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Mouse intestinal villi as a model system for studies of norovirus infection

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Summary. – Noroviruses (NoV) are enteric caliciviruses that have been detected in multiple species of mammals, including humans. Establishing an efficient *in vitro* cell culture system for human norovirus (HuNoV) remains a challenge; however, its replication has been reported in 3D cultured Caco-2 cells and a clone of Caco-2 cells (C2BBe1), human enteroids and human B cells. Isolated mouse intestinal villi, with large diversity of intestinal epithelial cells, are a primary cellular model that has been shown to be permissive for the infection and replication of enteric viruses such as rotaviruses. We hypothesized that they could allow the infection and replication of the human noroviruses. In this report, we indicate that the isolated villi model of the mouse intestine is effective for the infection study and replication of the human noroviruses from faeces and environmental matrices (water, vegetables and air). For successful infection, the virus needs to be activated with trypsin. The virus has an average replicative cycle of 10 h, although viral particles with infectious capacity are found already at 2 hours post infection (2 h.p.i.). The model is efficient in obtaining abundant biological material and is ideal for studying the biological activity of the human noroviruses in the same cell model or for generating antibodies.

Keywords: human norovirus; intestinal epithelial cells; intestinal villus isolation; norovirus isolated from water; norovirus isolated from plants; norovirus isolated from air

Introduction

Noroviruses belong to the *Caliciviridae* family, containing single-stranded positive sense RNA from 7.3 to 8.3 kb, with unenveloped virions of 38 to 40 nm in size (Robilotti *et al*., 2015). Virions are composed of two structural proteins, the main component of the capsid, VP1, organized into 90 dimers, and VP2 of which only 6 copies are found per virion and is associated with VP1 on the surface (Hardy *et al*., 1995). VP2, due to its basic nature, is associated with the stabilization and packaging of RNA, making it essential for the viability and infectious capacity of the virion (Vongpunsawad *et al*., 2013).

Norovirus-related gastroenteritis is a self-limited disease characterized by diarrhea, vomiting, and general malaise (Rockx *et al*., 2002). Histopathology analysis revealed, that it affects the proximity of the small intestine presented by shortening of the villi, hypertrophy of the crypt and inflammation of the mucosa at the tissue level and intracellular vacuolization in intestinal cells (Dolin *et al*., 1975; Schreiber *et al*., 1973), suggesting that the virus replicates in the duodenum and jejunum. However, the cell types in which HuNoV replicate in immunocompetent individuals are still unknown. HuNoV replicates in intestinal enterocytes of transplant and immunocompromised patients, as the structural protein VP1 and nonstructural antigens (RNA polymerase and viral genome-

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Abbreviations: h.p.i. = hours post infection; ICC = immunocytochemistry; IFA = immunofluorescence assay; HuNoV = human norovirus; LTP = leachate treatment plant; MNV = murine norovirus; NoV = Noroviruses

linked protein, VPg) were detected, providing direct evidence of viral replication in these cells (Karandikar *et al*., 2016). After introduction of the rotavirus vaccine, HuNoV, became the main cause of acute non-bacterial gastroenteritis affecting all age groups around the world but with special relevance in children under 5 years, adults over 65 years and immunocompromised persons (Kõivumägi *et al*., 2020; Morioka *et al*., 2017). Each year noroviruses cause 64,000 episodes of diarrhea requiring hospitalization being in turn responsible for 900,000 severe infections of children in industrialized countries and more than 1 million infections and 200,000 deaths of children <5 years of age in developing countries (Patel *et al*., 2008). In Latin America, they are responsible for 1 out of 6 cases of hospitalization for acute diarrhea associated with norovirus Genogroup II.4 (O'Ryan, 2017; Zheng *et al*., 2006). Transmission is typically through contaminated food, water, environment, or person-to-person contact (Patel *et al*., 2009). However, the most common food vehicle for HuNoV remains to be ready-to-eat foods (Patel *et al*., 2009) and fresh produce such as vegetables and fruits (Callejón *et al*., 2015; Berger *et al*., 2010), particularly those that require handling but no subsequent cooking (Widdowson *et al*., 2005). Study evaluating 972 epidemiological clusters associated with vegetables eaten raw in the U.S.A. between the years 1998–2013 showed that the most commonly identified etiological agents were noroviruses (54%), *Salmonella* (21%) and shigatoxin-producing *E. coli* (STEC) (10%) (Bennett *et al*., 2018).

Despite their importance, the study and possible development of vaccines and treatments has been difficult, due to the lack of models for *in vitro* culturing and replication, although traditionally used cell lines for enteric viruses such as Caco-2 or VERO have been tested with little success (Oka *et al*., 2018; Straub *et al*., 2011; Lay *et al*., 2010; Duizer *et al*., 2004). Dendritic cells and macrophages have been reported to be permissive for murine norovirus infection (Karst *et al*., 2014; Wobus *et al*., 2004). In more recent studies, a chimpanzee model demonstrated HuNoV infection of B cells (Bok *et al*., 2011), HuNoV infection was reported in a murine animal model (Taube *et al*., 2013) and murine norovirus (MNV) infection of B cells *in vivo* has been demonstrated (Grau *et al*., 2017), however these models are not capable of recreating all aspects of norovirus infection (Oka *et al*., 2018; Jones *et al*., 2015). Important advances in *in vitro* cell culture systems have been achieved for MNV using murine macrophage cell lines (e.g., RAW264.7), primary macrophages and dendritic cells (Wobus *et al*., 2004), and mouse B cell lines (Jones *et al*., 2014). Similarly, a method using human intestinal enteroids has been reported for the spread of HuNoV *in vitro* with high levels of RNA (Ettayebi *et al*., 2016). The study of MNV has provided knowledge of the biology of these viruses and made it possible to determine tropism for tuft cells determining immune promotion of norovirus pathogenesis (Wilen *et al*., 2018). Thus, it has been suggested that CD300lf-expressing immune cells in gastrointestinal lymphoid tissues are the main cellular targets during the acute phase of MNV infection, whereas persistent MNV hides in CD300lf-expressing tuft cells (Wobus, 2018). In turn, only one strain of MNV (CR6) has tropism for tuft cells (Wilen *et al.,* 2018). MNV also has been found to use M (microfold) cells to pass through the gastrointestinal epithelium without replicating in these cells (Gonzalez-Hernandez *et al*., 2013). In addition, it has been reported that acute MNV strains infect immune cells *in vivo* (Graziano *et al*., 2021; Van Winkle *et al*., 2018; Grau *et al*., 2017).

Isolated mouse intestinal villi are a primary cellular model that has been shown to be permissive for infection and replication of enteric viruses such as rotaviruses (Guerrero *et al*., 2010). Intestinal villi model allows the study of the virus in tissue cells where it naturally causes pathological changes. Also, this model shows high diversity of epithelial cells found in intestine. Due to these advantages, we have hypothesized that it could allow efficient infection and replication of HuNoV. Therefore, this work sought to establish and identify the conditions for HuNoV infection in the mouse isolated intestinal villus model. In this report, we show that the mouse isolated intestinal villus model is effective for the study of HuNoV infection and replication from faeces and environmental matrices (water, vegetables and air). For efficient infection, the virus needs to be activated with trypsin, presenting an average replicative cycle of 10 h, although there are viral particles with infectious capacity from 2 h.p.i. The model is efficient to obtain abundant biological material and is ideal for virus biological activity study in the same cellular model or to generate antibodies.

Materials and Methods

Sample collection and preliminary human enteric virus detection. Study participants were requested to submit stool sample to Virus Molecular Biology Laboratory of the National University of Colombia within 48 h after onset of diarrhea. The patients (34 children under 5 and 3 adults over 75 years) received a physical examination in the Usme hospital of the Bogotá City Colombia, and both the consistency of faeces and the number of diarrheal episodes were recorded. Aliquots of diarrheal faeces were stored at -20°C and transported on ice. Thirty-seven faecal samples were collected, and the viruses were partially purified following established procedure (Guerrero *et al*., 2014). The partially purified viruses were kept at -20°C until further use. Faeces samples were evaluated for parasites in microscopic

examination, and the absence of different pathogens that cause diarrhea (rotavirus type A (RV-A), astrovirus (AstV), *Salmonella* spp, *Shigella* spp and *Cryptosporidium* spp (*Crypto.* spp) was verified with RT-qPCR and qPCR.

Infectious HuNoV presence was assessed on 54 environmental samples collected in low, medium and high rainfall months between July 27, 2017 and August 11, 2018 within and in the surroundings of Bogota city. Colombia has no changing seasons, so the viral infections are more associated with rainy periods. We have used, 7 air samples from about 4 m^3 of air collected by biocollectors into 50 ml of phosphate buffered saline (PBS). Two samples were from two disposition sites inside of the Doña Juana landfill, one from the landfill leachate treatment plant (LTP) and 4 from terraces of houses around 7–10 m above the streets and located approximately ~3 km north northwest of the LTP and ~0.7 km northeast, east and southeast, of the LTP, respectively (Fig. 1c); 32 water samples (10 from Tunjuelito river on its way through Usme district of Bogotá city, 10 from a storm-water open collector combined with wastewater that discharged its waters into the river, 2 from leachate treated in the LTP, 6 drinking water samples from a primary school (3), a house (1) and the National University Campus (2) located at approximately 2.9 km southeast, 3.3 km northeast and 16.8 km northeast of the landfill, respectively (Fig. 1b,c), and 2 and 2 samples of irrigation water from La Ramada rural area near Mosquera municipality (~24 km northwest of the landfill) and Cota municipality (~34 km north of the landfill), respectively (Fig. 1b), that were trapped on HA filters doped with Al^{3+} after a prefiltration through 8–12 µm paper or 0.2 µm polyamide filters (Jordan-Lozano, 2020). Fifteen chard samples (leaves and petioles) were collected at La Ramada rural area, of which noroviruses were eluted with sterilized water from external surface (including 2, 2 and 2 samples during cultivation, harvest and transport, respectively; 2 and 1 samples during cultivation at Mochuelo Alto rural area in Ciudad Bolivar district (~1.3 km west to landfill) and at Cota, respectively; 4 samples of vegetable nearby stores in 3.6 km perimeter of the landfill, and 2 samples from Corabastos wholesale market in Bogotá at ~15 km north of the landfill). Vegetables were taken as a model because they are grown in dry and rainy periods of the year and because they account for approximately 90% of the crops in the area.

