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Human Noroviruses in the Feces of Wild Birds and Rodents – New Potential Transmission Routes

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SUMMARY

Human noroviruses (HuNoVs) are one of the leading global causes of diarrheal diseases, and are transmitted mainly from person to person but also through contaminated food, water, and fomites. The possible zoonotic nature of NoVs has occasionally been discussed, though the viruses are generally considered to be host-species-specific. We investigated whether wild birds and rodents could serve as carriers of HuNoVs, thereby transmitting the virus to humans directly or indirectly by contaminating foods. All samples, 115 avian and 100 rat feces collected in springs 2009-2013 from dump sites, and 85 feces from yellow-necked mice trapped in late autumn 2008 and 2009 after the rodents entered human settlements due to the first night frosts, were screened for HuNoV using real-time reverse transcription-PCR. HuNoVs were detected in 31 (27%) fecal samples of wild birds, in two (2%) fecal samples of rats, and in no samples of mice. Most (25) of the positive bird samples and both rat samples contained genogroup II, and six positive bird samples contained genogroup I HuNoV. The avian species shedding feces containing HuNoVs were identified as gulls and crows using DNA barcoding. Our results show that wildlife, birds and rats in particular, is capable of spreading HuNoVs in the environment.

Keywords

Human norovirus, reverse transcription-PCR, transmission route, carrier animal

Bullet points

We studied if wild birds and rodents could carry human noroviruses, and thereby transmit the virus to humans directly or indirectly by contaminating foods.

Human noroviruses were detected in 31 (27%) fecal samples of wild birds, in two (2%) fecal samples of rats, and in no samples of mice.

41 The bird species shedding feces containing human noroviruses were identified as gulls and
42 crows.

43

1. INTRODUCTION

Human noroviruses (HuNoVs) are globally one of the major causes of acute diarrhoeal diseases in all age groups, as summarized in a recent review (Mathijs et al. 2012). Virus transmission mostly occurs from person to person via the fecal-oral route, but also commonly via contaminated food, water, or surfaces. A HuNoV infection usually causes strong symptoms, such as nausea, vomiting, and diarrhoea, which can appear within 12–48 hours of virus ingestion and disappear within 1–3 days. NoVs, members of the *Caliciviridae* family, are genetically diverse and classified into seven genogroups (Vinjé 2015), with HuNoVs belonging to genogroups I, II, and IV (Zheng et al. 2006). The most common HuNoVs, which also spread from person to person the easiest, belong to genogroup II.

NoVs are generally regarded host-species-specific, but the option of their zoonotic nature has been discussed (Bank-Wolf et al. 2010, Mathijs et al. 2012, Mattison et al. 2007, Mesquita et al. 2010, Scipioni et al. 2008, Wang et al. 2005, Wilhelm et al. 2015). The known animal NoVs in cattle, sheep, and mice (genogroups GIII and GV, respectively) differ considerably from human strains (Mathijs et al. 2012, Scipioni et al. 2008, Wolf et al. 2009). However, certain genogroup II NoVs in pigs closely resembled HuNoVs, raising debate about animal reservoirs and new recombinants (Wang et al. 2005, L’Homme et al. 2009). Mattison et al. (2007) have detected GII.4-like HuNoVs in swine and cattle feces and Summa et al. (2012) detected GII.4 and GII.12 HuNoVs in the feces of pet dogs. Caddy et al. (2015) have shown some evidence of HuNoVs occurring in dogs, and genogroup IV NoVs genetically resembling those found in dogs and lions have been detected in humans (Martella et al. 2007, Martella et al. 2008, Mesquita et al. 2010). All these findings point to the possibility of species-to-species transmission of NoVs.

Wildlife is known to serve as a source of many zoonotic pathogens such as salmonella and rabies (Kruse et al. 2004). Rats and other small rodents are commonly found to spread various human diseases, such as salmonella and plague, but can also carry hepatitis E and hanta viruses (Kosoy et al. 2015, Leibler et al. 2016, Vaheri et al. 2013). Furthermore, wild birds reportedly transmit at least salmonella, mycobacteria, West Nile Virus, and Influenza viruses to humans (Tsiodras et al. 2008). The birds can also serve as reservoirs for many foodborne pathogens such as *Listeria monocytogenes* and *Campylobacteria*. Gulls and corvids (including crows and jackdaws) in particular are able to transmit numerous human pathogens (Tsiodras et al. 2008). So far, to our knowledge, HuNoVs have been detected in wild rodents only once (Wolf et al. 2013), and never in wild birds.

