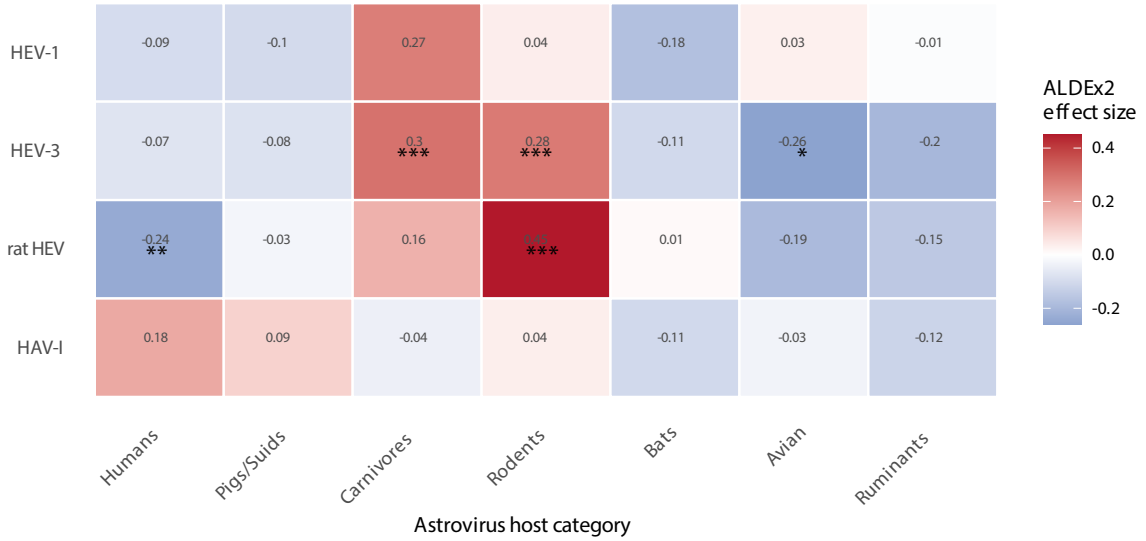


Figure 6. Compositional analysis of astrovirus host profiles by hepatitis virus detection.

Heatmap of ALDEx2 effect sizes from centered log-ratio (CLR)-transformed astrovirus host compositions, comparing samples positive versus negative for each hepatitis virus type. Each row represents a virus type (HEV-1, HEV-3, *Rocahepevirus ratti* C1 (rat HEV), and HAV-I) and each column an astrovirus host category (Humans, Pigs/Suids, Carnivores, Rodents, Bats, Avian, Ruminants). Cell values show the median ALDEx2 effect size across 128 Dirichlet-multinomial Monte Carlo instances; positive values (red) indicate a higher proportional contribution of that host category relative to the overall astrovirus composition in virus-positive samples; negative values (blue) indicate a lower proportional contribution. Statistical significance was assessed by Wilcoxon rank-sum test on CLR-transformed instances, with Benjamini–Hochberg correction applied globally across all 28 tests. Asterisks denote significance thresholds: * FDR < 0.05, ** FDR < 0.01, *** FDR < 0.001.

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