Virus purification. We followed procedure for the partial virus purification from faeces (Guerrero *et al.*, 2014) with some modifications: faeces were taken (0.1 g) and diluted in 0.6 ml of Tris-Base buffer (pH 8, 0.26 M Tris + 0.68 M NaCl) and vortexed for 2 min. The 600 µl of chloroform was added, vortexed for 5 min and centrifuged at 13,000 x *g* for 20 min. The supernatant was collected and 100 µl of Tris-Base buffer was added.

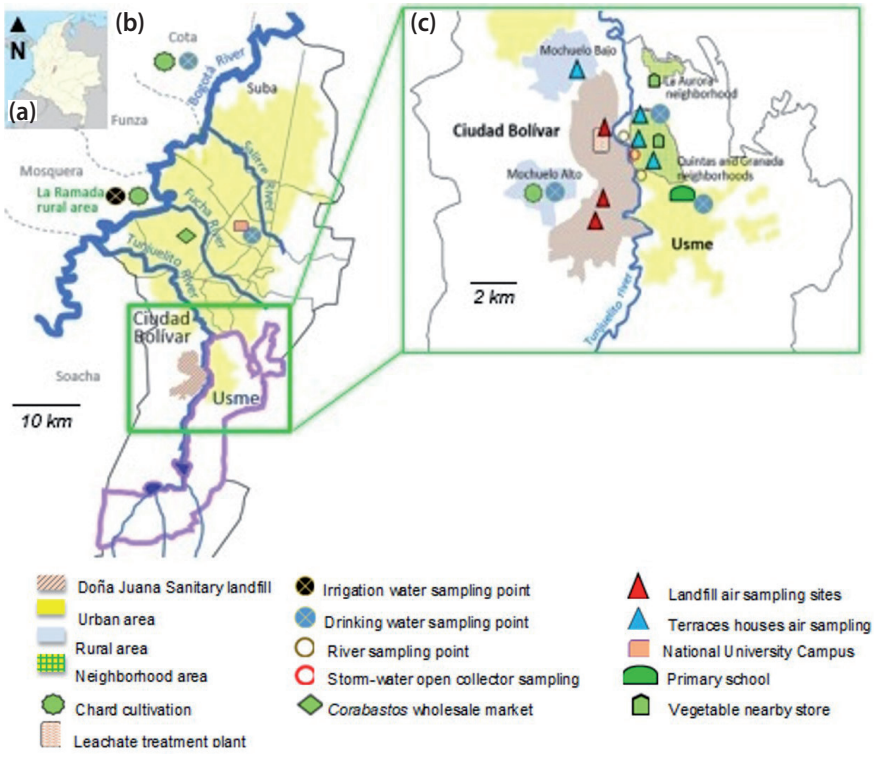

Fig. 1

Map of sample collection

Location of environmental sampling sites **(a)** at the Colombian scale, **(b)** at the Bogota scale, **(c)** at the Usme and Ciudad Bolivar scale.

Samples were vortexed for 2 min and centrifuged for 10 min at 13,000 x *g*. The supernatant was recovered and mixed with the previous one. The procedure was repeated twice. The recovered supernatant mixture was kept at -80°C. For the partial virus purification from environmental samples: (i) airborne viruses previously trapped by impaction (of about 4 m^3 air in 50 ml of PBS) in 6–h interval in a 125 ml glass Impinger (AGI-30 7540-10, Air Sampling, ACE GLASS Inc., USA) were initially concentrated by mixing with 9% polyethylene glycol 8000 (PEG-8000) (p: v) in PBS, stirred gently at 4°C for 12 h using a rocking shaker, then centrifuged in 50 ml tubes at 4°C and centrifuged at 10000 rpm for 30 min. Each pellet was resuspended in 2 ml of Tris-Base buffer; (ii) RNA from water samples was obtained using concentration methods (Haramoto *et al*., 2004, 2005) for tap water and other samples (river water, storm water combined with wastewater, irrigation water, leachates and water produced from washing of chard-leaves surface) adding the same quantities of PEG and Tris-Base buffer to 10–30 ml of sample previously eluted with 1 mM NaOH with pH 10.8 from filters with 0.45 µm pores after being doped with Al^{3+} to give it a positive charge. Each of the 2 ml suspension was concentrated in a Centriprep-YM-50 tube (Merck Millipore Sigma, USA) by subjecting the tube to four centrifugation cycles at 2,500 rpm and 4°C for 10 min, 5 min, 5 min and 3 min until the suspension was reduced to 260–460 µl. The samples were stored at -20°C.

Immuno-dot blot assays. Virus suspensions (20 µl) were applied onto PVDF membrane discs and incubated for 1 h at 37°C with 0.2 µg/ml of anti-VP1 monoclonal antibody (Norwalk Virus (1D9), sc-53557 Santa Cruz Biotechnology Inc., USA), 0.2 µg/ml of monoclonal anti-rotavirus antibody (SC53560, Santa Cruz) or polyclonal rabbit anti-astrovirus antibody. Secondary HRP-conjugated goat anti-mouse antibody (0.4 µg/ml, SC 2005, Santa Cruz) or HRP-conjugated goat anti-rabbit secondary antibody (0.4 µg/ml, sc-2004, Santa Cruz) were added. Peroxidase activity was revealed with aminoethylcarbazole substrate (Sigma, USA) in acetate buffer and hydrogen peroxide (Sigma).

RNA isolation. For viral RNA extraction from faeces samples or isolated mouse intestinal villi, we used disruptor buffer with pH 8 (0.26 M Tris, 0.09 M EDTA, 0.04 M SDS, 0.68 M NaCl and 1.3% β-mercaptoethanol). We mixed 20 µl of disruptor buffer and 4 µl of internal extraction control from Norovirus Genogroups 1 and 2 or Human Rotavirus A genesig advanced kit (PrimerdesignTM Ltd, Eastleigh, UK). Then, the disruptor buffer with the internal extraction control was added to sterile centrifuge tube containing 200 µl of viral suspension and shaked for 30 s. Then, 200 µl of phenol was added and vortexed for 2 min., centrifuged at 12,000 x *g* for 8 min at room temperature and 200 µl of the supernatant were recovered. The 99.8% ethanol was added to the supernatant and precipitated for 12 h at -80°C. The sample was centrifuged at 13,000 x *g* for 12 min at room temperature. The pellet was resuspend in 50 µl of deionized water and stored at -80°C. To extract viral RNA from suspensions of air samples, river water, storm water combined with wastewater, irrigation water, leachates and water produced from washing of chard-leaves surface, we used PureLink™ viral RNA/DNA mini kit (Invitrogen, Thermo Fisher Scientific, USA) following the manufacturer's instructions.

Real-time PCR. Viral RNA was detected using Norovirus Genogroups 1 and 2 and Human Rotavirus A genesig advanced kit (PrimerdesignTM Ltd, UK) according to manufacturer's instructions. The PCR was run in CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, USA) with following programme: 10 min at 55°C for reverse transcription, 2 min at 95°C, followed by 50 cycles with 10 s at 95°C and 60 s at 60°C. The cycle threshold (Ct) value from the real time RT-PCR was used as a proxy viral load measure of faecal and environmental matrices (water, vegetables and air) or NoV-positive villi culture; Ct <37 was considered positive.

Partial DNA purification. For partial purification of bacterial DNA (Salmonella spp., Shigella spp.) and parasite DNA (Cryptosporidium. spp.) from faeces samples, we used the phenol-chloroform method. The 0.1 g of stools was dilute in 0.6 ml of Tris-EDTA buffer (0.09 M Tris + 0.009 M EDTA, pH 8), vortexed for 5 min, add 800 µl of phenol/chloroform/isoamyl alcohol was added. The samples were vortexed for 2 min, then centrifuged at 13,000 x *g* for 20 min and 700 µl of supernatant was recovered. Posteriorly, we added 100 µl of Tris-EDTA to first tube, vortexed and centrifuged the sample to recover 100 µl of supernatant. In the second tube, DNA was precipitated by adding 80 µl of 3 M $C_2H_3NaO_2$ (pH 5.2), 1200 µl of 99.8% ethanol and incubated at -80°C for 6–8 h. The samples were thawed at room temperature for 3 to 5 min, centrifuged at 13,000 x *g* for 20 min, and the pellet was resuspended in 200 µl of Tris-EDTA buffer. Each tube with the partially purified DNA was stored at 4°C until further use.