We investigated whether wild birds and rodents could carry HuNoVs and thereby transmit the virus, posing a direct infection risk to humans or a contamination risk to food and water.

2. MATERIALS AND METHODS

2.1 Fecal samples

A total of 115 avian fecal samples were collected during late winter (at the end of March) 2009 (50 samples), 2010 (30 samples), and 2011 (35 samples) from a dump site, which was used for household waste from the entire metropolitan area of Southern Finland. Bright days with new pure snow on the ground were chosen as the sample collection days. Fecal samples were collected aseptically from the untouched snow surface at sites with birds present and then frozen at -20 °C. Gulls, crows, and jackdaws were the main bird species observed at the dump site.

Rat (*Rattus norvegicus*) samples comprised carcasses (8 samples) and droppings (92 samples). The samples were collected in late winter 2012 and 2013 from three dump sites in Southern

Finland. The carcasses were found from different parts of the dump site and the droppings were mainly found near rat poison bait stations. Three mouse carcasses were also concurrently collected from the dump sites. Droppings and carcasses were frozen at -20 °C. Carcasses were melted at room temperature and dissected to analyze the fecal samples collected from the gut.

All other mouse samples (85 samples, yellow-necked mouse *Apodemus flavicollis*) were obtained during studies on rodent-borne viral diseases by the Finnish Forest Research Institute, currently the Natural Resources Institute Finland. The mice were trapped in two localities in Southern Finland in late autumn 2008 and 2009 after the rodents entered human settlements due to the first night frosts. The mice were frozen at site, thawed at the later date, and their guts were dissected and frozen at -20 °C. We excised the feces from the guts and used them for preparing a fecal suspension (see 2.2 detection of HuNoV genome).

2.2 Detection of the HuNoV genome

Fecal samples were diluted in phosphate-buffered saline (PBS) to a 10% suspension, and the viral RNA was extracted from the supernatant of the suspension using a QIAamp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to instructions of the manufacturer. All samples were screened for HuNoV genogroups GI and GII using TaqMan real-time reverse transcription (RT)-PCR for polymerase-capsid junction as described by Summa et al. (2012) (incubation at 50 °C for 25 min for RT, preheating at 95 °C for 15 min, amplification of 50 cycles at 95 °C for 3 sec, at 54 °C for 25 sec, at 72 °C for 25 sec, and final incubation at 40 °C for 30 sec). In brief, the screening was performed using a QuantiTect Probe RT-PCR kit (Qiagen GmbH, Hilden, Germany) with primers and probes COG2R/QNIF2 and QNIFS for GII and QNIF4/QNIF3 and JJV1P for GI analysis. Each series in the RNA extraction and PCR run included blank reactions to rule out the possibility of cross-contamination between the

samples. Theoretical limit of detection for the whole procedure was approximately 100 genome copies/ml in a 10% stool suspension.

2.3 Sequencing and phylogenetic analysis

The avian samples tested positive with PCR, a capsid portion of 377 nt (region C) was amplified using conventional RT-PCR with primers QNIF2 and G2SKR (Kojima et al. 2002) and a Qiagen One Step RT-PCR kit (Qiagen GmbH, Hilden, Germany). The nucleic acid sequences of the amplicons were determined at the DNA Sequencing Service, Institute of Biotechnology, University of Helsinki (www.biocenter.helsinki.fi/bi/dnagen/index.htm). The HuNoV genotypes were determined using a norovirus genotyping tool (www.rivm.nl). The sequences were aligned using the Clustal W software, and the phylogenetic tree was constructed by the neighbor-joining method with Kimura's correction using the NJplot software. GenBank accession numbers for sequences of avian samples are presented in the Results section.