DNA isolation. DNA partially purified and resuspended in 200 µl of Tris-EDTA buffer was isolated by adding 400 µl of lysis solution from Genomic DNA purification kit (K0512, ThermoFisher Scientific, USA) and 4 µl of internal extraction control solution from Internal DNA extraction control kit (PrimerdesignTM Ltd, UK) following the manufacturer's instructions. The isolated DNA was stored at -20°C until further use.

qPCR assays. DNA isolated from stool samples was used for *Salmonella* spp., *Shigella* spp. and parasite *Cryptosporidium* spp. detection, following the manufacturer's instructions of three quantification kits respectively: (i) All pathogenic Salmonella species, Invasion protein (invA) gene – genesig standard kit (Primerdesign™ Ltd, UK); (ii) Shigella species, virulence plasmid pCP301 (VirA) – genesig standard kit (Primerdesign Ltd), and (iii) Cryptosporidium Oocyst wall protein (COWP) gene – genesig standard kit (Primerdesign Ltd), using the amplification Internal DNA extraction control kit (Primerdesign Ltd) and Oasig™ lyophilised 2x qPCR master mix (Primerdesign Ltd). Each reaction mixture was analyzed on CFX96 Touch™ Real-Time PCR detection system (Bio-Rad) with the following programme: 15 min at 37°C, 2 min at 95°C, and 50 cycles of 10 s at 95°C and 60 s at 60°C.

Intestinal villi isolation. To obtain intestinal villi, we used established protocol (Guerrero *et al*., 2010). Animal experiments were approved by the Ethics Committee of Medicine School, National University of Colombia (Bogotá, Colombia), according to established guidelines (number of the permission 008-071-16). Adult male and female ICR mice (older than 6 months age) obtained from Faculty of Veterinary Medicine, National University of Colombia, were killed by cervical dislocation. The villi-enriched preparation in minimum essential medium (MEM) containing antibiotic/antimycotic solution was kept at 4°C.

Determination of trypsin concentration. Some authors suggest that VP1 capsid proteins need to be digested for proper virus infection (Ma *et al*., 2018; Tan and Jiang, 2006). Intestinal villi-enriched preparation was plated in 96-well cell culture plates with 100 µl of MEM without fetal bovine serum (FBS). The inoculated villi were seeded in each well. The infection was standardized with NoVGII isolated from a stool sample (N° 1 in Table 1) that was negative for NoVGI, RV-A, AstV, *Shigella* spp., *Salmonella* spp. and *Cryptosporidium* spp. The sample was filtered through a 0.2 µm cellulose nitrate membrane and ideal trypsin concentration to activate the virus, was determined. The procedure was performed using different concentrations of trypsin (0 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 100 µg/ml and 200 µg/ml in PBS) added to the NoVGII sample and incubated at 37°C for 20 min (Hardy *et al*., 1995). Then, trypsin inhibitor was added in the same concentration as trypsin. The NoVGII dilutions 1:25, 1:50, 1:100, 1:200 and 1:400 were added to 100 µl of MEM, in 96-well plates containing the isolated intestinal villi. The cells were cultured at 37°C and 5% CO_2 for 12 h, harvested and fixed in suspension with methanol-acetic acid (3:1 v/v) for 20 min at 0°C. The infection was evaluated by immunocytochemistry or immunofluorescence using anti-norovirus monoclonal antibody.

In vitro cellular infection with HuNoV. Intestinal villus-enriched preparation was plated in 96-well cell culture plates with 100 µl of MEM without FBS and then inoculated by adding 10 µl suspension of HuNoV and trypsin-activated. Infectious norovirus was recovered from 36 environmental and 5 human feces samples that were positive by immunodot-blot, RT-qPCR or both methods. The villi were cultured in incubator at 37°C in 5% CO_2, until complete cell lysis was observed in approximately 12–24 h. To evaluate the norovirus infection in the villi, lysate was frozen and thawed twice, then 10 µl of the lysate was activated with trypsin (10 µg/ml) and applied to 100 µl of a new villus culture with MEM, without FBS. To evaluate norovirus infection of the villi, we used immunocytochemistry (ICC) or immunofluorescence (IFA) and capture ELISA assay. The percentage of infected cells was estimated on samples from three independent experiments with each sample being analyzed twice. Mock-infected villi cells were used as control.

Analysis of HuNoV replicative cycle. The intestinal villus-enriched preparation with 100 µl of MEM without FBS was plated in 96-well cell culture plates and infected with NoVGII at 1:50 dilution previously activated with trypsin. Infected villi were harvested at different time's post-infection (2 h, 4 h, 6 h, 8 h, 10 h and 12 h.p.i). The harvested villi were centrifuged at 700 x *g* and the supernatant was recovered. MEM without FBS was added to the pellet and lysed by two cycles of freezing and thawing. The cell lysate or the supernatant was activated with trypsin, added to freshly isolated intestinal villi, and harvested at 12 h.p.i. Infectious titers of cell lysates or the supernatant were determined using ICC and indirect ELISA assay.

Tumor cell lines infection. Two tumor lines were tested based on reports that noroviruses infect monocyte-macrophages and B cells (Bhar and Jones 2019; Jones *et al*., 2015; Wobus *et al*., 2004). We have used tumor line U937 (ATCC CRL-1593.2), derived from human pleural effusion (histiocytic lymphoma monocyte) o REH (acute lymphoblastic leukemia (ALL), B-cell). First, the infection was standardized with norovirus isolated from stool sample NoVGII (Table 1). In to a 96-well plate 5 x 10^4 U937 or REH cells were seeded in 100 µl of RPMI and infected with 10 µl of NoVGII suspension activated with trypsin with different dilutions (1:12.5, 1:25, 1:50, 1:100) and incubated for 36 h at 37ºC in incubator with 5% CO_2. The infection was evaluated by IFA. Villi cells inoculated with rotavirus Wt1-5 as control, were infected at 1:50 dilution. The REH and U937 tumor cell lines were also infected with rotavirus, adapted to these tumor lines (Guerrero *et al*, 2016).

Labeling cells with uridine analogs. The intestinal villi-enriched preparation was plated in 96-well cell culture plates with 100 µl of MEM without FBS. The villi were infected with NoVGII stool sample in dilution 1:25 as described above, and incubated at 37°C for 20 min. The cells were cultured 12 h, harvested and incubated for 1 h with 1 mM of 5-ethynyl uridine (EU; Invitrogen) in MEM. The cells were fixed using a 4% paraformaldehyde (PFA) solution in PBS. The fixed villi were washed with PBS and permeabilized using 0.1% Triton X-100 for 10 min at room temperature. EU labeling of villi was visualized according to the manufacturer's instructions (Invitrogen; Click-iT RNA imaging kits). Briefly, the samples were incubated with a 1× working solution of Click-iT reaction cocktail, containing Alexa Fluor 594, for 30 min at room temperature. The cells were washed once with Click-iT reaction rinse buffer, followed by wash with PBS. Cells were incubated for 15 min in blocking buffer (PBS 10% normal goat serum), followed by 60 min of incubation with 0.2 µg/ml anti-VP1 monoclonal antibody. RNasin (0,125 U/ml) was added to all incubation and washing steps. After washing the cells with PBS containing 0.05% Tween 20, the cells were incubated for 60 min with 0.4 µg/ml HRP-conjugated goat anti-mouse antibody or BP-CFL 488-conjugated anti-mouse antibody for 20 min. After four washes, the samples were mounted on glass slides in glycerol (70% in PBS). The samples were examined using a transmitted light or fluorescence microscope (VanGuard, Nova-Tech International, Inc., USA). The different fluorescent signals were determined using the JACoP plugin within the ImageJ processing and analyses software, version 1.43 (Ferreira and Rasband, 2010).

Table 1. Pathogen detection from human faeces samples using monoclonal antibodies and real time PCR

N° stool sample	Age	Immuno-dot blot assay			RT-qPCR (GC.g^{-1} human stool)		qPCR (GC.g^{-1} human stool)	
		RV-A (+)/(-)	HuNoV (+)/(-)	AstV (+)/(-)	NoVGII	RV-A	*Shigella* spp.	*Cryptosporidium* spp.
1*	4	(-)	(+)	(-)	$2.8\,10^{+2}$			
2	4	(+)	(+)				$2.1\,10^{+3}$	
3	4.2	(-)	(-)					
4	0.91	(-)	(-)				$3.1\,10^{+4}$	
5	1.6	(-)	(-)					
6	2	(-)	(-)					
7	2	(-)	(-)				$1.4\,10^{+3}$	
8	3	(-)	(-)					
9	4	(+)	(-)				$5.4\,10^{+5}$	
10	3	(-)	(-)					
11	4.96	(-)	(-)				$2.0\,10^{+5}$	
12	3	(+)	(+)				$4.4\,10^{+3}$	
13	5	(-)	(-)					
14	0.08	(-)	(-)				$4.8\,10^{+4}$	
15	3	(-)	(-)					
16	3	(+)	(+)		$4.9\,10^{+5}$			
17	2	(-)	(-)					
18	2	(-)	(-)					
19	2	(-)	(-)				$1.3\,10^{+5}$	
20	2	(-)	(-)					
21	2	(-)	(-)					
22	4.96	(-)	(-)				$5.7\,10^{+4}$	
23	5	(-)	(-)					
24	1	(-)	(-)			$7.7\,10^{+4}$		
25	1	(-)	(-)				$1.3\,10^{+5}$	
26	3	(-)	(-)				$1.5\,10^{+3}$	
27	0.67	(-)	(-)				$4.3\,10^{+5}$	
28	1	(+)	(-)					
29	3	(-)	(+)				$2.6\,10^{+8}$	
30	1	(-)	(-)				$9.4\,10^{+3}$	
31	4	(+)	(-)					$1.8\,10^{+2}$
32	0.92	(+)	(-)			$5.7\,10^{+5}$	$2.3\,10^{+5}$	
33	2	(+)	(-)			$7.5\,10^{+2}$	$8.0\,10^{+8}$	
34	3	(-)	(-)				$4.4\,10^{+4}$	
Elderly 1	83	(+)	(-)				$3.4\,10^{+3}$	
Elderly 2	81	(+)	(-)				$9.7\,10^{+6}$	
Elderly 3	87	(+)	(-)				$1.2\,10^{+4}$	

The pathogen concentrations in faeces samples (GC.g^{-1}) are given when they are above the detection threshold (about 300–1200 GC.g^{-1} human faeces). *: Sample used for trypsin concentration standardization for HuNoV activation and for NoVGII replicative cycle study.

Antisera preparation. Hartley guinea pig were subcutaneously immunized with 1 ml of a Freund's incomplete adjuvant emulsion containing CsCl (1 mg/ml) purified NoVGII from an air sample of a house terrace (Table 2). The emulsion was previously boiled for 5 min in a water bath, and then mixed with FIS peptide (FISEAAIIHVLHSR) (0.5 mg/ml) as an immunomodulatory agent (Prieto *et al*., 1995). The same amount of each antigen emulsified in Freund's incomplete adjuvant was inoculated to guinea pig twenty and forty days later. Bleeding of guinea pig was performed on day 60 after the first injection. Sera containing sodium azide (0.04%) and diluted two-fold with glycerol were kept at -20°C. Control pre-immune sera were collected before immunization. (Number of permission 008-071-16).

Immunocytochemistry and immunofluorescence. Villi infected with HuNoV were harvested and fixed in suspension with methanol-acetic acid (3:1 v/v) for 20 min at 0°C. After washing with PBS three times, fixed cells were placed onto coverslips and allowed to dry for 1 h at 50°C. Guinea polyclonal antibodies (1:250; 1:500, 1:1000, 1:2,000, 1:4,000 dilution) against norovirus or 0.2 µg/ml of anti-VP1 monoclonal antibody were added. Following incubation for 1 h at 37°C, the cells were washed twice with PBS and incubated with 0.4 µg/ml HRP-conjugate appropriate secondary antibody Peroxidase activity was revealed with aminoethylcarbazole substrate (Sigma). Ten representative fields on coverslips were acquired at 40× magnification, and red-stained cells were recorded as positive. A double-blind counting of cells was carried out, and the proportion of positive cells was presented as the percentage of total cells observed. For IFA, the villi were fixed and permeabilized with methanol-acetic acid. Blocking was performed with 1% BSA in PBS for 1 h at 37°C. Permeabilized villi were incubated with primary monoclonal antibodies Norwalk virus (1D9) (0.2 µg/ml) or guinea pig polyclonal antibodies (1:250; 1:500, 1:1000, 1:2,000, 1:4,000 dilution). Villi were washed three times with PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG secondary antibody (0.4 µg/ml) for 20 min at 4°C. Coverslips were mounted onto glass slides and observed in light microscope (VanGuard) and the percentages of immunoreactive cells were determined as mentioned above. To eliminate autoflorescence, Sudan Black 0.1% was used in 70% ethanol, applied for 5 min after the primary antibody (Oliveira *et al*., 2010), then the villi were washed 3 times with PBS. Additionally, the cell nucleus was labeled with 1 µg/ml DAPI.

U937 or REH cells infected with norovirus were harvested after 36 h.p.i. and fixed with paraformaldehyde at 4% for 20 min at 20°C. After PBS washing, fixed cells were placed onto coverslips and allowed to dry for 1 h at 50°C. Then 0.2 µg/ml of anti-VP1 monoclonal antibody was added, following by incubation for 1 h at 37°C, the cells were washed with PBS and incubated with HRP-conjugate-goat anti-mouse secondary antibody. Peroxidase activity was revealed with aminoethylcarbazole substrate (Sigma). Ten representative fields on coverslips were photographed at 40× magnification, and red-stained cells were recorded as positive. A double-blind counting of cells was carried out, and the proportion of positive cells was presented as the percentage of total cells observed. For phycoerythrin staining, anti-mouse labeled phycoerythrin secondary antibody was used. The percentages of immunoreactive cells were determined. As a positive control of the infection, the same tumor lines were infected with rotavirus Wt1-5 in a multiplicity of infection (MOI 0.8), adapted to those tumor cell lines (Guerrero *et al*., 2016). As another control, we used isolated villi infected with 1:50 dilution of norovirus. Villi were harvested at 12 h.p.i. and then collected and examined by ICC for the percentage of villus cells positive to norovirus structural antigens, using 0.2 µg/ml of anti-VP1 monoclonal antibody.

Virus purification by cesium chloride. NoVGII from a house terrace air sample (Table 2), coming from the fourth infection passage and villi obtained from three mice, was purified from the intestinal villus-enriched preparation, mixed and collected in a single 60 ml bottle of culture medium, showing a complete cytopathic effect. The lysate was emulsified by vortexing with one-third volume of trichlorothrifluoroethane (Sigma); the phases were separated by low-speed centrifugation. The organic phase was extracted three times with an equal volume of Tris-buffered saline (TBS) (10 mM Tris–HCl pH 7.4, 15 mM NaCl, 1 mM $MgCl_2$ and 5 mM $CaCl_2$); the aqueous phase was centrifuged through 1 ml 30% (w:v) sucrose in TBS at 150,000 x *g* (Sorvall TST 60.4 rotor) for 1.5 h at 4°C. Viral particles were resuspended in TBS and then layered onto a discontinuous gradient consisting of 1.4157 g/cm^3 (0.5 ml), 1.3039 g/cm^3 (1 ml) and 1.2070 g/cm^3 (0.5 ml) CsCl and an upper layer of 30% (w:v) sucrose. Isopycnic centrifugation was carried out in a Sorvall TST 60.4 rotor at 280,000 x *g* for 1.5 h at 4°C. Visible band was collected and then diluted with TBS before pelleting at 100,000 x *g* for 1.5 h at 4°C. Virus pellet was resuspended in PBS.

ELISA. In order to determine NoVGII infection curve in isolated mouse gut villi, we used indirect ELISA. Each well of a 96-well ELISA plate was coated with cell lysate samples of norovirus (400 ng/well/100 µl) for 2 h at 37°C. Wells were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with 5% skimmed milk in PBST. Anti-norovirus monoclonal antibody was used as primary antibody and HRP-conjugated goat anti-mouse as secondary antibody. The reaction was developed using ortho-phenylenediamine (OPD) (1 mg/ml) (Pierce) and H_2O_2 (0.04%) in 100 mM sodium citrate buffer, pH 5.0. Similarly, 96-well ELISA plates were coated with polyclonal guinea pig-antibodies (1:1000 dilution) for 12 h at 4°C. Coated plates were blocked with 5% skimmed milk in PBS for 1 h at 37°C. The norovirus-infected villi and non-infected villi were frozen and thawed twice prior to being lysed in modified RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X1 000, 1.0% Nonidet P-40, 0.5% DOC, and 50 mM Tris-HCl, pH 7.5). Villus lysates were added to wells (400 ng/well/100 µl); plates were incubated for 2 h at 37°C and then washed three times with

PBST, before adding 0.2 µg/ml of anti-VP1 monoclonal antibody. After three PBST washes, a bovine anti-mouse HRP-conjugate was added to plates and incubated for 1 h at 37°C. Plates were washed three times with PBST and developed by OPD (Pierce, USA) peroxidase substrate (0.4 mg/ml) in 0.1 M citrate buffer, pH 5.0. Absorbance was read at 492 nm after 20 min. Absorbance values corresponding to cell lysate without virus antigen were subtracted from all norovirus-infected sample readings. The results were presented as the mean of duplicates; all experiments were repeated three times.

Cell viability test. Villus cell viability was measured using Trypan blue solution (Sigma–Aldrich) in an exclusion assay. The norovirus-infected villi and non-infected villi were assessed after different post inoculation times. Trypan blue (0.4% in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic) solution was combined with intestinal isolated villi suspension in a 1:1 ratio, and the percentage of dead blue cells in the population was quantified in a Neubauer chamber under a light microscope.

Western blotting. Gradient purified norovirus suspension was treated with trypsin. As a control, a second aliquot was left without treatment. Rotavirus Wt1-5 inoculated villi were used as positive control. The samples were mixed with Laemmli buffer and separated on a 12% SDS-PAGE gel. PVDF membrane was probed with 0.2 µg/ml of anti-VP1 monoclonal antibody followed by incubation with HRP-conjugated bovine anti-mouse antibody. Peroxidase activity was revealed with aminoethylcarbazole substrate.

Statistical analysis. Results are expressed, as mean ± SE; n refers to the number of experiments performed in each analysis generally in triplicate and repeated twice. We used Students'*t*-test of two-tailed for comparisons and statistical significance with the value of $P<0.05$ indicating a significant difference.

Results

HuNoV detection from human stools and environmental samples

Using the immunodot-blot assay with a specific monoclonal antibody, we analyzed 37 faeces samples (including 34 samples from children <5 years and 3 from elderly >75 years). Thirteen of these samples were positive for enteric viruses, from which 8 were RV-A only, 2 HuNoV only and 3 were both viruses. Performing subsequent real-time PCR assays to identify HuNoV by genome group, we observed that of 5 samples were positive for HuNoV by immunodot-blot, 2 contained genome copies (GC) of NoVGII above the calculated detection threshold (300–1200 $GC.g^{-1}$ human stool), while three of them did not reach the threshold for neither NoVGI nor NoVGII. Of the 2 positive samples for NoVGII, one did not contain other tested viruses when testing with immunodot-blot, i.e. RV-A and AstV. This sample was used to standardize trypsin concentration to activate HuNoV, and also, in replicative cycle study in isolated villi of mouse intestine. Table 1 presents the different pathogens analyzed in the 37 stool samples using immunodot-blot, qPCR and RT-qPCR assays. We estimate a threshold of Ct <37 to distinguish NoV-positive stool samples. We evaluated the validity of the threshold through comparisons with NoV antigen immunodot-blot results (Table 1).