2.4 Definition of bird species

Nucleic acid extraction was carried out on avian samples that tested positive for HuNoV, using 150 µl of supernatant from the stool suspension using a MiniMag RNA extraction system (MM) and a NucliSens Magnetic Extraction Reagents kit (BioMerieux, France) according to the manufacturer's instructions. The nucleic acid was eluted in 70 µl of elution buffer and then frozen at -20°C. For gene sequencing, the nucleic acid was amplified using conventional PCR (preheating at 94 °C for 10 min, five cycles at 94 °C for 1 min, at 45 °C for 1.5 min, and at 72 °C for 1.5 min, amplification of 30 cycles at 94 °C for 1 min, at 51 °C for 1.5 min, and at 72 °C for 1.5 min and final incubation at 72 °C for 15 min) with primers BirdR2 and BirdF1 (Hebert et al. 2004) and the Qiagen HotStarTaq Plus DNA Polymerase kit (Qiagen GmbH, Hilden, Germany). Each reaction consisted of 2.5 µl PCR Buffer (10x), 0.5 µl dNTP (10 mM), 2.5 µl

of each primer (10 μ M), 0.5 μ l DNA Polymerase (2.5 units), 13.5 μ l water, and 3 μ l of the sample. The nucleic acid sequences of the 750 bp portions were determined at the DNA Sequencing Service. Bird species were determined using BLAST (U.S. National Library of Medicine, <http://blast.ncbi.nlm.nih.gov>).

3. RESULTS

Of the 115 avian fecal samples, 31 (27%) tested positive for HuNoV, 25 samples for GII, and six samples for GI (Table 1). HuNoVs were found during each year that samples were collected. Positive results could be confirmed by repeating the real-time PCR for 12 samples and also by sequencing for six samples. Four samples contained genotype GII.4 and two contained GII.3 NoVs based on the capsid gene region (Table 2). The quantification cycle (Cq) values of all positive samples varied from 35 to 41 meaning allover weak positivity (see Table 2), and thus the PCR amplification of the longer PCR product for genotyping failed for most of the samples. All GI isolates remained without a genotype.

A phylogenetic tree (Figure 1) was formed using the 223 overlapping nucleic acid sequences from the capsid region C of the six HuNoV strains found in bird feces, 27 HuNoV GII strains in Genbank, and four GII strains found in dog feces (JF746890–JF746893, Summa et al. 2012). The sequences of isolates 7-09 and 10-09 were 98.7% and 99.1% identical at the nucleic acid level, respectively, and 100% identical at the amino acid level, to the GII.4-2006b variant (EF126965) by the manual comparison of aligned sequences; the norovirus genotyping tool also recognized them as GII.4-2006b. The sequence of isolate 3-09 was closely related to GII.4-2006a variant (EF126964; 99.6% identical in nucleic acids and 100% in amino acids) and isolate 5-11 to the GII.4-2009 variant (JN595867; 98.7% identical in nucleic acids and 100% in amino acids). The sequences of isolates 1-10 and 9-11 were 93.6% and 96.9% identical in the nucleic acids and 97.3% and 95.9% in the amino acids to GII.3 strains U02030 and

EU187437, respectively. Interestingly, the GII.4-2006b sequences of isolates 7-09 and 10-09 were also closely related to one of the isolates found in dog feces (JF746892, 98.2% and 98.6% identical in nucleic acids, respectively, and 98.6% in amino acids) in Finland. The GII.4-2009 sequence of 5-11 was 97.3% identical in nucleic acids and 100% in amino acids to two isolates from dog feces (JF746890, JF746891) collected in 2009 and 2010.

Bird species could be identified in 10 HuNoV positive fecal samples after partial nucleic acid sequence determination of their cytochrome c oxidase I (COI) genes (Table 2); the remaining 21 did not produce any PCR product. Eight samples were collected from gulls (*Larus hyperboreus*, *Larus fuscus*, and *Larus argentatus*) and two from crows (*Corvus corone cornix*).

Two (one from each year) out of the total of 100 fecal rat samples from dump sites tested weakly positive (Cq values of 38.30 and 35.90) for HuNoV GII with real-time PCR (Table 1), but the positive results could not be repeated. All 88 fecal samples from mice mainly trapped in human settlements tested negative for HuNoV.

4. DISCUSSION

We detected HuNoV genome in avian and murine fecal samples, showing that the viral genome can pass through the gastrointestinal tract of birds and rats. Consequently, wild birds and rodents may potentially carry HuNoV genomes from one place to another. All six partial NoV sequences detected in avian fecal samples were identical or nearly identical to previously published sequences from human samples and differed from avian or murine NoVs. During the NoV epidemic seasons of 2009 to 2011, HuNoV variants GII.4-2006b, GII.4-2006a, and genotype GII.3 circulated commonly in the human community in Finland (Hulkko et al. 2010, 2011, Jaakola et al. 2012) and were also present in avian fecal samples. The possibility of animal transmission of the HuNoV genome is also supported by previous studies, which

184 observed HuNoVs in the feces of cattle, pigs, dogs, and a rat (Caddy et al. 2015, Mattison et
185 al. 2007, Summa et al. 2012, Wolf et al. 2013).