When evaluating the 54 samples obtained from different environmental matrices (water, plants and air) by RT-qPCR and immunodot-blot, we found 40 HuNoV-positive samples, including 26, 10 and 4 samples positive by immunodot-blot only, RT-qPCR only and both methods, respectively (Table 2). We estimated a threshold of Ct <37 to distinguish NoV-positive environmental matrices (water, plants and air). We evaluated the validity of the threshold through comparisons with NoV antigen immunodot-blot results. Of the 14 samples, that were HuNoV-positive by RT-qPCR, 6 samples were positive for NoVGI only, 6 for NoVGII only, and 2 for both viruses. Out of the 54 environmental samples tested by immunodot-blot for HuNoV, 45% (13/29), 80% (12/15) and 50% (5/10) were positive during low, medium and high rainfall periods, respectively (Table 2).

In the area where this research was conducted, there is a sanitary landfill (called Doña Juana), where about 8.6 million inhabitants of the Bogotá city and 17 surrounding municipalities (Rojas *et al*., 2015) dump all their waste including dirty disposable diapers of children and adults and the used toilet paper generated by the inhabitants of this region (Jordan-Lozano, 2020). In turn, leachate from this landfill previously treated is discharged into nearby Tunjuelito river (Jordan-Lozano, 2020) and landfill gases are emitted in air ambient containing potentially contaminated biological material. For these reasons, HuNoV presence from different environmental matrices (water, air and vegetables) was evaluated by immunodot-blot immunological test with respect the distance to sanitary landfill. 61% of the samples (25/41) were positive for immunodot-blot within 3 km radius of the landfill which included air of the landfill, housing terraces and from leachate treatment plant, waters from Tunjuelito river, stormwater combined with wastewater, leachate treated in wastewater plant, drinking water from a school and chards grown near to the landfill, while 38% (5/13) were positive for immunodot-blot in sources located beyond (Corabastos wholesale market and La Ramada rural area) with norovirus contamination in irrigation water and chard-leaves on cultivation, harvest, transported in truck and wholesale market.

Culture and norovirus infection in isolated villi

HuNoV infects and replicates in the intestine, so for this reason we explored whether the model described for rotavirus (Guerrero *et al.*, 2010) was useful for adequately infecting and replicating human norovirus, obtained from various sources: faeces, air, water and vegetables. Initially, the model of isolated mouse villi was explored, infecting these with HuNoV from human feces and analyzed by the IFA (Fig. 2b). Different concentrations of trypsin were used to determine the ideal concentration to activate norovirus, finding that from 10 µg/ml there was no difference in the infective capacity (Fig. 2c). Similarly, an exclusion of blue trypan of about 50% at 24 h of culture in the non-infected and 35% in the infected samples was found in the isolated villi, both infected and non-infected (Fig. 2d). Cell viability test showed a degeneration of infected villi cells to a lesser extent compared to non-infected cells; this allows us to deduce that the villi are viable for a sufficient time to allow replication of the norovirus. In our study, the infected cells were 100% viable at 10 h and decayed to 40% at 24 h suggesting at least two replicative cycles of human norovirus in isolated villi of mouse as explained in section 3.3.

Subsequently, to confirm that the isolated mouse gut villus model is suitable for infecting and replicating HuNoV, the villi were infected with 10 µl of HuNoV isolated and concentrated from each of the environmental sources, as described in materials and methods. The infection was evaluated by ICC and IFA, using specific monoclonal antibodies. All isolated viruses were found to adequately infect villi, with an infection rate of 75%–90% when tested by ICC, showing high levels of HuNoV saturation with MOI >1 in samples of cultivated and harvested chard irrigated with wastewater, rainwater contaminated with wastewater and air from a sanitary landfill (Fig. 3a,b).

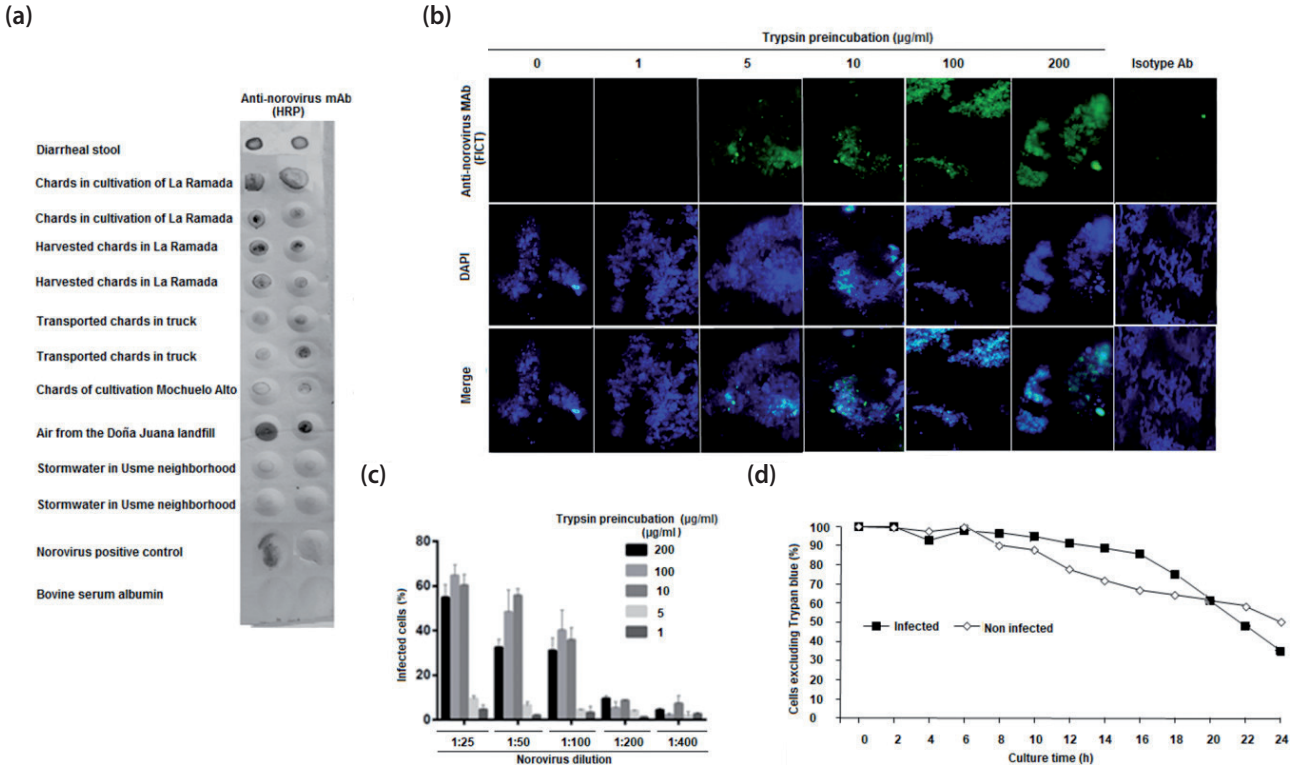

Fig. 2

Human norovirus detection in faecal and environmental samples and standardization of the infection in isolated villi

(a) The infection was standardized with NoVGII isolated from a diarrheal stool that was negative for NoVGI, RV-A, AstV, *Shigella* spp., *Salmonella* spp. and *Cryptosporidium* spp. HuNoV were detected on environmental samples. **(b)** Illustration of isolated villi from intestinal sections (duodenum, jejunum and ileum) seeded into 96-well culture plates, inoculated with trypsin-activated norovirus at different concentrations in a dilution 1:25. Villi were harvested at 12 h.p.i. The samples were examined using a fluorescence microscope. **(c)** Villi were collected and examined by ICC, for the percentage of villus cells positive to norovirus structural antigens. Infection was expressed as the percentage of cells being positive to norovirus structural antigens. Error bars represent the standard error of the means from triplicates (n = 3 wells). Mock-inoculated villi were used as control. **(d)** Villus cell viability was measured using Trypan blue solution in an exclusion assay in a Neubauer chamber.

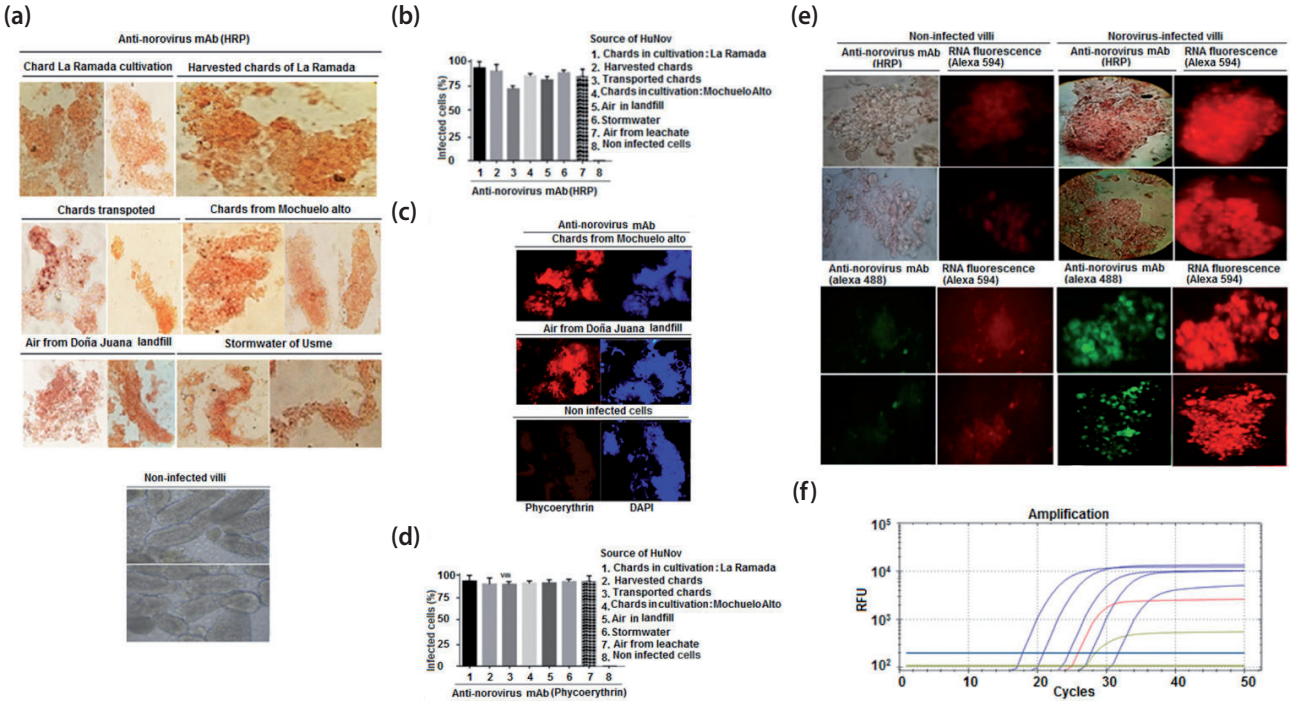

Fig. 3

Isolated mouse intestinal villi infected with HuNoV isolated from water, plants, and air in dilution 1:25

(a-b) Isolated villi from intestinal sections were seeded into 96-well culture plates and inoculated with trypsin-activated norovirus. Villi were harvested at 12 h.p.i. and then collected and examined by ICC for the percentage of villus cells positive to norovirus structural antigens. Representative fields on coverslips were photographed at 40× magnification, and red-stained cells were recorded as positive. Mock-inoculated villi were used as control. **(c-d)** Villi were collected and examined by IFA for the percentage of villus cells positive to norovirus structural antigens. Mock-inoculated villi were used as control. Error bars represent the standard error of the means from triplicates (n = 3 wells). **(e)** Norovirus - or mock-infected villi after 12 h were fed with 1 mM of 5-ethynyl uridine (EU). **(f)** RT-qPCR detection of newly synthesized viral RNAs. In red, NoVGII detection from 5 µl of infected villi. In blue, dilutions of standard curves; in green, internal extraction control amplification.

Similar results were obtained when tested by IFA, with a percentage of villus infection near or exceeding 90% for noroviruses from all sources examined (Fig. 3c,d). To add, effectiveness of the mouse intestinal villi model for noroviruses including both NoVGI and NoVGII was for all sources, because both genotypes were present in the viral isolates from all the samples (Table 2).

We analyzed the newly synthesized viral RNAs by metabolic labeling of villi with the uridine analog EU. To this end, villi were either infected with norovirus or mock non-infected during 12 h at 37°C. Villi were incubated with 1 mM EU for 1 h, fixed and prepared for detection of the incorporated alkyne-modified uridine analog by using click chemistry with azide-derivatized Alexa 594 fluorophores. As shown in figure 3e, in mock non-infected villi, EU was incorporated into RNA in low quantity, compared to infected villi. In infected cells, EU was incorporated into perinuclear cytoplasmic foci, besides the nuclei. Apparently, this cytoplasmic staining corresponds to newly synthesized viral RNA. Viral RNAs readily were detected after 60 min of labeling, which resulted in a staining pattern that was still very similar to the one obtained for nascent viral RNA in SARS-CoV-infected cells (Hagemeijer *et al.*, 2012). After this step, samples were washed with PBS and were incubated with anti-norovirus mAb. The cells were washed with PBS, and incubated with HRP-conjugated goat anti-mouse antibody or BP-CFL 488-conjugated anti-mouse antibody. The peroxidase activity was revealed with aminoethylcarbazole substrate. The samples were examined using a transmitted light or fluorescence microscope. Equally, we analyzed the newly synthesized viral RNAs by RT-qPCR. We estimated a threshold of Ct <25 to distinguish NoV-positive villi culture (Fig. 3f).

Two tumor cell lines (REH and U937) were also tested for HuNoV replication. For this purpose, 50,000 cells from the tumor lines in 100 µl of MEM without FBS were infected with HuNoV isolated from feces (stool sample 1 in Table 1), in dilutions 1:12.5, 1:25, 1:50, 1:100. Nevertheless, in dilution 1:12.5, the infection percentage

was very low for each tumor cell line, around 5% for U937 and 3%–10% for REH (Fig. 4a-c). As a positive control of the infection, the same tumor lines were infected with rotavirus Wt1-5 in a multiplicity of infection 0.8, adapted to those tumor cell lines (Guerrero *et al*., 2016) presenting an infection of 70% for REH and 75% for U937. As another control, we used isolated villi infected with 1:50 dilution of norovirus, which presented an infection of around 60% as evaluated by ICC, using anti-VP1 monoclonal antibody.

NoVGII growth curve in isolated intestinal villi

In order to determine the infectious curve of NoVGII in the isolated villi of mouse intestine, these were infected with a 1:50 dilution and harvested every 2 h. NoVGII was added to the cells and they were harvested at 0, 2, 4, 6, 8, 10 and 12 h.p.i. After incubation, the supernatant was removed, and cells were exposed to 3 cycles of freezing and thawing for lysis. The lysate or the supernatant harvested every 2 h was trypsin activated, added to new villi and harvested after 12 h.p.i. The infection was evaluated by ICC (Fig. 5a) and by indirect ELISA (Fig. 5b), using monoclonal antibody in both assays. By ICC a rising peak is observed up to 8 h.p.i., and it is stabilized at 12 h.p.i. By indirect ELISA, the rising peak is observed up to 10 h.p.i., both in the supernatant and in the cell pellet lysate. These results suggest that replicative cycle of HuNoV in isolated villi from wild mouse is between 8–12 h, with an average of 10 h and that the virions are released from enterocytes.

To determine that NoVGII HuNoV grown repeatedly in the mouse isolated villus model maintains its characteristics, it was analyzed by electrophoresis and western blotting. Rotavirus Wt1-5 inoculated villi were used as control. NoVGII was found to have two characteristic

Table 2. Number of environmental samples that were positive by immunodot-blot and/or RT-qPCR for the presence of HuNoV

Source	Low rainfall months*				Medium rainfall months*			High rainfall months*		
	HuNoV	NV GI [Ct]	NVGII [Ct]	NVGI & NVGII [Ct]	HuNooV	NVGI [Ct]	NoVGII [Ct]	HuNoV	NVGI [Ct]	NVGII [Ct]
	----------RT-qPCR (Immunodot-blot)----------									
Tunjuelito river water	1+2 (1+2)		1 (0) [34]	1 (0) [35] & [34]	5 (5)					
Stormwater	+1 (+1)		2 (0) [34] [34]	1 (0) [36]& [35]	3+1 (3+1)		1 (0) [34]			1 (0) [35]
Irrigation waters		1 (1) [17]						1 (1)		
Tap water	1 (1)									
Leachate treated in wastewater plant	2 (2)									
Chards during cultivation in farms		1 (0) [25]				1 (1) [24]		1 (1)		
Harvested chard leaves		1 (1) [27]			1 (1)					
Transported chard leaves in truck	1 (1)					1 (1) [24]				
Chard leaves from wholesale market								1 (1)		
Air from Doña Juana landfill		1 (0) [33]						1 (1)		
Air from leachate treatment plant	1 (1)									
Air from housing terraces	2 (2)		1 (0) ** [36]					1 (1)		

Outside parentheses, number of samples corresponding to none, one or two viruses detected by RT-qPCR; in parentheses, number of positive samples for the immuno-dot blot. Underlined numbers are for samples that did not infect mouse villi. *: Rainfall data month from Kennedy meteorological station. The thresholds between low, medium and high precipitation months are 70 and 140 mm.month^{-1} before sampling, respectively **: Sample used to antisera preparation and for polyclonal antibodies generation.