186 But, how do birds become infected by HuNoVs and can these birds transmit NoVs to humans?
187 Our avian samples were collected from a large dump site, which received all the household
188 garbage from human settlements in the metropolitan area of Southern Finland until 2014. NoV
189 infections, including asymptomatic ones, occur frequently in children younger than two years
190 old (Pringle et al. 2015, Robilotti et al. 2015), who usually wear diapers. HuNoV remains
191 infectious for several days unless inactivated e.g. using effective disinfectants (Weber et al.
192 2010), and therefore the virus is easily transferred to the dump site with discarded textiles,
193 tissues, and diapers that may be contaminated with high amounts of the virus by infected
194 persons. Consequently, the dumped rubbish among which the birds search for food may be
195 highly contaminated with HuNoV during epidemic seasons. Interestingly, GII.4 and GII.3
196 NoVs, the genomes also present in our avian samples, dominated globally (67% and 16%
197 respectively) in children in 2004–2012 (Hoa Tran et al. 2013).

198 The food industry generally regards wild birds as noxious animals that spread numerous
199 diseases (Tsiodras et al. 2008), and are therefore strictly controlled in primary food production
200 and food premises. Birds are recognized to contaminate food and water with salmonella or
201 campylobacter via their feces (Butterfield et al. 1983, Tsiodras et al. 2008), but our findings
202 show that HuNoV contamination could also be possible. The virus genome in the bird feces
203 could spread directly to vegetables and berries in fields, or indirectly via contaminated food
204 crates or irrigation and washing water. Contaminated surfaces (Oristo et al. 2017, Tuladhar et
205 al. 2013, Weber et al. 2010), such as outdoor tables, handrails, etc., could also be a possible
206 infection route from birds to humans.

Several NoV outbreaks occurred in Finland during summer 2014, and they were shown to originate on a few beaches (Kauppinen et al. 2017). The source of water contamination was not clear, but the authors suspected that bathers and other beachgoers spread the virus. According to our results, birds carrying HuNoV could also be one, albeit minor, possibility. However, large seagull flocks could be a risk for human health, as they may contaminate public beaches and bodies of water which are used as a raw water source for household water.

Our study does not demonstrate that HuNoVs can replicate in the gastrointestinal tract of birds, as only viral genome was screened in the samples. According to our results, the viral genome load in the avian samples was low compared to human samples during acute infections. Norovirus is proposed to bind with carbohydrates of histo-blood group antigens (HBGAs) when interacting with a host cell (Green 2013). As reviewed by Shirato (2011), the *FUT2* gene encodes HBGA carbohydrates in intestinal epithelial cells that are needed by HuNoV binding to the cells. According to Yamamoto et al. (2014), a large variety of organisms, including mice, rats, and dogs, carry the *FUT2* gene. However, none of the bird species mentioned in the study (flycatcher, zebra finch, turkey, duck, chicken) have the gene. These findings and the low viral genome load in our samples suggests no or very limited replication in birds.

Fecal samples containing host intestinal cells have been used to identify host species (Lee et al. 2010). We succeeded in determining the bird species from 10 of our 31 avian fecal samples positive for HuNoV using PCR that was originally optimized for muscle tissues (Hebert et al. 2004). This non-invasive method worked well with fresh fecal samples of our study, but the quality of the host DNA in the fecal samples kept frozen may have deteriorated up to the point that the method could not specify the bird species in all samples. All identified bird species were gulls and crows, which are known to transmit numerous human pathogens (Tsiodras et al. 2008) and which were also usually observed during visits to dump site.

Wolf et al. (2013) detected HuNoV in the intestinal content of a rat trapped in a sewer system. In addition to dump sites, sewer systems of urban areas are an important contamination source of HuNoV for rats. The low HuNoV genome load in our rat samples may partially be explained by the quality of our samples. The rat droppings were not fresh, so they could have been lying on the ground for an extended period. Because the droppings were collected near the rat poison bait stations, both feces and carcasses may have contained rat poison. We do not know if the rat poison could have damaged the virus particles in the gastrointestinal tract of rats.