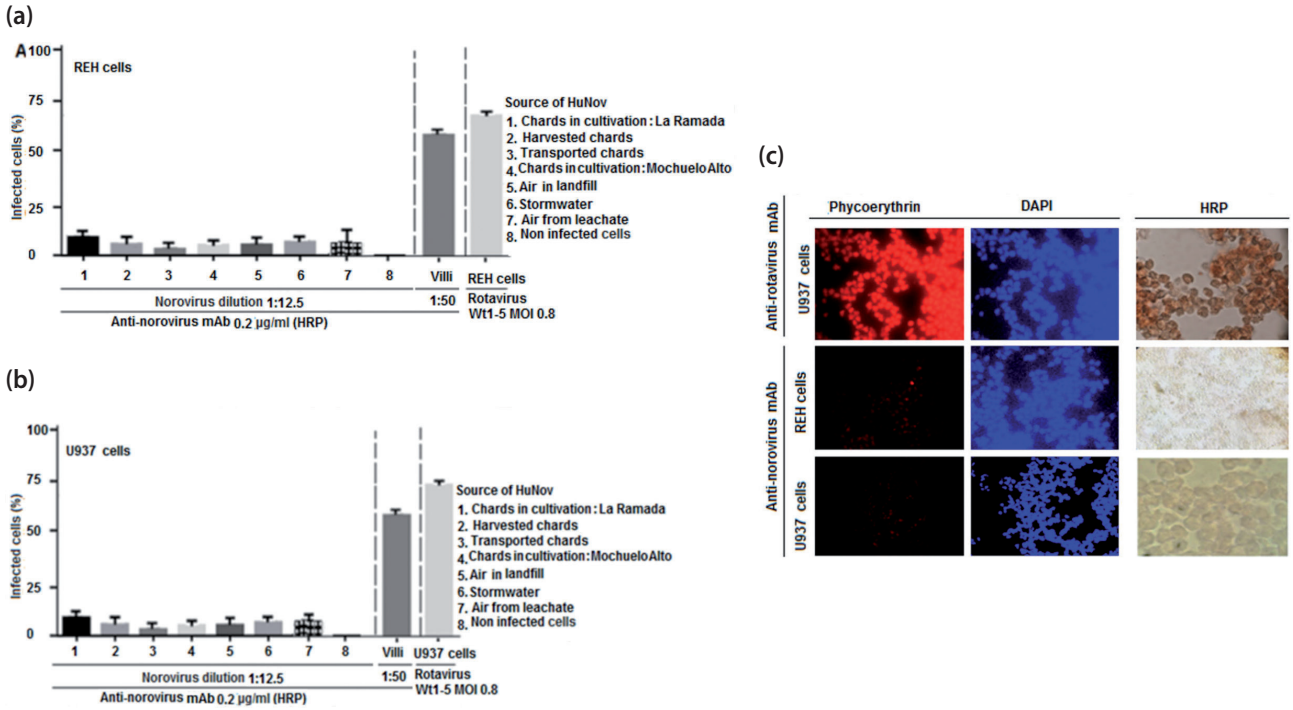

Fig. 4

REH or U937 cells do not allow norovirus infection

REH or U937 cell lines were inoculated with trypsin-activated norovirus. **(a-b)** Cells were harvested at 36h.pi., collected and examined by ICC for the percentage of cells positive to norovirus structural antigens. Villi cells or U937 cells were inoculated with norovirus (1:50 dilution) or rotavirus (MOI 0.8) respectively, as control. Error bars represent the standard error of the means from triplicates (n = 3 wells). **(c)** Illustration of tumor cell lines positive to norovirus or rotavirus Wt1-5 examined by IFA. Phycoerythrin/red – norovirus, HRP – rotavirus.

bands of 58 and 38 kDa after trypsin treatment (Fig. 5c) and rotavirus antigens were not detected.

Isolated villi from mouse intestine produce a good amount of HuNoV infectious virions

We sought to have enough virus for CsCl gradient purification and to carry out biochemical studies or to generate polyclonal antibodies. For this purpose, NoVGII isolated and concentrated from house terrace air sample (Table 2) was used to infect isolated intestinal villi from mouse. The NoVGII obtained in the fourth passage was used to infect isolated villi from three mice, collected in a single vial with 60 ml of culture medium. The villi were cultured until complete lysis of all cells, that was achieved in approximately 36 h. The lysates were emulsified with trichlorothrifluoroethane and purified by means of CsCl. The recovered NoVGII was used to generate polyclonal antibodies in guinea pig. The generated antibodies recognize HuNoV isolated from different environmental sources and were titrated in ICC assay with 1:4,000 dilution (Fig. 6a) and capture ELISA (Fig. 6b) with 1:1,000 dilution.

Discussion

HuNoV is a highly contagious virus, and the studies of these viruses has been hampered by the lack of *in vitro* culture methods or animal models, even though its discovery was in 1972 by immunoelectron microscopy (Kapikian *et al.*, 1972). Most routine cell cultures lack the characteristics of human intestinal epithelial cells. However, different gut-related lines have been tested as differentiated Caco-2 cells (White *et al.*, 1996) that resemble mature enterocytes, express H antigen, and were derived from an individual with 0 blood type (Amano and Oshima, 1999). We assume that successful replication of HuNoVs *in vitro* depends on finding a method that accurately mimics the receptors and basic cell biochemistry present on intestinal epithelial cells.

Based on the current understanding of HuNoV binding and replication sites *in vivo* (Bhar and Jones, 2019; Oka *et al.*, 2018; Ettayebi *et al.*, 2016; Marionneau *et al.*, 2002; Dolin *et al.*, 1975), we believe that the model that is closest to *in vitro* cell culture, which mimics *in vivo* conditions, are isolated villi of mouse small bowel. So far, there are no reports of a model that allows successful replication

of HuNoV collected from diverse sources such as air, water, or plants. None of the cell culture combinations reported so far have been successful in *in vitro* replicating of HuNoV. For this reason, attempts to detect and isolate environmental viruses have been limited to biochemical detection, using techniques such as immunoassays or RT-PCR, but without determining whether the virus is biologically active or infectious (Rodríguez *et al*., 2009; Duizer *et al*., 2004; Karim and LeChevallier, 2004). Using isolated villi from mouse small intestine, we found that 36 environmental samples and 5 from human faeces were positive. Of these, 22 environmental samples were positive for HuNoV by immunodot-blot assay only, 10 by RT-qPCR only, and 4 by both assays. On the other hand, the 5 human feces samples were all positive for HuNoV presence by immunodot-blot assay, of which 2 were subsequently confirmed for NoVGII with RT-qPCR. The most important thing to highlight is that the great majority of the samples (41/45 samples) was detected as positive by immunodot-blot or RT-qPCR, presented biologically active virions.

To visualize viral RNA synthesis in infected cells, in the present study we have employed novel method suitable for the detection of nascent RNAs, based on the biosynthetic incorporation of an alkyne-modified EU, followed by click chemistry to attach fluorescent azide derivatives to the synthesized RNA. We show that this method can be combined with the detection of viral proteins by using ICC or IFA. Our results indicate that foci of EU labeling correspond with active sites of norovirus RNA synthesis during the infection. Merged IFA images of viral proteins and EU labeled RNA show colocalization. The incorporation of EU into RNA in the infected cells suggests that this cytoplasmic staining corresponds to newly synthesized viral RNA as it coincides with the presence of the norovirus structural protein VP1. We used this assay because of its reliability and low cost compared to the RT-qPCR. However, we also analyzed the levels of RNA in the infected villi using the RT-qPCR. We did not use RT-qPCR for all samples due to limited economic resources.

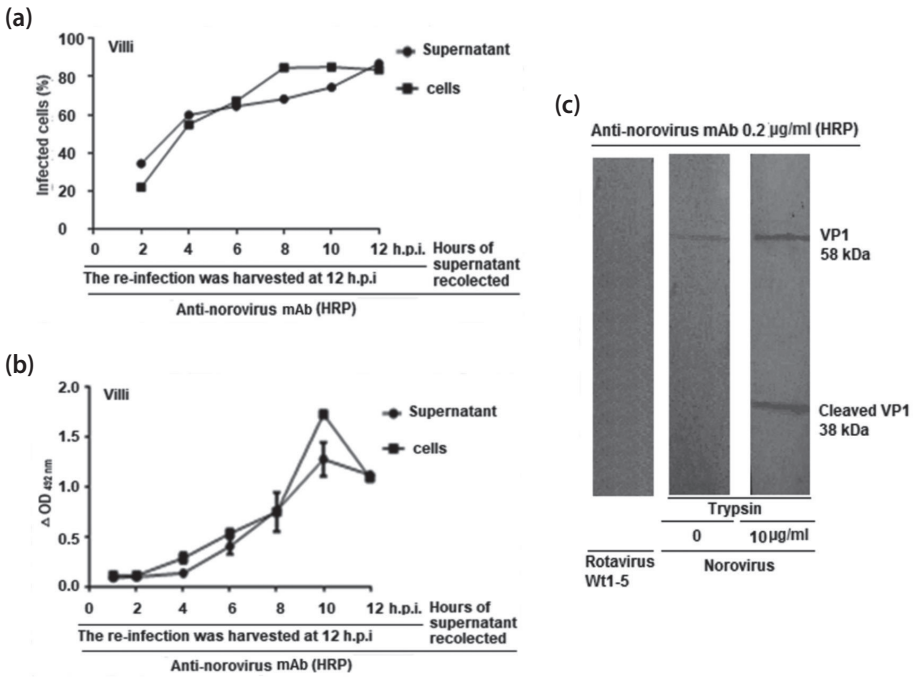

Fig. 5

Growth curve of the norovirus in isolated intestinal villi and viral protein analysis

The infection was evaluated with NoVGII isolated from stool sample N° 1 (Table 1). The isolated villi of mouse intestine were infected with trypsin activated NoVGII (1:50 dilution) and harvested every 2 h (0, 2, 4, 6, 8, 10 or 12 h.p.i.). The lysate or the supernatant harvested every 2 h was trypsin activated, added to new villi and harvested after 12 h.p.i. The infection was evaluated by ICC, ELISA assay and western blotting. **(a)** ICC for the percentage of villus cells positive to norovirus structural antigens. Infection was expressed as the percentage of cells being positive to norovirus structural antigens after challenge with supernatants or cell pellet lysates. Mock-inoculated villi were used as control. **(b)** Villi were collected and examined by indirect ELISA for the percentage of villus cells positive to norovirus structural antigens in supernatant or in cell pellet lysate. Mock-inoculated villi were used as control. Error bars represent the standard error of the means from triplicate samples (n = 3 wells). **(c)** Western blotting with anti-VP1 monoclonal antibody. Rotavirus Wt1-5 inoculated villi were used as control.

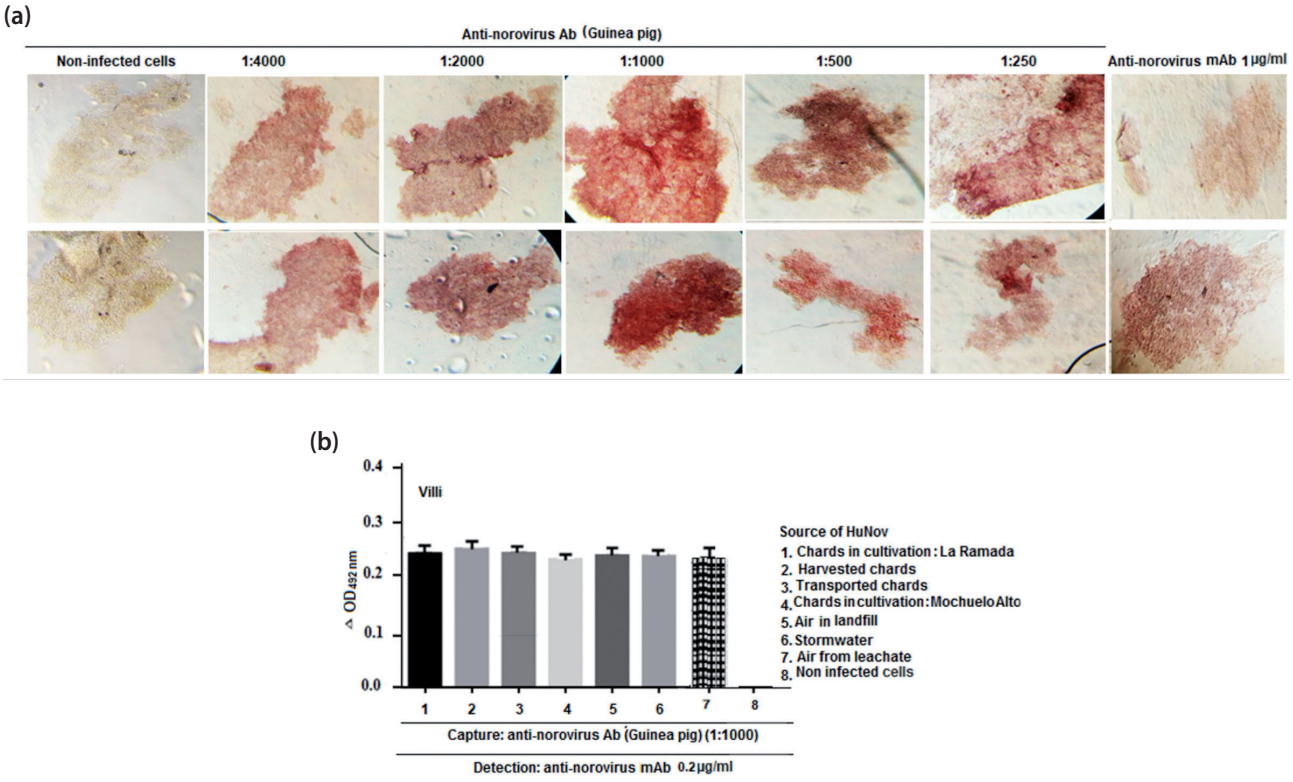

Fig. 6

Characterization of antibodies generated from harvested norovirus in isolated mouse intestinal villi and purified by CsCl
NVGII was isolated and concentrated from the air, amplified and obtained from the fourth passage in isolated mouse intestinal villi. Norovirus was purified by of CsCl gradient. Hartley guinea pig were subcutaneously immunized three times with 1 ml of a Freund's incomplete adjuvant emulsion containing the purified norovirus. **(a)** Different concentrations of monoclonal antibody stained with peroxidase. Representative fields were photographed at 40× magnification. **(b)** ELISA plates were coated with polyclonal guinea pig-antibodies against norovirus particles, villi lysates were added to wells, incubated and stained with anti-VP1 antibody. Absorbance values corresponding to samples without virus antigen were subtracted from all norovirus-infected samples. The results were presented as the mean of duplicates; all experiments were repeated three times.

The isolated villus model of the mouse small intestine is 100% effective and efficient to produce acute infection and to generate virions of human origin. This suggests that the receptors and basic cellular biochemistry necessary for efficient entry and replication of human norovirus are present in the mouse villi cells. The various combinations of cell cultures reported so far have probably not been as successful in *in vitro* norovirus replication, because it is difficult to determine which steps are critical for norovirus replication. Specific characteristics of host cells, the virus, or both are probably required. Perhaps, for this reason, we were not successful in trying to replicate norovirus in tumor cell lines such as U937 or REH. However, successful cultivation of multiple strains of NoV in enterocytes, in cultures of unprocessed human intestinal monolayers, derived from stem cells was reported (Ettayebi *et al*., 2016). In this model, bile was a critical factor needed for strain-dependent NoV replication and these authors are of the opinion that the lack of adequate expression of histo-blood group antigens (HBGA) in intestinal cells restricts virus replication. HBGAs (complex sugars) are now considered as attachment factors facilitating the infection of HuNoV into the host cell (Ettayebi *et al*., 2016; Hutson *et al*., 2002; Marionneau *et al*., 2002). Considering that both NoVGII and NoVGI genotypes from the environmental samples infected the villi cells, we suggest that our model is able to be recognized by both HBGAs receptors and the infection can be established by each or both genotypes. In our model, it was not necessary to use any reagents, except trypsin to activate the virus for successful infection. This suggest that cellular HBGAs receptors recognize both, NoVGII and NoVGI (from fecal or environmental samples), when viral antigen epitopes are exposed by the cleavage with trypsin in the major capsid protein facilitating the infection mediated by exosomes (i.e. ex-

tracellular vesicles whose size varies from 50 to 150 nm) carrying viral particles or NoV RNA (Chassaing, 2021). In addition, fluid-stable exosomes could allow NoVs to remain immunologically undetected and to aggregate increasing the infection probability (Todd and Tripp, 2020; Santiana *et al*., 2018; Koga *et al*., 2011). However, this was not analyzed in our study. Although we did detect infectious particles in similar amounts in the culture supernatant and cell pellet, suggesting that the virions are constantly released from the infected cells, probably due to cell lysis. In our study, the immunodot-blot assay resulted in a higher prevalence of human noroviruses in the environmental and human samples. Immunological assays are very sensitive for the full range and diversity of norovirus genotypes present in the sample, whereas RT-qPCR is more sensitive and specific for a given viral genotype (Chassaing, 2021). It has been reported that immunological assays did not correlate with RT-qPCR (Costantini *et al*., 2010). Thus, our study suggests that the immunological assay presented higher sensitivity to human noroviruses by revealing the two genotypes (NoVGI-NoVGII) present in the viral isolate. However, we do not rule out that the lower sensitivity obtained with the molecular technique for genome detection could have been affected by the presence of PCR inhibitory compounds. A similar situation was found with rotaviruses, which were also effectively detected in human feces with the immunological assay.

We observed, that some isolates showed longer period to lyse the cells (12–24h) and four norovirus positive isolates did not infect the mouse intestinal villi cells, probably due to the reduced number of virions present in the source material. Another possibility is that the samples did not have virions with infectious capacity. A disadvantage of this model is that the number of cells in each villi cannot be accurately quantified. Two hours after the infection of intestinal villi cells, the cells detach from the matrix leading to anoikis. This drawback makes it difficult to calculate the number of particles generated or the multiplicity of infection (MOI). This difficulty can lead to the administration of excessive amounts of the virus in the crop, saturating the system, as shown in Figure 6b. However, the infection of the villi allowed successful passages of viral replication and the purification of good amounts of virus using $CsCl_2$ gradient.

The air sample from house terrace, NoVGII, used to generate polyclonal antibodies in our study, could have been carried by the action of winds displacing the virus, which is consistent with the findings of different authors. One study showed a positive sample of NoVGII.17 genotype in ambient air at Yangjae meteorological station located in the southern Seoul (Korea) (Han *et al*., 2018). Another, evidenced 3 and 1 positive samples for NoVGII genotype in ambient air samples at urban areas of La Paz (Bolivia) and Kanpur (India), respectively (Ginn *et al*., 2021). It is worth mentioning that the house terrace air sample of our study and positive NoVGII, was only 0.7 km southeast of the landfill leachate treatment plant, that is consistent with finding of HuNoV in areas near to wastewater treatment plants (WWTP) as far as 0.3 km, 0.5 km and 1.0 km (Pasalari *et al*., 2019) and with the airborne presence of NoVGI as far as 1.0 km and >1.0 km of open wastewater canals in urban areas (Ginn *et al*., 2021). All $OD_{492\ nm}$ obtained in ELISA assays for the different environmental sources used to test the generation of polyclonal antibodies from the air sample showed villus system saturation (Fig. 5b).

According norovirus water contamination in our study, we believe that noroviruses contained in reusable diapers and toilet paper could end up in the sewage system of the landfill, then in the leachate treatment plant and then in the Tunjuelito river where this water is disposed of. In respect to noroviruses found in drinking water school, we believe that it could have been reached by cracks in drinking water pipes permitting entry of virus from the sewer system (combined with rainwater) and fed by neighborhoods upstream of the school. We do not rule out the possibility that noroviruses could have reached the school's drinking water via airborne contamination of the drinking water storage tanks located on the school's roof. Concerning noroviruses found in the irrigation water, these eventually arrive there through insufficiently treated wastewater in nearby municipalities such as Mosquera and Funza, and subsequently, chard irrigated with these waters is contaminated by norovirus, which is reflected in the production (cultivation and harvest) in La Ramada rural area and the marketing chain of the chard-leaves (transportation and Corabastos wholesale market) (Jordan-Lozano, 2020).

In conclusion, the model of intestinal villi isolated from mouse is effective for the study of human norovirus infection and replication from faeces and environmental matrices. Treatment with 10 µg/m trypsin is required to promote HuNoV infection in isolated mouse villi. In this model the human norovirus presents an average replicative cycle of 10 h although viral particles with infectious capacity are found already 2 h.p.i., followed by the virion release from the cell. The model is efficient in obtaining abundant biological material and is ideal for studying the biological activity of the virus in the same cell model or for generating antibodies. The virus induces cell destruction, with disaggregated cell debris observed in the culture well and a decrease in cell number. Uninfected cells lose luster and separate from the villi, but there is no cell debris and no decrease in number when compared to infected villi after 12 h of culture.

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