Nearly all our mouse samples were from the species *Apodemus flavicollis*. Our sampling occurred late in autumn, when mice invading human settlements could pose a disease risk. *Apodemus* mice eat human and animal food and food waste while visiting human settlements, but they may not come in direct contact with HuNoV contaminated waste, e.g. diapers or sewage. The mice were also trapped outside the NoV epidemic season. As far as we know, no one, us included, has been able to detect HuNoVs in mouse samples (Leibler et al. 2016, Lõhmus et al. 2013).

Our results show that wild birds and possibly also rats are involved in the transmission of HuNoVs. At the very least these groups can mechanically carry the virus genome from the original source to a new site. Therefore, it would be important to keep in mind the option of birds as a contamination source when investigating NoV outbreaks caused by food or water. Determination of the replication capability of HuNoV in these new potential carrier animals requires more investigation, for example viral antibodies in blood or whole virus particles in feces should be searched for.

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Conflict of Interest: None.

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Figure legends

A phylogenetic tree was formed using the 220-bp sequence alignments consisting of a partial
capsid gene from six HuNoV GII strains found in avian fecal samples (marked “BIRD feces”),
27 HuNoV GII genotypes in GenBank and four GII strains found in canine fecal samples
(Summa et al. 2012, JF746890–JF746893, marked “DOG feces”). Norwalk GI.1 was added as
an outgroup. Phylogenetic analysis was performed with the neighbor-joining method using the
Clustal W software. Bootstrap values (1000 replicates) are shown on the branches. The scale
of the tree is indicated by the bar.

Table 1. Fecal and carcass samples from birds, rats, and mice

	Total number	HuNoV positives		total	% positive
		GII	GI		
Bird	115	25	6	31	27
Rat	100	2	0	2	2
Mouse	88	0	0	0	0

Table 2. HuNoV-positive avian samples

Positive samples ^a	HuNoV GI / GII	Bird species	Confirmed ^b (yes/no /Cq value)	NoV genotype capsid region C	Accession no. in Genbank
1-09	GII	Gull (<i>Larus fuscus</i>)	Y /38.68	-	-
2-09	GII	Gull (<i>Larus argentatus</i>)	Y /39.02	-	-
3-09	GII		Y /39.48	GII.4 2006a	MF444290
4-09	GII		Y /39.20	-	-
5-09	GII	Crow (<i>Corvus corone cornix</i>)	Y /38.11	-	-
6-09	GII		Y /40.44	-	-
7-09	GII		Y /37.18	GII.4 2006b	MF444291
8-09	GII		Y /39.62	-	-
9-09	GII	Gull (<i>Larus hyperboreus</i>)	Y /39.34	-	-
10-09	GII		Y /38.64	GII.4 2006b	MF444292
11-09	GII		Y /39.85	-	-
1-10	GII	Gull (<i>Larus fuscus</i>)	Y /39.63	GII.3	- ^c
2-10	GII	Gull (<i>Larus hyperboreus</i>)	N /38.49	-	-
3-10	GII	Gull (<i>Larus hyperboreus</i>)	N /39.53	-	-
4-10	GII	Gull (<i>Larus fuscus</i>)	N /38.34	-	-
5-10	GI		Y /39.18	-	-
6-10	GI		N /39.48	-	-
7-10	GI	Gull (<i>Larus hyperboreus</i>)	Y /41.49	-	-
8-10	GI		N /40.63	-	-
9-10	GI		N /40.24	-	-
1-11	GII		N /40.42	-	-
2-11	GII		Y /38.91	-	-
3-11	GII		N /39.47	-	-
4-11	GII		N /38.33	-	-
5-11	GII		Y /38.16	GII.4 2009	MF444293
6-11	GII		N /38.64	-	-
7-11	GII		N /38.36	-	-
8-11	GII		N /40.72	-	-
9-11	GII	Crow (<i>Corvus corone cornix</i>)	Y /35.87	GII.3	MF444294
10-11	GII		Y /38.29	-	-
11-11	GI		N /39.71	-	-

a: running number of the sample- year of collection

b: positive PCR result on at least two occasions

c: nucleotide sequence too short for Genbank accession